The Fifth International Conference of the
IUFRO Unit 2.09.02:
Somatic Embryogenesis and Other Vegetative Propagation Technologies

Proceedings

Clonal Trees in the Bioeconomy Age: Opportunities and Challenges

September 10-15, 2018
University of Coimbra
Coimbra, Portugal

CENTRE FOR FUNCTIONAL ECOLOGY

Coimbra Tree designed by
Ricardo Costa

IUFRO Unit 2.09.02
Somatic Embryogenesis and Other Vegetative Propagation Technologies

Proceedings

of the 5th International Conference on

Clonal Trees in the Bioeconomy Age: Opportunities and Challenges

September 10 – 15, 2018
University of Coimbra
Coimbra, Portugal

Citation

In: Bonga JM, Park YS, Trontin JF (Editors) Proceedings of the 5th International Conference of the IUFRO Unit 2.09.02 on "Clonal Trees in the Bioeconomy Age: Opportunities and Challenges." September 10-15, 2018. Coimbra, Portugal.

Published online, April 5, 2019:
https://www.iufro.org/science/divisions/division-2/20000/20900/20902/publications/

http://www.iufro.org/publications/proceedings/proceedings-meetings-2018/

Organization

The 5th International Conference of the IUFRO Working Party 2.09.02 – Somatic Embryogenesis and Other Vegetative Propagation Technologies is an organization of the Centre for Functional Ecology (http://cfe.uc.pt) of the Department of Life Sciences of the University of Coimbra (www.uc.pt/fctuc/dcv), and of the IUFRO (www.iufro.org). The theme of this conference is: Clonal Trees in the Bioeconomy Age: Opportunities and Challenges. Other institutions involved in the organization are the FCBA (www.fcba.fr/), Neiker-Tecnalia (www.neiker.net/), SCION (www.scionresearch.com), Faculdad de Ciencias Agrarias e Florestales da la Universidad de La Plata (www.agro.unlp.edu.ar) and the National Institute of Forest Science in Korea – NIFoS (http://english.forest.go.kr).

Organizing Committee

Jean-François Trontin, FCBA Technological Institute, Biotechnology and Advanced Forestry Department, Bordeaux, France. Jean-francois.trontin@fcba.fr
Jorge Canhoto, Centre for Functional Ecology, Department of Life Sciences of the University of Coimbra. jorgecan@uc.pt
Sandra Correia, Centre for Functional Ecology, Department of Life Sciences of the University of Coimbra. sandraimc@ci.uc.pt
Paloma Moncaleán, NEIKER-TECNALIA, Centro de Arkaute. 01080 Vitoria-Gaistez, Spain. pmoncalean@neiker.eus
Jana Krajňáková, Department of Forest Genetics, Scion, New Zealand. Jana.Krajnakova@scionresearch.com
Sandra Sharry, Dep. Agricultural and Forestry Technology – Lab. research of wood (LIMAD) - Vegetative propagation of plants Unit (UPROVE), Faculty of Agricultural and Forestry Sciences, La Plata University, Buenos Aires, Argentina, ssharry@gmail.com
Yong Wook Kim, Division of Forest Biotechnology, National Institute of Forest Science. bravekim@korea.kr

Conference Executive Committee and Local Arrangements (DCV-UC)

Jorge Canhoto
Sandra Correia
João Martins
Ricardo Costa
Jorge Noro
André Caeiro
Cátia Pereira
Mariana Neves
Arlindo Cardoso

Scientific Committee

Célia Miguel (Portugal)
Elena Corredoira (Spain)
Iwar Wendling (Brazil)
Yill Sung Park (Canada)
Scott Merkle (USA)
Hailong Shen (China)
Glória Pinto (Portugal)
Mariano Toríbio (Spain)
Jan Bonga (Canada)
Miguel Guerra (Brazil)
Jean-François Trontin (France)
Sandra Sharry (Argentina)
Isabel Arrillaga (Spain)
Yong Wook Kim (Korea)
Sandra Correia (Portugal)
Jana Krajňáková (New Zealand)
Tsuyoshi Maruyama (Japan)
Cristina Marques (Portugal)
Paloma Moncaleán (Spain)
Jorge Canhoto (Portugal)

Sponsors

www.thenavigatorcompany.com/ ; www.foriente.pt ; www.fct.pt ; www.uc.pt ; www.santander.pt ; www.vistaalegre.com ; www.cm-coimbra.pt ; www.cm-obidos.pt ; We also would like to thank to the Mosteiro de Alcobaça (www.mosteiroalcobaca.gov.pt)

Foreword

Dear friends and colleagues,

First of all, we would like to warmly thank Jorge, Sandra and the whole executive organizing committee for hosting this successful 5th IUFRO 2.09.02 conference at the University of Coimbra (Department of Life Sciences, Center for Functional Ecology), Coimbra, Portugal. We had a wonderful time in beautiful, secret and historical Coimbra, reuniting with old friends and meeting with new ones – we had 122 attendees! The conference provided us with important scientific updates (see the Summary Report at the end of these proceedings) as well as many opportunities for social networking (see the Photo Gallery). Incidentally, this conference marks the 10th anniversary of the founding of our IUFRO working unit. Through these conferences and publications, we have established a fine tradition of excellent communication and progress in a friendly atmosphere. We sincerely hope that we will continue this tradition into the future years, organizing our conferences on different continents to promote networking and friendship.

Thank you for your enthusiastic contributions for the conference proceedings. We have received a total of 63 submissions including, 24 full length articles, 14 extended abstracts, and 25 short abstracts. These articles cover a wide range of topics including somatic embryogenesis, micropropagation, rooting of cuttings, molecular biology, genomics, epigenetics, genetic resource conservation, tree breeding, and even more! Sustainable plantation forestry and in particular multi-varietal forestry based on vegetative propagation technologies obviously could make an important contribution to the green bio-economy in the context of climate change. We believe these proceedings will provide us with a wonderful opportunity to glean into one another's scientific interests and progress. This in turn offers an opportunity to network and collaborate. There are now many examples of international collaborations initiated as part of our group's activities!

During this conference, we were especially pleased to see the strong participation of graduate students and postdoctoral fellows, including to our third and well-contested international scientific competition (9 candidates from 8 countries). Thank you to those who presented their work as well as those who accepted to co-chair conference sessions! We hope that our conferences provide young participants with a forum that stimulates future endeavors.

Our next conference will be in Harbin, China, hosted by the Northeast Forestry University and organized by Prof. Hailong Shen. This will be the second time in Asia, after our inaugural conference in Suwon (2010). We fully expect that this meeting again will be a very fruitful one and we hope you will be able to attend Harbin in 2020 and discover and enjoy the "Ice City". It surely will provide us again with an opportunity to further our knowledge of our favorite research field and establish new cooperative projects.

Once again, we wish to express our gratitude to the University and City of Coimbra for providing us with the facilities needed and also to all other organizations and institutes that provided funding or supported us otherwise. Enjoy the Proceedings of Coimbra 2018!

Jan Bonga, Yill-Sung Park, and Jean-François Trontin
Editors

April 5, 2019

Contents

Foreword vi

Full Articles

***In vitro* propagation and *ex situ* conservation of *Phytolacca tetramera*, an endemic component of the 'Talares' of Buenos Aires, Argentina**
María de los Ángeles Basiglio-Cordal, Nicolás Dobler 3

Production of cell suspensions of peanut (*Arachis hypogeae*)
Hajer Ben Ghozlen, Mohamed Gouiaa, Lotfi Fki, Stefaan Werbrouck 9

What technical improvements are needed to achieve industrial application of conifer somatic embryogenesis?
Jan Bonga, Yill-Sung Park, Chen Ding 14

Performance of culture lines established *in vitro* from a monumental birch tree
Purificación Covelo, Nieves Vidal , Saleta Rico, Jesús M. Vielba , Martín Reggiardo, Conchi Sánchez 25

Improvement of micropropagation by micrografting *in vitro* cultured mature scions of *Castanea sativa* Mill. onto physiologically juvenile rootstocks of the same genotype
Santiago Crecente-Campo, Juan L. Fernández-Lorenzo, María R. Mosquera-Losada, Antonio Rigueiro-Rodríguez Nuria Ferreiro-Domínguez, María P. González-Hernández, José J. Santiago-Freijanes, Rosa Romero-Franco 34

Micropropagation of *Tilia cordata* Mill. and verification of genetic diversity of donor trees
Helena Cvrčková, Martina Komárková, Olga Trčková, Pavlína Máchová 43

Propagation and rooting of *Prunus avium* by temporary and continuous immersion systems
Damián Dasilva, Conchi Sánchez, Anxela Aldrey, Jesús M Vielba, Ramiro Martínez, Nieves Vidal 47

A brief review of combining genomic selection and somatic embryogenesis for tree improvement
Chen Ding, Yill-Sung Park, Jan Bonga, Benjamin Bartlett, Yang Li, Fred Raley 55

Induction of embryogenic calli in BC1 zygotic embryos (*Elaeis oleifera* x *Elaeis guineensis*) x *Elaeis guineensis*
Cibelle A. dos Santos, Pamela K. Harada, Ricardo Lopes, Regina C. Quisen 70

Effect of sucrose supplementation and culture system on growth and stress status of *Prunus domestica* and *Castanea sativa* x *C. crenata* shoots
Diego Gago, Mª Ángeles Bernal, Conchi Sánchez, Anxela Aldrey, Beatriz Cuenca, Nieves Vidal 74

Conservation and vegetative propagation of forest genetic resources from Talares and Monte Blanco ecosystems in Argentina
Sebastián P. Galarco, Maite Romero Alves, Patricia Boeri, Luciano Roussy, Marina Adema, Blanca Villarreal, María V. Briones, María de los A. Basiglio Cordal, Tatiana Cinquetti, Diego I. Ramilo, Sandra E. Sharry 84

Genetic characterisation of oak trees with limited natural recovery potential and their effective reproduction by the organogenesis
Martina Komárková, Eva Pokorná, Martin Fulín, Radek Lefnar, Pavlína Máchová 89

Regeneration of a unique grey poplar population by organogenesis and characterisation of individuals by SSR analysis
Pavlína Máchová, Eva Pokorná, Martina Komárková, Helena Cvrčková 94

Putative rejuvenation through tissue culture in the success of *Eucalyptus globulus* vegetative propagation
Cristina M. P. Marques, António R. Teixeira, Ana F. Rodrigues, Joana S. P. N. Costa, Nuno M. G. Borralho, Ana M. Pires 99

Epigenetics in trees: a source of plasticity and adaptation in the context of climate change
Stéphane Maury, Anne-Laure Le Gac, Clément Lafon-Placette, Mamadou Dia Sow, Régis Fichot, Alain Delaunay, Isabelle Le Jan, Marie-Anne Lelu-Walter, Vincent Segura, Odile Rogier, Jean-François Trontin, Christophe Plomion, Grégoire Le Provost, François Ehrenmann, Jérome Salse, Christophe Ambroise, Svetlana Gribkova, Marie Mirouze, Cristoph Grunau, Cristian Chaparro, Steeven H. Strauss, Daniel Conde, Isabel Allona, Jörg Tost 110

Hybrid pine (*Pinus attenuata* x *Pinus radiata*) seeds: what do you prefer, mother or nurse?
Itziar A. Montalbán, Cathy L. Hargreaves, Keiko Gough, Cathie B. Reeves, Shaf van Ballekom, Tomás Goicoa, María Dolores Ugarte, Paloma Moncaleán 116

Comparison of rooting rates of cuttings from different mother stocks of *Eucalyptus pellita* ID30 clone growing in different conditions
Heung-Kyu Moon, Nelly S. Aggangan 123

Growth and mineral uptake of *Eucalyptus pellita* in response to inoculation with different mycorrhizal inoculants
Heung-Kyu Moon, Nelly S. Aggangan 127

Use of liquid medium and biofortificants for improving micropropagation and acclimation of *Musa* AAA cv. Williams
Alexander Moreno, Ángeles Bernal, Francisco Ugarte, Kevin Lima, Mónica Coig , Conchi Sánchez, Anxela Aldrey, Nieves Vidal 138

Using *in vitro* culture for conservation of genetic resources: micropropagation of a monumental *Prunus dulcis* tree
Conchi Sánchez, Saleta Rico, Anxela Aldrey, Damián Dasilva, Jose María Rey-Benayas, Nieves Vidal 146

Micropropagation of willow shoots under photomixotrophic and photoautotrophic conditions: proliferation in liquid medium and acclimation in different soils
Conchi Sánchez, Carmen Trasar, Mar Casalderrey, Diana Bello, Anxela Aldrey, Nieves Vidal 151

***In vitro* propagation and callus formation of *Morus alba*.**
Juan J. Silva, Yanexi Y. Fonseca, Marisel Bahi, Jorge L. Pérez, Stefaan Werbrouck 159

Somatic embryogenesis of Norway spruce in Finland – seven years from start to first commercial pilots
Mikko A. Tikkinen, Saila A. Varis, Sakari M. Välimäki, Teijo O. Nikkanen,Tuija S. Aronen . 165

Flowering traits as a component of reproductive success in maritime pine clonal seed orchards
Jean-François Trontin, Pierre Alazard, Sandrine Debille, Laurent Bouffier 173

Extended Abstracts

Biotechnology and bio-prospection of native species from Monte desert Patagonia, as strategies for the development of regional bio-economy
Patricia Boeri, Lucrecia Piñuel, Irina Lopez Dumrauf, Daniela Dalzotto, Inti Sabanes, Sandra Sharry 185

Comparative analysis of primary metabolome and secondary metabolites in the somatic embryogenesis of tamarillo (*Solanum betaceum* Cav.)
André Caeiro, Ivana Jarak, Sandra Correia, Rui Carvalho, Jorge Canhoto 191

Cork oak somatic embryogenesis as a system model to study QsMYB1, a transcription factor highly expressed in phellem
Tiago Capote, Leonar Morais-Cecílio, Sónia Gonçalves, Liliana Marum 194

***In vitro* germination of *Brachychiton populneus* (Schott & Endl.) R.Br.**
Tatiana Cinquetti , Maite Romero Alves, Sandra Sharry 197

A method of initiation and multiplication by - culture - cycling of somatic polyembryogenesis of Loblolly pine.
Pramod K Gupta 200

Effects of exogenous phytosulphokine and polyamines in somatic embryogenesis of stone pine
Inmaculada Hernández, Mar Ruiz-Galea, Dolores López-Vela, Mariano Toribio, Cristina Celestino 204

Detection and expression of somatic embryogenesis regulatory genes in *Tilia amurensis, Tilia insularis* and *Tilia mandshurica*
Hae-In Kang, Kyu-Suk Kang 210

Biochemical characterization of cryopreserved *Pinus radiata* embryogenic tissue
Yenny Lineros, Darcy Ríos, Ximena Muñoz, Claudio Balocchi 214

Improving somatic embryogenesis in grapevine
Óscar Martínez, Verónica Arjones, Yosvanis Acanda, Cristina Domínguez, María Victoria González, Manuel Rey 219

Strawberry tree (*Arbutus unedo* L.) breeding: hybridization and polyploidization
João F. Martins, Glória Pinto, Jorge M. Canhoto 223

The ups and downs of developing hybrid sweetgum varieties for the U.S. bioenergy and pulp and paper industries: a 20-year case study
Scott Merkle 225

Implementing genomic selection for multi-varietal forestry of white spruce (*Picea glauca*) in New Brunswick, Canada
Yill-Sung Park, Chen Ding, Patrick Lenz, Simon Nadeau, Greg Adams, Shona Millican, Jean Beaulieu, Jean Bousquet 230

Induction of somatic embryogenesis in *Chorisia speciosa (A.St.-Hil.)* Ravenna
Maite Romero Alves, Tatiana Cinquetti, Sandra E. Sharry 234

***Ex situ* conservation of the Brazilian conifer *Podocarpus lambertii* (Podocarpaceae) by means of cryopreservation**
Leila N. Vieira, Patrick D. Marques, Angelo S. Heringer, Hugo P.F. Fraga, Daniela Goeten, Miguel P. Guerra 237

Short Abstracts

Transcriptional identification and characterization of differentially expressed miRNAs involved in conifer embryogenesis
Ana Alves, Andreia Rodrigues, Susana Lopes, Pedro Perdiguero, Bruno Costa, Inês Chaves, Ana Confraria, Célia Miguel 245

An automated active compounds screening system allows high-throughput optimization of somatic embryogenesis in *Coffea Arabica*
Rayan Awada, Dorothée Verdier, Solène Froger, Eric Brulard, Hervé Etienne, David Breton 247

Effect of priming during SE maturation on the phenotype of maritime pine plants after a short-term heat treatment
Eva Cañizares, Alex Alborch, María Cano, Alberto Guillén, Juan Segura, Ester Sales, Isabel Arrillaga 248

Dynamics of DNA methylation and effects of de-methylating agents on somatic embryogenesis of *Quercus suber* L
Elena Carneros, Yolanda Pérez-Pérez, Ivett Bárány I, Beatriz Pintos, Aránzazu Gómez-Garay, María C. Risueño, Pilar S. Testillano 249

Characterization of the influence of nitric oxide donors on metabolism of polyamines and amino acids in embryogenic cell cultures of Brazilian pine (*Araucaria angustifolia* (Bertol.) Kuntze)
André L.W. dos Santos, Giovanni V. Cerruti, Alana S. Silva, Ana S.R.L.A. Duque, Manuel P.S. Fevereiro, Eny I.S. Floh 251

Field performances of hybrid poplar leaf and wood traits in both micropropagated plants and plants propagated from root cuttings: ecophysiological, vascular, nanomechanical and cell wall compositional assessments
Jaroslav Ďurkovič, Hana Husárová, František Kačík, Rastislav Lagaňa, Ingrid Čaňová . . . 252

The effect of different air humidity during desiccation on the development of Norway spruce somatic embryos
Lucie Fischerová, Lenka Gemperlová, Jan Kuderna, Alena Trávníčková, Jiří Malbeck, Kateřina Eliášová, Zuzana Vondráková, Milena Cvikrová, Martin Vágner 253

Effect of megagametophyte priming on short-term response to high temperatures in somatic embryogenesis-derived plants of maritime pine
Juan G. Haro, Juan Segura, Alex Alborch, María Cano, Ester Sales, Isabel Arrillaga 254

Recent progress in somatic embryogenesis of Japanese conifers
Tsuyoshi E. Maruyama, Hosoi Yoshihisa 255

Development of an *in vitro* screen for *Phytophthora cinnamomi* resistance in hybrid and transgenic chestnut trees
Scott A. Merkle, Marjorie G. MacKnight, Heather J. Gladfelter, Steven N. Jeffers 257

CYTED NET: BIOALI, Biotechnology to improve breeding programs of species of socioeconomic interest
Paloma Moncaleán, BIOALI Consortium 258

Current results in somatic embryogenesis for *Pinus koraiensis*, an ecologically and economically very important pine species in East Asia
Chunxue Peng, Fang Gao, Hailong Shen, Ling Yang, Peng Zhang, Chunping Liu 259

Clonal test of *Sequoia sempervirens* (D. Don) Endl. in southern Brazil
Pereira MO, Navroski MC, Angêlo AC 260

Cell wall remodeling by pectin esterification and AGP expression underlies somatic embryogenesis of cork oak
Yolanda Pérez-Pérez, Elena Carneros, María-Teresa Solís, Ivett Bárány, Beatriz Pintos, Aránzazu Gómez-Garay, María C. Risueño, Pilar S. Testillano 261

The transcriptome of maritime pine across embryo development
Andreia S. Rodrigues, José J. De Vega, Célia M. Miguel 265

***Ex situ* conservation of *Prunus lusitanica* by micropropagation techniques**
Alberto Rodríguez, Nieves Vidal, Purificación Covelo, Saleta Rico, Conchi Sánchez 261

Effect of genotype, sucrose concentration and glutamine supply on the protein and lipid content in embryogenic suspension cultures of cork and holm oak
Mar Ruiz-Galea, Inmaculada Hernández, Celia Cano, Eva Friero, Cristina Celestino, Mariano Toribio 267

Public perception in university and educational institutions about clonal forestry and forest biotechnology in Argentina and Venezuela
Sandra Sharry, Iselen Trujillo, Maite Romero Alves, Tatiana Cinquetti, Sebastian Galarco, Patricia Boeri 268

What the Latin American people think about clonal forestry and forest biotech?
Sandra Sharry, Iselen Trujillo, Maite Romero Alves, Tatiana Cinquetti, Sebastian Galarco, Patricia Boeri 269

Somatic embryogenesis and other vegetative propagation techniques for native forest tree species in north China: current status and issues that should be resolved in the near future
Hai-Long Shen 270

Repetitive somatic embryogenesis induced cytological and proteomic changes in embryogenic lines of *Pseudotsuga menziesii*
Caroline Teyssier, Florian Gautier, Kateřina Eliášová, Jean-Charles Leplé, Zuzana Vondráková, Anne-Marie Lomenech, Claire Le Metté, Philippe Label, Guy Costa, Jean-François Trontin, Marie-Anne Lelu-Walter 272

Temperature affects somatic embryo development in maritime pine
Jean-François Trontin, Isabelle Reymond, Francis Canlet, Mamadou D. Sow, Alain Delaunay, Stéphane Maury, Claire Le Metté, Caroline Teyssier, Marie-Anne Lelu-Walter 274

Primordial shoots of Norway spruce (*Picea abies*) as explants for somatic embryogenesis
Saila A Varis, Krystyna K Klimaszewska, Tuija S. Aronen 275

Low cost media for improving *in vitro* propagation of woody plant species
Nieves Vidal, Saleta Rico, Salvatore Casano, Verónica Codesido, Conchi Sánchez 276

Relationship between H_2O_2 accumulation and NO signal synthesis in osmotic stress-induced somatic embryogenesis of *Fraxinus mandshurica*
Ling Yang, Hailong Shen 278

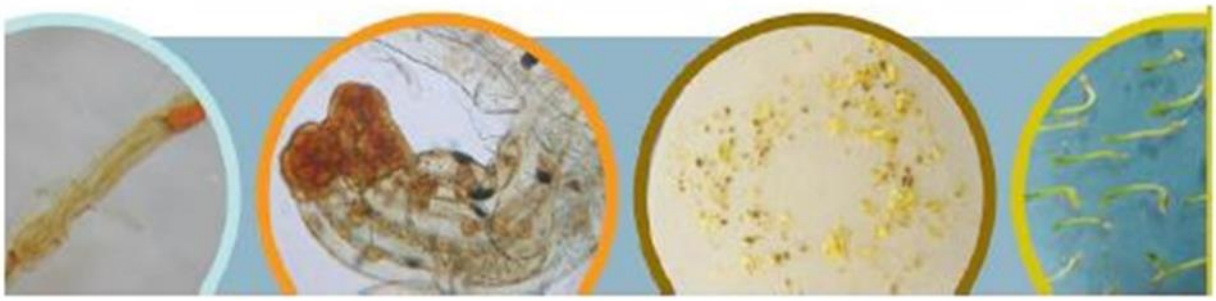
Photo Gallery

The wonderful memories of 'Coimbra2018' conference: Our group, speakers, sessions, visits, next conference, and supportive message 279

Participants & Contact Information 300

Summary Report of Coimbra 2018 Conference 310

Full Articles



In vitro propagation and *ex situ* conservation of *Phytolacca tetramera*, an endemic component of the 'Talares' of Buenos Aires, Argentina

María de los Ángeles Basiglio-Cordal, Nicolás Dobler*

Laboratorio de Cultivo in vitro, CEAMSE
(Coordinación Ecológica Área Metropolitana Sociedad del Estado). Buenos Aires, Argentina.
**Corresponding author: ndobler@ceamse.gov.ar*

Abstract

The native forest formation in the northeast of the Province of Buenos Aires on hills composed of shell deposits of the alluvial valley of the Río de La Plata is called 'talar.' *Phytolacca tetramera* Hauman, or 'Ombusillo', is a shrub endemic to the Talares that belongs to the Phytolaccaceae Family. By anthropic action, it was included in the category CR (critic risk) of threatened species. It has fungicide active ingredients. Moreover, the methanolic extract of its berries possesses antifungal activity against opportunistic pathogenic fungi. With the aim of conserving this species, in CEAMSE´s *in vitro* culture laboratory, we´re focused on the development of propagation protocols via organogenesis and somatic embryogenesis, plant nursery culture and ground planting. After production of plants and the subsequent acclimation period in the greenhouse, 20 individuals we´re selected to carry out survival and development tests in the soil cover of the Villa Dominico Sanitary Landfill (Province of Buenos Aires, Argentina). At the end of its dormancy period, between the months of September and December, the status of the individuals will be evaluated, taking into account their health and air development. The following actions prepared for the conservation of *Phytolacca tetramera*, will include collaboration with public welfare organizations dedicated to its protection and raising of awareness of the state of its condition in its habitat involving Botanical Gardens, Urban Nature Reserves, educational entities.

Keywords: native forest, conservation, endemism, organogenesis, *Phytolacca tetramera*, 'talares'

Abbreviations: CR (critic risk), BAP (6-Benzylaminopurine), IBA (Indolbutyric Acid)

Introduction

The native forest formation in the northeast of the Province of Buenos Aires on hills composed of shell deposits of the alluvial valley of the Río de La Plata is called 'talar'. Anthropic pressure is a great danger that confronts these woods in the most populated part of the country, and that is why their situation is critical. *Phytolacca tetramera* Hauman or 'Ombusillo' is a shrub endemic to the Talares from Buenos Aires that belongs to the Phytolaccaceae family. It has 18 genera and 65 species globally with 7 genera and 12 species being present in Argentina (Zuloaga y Morrone, 1999). Its most widespread genus is Phytolacca with at least 25 species. Many of them were used in oriental medicine for a long time for the treatment of various conditions such as edema, rheumatism (*P. americana, P. insulares*) (Kang and Woo, 1987), dermatitis. (*P. octandra*) (Moreno and Rodriguez, 1981), emetics, purgatives and 'antisifilica' (Jolliffe, 1982), among others (Galarraga, 2011). Also *Phytolacca tetramera* has fungicide active ingredients. Present in their berries are 3 saponin triterpenoids, the phytolacosides B, E and F (Escalante et al. 2002). The Phytolacoside B showed a medium antifungal activity against of a series of

dermatophytes such as *Trichophyton mentagrophytes* and *Candida albicans*, using ketoconazol as a control (Galarraga, 2011). As for its conservation status, *Phytolacca tetramera* it is included on the list PlanEAr (Endemic Plants of Argentina) in the category 5 according to the update of the year 2008, which states for this species 'restricted distribution of plants with scarce populations or about which there are one or more threat factors' (habitat destruction, overexploitation, biological invasions, etc). In 2006, it was proposed to review the threat situation of vascular plant species of the province of Buenos Aires; in said work, Phytolacca tetramera, is included in the category 'Critic risk' (CR), whereby a taxón faces an extremely high risk of extinction in the wild state in the immediate future (Delluchi, 2006). According to the Global Environment Facility (Global Environment Facility, 1986), the accelerated loss of biodiversity raises a serious threat to the welfare of humanity. The magnitude of the human impact on the biological diversity increases exponentially due mainly to the global patterns of consumption, production and trade, to agricultural, industrial and human settlements, and the development and growth of population. It is here where biotechnology appears as a tool to reverse this delicate situation. *In vitro* culture has emerged as a powerful alternative for the reproduction and conservation of species that are difficult to grow by traditional methods. Several thousand publications have reported propagation of hundreds of species, including medicinal and aromatic plants (Alfermann and Petersen, 1995). They have emerged as strong tools widely used both to support breeding programs and *in situ*, and *ex situ* conservation. Biotechnology and biodiversity go together because of multiple factors, since the former provides new techniques in addition to classical breeding to meet two basic objectives: maintaining natural diversity and genetic improvement of agricultural plantations. In 2011, the first advances in the conservation and *in vitro* propagation of *Phytolacca tetramera* were achieved, with the disinfection of nodal and internodal sections and by studying their response to different culture media for their *in vitro* vegetative propagation (Basiglio- Cordal et al. 2011). Later, the induction of somatic embryos in this species was studied. (Basiglio- Cordal et al. 2013).

With the aim of conserving this species, in CEAMSE´s *in vitro* culture laboratory, we focus on the development and optimization of propagation protocols via organogenesis and somatic embryogenesis. In addition, acclimation has been achieved as well as plant growth in the nursery and subsequent planting in the field. We also conducted studies to learn their adaptation to different terrains and thus ensuring the survival of the species.

Materials and methods

Mother plants in good health and with optimal growth were selected. Thirty explants were used per treatment, obtained from nodal and internodal sections of 1 to 1.5 cm in length and 1 to 2 mm in diameter. Its disinfection was developed following the previously adjusted protocol (Basiglio- Cordal et al. 2011). Briefly, it consisted of immersing the explants in commercial sodium hypochlorite (55 gL-1 of active chlorine) at 30% with a few drops of Tween 20® for 30 min. Later, the explants were washed with sterile water and submerged in 0.6% of Erythromycin. The nodal and internodal disinfected sections were placed, maintaining their polarity, in the basal culture medium of Murashige and Skoog (1962) supplemented with 3% sucrose. We tested different concentrations of 6-Benzylaminopurine and Indolebutyric Acid: Without growth regulators (MS1), 0,002 µM of BAP and IBA (MS2), 0004 µM of BAP and IBA (MS3). Two explants were cultured per container, with up to 20 per treatment. A total of 420 explants were cultivated, in a culture chamber at 24 ° C ± 2 ° C with 16: 8 photoperiod. The lighting was by white LEDs achieving at light intensity of 60, 70 $\mu mol.m^{-2}s^{-1}$. The explants were subcultured every 35 days. After 70 days in culture, once elongated plants were obtained, they were subjected to a rooting process in MS medium with the macronutrients diluted in half and without the addition of growth regulators. After 35 days in rooting medium, the micro-shoots that had rooted were acclimatized to *ex vitro* conditions in the greenhouse by gradually decreasing the relative humidity for 1 month. Later, the plants continued their development under conventional conditions in the nursery for two years (Figure 1).

In June 2018, 20 individuals were selected from those obtained by micropropagation for testing survival and development in the Villa Dominico Sanitary Landfill (Province of Buenos Aires, Argentina). Within the Landfill, four sites were selected (A, B, C and D) that differ in their altitude above sea level (A= 9 m, B= 15 m, C= 19 m and D= 6 m). At each site an analysis of soil characteristics was carried out, evaluating the following parameters: PH, electrical conductivity (dsm^{-1}), sodium adsorption ratio ($meq\ l^{-1}$) and organic matter (Table1).

Figure 1. *Two-year plants of Phytolacca tetramera produced in vitro at the Vivero Experimental "Ing. José Luis Giannoni", at CEAMSE, Villa Dominico, Buenos Aires, Argentina.*

Table 1. *Characteristics of the four places chosen to carry out the plantation of the 'Ombusillos' in the Villa Dominico Landfill.*

Determination	A	B	C	D	Reference Values
pH	6,4	8,1	7,5	4,8	6 – 7,5
Electric conductivity (dsm^{-1})	0,79	0,98	0,88	4,2	< 4
Sodium absorption ratio ($meql^{-1}$)	0,2	1,8	0,1	0,5	< 13
Organic material	4,33	2,12	5,28	5,45	>3
Height (m)	9	15	19	6	-

Later, 5 individual were installed in each one of the selected sites (Picture 2A, B). Also, of each copy we recorded their sex, filiation, height and width of the stem prior to being planted.

Figure 2. *Phytolacca tetramera produced in vitro at the Vivero Experimental Villa Domínico installed in zone C (2A) and in zone D (2B) on the landfill.*

Results

The results obtained during the micropropagation process can be seen in Table 2. The culture medium MS2 was the one that showed the best results during the micropropagation process. After two subcultures in the MS2 medium, 453 plants were obtained, observing a multiplication index of more than two stems per stem cultivated *in vitro*. The percentage of acclimation was in all cases greater than 90%.

Table 2. *In vitro establishment (%), number of vitroplants established, number of stems obtained after two subcultures and acclimatization (%) of Phytolacca tetramera.*

Culture media	Establishment (%)	Vitroplants established (n)	Stems obtained after two subcultures (n)	Acclimatization (%)
MS 1	52	73	197	91
MS 2	73	102	453	94
MS 3	46	64	158	90

In June and December of 2018, the characteristics of the plants installed on the landfill were evaluated as shown in Table 3. The results showed that after six months in the field, 90% of the individuals survived at all the sites used. Site D has some edaphic limitations, either because of its high electrical conductivity, that can bring associated problems of salinity (FAO, 2016), or its low PH value, that can generate shortcomings of alkaline cations such as Calcium, Magnesium and Potassium, affecting the sensitive of the species to acidity (Kochba et al. 2004; Mueller et al. 2004; Shukla et al, 2004). Both sites A and C would present good conditions for future development, according to soil analysis observed in Table 1. With respect to Site B, although it would be the ideal site due to similarity in alkalinity with the area of endemism, the results obtained at the moment do not allow to conclude its suitability, because this site was affected by equine activity. It is important to remember that *Phytolacca tetramera* develops in the calcareous soils of a narrow strip of the Talares Bonaerenses. According to the measurements, a variation in the height of the stems occurred in the different planting zones, which, in some cases, may be due to animal activity and water scarcity. New data and results are needed in order to estimate the role of the characteristics of each site in the growth of individuals. Therefore, the trial will require follow-up testing during the next years.These first results are of particular interest given the vulnerability of the species at present, its importance as a unique endemism of the area and the variety of antifungal compounds found in it. This work forms the basis for strengthening actions of domestication in endangered species. The importance of implementing biotechnological techniques in this type of species is of great interest because of the high efficiency of these techniques in comparison with traditional ones and because it guarantees the obtaining of healthy and vigorous plants. The following actions prepared for the conservation of *Phytolacca tetramera* will include collaboration with public agencies that are dedicated to their protection such as Botanical Gardens, Urban Nature Reserves, Educational Entities, etc.

Table 3. *Height of stem (cm), height increase (M±E.E.), stem base (cm), increase of stem base (M±E.E.) of Phytolacca tetramera micropropagated and installed in four areas of the Villa Dominico sanitary landfill, Buenos Aires, Argentina*

.

		Height of stem (cm)			Increase	M ± E. E	Stem base		Increase	M ± E. E
Site	Code	June	December				June	December		
A	V4	2,0	19,0		17	10,8 ± 9,25	12,0	17,0	5	3 ± 1,58
	EC2	4,0	7,0		3		0,0	2,0	2	
	V3	0,0	12,0		12		7,0	10,0	3	
	EC6	19,0	6,8		0		9,0	10,0	1	
	EC6	13,0	35,0		22		10,0	14,0	4	
B	V4	12.5	25		12,5	14 ± 14,40	7,0	17,0	10	2,6 ± 4,33
	EC2	9,0	0		0		7,0	0,0	0	
	EC6	9,0	0,5		0		3,0	0,0	0	
	EC6	10.5	40		29,5		8,0	6,5	0	
	V3	9,0	37		28		4,0	7,0	3	
C	EC2	14,0	42,0		28	30,1 ± 8,81	3,5	11,0	7,5	6,5 ± 3,98
	V3	7,5	49,0		41,5		2,5	13,5	11	
	EC6	8,5	25,0		17		6,0	11,0	5	
	EC6	5,5	36,5		31		3,5	12,0	8,5	
	V4	7,0	40,0		33		4,0	4,5	0,5	
D	V3	13,0	23,0		10	5,8 ± 7,01	3,5	3,0	0	0,5 ± 0,70
	EC6	11,0	27,0		16		3,5	5,0	1,5	
	EC6	16,0	9,0		0		4,5	3,0	0	
	EC2	11,0	11,0		0		3,5	4,5	1	
	V4	6,0	9,0		3		3,5	2,5	0	

Acknowledgements: We are especially grateful to Paloma Moncaleán, PhD, Researcher of Neiker and Coordinator of Bioali-Cyted

References

Alfermann AW, Petersen M (1995) Natural product formation by plant cell biotechnology. Plant Cell Tissue Organ Cult 43: 199-205

Basiglio-Cordal MA, Roussy L, Briones V, Sharry S, Abedini W (2011) Strategies for *in vitro*

preservation and propagation of *Phytolacca tetramera* Hauman, an endemic medicinal species in danger of extinction. XX Italo Latinamerican Congress of Ethnomedicine. (SILAE) Fortaleza, Brasil 496: 394

Basiglio-Cordal MA, Adema M, Briones V, Villarreal B, Panarisi MH, Abedini W, Sharry S (2013) Induction of somatic embryogenesis in *Phytolacca tetramera*, medicinal species of Argentina. Emirates Journal of Food and Agricultura. XXII Italo Latinamerican Congress of Ethnomedicine. (SILAE) Puntarenas, Costa Rica 443: 67 p

Delucchi G (2006) The threatened plant species of the Province of Buenos Aires. An update. APRONA. 39: 19-31

Escalante AM, Santecchia CB, López SN, Gattuso MA, Gutierrez A, Delle Monache F, González Sierra, Zacchino SA (2002) Isolation of antifungal saponins from Phytolacca tetramera, an Argentinean species in critic risk. Journal of Ethnopharmacology 1: 29- 34.

Galarraga EM (2011) Phytochemical study of the species: Phytolacca rugosa (Phytolaccaceae), *Phytolacca icosandra* (Phytolaccaceae), *Cestrum ruizteranianum* (Solanaceae) and *Ganophyllum giganteum* (Sapindaceae). Universidad de Los Andes. Mérida,Venezuela 299: 28.

Global Environment Facility (1986) Operational Strategy of the Global Environment Facility. Chapter 2. "Biological Diversity". The Global Environment Facility, Washington, DC. http://www.gefweb.org/public/opstrat/ch2.htm#chapter2

Jolliffe G (1982) Phytolacca, a study of its properties and used. Br Homeopath J. 71: 31- 34.

Kang SS, Woo WS (1987) Two new saponins from Phytolacca Americana. Planta Med. 53:338-340.

Kochba M, Ritvo G, Avnimelech Y (2004) The effect of municipal solid waste compost (msw) on the replacement of sodium in sodic soil models. Soil Science. 169 (8): 567- 562.

Moreno M, Rodriguez VM, Yiamoloside B (1981) A fungistatic saponin of Phytolacca octandra. Phytochemistry 20: 1446-1447

Mueller TG, Mijatovic B, Sears BG, Pusuluri N, Stombaugh, TS (2004) Soil electrical conductivity map quality. Soil Science. 169 (12): 841- 851

Shukla MK, Lal R, Ebinger M (2004) Soil quality indicators for the north appalachian experimental watersheds in coshocton ohio. Soil Science. 169 (3): 195- 205

Zuloaga FO, Morrone O, Belgrano MJ (2008) Catalog of Vascular Plants of the Southern Cone.

Production of cell suspensions of peanut (*Arachis hypogeae*)

Hajer Ben Ghozlen[1,2], Mohamed Gouiaa[2], Lotfi Fki[3], Stefaan Werbrouck[1]

[1] Laboratory of Applied In Vitro Plant Biotechnology, Faculty of Bioscience Engineering - Ghent university, Belgium; [2] Research Unit on Conservation and Valorization of Plant Resources through the creation of Botanical Garden, Higher Agronomic Institute, Chott Mariem, IRESA – University of Sousse, Tunisia; [3] Laboratory of biotechnology for cultures amelioration, Faculty of sciences of Sfax-University of Sfax, Tunisia. E-mail: Hajer.BenGhozlen@ugent.be

Abstract

Peanut has been described as a potent producer of stilbenes. The present research focused on callus induction and establishment of cell suspension culture of three local Tunisian varieties. Leaf explants were used for callus induction in MS medium supplemented with 10µM picloram. Callus induction rate and morphology were recorded and cell suspension culture was established in 250 ml flasks containing 50 ml of the respective culture medium but lacking agar and with added 0,1 g l^{-1} citric acid and 50 µl l^{-1} PPM. Fresh weight, growth index and relative growth rate were quantified and cell viability was assessed with fluorescence diacetate. The callus showed different morphology in response to picloram. The growth curve of the cell suspension culture was genotype dependent. The best rate, stability and alive cells of culture were achieved with 'Chanfakhi'.

Keywords: peanut, picloram, callus, cell culture, cell viability

Introduction

Plants produce a high diversity of secondary metabolites that have been used throughout history as drugs, pesticides, pigments, flavors and fragrances. Amongst them, phenolic compounds are one of the most common and widespread groups (El Gharras.2009). They are divided into several classes among which stilbenes exemplified by resveratrol, are the most intensively studied. Resveratrol and its derivatives are regarded as important health promoting compounds (Wang et al.2018). So, demand is continuously increasing, which has led to ascertain diverse biotechnology approaches for the large-scale production (Carolina et al.2017). Plant cell tissue and organ cultures can produce a high amount of phytoconstituents in the in vitro microenvironment and are an alternative of extensive plants exploitation. The production of bioactive metabolites has been studied in some *Arachis* species, including phenolic acids, phytosterols and stilbenoids by calluses as an easy to obtain source. In this study, cell cultures of peanut are established from callus derived from leaf callus.

Methodology

Plant material and establishment of callus cultures

Mature and fresh seeds of three peanut varieties ('Trabelsia', 'Chanfakhi' and 'American') were peeled, rinsed in 70% ethanol and sterilized for 15 min in 10% NaOCl solution with 0.005% detergent (tween). They were washed three times and soaked for 2h in in sterile distilled water, Then the seeds were germinated in Gamborg B5 medium (1968) with 3% sucrose and 0.2% Gelrite, with an pH set to 5,8 before autoclaving. The cultures were maintained at 25 ± 2° C under a 16 hours photoperiod. Leaves discs of 0.5 cm^2 from 15 days old seedlings were placed on Murashige and Skoog (1962) (MS) medium

supplemented with 10 µM picloram and incubated under a 16h photoperiod. At least 3 replicates were performed with 6 explants each.

Cell suspension culture

Cultures were established in 250 ml erlenmeyers by transferring callus separated from the initial explant to fresh MS liquid medium supplemented with 10 µM picloram, citric acid and Plant Preservative Mixture (PPM) (Plant Cell Technology, USA). Suspensions were maintained in an orbital shaker at 110 rpm at 25°C in constant darkness. They were subcultured every 10 days to collect cells and small callus clumps by mass filtration. The callus was harvested and weighted as Fresh Weight (FW). Growth Index (GI), and relative growth rate (RGR) were measured and calculated according to following formulas :

$GI = (wt_F - wt_I) / wt_I$

$RGR = Ln\ wt_F - Ln\ wt_I / cp$

Where wt_F is the final weight of the callus, wt_I is the initial weight and cp is the culture period in days.

Determination of total cell count and cell viability

Cell viability was analysed by incubating the cell suspension for 5 minutes in fresh medium containing 100 µg/ ml fluorescent diacetate (FDA) and observing the fluorescence emission of the living cells under ultraviolet light with an inverted microscope Nikon ECLIPSE TE2000-S (Navarro et al., 2012). The number of viable cells per µl (N) was assessed in accordance with this formula:

Number of viable cells per µl volume = viable cells counted / (counted surface (mm2) x chamber depth (mm) x dilution factor.

Results and discussion

Establishment of callus culture

The present study investigated the influence of picloram, naturally a systemic herbicide, on growth in callus cultures. The leaf explants of different varieties developed some morphological variation in callus such as white yellowish friable callus with nodular structures and compact, green callus (Fig. 1). 91 to 100% of the explants responded to 10 µM picloram by a relatively rapid growth of callus. Our results are in harmony with Isabela et al. (2018), who reported that picloram stimulated callus growth in *Arachis pintoi Krapov*. Likewise, Salma et al. (2018) concluded that adding 2 mg/ml of picloram to MS medium induced the highest percentage of callus in *Eclipta alba.* Also, Bekhet et al. (2014) mentioned that 3 mg/ml picloram increased responses of explants towards callus growth in artichoke and milk thistle.

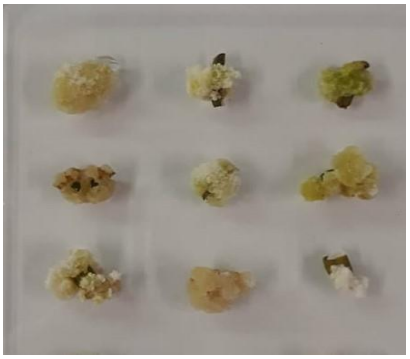


Figure 1. *Response of leaves explants of peanut to picloram ('Trabelsia' (top), 'Chanfakhi' (middle), 'American' (bottom))*

The friable callus was used for the suspension culture experiments. The reaction was genotype dependent. As shown in Fig 2, the highest biomass growth of cell aggregates was obtained with 'Chanfakhi' while the 'American' cell culture easily browned and died rapidly. Moreover, the pattern curve of growth index and relative growth rate (Fig. 3) demonstrated the rapid growth of cells derived of 'Chanfakhi' and 'Trabelsia'. A similar reaction was observed for cell suspension cultures of *Eysenhardtia polystachya* (Bernabé-Antonio et al., 2017), where picloram was more efficient than NAA and yielded the most suitable callus from leaf explant in order to establish cell suspension culture. Likewise, this result agrees with the findings of Solange et al. (2000) in which they registered the high efficiency of picloram for callus induction and cell biomass growth in suspension culture of *Rollina mucosa*.

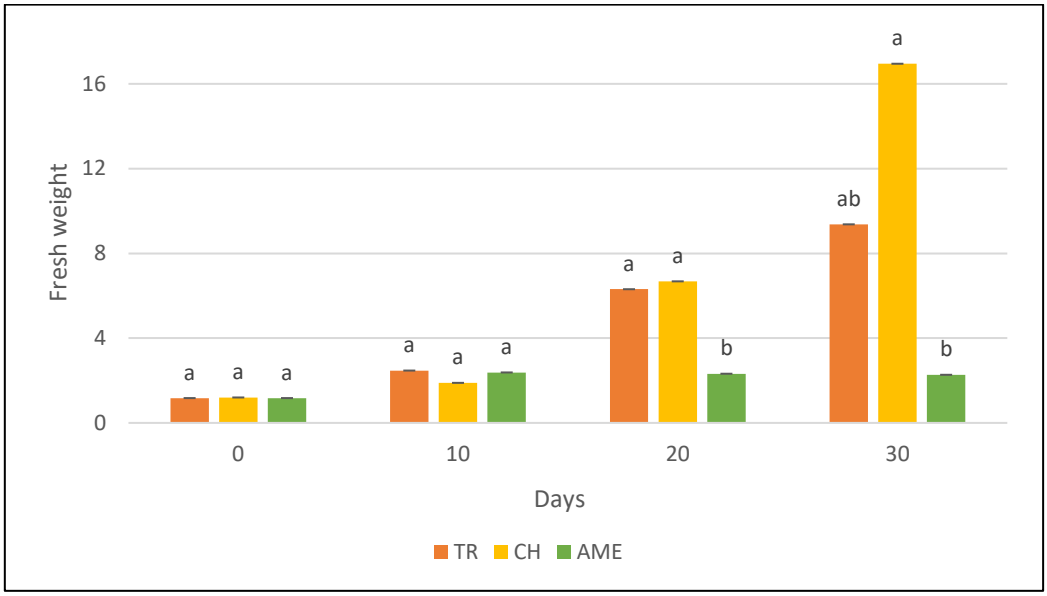
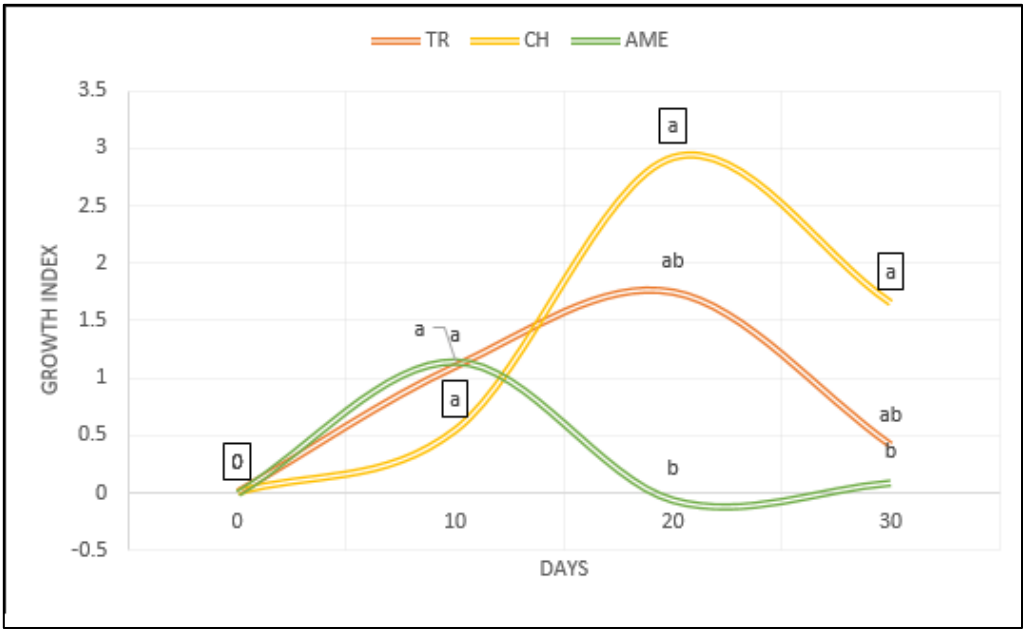


Figure 2. *Effect of picloram on biomasses accumulation in calluses and cell aggregates in cell suspension culture of Arachis hypogeae (TR :'Trabelsia', CH :'Chanfakhi', AME :'American')*



(Continue to next page)

(Figure continued from previous page)

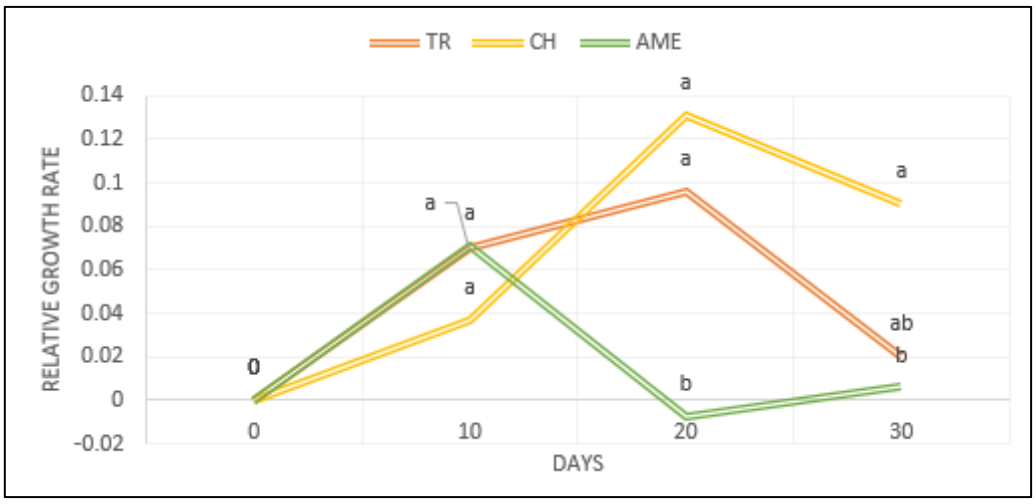


Figure 3. *Effect of picloram on growth index and relative growth rate of cell suspension culture of Arachis hypogeae (TR :'trabelsia', CH :'chanfakhi', AME :'american')*

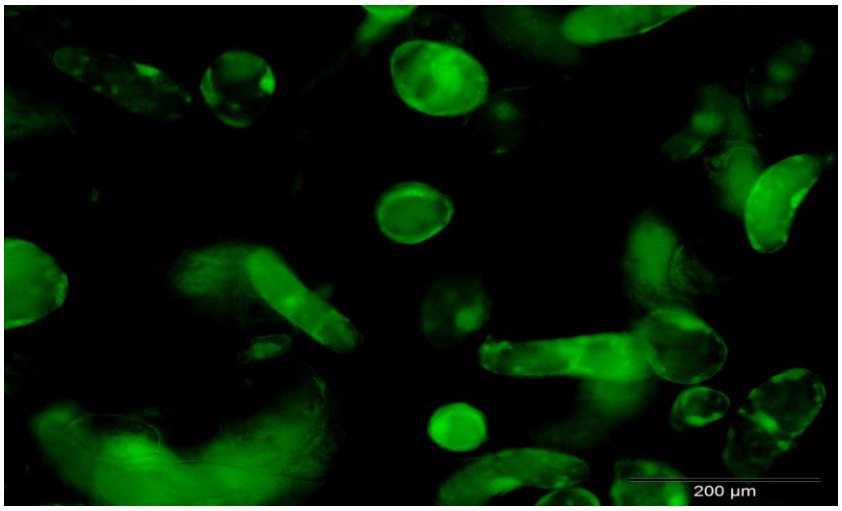


Figure 4. *Vital staining of cells with FDA after 30 days of cell suspension culture of Arachis hypogeae*

The cell viability was determined via vital staining with FDA (Fig. 4). The accurate assessment of the number of viable cells in a population is very important and a basic parameter for the evaluation of culture response to auxin and elicitor added. FDA allowed to assess cell viability of the 30-day-old culture cells, cell clusters and aggregates of various sizes as 3.06 x 10^4 viable cells / µl. Naoko et al. (2013) reported the utility of FDA for quantification of cellular metabolites activity and cell viability. They concluded that differences in fluorescein production are related to the differences in cellular esterase activities.

Conclusion

The callus formed from leaf explants of three varieties of peanut was used to establish cell suspension cultures. The most vigorous and stable suspension culture that could be prepared was 'Chanfakhi'. This cell suspension will further serve for intracellular and extracellular extraction of stilbenes as resveratrol and its derivatives.

References

Bernabé-Antonio A, Maldonado-Magaña A, Ramírez-López C.B, Salcedo-Pérez E, Meza-Contreras J.C, González-García Y, López-Dellamary Toral F.A, Cruz-Sosa F (2017) Establishment of callus and cell suspension cultures of *Eysenhardtia polystachya* (Ortega) and fungistatic activity of their extracts. South African J Bot 112: 40-47

Bekheet SA, El-Bahr MK, Ali SA, Hamed MA (2014) Callus production of Globe artichoke and Milk thistle: in vitro hypolipidemic and antioxidant activities. World J Pharma Resources 3: 1–71

Carolina S, Evelyn B, Claudia P, Jose B, Roque B, Matilde U (2017) Callus Induction and Cellular Suspensions from Murtilla (*Ugni molinae Turcz*.) for trans-resveratrol Production. Amer J Biochem Biotechnol 13: 7-14

El Gharras H (2009) Polyphenols: food sources, propreties and applications-a review. International J Food Sci Technol 44: 2512–2518

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Experim Cell Res 50: 151-158

Isabella B, Tayanne F, Renata G, Georgia P, Davyson M, Elisabeth M (2018) Total phenolics, resveratrol content and antioxidant activity of seeds and calluses of pinto peanut (*Arachis pintoi Krapov*.& W.C. Greg.). Plant Cell Tissue Organ Cult 134: 491-502

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473-497

Naoko S, Yurina S, Tomoya A, Takumi N, Hamako S, Hiroaki K (2013) Quantification of metabolic activity of cultured plant cells by vital staining with fluorescein diacetate. Analytical Biochem 441: 58-62

Navarro S, Almagro L, Lijavetzky D, Bru R, Pedreno M (2012) Enhanced extracellular production of trans-resveratrol in *Vitis vinifera* suspension cultured cells by using cyclodextrins and methyljasmonate. Plant Cell Reproduction 31: 81–89

Salma U, Kundu S, Ali N, Mandal N (2018) Elicitor mediated enhancement of wedelolactone in cell suspension culture of *Eclipta alba* (L.) Hassk. Plant Cell Tissue Organ Cult 134: 409-421

Solange FL, Claudia S, Norma A, Vera Regina C (2000) *Rollina mucosa* cell suspensions cultures: establishment and growth conditions. Plant Cell Tissue Organ Cult 63: 85-92

Wang J, Yang Y, Yan Y (2018) Bioproduction of resveratrol. In: Biotechnology of Natural Products. Springer International Publishing AG 61-79

What technical improvements are needed to achieve industrial application of conifer somatic embryogenesis?

Jan Bonga[1], Yill-Sung Park[1*], Chen Ding[2,3]

[1]Scientist Emeritus, Natural Resources Canada - Canadian Wood Fibre Centre, Fredericton, New Brunswick, Canada; [2] Canada Research Chair in Forest Genomics, Université Laval, Québec, Canada; [3]West Gulf Forest Tree Improvement Program, TAMU, College Station, TX, USA.
**Corresponding author: yillsung.park@canada.ca*

Abstract
Even though somatic embryogenesis (SE) of conifers has long been considered to be a potentially useful technique to mass produce high quality planting stock, it has been difficult to develop the technique to a level where it can be applied effectively on an industrial scale. In the following we outline a number of problem areas and we indicate a few areas of research focus that need attention in our efforts to improve the situation. The SE initiation rates are low for many conifers, and often the capacity is restricted to only a small number of genotypes, except for in a few spruce species. The basal medium primarily used in initiation may not be optimal for the maturation step and thus may result in embryo abnormalities and vitrification. As generally practiced today, manual transfer of germinating embryos to soil is labor intensive and expensive and, therefore, requires automation. Furthermore, long-term cryopreservation of clones during lengthy field testing is expensive. We suggest potential solutions to these problems, including media modifications, the use of capable genotypes obtained by breeding to improve SE initiation rate, automated SE production systems, and the implementation of genomic selection to circumvent or reduce the length of the field testing period.

Keywords: conifer SE, cost, automation, cryopreservation, genomic selection

Introduction

The implementation of somatic embryogenesis (SE) on an industrial scale for conifers has long been a desired goal because it has the potential to increase the forest productivity dramatically. When used in conjunction with tree breeding it, in theory, provides the means to clonally mass-propagate the best genotypes within the breeding population (Park 2002). Because the genetic variation within breeding populations for most conifers is substantial, clonal propagation of the best genotypes within the breeding population can result in large genetic gain over that obtained by traditional seed orchards. Implementing the current "breeding-clonal deployment" strategy involves maintenance of the clonal cell lines produced from the improved seed provided by breeding in cryopreservation while propagules produced by the cell lines are field tested, generally for 10 – 15 years. After the field test has determined what are the best genotypes, the corresponding cell lines are thawed from cryopreservation and used for mass producing planting stock, which is also known as multi-varietal forestry (MVF) (Park et al. 2016). For a small number of species, mostly spruces, this has found industrial application (Adams et al. 2016) but for most conifers the technology has so far failed to achieve that objective. The main problems that still have not yet been mastered are: cell line initiation rates that are too low for a number of species, formation of abnormal embryos, poor germination and acclimation, overall high production cost of SE

seedlings and the necessity of cryogenic storage. In the following we will tackle these issues and suggest a few potential solutions and alternative methods to overcome these problems.

Problems and possible improvement

Low initiation rates

Generally a SE initiation rate higher than about 50% is desired to provide a desired level of clonal variation from which to choose the best genotypes. However, this is difficult to achieve for many conifer species, for example in the pines (Klimaszewska et al. 2016, Abrahamsson et al. 2017). In our experience good initiation has been possible with a number of spruces but was more problematic with other genera.

Table 1. *Some examples of somatic embryogenesis initiation and maturation percentages in spruces and pines as obtained at the Canadian Forest Service including collaborative work.*

Species	Base Media	Plant growth Regulators	Zygotic embryo explant Immature	Zygotic embryo explant Mature	Maturation
Spruce spcies					
Picea glauca	mLV	2,4-D+BA	68	20	89
P. mariana	mLV	2,4-D+BA	65	21	85
P. abies	mLV	2,4-D+BA	75	29	80
P. sitchensis	mLV	2,4-D+BA	70	-	80
Pine species					
Pinus strobus	mLV	2,4-D+BA	61	2	76
P. taeda	WV5	2,4-D+BA	36	0	66
P. pinaster	mLV	CPPU	76	0	58
P. sylvestris	mLV	2,4-D+BA	20	0	85
P. albicaulis	mLV	2,4-D+BA	15	-	75
P. flexilis	mLV	2,4-D+BA	15	-	-
P. patula	mLV	2,4-D+BA	13	-	-
P. monticola	mLV	2,4-D+BA	6	0	75
P. banksiana	mLV	2,4-D+BA	4	0	90
P. contorta	mLV	2,4-D+BA	>1	-	-
P. maximinoi	mLV	2,4-D+BA	11[a]	-	-
P. tecunumanii	mLV	2,4-D+BA	4 [a]	-	-

mLV: modified Litvay's medium (Litvay et al. 1985); 2,4-D: 2,4-dichlorophenoxyacetic acid; BA: benzyladenine; WV5: Coke JE (1996) and Duchefa Biochimie (2003); CPPU: N-(2-chloro-4-pyridyl)-N'-phenylurea; [a] Immature cones damaged somewhat during the shipment to Canada; - not attempted.

The most commonly used initiation media for conifers are Litvay et al. (1985) (LM) or Murashige and Skoog (1962) (MS) media or variations thereof. For many conifer species these media provide a good SE initiation response. Of course it is possible that these two media are inadequate for species that fail to initiate SE on these and that other media have to be devised before proper initiation will occur. However, in our opinion it is more likely that the media are adequate for initiation but that the explant cells are either not in a responsive state or that they cannot express their embryogenic potential because of inhibition by neighboring tissues. Whenever the latter is the case, removal of the offending tissues by microdissection or separation of protoplasts may be needed to resolve the problem (Bonga 2017). In case no cells capable of responding are available it may be necessary to make cells more responsive by applying a finely tuned stress treatment (Zavattieri et al. 2010, Horstman et al. 2017, Bonga 2018).

Stress results in autophagy and autophagy is involved in the rejuvenation process that occurs during sexual reproduction and apomixes. Furthermore, artificial stress will induce meiosis and, presumably, is sometimes the driving force in SE (Bonga 2017). It is of interest to note that 2,4-D, the auxin most commonly used to initiate SE causes oxidative stress, which may in part account for its effectiveness (Zavattieri 2010). Sometimes 2,4-D is replaced by other auxins such as N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU) (Park et al. 2006) and Picloram (Park et al. 1998). It is further possible that the LM and MS media, commonly used for SE induction in conifers, are in part effective in that respect because they act as stressors. For example, LM is very low in calcium (Teasdale 1987) which could well result in nutritional deprivation and this stress could, we speculate, be an additional SE inducing factor, especially when LM is used at half strength. This assumption, however, has to be considered with caution since, at least in some cases, low calcium availability inhibits SE initiation (Smertenko and Bozhkov 2014). Nevertheless, short-term application of stress by starvation or by heat or cold shock etc. has been helpful in initiating androgenesis (Bonga et al. 2010) and perhaps could, in some cases, help in initiating conifer SE. On the other hand, stress, if of the wrong kind, can result in abnormal development during SE (Abrahamsson et al. 2017). The precise nature of stress treatments that could be effective in initiating normal SE remains to be determined.

Whether or not SE occurs is, among other factors, controlled by epigenetic controls, in which DNA methylation plays a major role. DNA methylation is generally lower in embryogenic than non-embryogenic cultures (Osorio-Montalvo et al. 2018), including those of conifers (Teyssier et al. 2014). In order to achieve demethylation in cultures in vitro, the DNA methylation inhibitor 5-Azacytidine has been used, sometimes with a positive and often with a negative effect on the formation of somatic embryos (Osorio-Montalvo et al. 2018).

Improved SE initiation by breeding

It is well known that SE is influenced by genetics (Park et al. 1996, Cyr and Klimaszewska 2001, MacKay et al. 2006). Thus, an understanding of genetic control is an important element of improving the SE process. Park et al. (1993) demonstrated that, in a quantitative genetic analysis of white spruce SE that the initiation stage of SE is under strong additive genetic control amounting to 69% of total genetic variance. However, the genetic influence declined steadily to the subsequent proliferation (38%), maturation (9%) and germination (3%) stages. Similarly, in loblolly pine, MacKay et al. (2006) have shown that, during the initiation, about 45% of total variation was accounted for by additive variation. Therefore, it is the initiation of SE that can be most effectively be improved by breeding. However, only limited improvements in maturation and germination can be expected by breeding.

Improve embryogenic capacity by secondary and serial SE

Secondary somatic embryogenesis from mature somatic embryos have been obtained in pines (Klimaszewska et al. 2007). Furthermore, serial somatic embryogenesis from cotyledonary somatic embryos has been obtained in Douglas-fir and this serial somatic embryogenesis resulted in significantly higher embryogenic potential when compared to primary and secondary SE lines (Gautier et al. 2018). These authors showed that cells have the ability to use different protein regulatory pathways and that this resulted in the increased embryogenic potential. This aspect may be further exploited as a means of improving initiation rates.

Poor maturation or germination

Poor maturation, resulting in abnormally formed embryos, is still a problem with several conifers. Often these are the result of culture proceedings that are not optimal. Sometimes abnormalities arise early in the initiation stage of SE (Abrahamsson et al. 2017) in which case they are an initiation, not a maturation

problem. However, generally abnormalities occur later, i.e., in the maturation process (Tereso et al. 2007). This may in part be due to the basal media being less suitable for maturation than for initiation, or to suboptimal physical factors (Etienne et al. 2013). The commonly used LM and MS initiation media, in comparison to a list of other nutrient media used for woody species, have a high mineral ionic strength. High ionic strength media are less suitable than lower strength ones for in vitro shoot growth of several woody species (McCown and Sellmer 1987, Nas and Read 2004) and thus, presumably, are less suitable for maturation, which is the stage of development when meristems develop. However, since LM is often used at half instead of full strength of its macro minerals, ionic strength in that case may not be problematic. However, ½ strength LM even further lowers its already low calcium content. Therefore, LM and certainly ½ LM may not optimal for the shoot and root development during maturation. Low calcium, for example, often results in debilitating shoot tip necrosis (McCown and Sellmer 1987, Poothong and Reed 2014). It has been demonstrated that for some species, for example Eucalyptus dunnii (Oberschelp and Gonçalves 2018), even a minor deviation from the optimal media composition can result in shoot tip chlorosis and necrosis. Complex interactions between mineral elements have to be considered since these can affect the suitability of the culture medium (Teasdale 1987, Oberschelp and Gonçalves 2018, Poothong and Reed 2014). Even varieties within a species can have different mineral requirements for proper in vitro growth (Poothong and Reed 2014). Therefore, one may expect that for each species the mineral requirements for in vitro development at different developmental phases may differ. Clearly, proper maturation may require a finely tuned mineral medium that is considerably different from the optimal initiation medium.

Both LM and MS are high in inorganic nitrogen. This could be a stress factor needed to promote SE initiation, but could inhibit proper meristem development, i.e., maturation, in the embryos. High nitrogen was sub-optimal in shoot cultures of some woody species (McCown and Sellmer 1987, Nas and Read 2004). Increased calcium, magnesium and phosphate has benefitted shoot growth in several species (Poothong and Reed 2014). Although ammonium is often an essential element in SE initiation (Durzan 1987), the high ammonium content of LM and MS could also, in some cases, be a contributing factor to the hyperhydricity (Gaspar et al. 1987, Klimaszewska et al. 2001, Ivanova and Van Staden 2009) that often occurs during maturation. Important nitrogen sources during conifer SE are arginine (Rodriguez et al. 2006) and especially glutamine (Dahrendorf et al. 2018). For *Pinus pinaster* more mature embryos were produced with a mixture of amino acids than with casein hydrolysate, which is often used as a nitrogen source (Alvarez et al. 2013). Glutamine as the sole nitrogen source sometimes promotes shoot growth and reduces hyperhydricity (Ivanova and Van Staden 2009). Germination of Norway spruce somatic embryos was improved by cold storage prior to germination, lowering the nitrogen content in the germination medium and reducing the in vitro germination phase to one week (Tikkinen et al. 2018a). Accumulation of nitrogen containing storage products in the maturing embryos no doubt play an important role in subsequent proper germination and acclimation. In an experiment with Norway spruce, glutamine in the nutrient medium accounted for 67% of the assimilated nitrogen in the free amino acid pool (Carlsson et al. 2017).

It has been observed by Poothong et al. (2017) that optimization of the macro mineral contents of the medium can result in a reduction in metabolic markers that indicate oxidative stress. Reduction of abiotic stress by optimization of the culture medium, subsequent to SE induction, appears to be essential for normal maturation.

A common problem in conifer SE germination is root formation (Ragonezi et al. 2010, Montalbán and Moncaleán 2018), which, in part, could be due to stress and thus requiring adjustment in the culture protocol (Gaspar and Coumans 1987). On the other hand it has also been observed that stress, i.e., water stress, can sometimes have a beneficial effect on rooting (Gaspar and Coumans 1987). According to Gaspar and Coumans (1987) root induction has requirements that are different from those of root elongation.

A major factor in root initiation and development are specific requirements for nitrogen. For example. Root formation during the germination of SEs of the *Pinus strobus* x *P. wallichiana* hybrid was improved by the presence of casein hydrolysate and glutamine and the absence of inorganic nitrogen in the germination medium (Llebrés et al. 2018). Many other physical and chemical factors are involved in the rooting process (review Ragonesi et al. 2010). For proper maturation and prevention of precocious germination of *Picea glauca* somatic embryos Sutton et al. (2004) adjusted the ABA concentration and osmolality of the medium at 2 week intervals, again indicating that the culture conditions have to be adjusted for each stage of SE development.

An important aspect of increasing the production of properly developed mature embryos is the breaking up of pro-embryogenic masses into much smaller ones by shaking in liquid medium. The small clumps are then dispersed on filter paper and transferred to maturation medium. With Norway spruce this procedure not only greatly increased the number of mature embryos being formed but also their synchronization in development (Mamun et al. 2018).

Lately, image processing combined with artificial neural network-based models has been used to optimize culture conditions for callus and SE production. This requires complex statistical models but, if properly executed, can be highly effective (Niazian et al. 2018).

Cryopreservation

Currently, cryopreservation is a key element of MVF strategy. The use of alcohol-insulated freezing containers (Nalgene™ Cryo Freezing Containers) makes cryopreservation a simple process with a good success rate. In general, successful cryopreservation is dependent on the vigor of the embryogenic tissue. Thus far, a recovery rate of about 95% without any deleterious effects after 20 years of cryo-storage was obtained (unpublished data). However, cryopreservation can be costly, especially when maintaining a large number of lines for a long period of time, which is often required for the field testing phase of tree breeding. Therefore, a reduction in the number of lines and length of the cryopreservation period should result in cost savings, and GS could be used for these purposes.

Automation

Even though the number of SEs produced is relatively high for several conifers, the commonly practised SE deployment is today still labor intensive and thus expensive, and, therefore, has so far found only limited industrialization (Sutton et al. 2004, Thompson 2014). The current process involves proliferation of embryogenic tissue, embryo maturation and germination of somatic embryos on either semi-solid or liquid media. Automation within each of these phases would be beneficial. To reduce handling costs it is attempted to culture the various stages of SE in liquid rather than on solidified media, culture in liquid media being more amenable to automation than the traditional culture on semi-solid media. However, even though multiplication of embryogenic tissue (ET) in liquid culture (Etienne et al. 2013, Mamun et al. 2015) is often possible, it can lead to problems such as, for example, failure to establish polarity or hyperhydricity. The latter can be due to impeded gas exchange during immersion (Albarrán et al. 2005). It is also often due to the use of cytokinin in the culture medium, a problem that can sometimes be mitigated by the use of salicylic acid (Ma et al. 2018). The use of bioreactors for the multiplication of conifer SEs has been described by Ingram and Mavituna (2000), Sutton et al. (2004) and Egertsdotter (2018).

One problem with bioreactors is that it is often difficult to supply enough light to the maturing embryos due to the compactness of the masses of embryos and the vertical design of most bioreactors. Use of bioreactors designed to disperse embryos and improve light availability have improved embryo-to-plantlet conversion rates (Ducos et al. 2007, Etienne et al. 2013). Light is a critical factor especially during germination. Growth chambers do not provide light uniformly. Therefore, culture vessels have been designed with LED lighting in the vessel lids, thus improving uniformity in light distribution and

saving in energy cost. In addition this allowed for stacking of the culture vessels, thus saving space. Excessive heating at a light intensity of 35 µmol m^{-2} s^{-1} did not occur (Shukla et al. 2017).

Efforts to automate are not confined to mass production of embryogenic masses and mature embryos. A process that requires a lot of manual handling is the selection and removal of mature embryos from the culture medium and their transfer to germination trays. JD Irving Ltd has developed a robot that can do this with white and Norway spruce SEs (Adams, personal communication). This company is continuing to pursue automation or semi-automation for planting germinants into substrate plugs.

In other efforts to automate and reduce costs it has been attempted to sow germinating embryos encapsulated in agarose or other capsule (synseed) directly on a soil substrate. Achieving this would make planting of germinating SEs, which is currently almost exclusively done manually, much more efficient and less expensive. However, even though germination of synseed on nutrient media in vitro has been achieved for many species, germination in non-sterile soil still is a major obstacle (Sharma et al. 2013, Thompson 2014, Rihan et al. 2017). Another problem with synseed that currently limits its commercialization is the fact that encapsulation is labor intensive and thus expensive (West and Preece 2009). With regard to conifers, synseed germination in vitro has been reported, for example, *for Picea glauca* (Sutton et al. 2004) and *Pinus radiata* (Aquea et al. 2008). The process has been patented by Weyerhaeuser (https://patentimages.storage.googleapis.com/eb/27/d2/ac45d5f98e5c77/US7555865.pdf).

Rooting of cuttings or organogenesis from SE trees

Although planting stocks can be produced by SE, for many species it is not cost effective for commercial production due to low initiation rates, abnormal or poor maturation and germination and lack of automation. However, as long as a cell line produced by SE forms a number of plants large enough for a field test and a few plants can be recovered from the cryopreserved part of the line, these recovered plants can be mass produced by rooting of cuttings (Park et al. 1998). In that case, SE is used to create new, improved clonal varieties while the rooting of cuttings provides the means of mass producing offspring of the new variety. This procedure has been practised for example with *Picea sitchensis* (Lelu-Walter et al. 2013) and Norway spruce (Tikkinen et al. 2018b). Another option is to use plants produced by SE as a source of explants that can be used in propagation by organogenesis. This is only practical for species for which organogenesis is an effective way of cloning, i.e., *Pinus radiata* (Montalbán et al. 2011 Moncaleán et al. 2016).

Genomic selection

Somatic embryogenesis is a useful biotechnology that deploys genetically improved material in commercial plantations and is closely linked to tree breeding. One of the breeding strategies using SE is multi-varietal forestry (MVF), defined as the deployment of genetically tested clonal varieties in plantation forestry (Park 2004). SE is the key enabling technology for deploying new varieties that are of high value and that provide uniform planting stock (Trontin et al. 2016). Owing to development of new genotyping technology, genomic selection (GS) in forestry has become an important tool, especially with genotyping cost falling steadily. Park et al. (2016) discussed integration of GS in MVF to gain a large genetic gain at a significantly reduced time span, e.g., varietal deployment in 4 years instead of the 19 years required for seed production as in the case of white spruce breeding in eastern Canada (Park et al. 2016).

A GS application scheme has two phases: (1) the development of a GS model from an existing genetic test as a training population and (2) the application of the GS model in the offspring/juvenile generation for deployment. The GS models can be developed by using the trait measurements (phenotype) data from the existing genetic field testing plantation and the genotyping data based on molecular markers such as single-nucleotide polymorphism (SNP) (Resende et al. 2012a, Resende et al. 2012b). In the GS models, the phenotype is considered as the sum of all marker effects (SNPs) for quantitative traits such

as height and volume growth as well as the traits for pest and pathogen resistance, wood quality controlled by major gene effects (Beaulieu et al. 2014, Resende et al. 2012a). Therefore, the GS model calculates the genomic estimated breeding value (GEBV) of elite individuals in the current test (training) population. Subsequently, the GEBV individuals in the test population are used as the parents of the next generation (offspring) population, and is considered to be the breeding population (BP), where the selection for deployment in MVF is to be made. In order to apply the GS model in the BP, it requires only genotyping data of the individuals but no phenotyping efforts, thus circumventing a need for field testing. Once genotyping data are obtained for the BP, they can be substituted into the GS model developed from the training population to obtain GEBV. The top GEBV individuals are then selected to be mass propagated either by SE or by rooting of cuttings using juvenile SE plants as the donor plants, i.e., forward deployment (Park et al. 2016, Resende et al. 2012b). The vegetative propagation technology such as SE and/or rooting of cuttings are required to mass-produce selections without waiting for seed production or gene recombination. Conifers benefit more from the GS integrated with SE (GS-SE), due to the potential gain per time investment in breeding and the bypassing of sexual maturation to produce seeds (e.g., about 15-40 years for *Picea glauca*). Therefore, vegetative propagation is the key to optimize GS gain in conifer breeding and deployment.

GS is still in the developing stage and not widely applied in all breeding programs; however, there are pilot cases of applying GS-SE in tree improvement, e.g., FastTRAC in Canada (http://fasttracproject.ca/) and PineSNPchip consortium in North Carolina State University led by Dr Fibret Isik (Towards Genomic Selection in Forest Trees 2015-2019, USDA-NIFA Award #: 2016-67013-24469). The hurdles of applying GS-SE in most tree breeding programs are: (1) the limitation of SE production efficiency and cost of mass production; (2) the initial cost of genotyping; and (3) the availability of SNP genotyping chips for commercially important conifers. However, these issues are likely to be solved in the near future. In addition to obtaining a large genetic gain at significantly reduced time, the application of the GS model would also result in the circumvention of genetic field testing or its streamlining and in the reduction in the periods of storage and the number of varietal lines that need to be stored in cryopreservation.

Conclusion

Conifer biotechnology such as SE has the potential to be of benefit in tree improvement programs but the challenge remains to achieve this on a cost effective, industrial scale for many conifer species. However, for some species, primarily Picea glauca, industrial application has been attained, and, presumably, will become possible for other commercially important species in the not too distant future as well. The genomic selection is becoming an important tool for forest tree breeding; however, it is required to have a functioning mass vegetative propagation system in place in order to realize all the benefits and efficiency of GS.

Acknowledgements: We thank Greg Adams and Andrew McCartney (JD Irving Limited) for their comments regarding this manuscript. We thank Dr Fred Raley (Western Gulf Forest Tree Improvement Program, Texas AM Forest Service) for assisting the writing and editing of the genomic selection section.

References

Abrahamsson M, Valladares S, Merino I, Larsson E and von Arnold S (2017) Degeneration pattern in somatic embryos of Pinus sylvestris L. In Vitro Cell Dev Biol-Plant 53: 86-96 DOI 10.1007/s11627-016-9797-y

Adams GW, Kunze HA, McCartney A, Millican S and Park YS (2016) An industrial perspective on the use of advanced reforestation stock technologies. In: Park YS, Bonga JM and Moon HK (eds) Vegetative Propagation of Forest Trees. National Institute of Forest Science (NiFoS). Seoul, Korea. pp 323-334

Albarrán J, Bertrand B, Lartaud M and Etienne H (2005) Cycle characteristics in a temporary immersion bioreactor affect regeneration, morphology, water and mineral status of coffee (Coffea arabica) somatic embryos. Plant Cell Tissue Organ Cult 81: 27-36 DOI 10.1007/s11240-004-2618-8

Alvarez JM, Bueno N, Cortizo M and Ordás (2013) Improving plantlet yield in Pinus pinaster somatic embryogenesis. Scan J For Res 28: 613-620 https://doi.org/10.1080/02827581.2013.821516

Aquea F, Poupin MJ, Matus JT, Gebauer M, Medina C and Arce-Johnson P (2008) Synthetic Seed production from somatic embryos of Pinus radiata. Biotechnol Lett 30: 1847-1852 DOI 10.1007/s10529-008-9754-x

Beaulieu J, Doerksen T, Clément S, Mackay J, and Bousquet J (2014) Accuracy of genomic selection models in a large population of open-pollinated families in white spruce. Heredity 113: 343-352

Bonga JM, Klimaszewska KK and von Aderkas P (2010) Recalcitrance in clonal propagation, in particular of conifers. Plant Cell Tissue Organ Cult 100:241-254 DOI 10.1007/s11240-009-9647-2

Bonga JM (2017) Can explant choice help resolve recalcitrance problems in in vitro propagation, a problem still acute especially for adult conifers? Trees 31: 781-789 DOI 10.1007/s00468-016-1509-z

Bonga JM (2018) Can mimicking sexual reproduction solve problems with recalcitrance in in vitro propagation of tree species. Can J For Res 48: 735-739 doi: 10.1139/cjfr-0020

Carlsson J, Svennerstam H, Moritz T and Egertsdotter U (2017) Nitrogen uptake and assimilation in proliferating embryogenic cultures of Norway spruce – Investigating the specific role of glutamine. PLOS ONE https://doi.org/10.1371/journal.pone.0181785

Coke, JE 1996. Basal nutrient medium for in vitro cultures of loblolly pine. US Patent 5,534,433.

Cyr DR and Klimaszewska K (2002) Conifer somatic embryogenesis: II. Applications. Dendrobiology 48: 41-49

Darendorf J, Clapman D and Egertsdotter U (2018) Analysis of nitrogen utilization capability during the proliferation and maturation phases of Norway spruce (Picea abies (L.) H. Karst.). Forests 9, 288 doi:10.3390/f9060288

Duchefa Biochemie 2003. Biochemicals, plant cell and tissue culture, plant molecular chemicals. Catalog 2003-2005. Haarlem, The Netherlands

Ducos J-P, Lambot C and Pétiard (2007) Bioreactors for coffee mass propagation by somatic embryogenesis. Int J Plant Dev Biol 1: 1-12

Durzan DJ (1987) Ammonia: Its analogues, metabolic products and site of action in somatic embryogenesis. *In*: Bonga JM and Durzan DJ (eds) Cell and Tissue Culture in Forestry. Vol 2. Specific Principles and Methods: Growth and Developments. Martinus Nijhoff Publ, Dordrecht/Boston/Lancaster, pp 92-136

Egertsdotter U (2018) Plant physiological and genetical aspects of the somatic embryogenesis process in conifers. Scan J For Res DOI: 10.1080/02827581.2018.1441433

Etienne H, Bertrand B, Georget F, Lartaud M, Montes F, Dechamp, Verdeil J-L and Barry-Etienne D (2013) Development of coffee somatic and zygotic embryos to plants differs in the morphological, histochemical and hydration aspects. Tree Physiol 33: 640-653 doi: 10.1093/treephys/tpt034

Gaspar Th and Coumans M (1987) Root formation. In: Bonga JM and Durzan DJ (eds) Cell and Tissue Culture in Forestry. Vol 2. Specific Principles and Methods: Growth and Developments. Martinus Nijhoff Publ, Dordrecht/Boston/Lancaster, pp 152-166

Gaspar Th, Kevers C, DeBergh P, Maene L, Paques M and Boxus Ph (1987) Vitrification: Morphological, physiological and ecological aspects. In: Bonga JM and Durzan DJ (eds) Cell and Tissue Culture in Forestry. Vol 1. General Principles and Biotechnology. Martinus Nijhoff Publ, Dordrecht/Boston/Lancaster, pp 152-166

Gautier F, Eliášová K, Leplé JC, Vondrákova Z, Lomenech AM, Le Metté C, Label P, Costa G, Trontin JF and Teyssier C (2018) Repetitive somatic embryogenesis induced cytological and proteomic changes in embryogenic lines of *Pseudotsuga menziessii*. BMC Plant Biol 18:164 https://doi.org/10.1186/s12870-018-1337-y

Horstman A, Bemer M and Boutilier K (2017) A transcriptional view on somatic embryogenesis. Regeneration 4: 201-216 DOI: 10.1002/reg2.91

Ingram B and Mavituna F (2000) Effect of bioreactor configuration on the growth and maturation of *Picea sitchensis* somatic embryo cultures. Plant Cell Tissue Organ Cult 61: 87-96

Ivanova M and Van Staden J (2009) Nitrogen source, concentration, and NH_4^+:NO_3^- ratio influence shoot regeneration and hyperhydricity in tissue cultured Aloe polyphylla. Plant Cell Tissue Organ Cult 99: 167-174

Klimaszewska K, Park YS, Overton C, MacEacheron I and Bonga JM (2001) Optimized somatic embryogenesis in Pinus strobus L. In Vitro Cell Dev Biol-Plant 37: 392-399

Klimaszewska K, Trontin J-F, Becwar MR, Devillard C, Park YS and Lelu-Walter M-A (2007) Recent Progress in Somatic Embryogenesis of Four Pinus spp. In: Tree and Forestry Science and Biotechnology ©2007 Global Science Books, pp 11-25

Klimaszewska K, Hargreaves C, Lelu-Walter M-A and Trontin J-F (2016) Advances in conifer somatic embryogenesis since year 2000. In: Germanà MA and Lambardi (eds). In Vitro Embryogenesis in Higher Plants, Methods in Molecular Biology. Vol 1359, Springer, pp 131-166 DOI 10.1007/978-1-4939-3061-6_7

Lelu-Walter MA, Thompson D, Harvengt L, Sanchez L, Toribio M and Pâques LE (2013) Somatic embryogenesis in forestry with a focus on Europe : state-of-the-art, benefits, challenges and future direction. Tree Genet Genom 9: 883-899 DOI 10.1007s11295-013-0620-1

Litvay JD, Verma DC, Johnson MA (1985) Influence of a loblolly pine (Pinus taeda L.) culture medium and its components on growth and somatic embryogenesis of the wild carrot (Daucus carota L.). Plant Cell Rep 4: 325-328

Llebrés MT, Avila C, Cánovas FM and Klimaszewska K (2018) Root growth of somatic plants of hybrid Pinus strobus (L.) and P. wallichiana (A.B. Jacks.) is affected by the nitrogen composition of the somatic embryo germination medium. Trees 32: 371-381 https://doi.org/10.1007/s00468-017-1635-2

Ma Z, Ji J, Zhu X, Yi L, Li Q, Wang G, Jin C and Guan C (2018) Salicylic acid mitigates hyperhydricity in newly developed potato shoots through reduced oxidation. Botany 96: 793-803 doi.org/10.1139/cjb-2018-0041

Mamun NHA, Egertsdotter U and Aidun CK (2015) Bioreactor technology for clonal propagation of plants and metabolic production. Front Biol 10: 177-193 DOI 10.1007/s11515-015-1355-1

Mamum NHA, Aidun CK and Egertsdotter U (2018) Improved and synchronized maturation of Norway spruce (Picea abies (L.) H. Karst.) somatic embryos in temporary immersion bioreactors. In Vitro Cell Dev Biol –Plant. https://doi.org/10.1007/s11627-018-9911-4

MacKay JJ, Becwar MR, Park YS, Corderro JP and Pullman GS (2006) Genetic control of somatic embryogenesis initiation in loblolly pine and implications for breeding. Tree Genet Genome 2: 1-9 DOI 10.1007/s11295-005-0020-2

McCown BH and Sellmer JC (1987) General media and vessels suitable for woody plant culture. In: Bonga JM and Durzan DJ (eds). Cell and Tissue Culture in Forestry, Vol 1, General Principles and Biotechnology. Martinus Nijhoff Publ. Dordrecht, Boston, Lancaster pp 4-16

Moncaleán P, Montalbán IA, García-Mendiguren O, Reeves C, Hargreaves C (2016) Optimisation of somatic embryogenesis of Pinus radiata for production forestry. In: Park YS, Bonga JM and Moon HK (eds) Vegetative Propagation of Forest Trees. National Institute of Forest Science (NiFoS). Seoul, Korea. pp 607-622

Montalbán IA, De Diego N, Aguirre Igartua E, Setién A and Moncaleán P (2011) A combined pathway of somatic embryogenesis and organogenesis to regenerate radiate pine plants. Plant Biotechnol Rep 5:177-186

Montalbán IA and Moncaleán P (2018) Rooting of Pinus radiata somatic embryos: factors involved in the success of the process. J For Res https://doi.org/10.1007/s11676-018-0618-5

Murashige T and Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473-497 https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nas MN and Read PE (2004) A hypothesis for the development of a defined tissue culture medium of higher plants and micropropagation of hazelnuts. Sci Hortic 101: 189-200

Niazian M, Sadat-Noori SA, Abdipour M, Tohidfar M and Mortazavian SMM (2018) Image processing and artificial neural network-based models to measure and predict physical properties of embryogenic callus and number of somatic embryos in Ajowan (Trachyspermum ammi (L.) Sprague). In Vitro Cell Dev Biol – Plant 54: 54-68 https://doi.org/10.1007/s11627-017-9877-7

Oberschelp GPJ and Gonçalves AN (2018) Analysis of nutrient deficiencies affecting in vitro growth and development of Eucalyptus dunnii Maiden. Physiol Mol Biol Plants 24: 693-704

Osorio-Montalvo P, Sáenz-Carbonell and C De-la-Peña (2018). 5-Azacytidine : A promotor of epigenetic changes in the quest to improve plant somatic embryogenesis. Int J Mol Sci 19: 3182 doi:10.3390/ijms19103812

Park YS, Pond SE and Bonga JM (1993) Initiation of somatic embryogenesis in white spruce (Picea glauca): genetic control, culture treatment effects, and implications for tree breeding. Theor Appl Genet 86: 427-436

Park YS (2002) Implementation of conifer somatic embryogenesis in clonal forestry: technical requirements and deployment considerations. Ann For Sci 59:651-656

Park YS (2004) Commercial implementation of multi-varietal forestry using conifer somatic embryogenesis. In Proc IUFRO joint Conf Div 2 on Forest genetics and tree breeding in the age of genomics: Progress and future. Charleston, SC Nov 1-5 p 139

Park YS, Barrett JD and Bonga JM (1998) Application of somatic embryogenesis in high-value clonal forestry: deployment, genetic control, and stability of cryopreserved clones. In Vitro Cell Dev Biol-Plant 34: 231-239

Park YS, Lelu-Walter MA, Harvengt L, Trontin JF, MacEacheron I, Klimaszewska K and Bonga JM (2006) Initiation of somatic embryogenesis in Pinus banksiana, P. pinaster, and P sylvestris at three laboratories in Canada and France. Plant Cell Tissue Organ Cult. 86:87-101

Park YS, Beaulieu J and Bousquet J (2016) Multi-varietal forestry integrating genomic selection and somatic embryogenesis. In: Park YS, Bonga JM and Moon H-K (eds) (2016) Vegetative Propagation of Forest Trees. National Institute of Forest Science (NiFoS). Seoul, Korea, pp 302-322

Poothong S and Reed BM (2014) Modeling the effects of mineral nutrition for improving growth and development of micropropagated red raspberries. Sci Hortic 165: 132-141

Poothong S, Morré J, Maier CS and Reed BM (2017) Metabolic changes and improved growth in micropropagated red raspberry "Indian summer" are tied to improved mineral nutrition. In Vitro Cell Dev Biol-Plant 53: 579-590

Ragonezi C, Klimaszewska K, Castro MR, Lima M, Oliveira P de and Zavattieri MA (2010) Adventitious rooting of conifers: influence of physical and chemical factors. Trees 24: 975-992 DOI 10.1007/s00468-010-0488-8

Resende MF, Munoz P, Resende MD, Garrick DJ, Fernando RL, Davis JM, and Kirst M (2012a) Accuracy of genomic selection methods in a standard data set of loblolly pine (*Pinus taeda* L.). Genetics, 190(4): 1503-1510 doi:10.1534/genetics.111.137026

Resende MF, Munoz P, Acosta JJ, Peter GF, Davis JM, Grattapaglia D, Kirst M (2012b) Accelerating the domestication of trees using genomic selection: accuracy of prediction models across ages and environments. New Phytologist 193: 617-624

Rihan HZ, Kareem F, El-Mahrouk ME and Fuller MP (2017) Artificial seeds (Principles, aspects and applications). Agronomy 2017,7,71 doi:10.3390/agronomy7040071

Rodriguez MJ, Suárez, Heredia R, Avila C, Breton D, Trontin J-F, Filonova L, Bozhkov P, von Arnold S, Harvengt L and Cánovas FM (2006) Expression patterns of two glutamine synthethase genes in zygotic and somatic pine embryos support specific roles in nitrogen metabolism during embryogenesis. New Phytol 169: 35-44

Sharma S, Shahzad A and Teixeira da Silva JA (2013) Synseed technology – A complete synthesis. Biotechnol Adv 31: 186-207 http://dx.doi.org/10.1016/j.biotechadv.2012.09.007

Shukla MR, Singh AS, Piuno K, Saxena PK and Jones AMP (2017) Application of 3D printing to prototype and develop novel plant tissue culture systems. Plant Methods 13: 6 DOI 10.1186/s13007-017-0156-8

Smertenko A and Bozhkov PV (2014) Somatic embryogenesis: life and death processes during apical-basal patterning. J Exp Bot 65: 1343-1360 doi: 10.1093/jxb/eru005

Sutton BCS, Atree SM, El-Kassaby YA,, Grossnickle SC and Polonenko DR (2004) Commercialisation of somatic embryogenesis for plantation forestry. In: Walter C and Carson M (eds) Plantation Forest Biotechnology for the 21st Century. Research Signpost 37/661 (2), Fort PO, Trivandrum-695 023, Kerala, India ISBN: 81-7736-228-3

Teasdale RD (1987) Micronutrients. In: Bonga JM and Durzan DJ (eds). Cell and Tissue Culture in Forestry, Vol 1, General Principles and Biotechnology. Martinus Nijhoff Publ. Dordrecht, Boston, Lancaster pp 17-49

Tereso S, Zoglauer K, Milhinhos A, Miguel C and Oliveira M (2007) Zygotic and somatic embryo morphogenesis in Pinus pinaster: comparative histological and histochemical study. Tree Physiol 27: 661-669

Teyssier C, Maury S, Beaufour M, Grondin C, Delaunay A, Le Metté C, Ader K,Cadene M, Label P and Lelu-Walter M-A (2014) In search of markers for somatic embryo maturation in hybrid larch (Larix x eurolepis) : global DNA methylation and proteomic analyses. Physiol Plant 150: 271-291

Thompson D (2014) Challenges for the large-scale propagation of forest trees by somatic embryogenesis - a review. In: Park YS and Bonga JM (eds) Proceedings of the 3rd international conference of the IUFRO unit 2.09.02 on "Woody plant production integrating genetic and vegetative propagation technologies." September 8-12, 2014. Vitoria-Gasteiz, Spain. Published online: http://www.iufro20902.org.

Tikkinen M, Varis S, Peltola H and Aronen T (2018a) Improved germination conditions for Norway spruce somatic cotyledonary embryos increased survival and height growth of emblings. Trees https://link.springer.com/article/10.1007/s00468-018-1728-6

Tikkinen M, Varis S, Peltola H and Aronen T (2018b) Norway spruce emblings as cutting donors for tree breeding and production. Scand J For Res 33: 207-214 https://doi.org/10.1080/02827581.2017.1349925

Trontin JF, Teyssier C, Morel A, Harvengt L, and Lelu-Walter M-A (2016) Prospects for new variety deployment through somatic embryogenesis in maritime pine. In: Park YS, Bonga JM and Moon (eds) Vegetative Propagation of Forest Trees. National Institute of Forest Science (NiFoS). Seoul, Korea. pp 572-606

West TP and Preece JE (2009) Bulk aliginate encapsulation of Hibiscus moscheutos nodal segments. Plant Cell Tissue Organ Cult 97: 345-351 DOI 10.1007/s11240-009-9525-y

Zavattieri MA, Frederico AM, Lima M, Sabino R and Arnholdt-Schmitt B (2010) Induction of somatic embryogenesis as an example of stress-related plant reactions. Electron J Biotechnol 13: 1-9 DOI: 10.2225/vol13-issue1-fulltext-4

Performance of culture lines established *in vitro* from a monumental birch tree

Purificación Covelo[1], Nieves Vidal [1], Saleta Rico[1], Jesús M. Vielba [1], Martín Reggiardo[2], Conchi Sánchez [1]

[1] Instituto de Investigaciones Agrobiológicas de Galicia. CSIC. Avda. de Vigo s/n. Apdo 122. 15780 Santiago de Compostela, Spain; [2]Centro de Estudios Fotosintéticos y Bioquímicos (CEFOBI), CONICET, Universidad Nacional de Rosario, Rosario, Argentina.
pcovelo@iiag.csic.es, nieves@iiag.csic.es, saleta@iiag.csic.es, jmvielba@iiag.csic.es, reggiardo@cefobi-conicet.gov.ar, conchi@iiag.csic.es

Abstract
This study aims to evaluate the origin- and topophysical- effects of the initial explants excised from crown branches on the in vitro clonal production of mature birch. Seven culture lines were successfully established in vitro from single buds and nodes taken from top crown branches and epicormic shoots of a monumental mature white birch tree (*Betula pubescens* ssp. celtiberica). Murashige and Skoog medium supplemented with meta-topoline was used for shoot proliferation. The effects of explant type (apical or basal segment), vessel type and subculture period during the proliferation stage were also investigated. Results indicated that the number of shoots per explant and the height of the tallest shoot were influenced by the type of explant. Basal nodal segments performed better than apical explants and their morphogenetic ability was independent of the vessel type. Proliferation rates of shoot lines were influenced by the position of the initial shoots on the explant sources. Without considering the position of the initial explant, there were no differences in shoot proliferation between lines established from two different origins. Annual new shoots developed on upper branches and epicormic shoots originated on lower crown branches. Differences related to the ontogenetic aging between the two explant sources were not reflected in the proliferation ability of crown-derived lines. The highest proliferation rates were achieved in two lines (C3 and E7-n2), each of them established from different explant sources (upper branches *versus* lower branches). In epicormic shoots-derived lines, the highest number of shoots was produced in line E-7n2, which was initiated from the nodal explant closest to the apical meristem. These results indicated that clonal production of mature birch trees was influenced by the position of the initial explants on the mother shoot/branch as well as by the physiological stage of the explant source. A six-week proliferation period improved the proliferation and rooting rates. After four weeks of culture, adventitious roots were developed spontaneously in shoots maintained in the multiplication medium. Spontaneous rooting ranged from 87 to 100% depending on the culture line. Auxin induction of adventitious roots improved the root system of rooted shoots.

Keywords: *Betula pubescens*, mature, meta-topoline, subculture period, rooting, topophysis

Introduction

Biotechnological approaches, by using in vitro culture techniques, can be applied for clonal propagation and genetic transformation. Furthermore, they offer an alternative for ex situ conservation of elite genotypes, including monumental trees which are characterized by their high biological, ecological, historical and cultural values (Reed et al. 2011).

It is well known that clonal propagation and in vitro morphogenesis of mature trees is negatively affected by the maturation-related loss of rooting and regeneration abilities (Sánchez and Vieitez 1991; Hackett and Murray 1993). New shoots developed from the upper parts of a tree are chronologically young tissues but they are ontogenetically older than basal shoots and stump sprouts, which retain juvenile characteristics (Hackett 1985; Bonga, 1987). Epicormic shoots emerging from dormant or latent buds on older branches are also ontogenetically younger than the new year´s growth shoots of the crown (Chalupa 1984; Vieitez et al. 1985)

Birches are important components of the forest and landscape. Among birches, white birch (*B. pubescens*) and silver birch (*B. pendula*) are two commercially important trees widely distributed through almost the whole of Europe (Hynynen et al. 2010). Micropropagation of birch species from juvenile and mature trees has been previously reported (Chalupa 1981; Welander 1988; Perez and Postigo 1989; Welander 1993). Micropropagated mature birches were used for forest cultivation and their production and field performance has been evaluated (Meier-Dinkel 1992; Jones et al. 1996; Viherä-Aarnio and Welling 2001). In *B. pendula* Roth, micropropagated trees proved to be more uniform than seedling-derived trees (Jones et al. 1996). Moreover, micropropagation techniques have been applied for in vitro conservation of threatened *Betula* species (Gaidamashvili et al. 2015; Rathwell et al. 2016). The effect of different growth regulators, such as benzylaminopurine (BA), kinetin or zeatin at different concentrations have also been tested in the micropropagation of birches (Meier-Dinkel 1992; Girgze and Samsone 2017). However, little is known about the effect of the position of the initial explants (based in their location or topophysis on the branch) on the *in vitro* performance of *Betula* species.

The aim of this work is to micropropagate a mature white birch tree (*B. pubescens* ssp. celtiberica), and to study the origin and topohysical effects of the initial explants on the in vitro response of shoot cultures established from different explant sources.

Materials and Methods

Initiation of cultures

Plant material was collected from the crown of an adult birch tree (*B. pubescens* ssp. celtiberica), that is included in the catalogue of monumental trees (https://www.monumentaltrees.com) with nº 17602. For in vitro establishment newly formed shoots were collected in June. These shoots belonged to two categories: i) Shoots developed on upper crown branches of the last growing season, which bore one apical bud (designated as C), and ii) New epicormic shoots (seven to eight nodes) originated directly on lower- and thick- crown branches situated close to the trunk junction (designated E). The positon of the shoots on the crown branches was not documented and shoots were randomly labelled 1 to n. Epicormic shoots and apical buds were surface-sterilized with 70% (v/v) ethanol (50 s) first and 25 % commercial bleach (4% available chlorine) second for 12 min, and then rinsed with sterile distilled water, three times of 10 min each. Nodal segments (bearing one axillary bud) from epicormics shoots were labelled "n1 to nn" from the first uppermost (1) node position to the bottom (n) of the shoot, and used to establish different culture lines that were maintained separately in subsequent proliferation cycles. The materials used for in vitro establishment of culture lines are listed in Table 1 and Fig. 1. Buds and nodes were inoculated into culture tubes containing 20 mL of Woody Plant medium (WPM, Lloyd and McCown 1980) supplemented with 7 gL^{-1} agar, 30 gL^{-1} sucrose, 2.2 µM BA and 0.0054 µM naphthalene acetic acid (NAA). The pH of the medium was adjusted to 5.5-5.6 before autoclaving. All cultures were maintained in a growth chamber under standard conditions of photoperiod, light and temperature (Sánchez and Vieitez 1991). Six weeks after establishment, the percentage of reactive explants as well as bacterial or fungal contamination were determined.

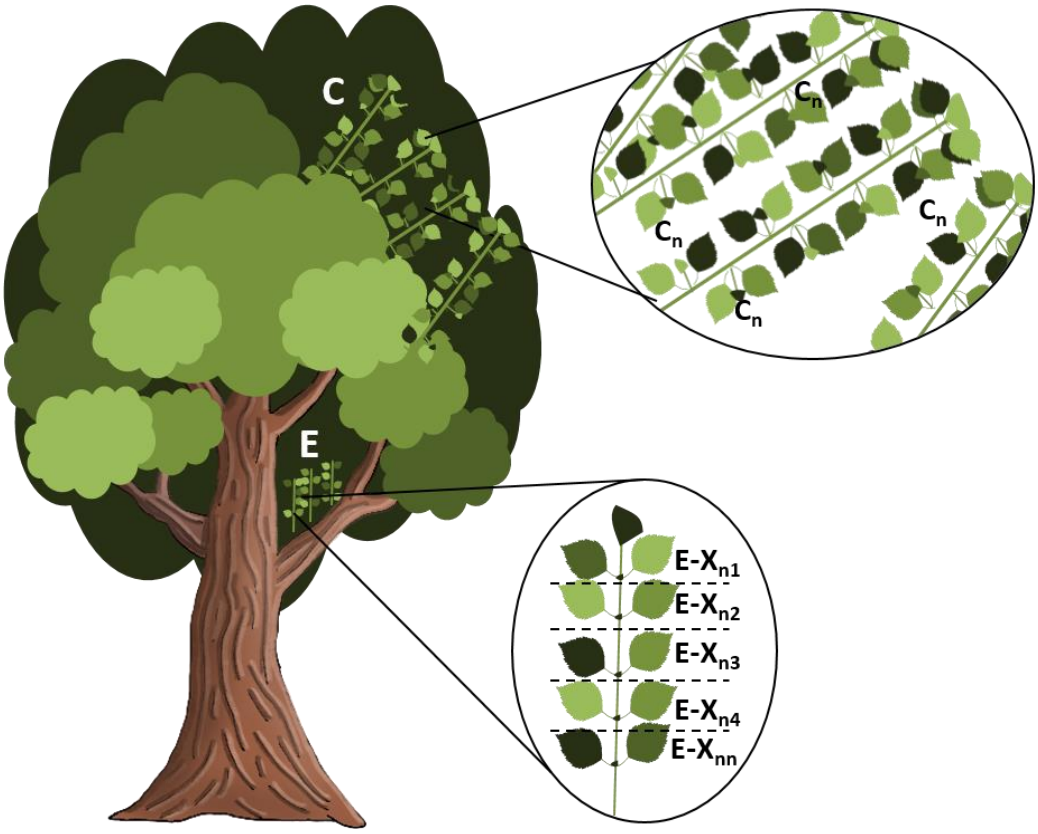


Figure 1. *Experimental system used for in vitro establishment of culture lines from newly growing shoots of a mature birch tree. (C): upper crown branches; (E): epicormics shoots; (Cn): apical buds from upper crown branches; E-Xn1-E-Xnn: nodes from epicormics shoots.*

Table 1*. Source, origin and position of the initial explants used for the establishment of birch culture lines*

Culture line	Explant source	Origin of initial explants	Position in the shoot
C1	upper crown branches	annual new growth	apical
C2	upper crown branches	annual new growth	apical
C3	upper crown branches	annual new growth	apical
E-1n3	lower crown branches	epicormic shoot	node 3
E-1n4	lower crown branches	epicormic shoot	node 4
E-5n3	lower crown branches	epicormic shoot	node 3
E-7n2	lower crown branches	epicormic shoot	node 2

Shoot multiplication and rooting experiments

Reactive explants were used for the initiation of seven culture lines (each line derived from one initial explant) and further evaluation of their in vitro performance. New shoots that had originated from each reactive bud were transferred to new tubes containing Murashige and Skoog (MS, Murashige and Skoog 1962) medium supplemented with 1.6 µM meta-topolin (mT) and 0.054 µM NAA. To determine the

effect of the vessel type, explants were grown in culture tubes or in 500 mL glass jars containing 70 mL of multiplication medium. Additionally, the effect of explant type on shoot multiplication and elongation was tested by comparing apical part of the shoots with basal stem segments. The effects of using a four- and six-week subculture cycle on shoot production were also evaluated in all seven culture lines. The following variables were determined at the end of the 4- and 6-week multiplication periods: the number of shoots longer than 1 cm per explant; the height of the tallest shoot; the percentage of rooted explants; the number of roots and the length of the longest root. Twenty explants of each line were used per culture period and, experiments was repeated at least twice.
For root induction, individual shoots longer than 2 cm were isolated from cluster shoots at the end of a six week- period cycle. Shoots were cultured on MS ½ medium supplemented with 25 µM of indole butyric acid (IBA) for 5 days and then transferred to IBA-free medium. Rooting percentage, mean number of root and lengh of the longest root were recorded at the end of the experiment.

Results and discussion

Establishment and proliferation of culture lines

We successfully established seven culture lines from crown branches of a mature white birch tree, three of them from upper branches and the other four from lower branches. The rates of contamination were high (50 to 70%) because of the nature of the starting material collected in the field (Almeida et al. 2008; Rathwell et al. 2016). Contamination was higher in basal nodes than in the upper nodes of donor shoots. This was also shown for *Corylus avellana* where contamination increased with the distance from the shoot tip (Hand et al. 2016).

Explant survival was higher than 50%, irrespective of the explant source (upper- and lower- crown branches), suggesting that in vitro reactivity was not dependent on the explant origin (C or E). Similar survival rates were achieved in cherry birch using dormant buds from mature trees (Rathwell et al. 2016). Although donor epicormic shoots were more vigorous and exhibited more juvenile-like characteristics than the upper crown shoots, these differences were not reflected on the in vitro reactivity of initial explants. The effect of type of initial explant (apical or nodal segment) could not be inferred from our results as a consequence of the high contamination in basal nodes and the fact that only apical explants were used for the establishment of lines C. Each shoot line, initiated from one single apical or axillary bud, has been cultured and maintained separately.

In line C1, preliminary experiments had shown the positive effect of using mT (1.6 µM) instead of BA (1.8 µM) in the proliferation medium (data not shown) and all further experiments were carried out in mT-containing medium. The combination of MS medium supplemented with mT and NAA allowed high proliferation rates in the seven culture lines. MS medium has been used for propagating shoots excised from seedlings of *B. celiberica* (Perez and Postigo 1989), as well as for other birch species such as *B. pendula* (Jones et al. 1996), *B. platyphylla* and *B. papyrifera* (Magnusson et al. 1999) or *B. lenta* (Rathwell et al. 2016). In the micropropagation of one selected genotype of *B. pendula*, WPM medium supplemented with 1 mg L^{-1} BA gave the best results for shoot proliferation (Girgžde and Samsone 2017). To our knowledge, the use of mT in the micropropagation of birches has not been previously reported however, its beneficial effect has been described in pistachio (Benahioul et al. 2012) and paulownia (Clapa et al. 2014), among others species.

The effects of the explant type and the culture vessel on the proliferation stage were also investigated. The number of shoots per explant and the length of the tallest shoot were higher in basal segments than in apical explants (Table 2; Fig. 2), irrespective of the vessel type used. Regarding the expant type, similar results were obtained during the micropropagation of pedunculate oak (Vieitez et al. 1985) and mature chestnut shoots (Sánchez et al. 1997). Morphogenetic ability of basal explants, in terms of number and quality of shoots, was not affected by the culture vessel (Table 2). Therefore, further experiments were performed in glass jars as it becomes more efficient from a practical and economical

point of view, since eight explants were cultured in glass jars *versus* one explant per culture tube. Similarly, in *Pontederia cordata*, the multiplication rates and quality of shoots were not influenced by the culture vessel (Kane and Phylman 1992). In contrast, the effect of vessel type has been reported in other species (Monete 1983; Mohamed and Alsadon 2011).

Table 2*. Effect of explant type (apical and basal) on the number of shoots per explant and the length of the tallest shoot of explants of line C1 cultured in glass jars (GJ) or culture tubes (CT). Data were collected after 4-week multiplication period.*

Line	Vessel	Shoots per explant		Tallest shoot length (mm)	
		apical	basal	apical	basal
C1	CT	1.2±0.1	6.7±0.7	21.1±2.1	34.7±1.9
	GJ	2.0±0.2	7.1±0,5	27.5±1.9	38.8±1.6

Values represent means ± standard errors (SE) for three replications

Figure 2*. Proliferation of birch shoots by using apical (A) and basal explants (B)*

To test the influence of the explant source on the proliferation capacity of lines, the data were analyzed without considering the explant position on the donor branch/shoot from which the lines were initiated. After four weeks of subculture period, there were no differences in the number of shoots per explant and height of the tallest shoot between C- and E- derived lines (Table 3). Shoot lines C and E produced over 6 shoots per explant with the longest shoot length ranging from 33.1 to 37.5 mm (Table 3). As expected, after a six- week culture cycle there was a significant increase in number of shoots and length in C- and E- derived lines compared to the four-week period. Our results showed that the in vitro proliferation abilities of these crown-derived lines were not influenced by the maturation process occurring along the tree, which increases from the base to the crown, as highlighted by Hackett (1985). These results suggested that both types of shoots, new shoots developed from upper crown branches and epicormic shoots originated on lower crown branches, had the same chronological age and they exhibited a similar physiological stage in terms of in vitro reactivity. Similarly, no differences were observed in the morphogenetic ability between cultures initiated from top branches and from epicormic

Table 3*. Effect of the explant source (C or E) on the number of shoots per explant and the length of the tallest shoot at the end of a four- or six- week- (w) subculture period.*

Explant source	Shoots per explant		Tallest length shoot (mm)	
	4w	6w	4w	6w
C	6.0±0.6	10.9±1.4	37.5±3.3	51.8±1.7
E	5.7±0.4	11.3±1.2	33.1±0.8	53.8±1.2

Values represent means ± standard errors (SE) for three replications

shoots of *Robinia pseudoacacia* (Han et al. 1997). In contrast, explants from the upper crown of *Pinus ponderosa* produced more axillary buds than explants taken from the lower crown (Lin et al., 1991).

Table 4. *Effects of the explant position and period of subculture on the proliferation rates of different culture lines. Shoots were maintained on the proliferation medium for four (4w) or six (6w) weeks.*

Culture line	Subculture cycle (week)	Number of shoots	Tallest shoot length (mm)
C1	4	6.2±0.5	41,3±0.1
	6	8.7±0.3	50,0±0.0
C2	4	6.8±0.1	40,2±0.1
	6	10.5±0.1	49.5±0.3
C3	4	4.9±0.1	30.9±0.2
	6	13.6±0.4	56.0±0.5
E-1n3	4	4.6±0.6	32.9±0.7
	6	9.1±0.2	52.5±0.6
E-1n4	4	6,2±0.8	34.2±0.4
	6	10.2±0.5	57.3±0.0
E-5n3	4	5.6±1.1	31.0±0.6
	6	11.0±1.5	52.2±0.0
E-7n2	4	6.5±1.7	34.3±0.6
	6	14.7±0.9	53.7±0.0

Values represent means ± standard errors (SE) for two replications

To determine whether the position of the initial explants on the tree branch or in the donor shoot influenced the morphogenetic response of lines, we evaluated their proliferation rates at the end of four- or six- week subculture period. Differences in the number of shoots within lines derived from the same explant source (C or E) appeared to be higher than between lines derived from different sources (Tables 3, 4), indicating a positional effect of the initial explant on shoot proliferation. Although all lines exhibited high morphogenetic capacity, the highest proliferation rates were achieved in lines E-7n2 (Fig. 3A) and C3 (Fig 3B) initiated from epicormic shoots and top crown branches, respectively. On the other hand, lines E-1n3 and C1 derived from different branch types were the less morphogenetic (Fig. 3C, D). Regarding to the explant position on the shoot, the best proliferation rate was achieved in the line established from the node closest to the apical meristem (E-7n2), whereas a similar response was observed in lines initiated from middle nodes (3 and 4) of the same shoot. No great differences were detected in the height of the tallest shoot between all the lines. The effect of using explants from different topophysical positions on the seedling or mother shoot has been reported in *Q. robur* (Puddephat et al. 1997), chrysanthemum (Zalewska et al. 2010) and *Macadamia* spp. (Gitonga et al. 2010). In the case of Macadamia the differences seemed to be related to the explant age rather than to the explant position.

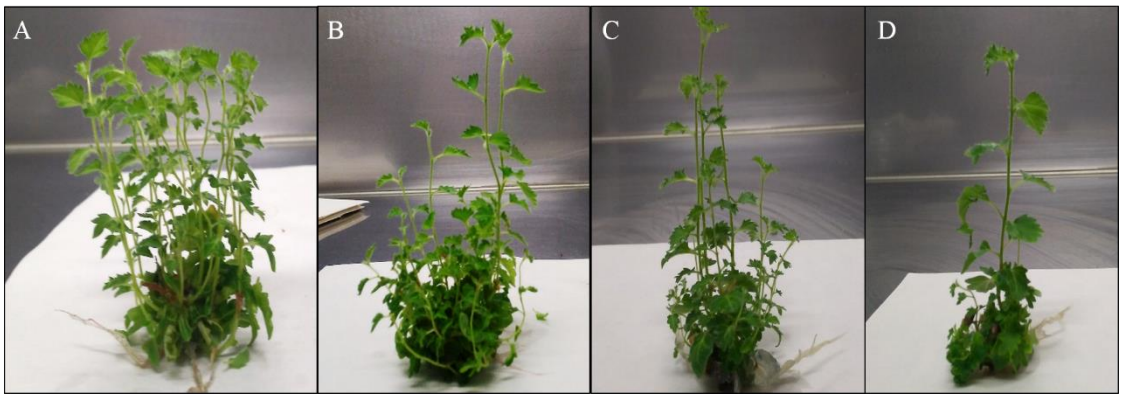


Figure 3*. Shoot lines derived from epicormic shoots and top branches of the crown of a mature birch tree. Line E-7n2 (A), C3 (B), E-1n3 (C) and C1 (D).*

Regarding the subculture cycle, shoot elongation was also influenced by the subculture period **(**Table 3, Table 4). An increase in the number of shoots was observed in all lines by changing the subculture period from four to six weeks .

Rooting

In all culture lines, high rates of spontaneous rooting occurred in most shoots maintained in the proliferation medium in a six week-culture cycle (Fig. 4, Fig. 5). The lowest rooting percentage (87.3 %) was recorded in line E-1n3. The number of roots ranged from 2.9 (line E-1n3) to 4.1 (line C3). High rooting rates were achieved in lines that had exhibited the highest proliferation rates (C3 and E-7n2) (Table 4).

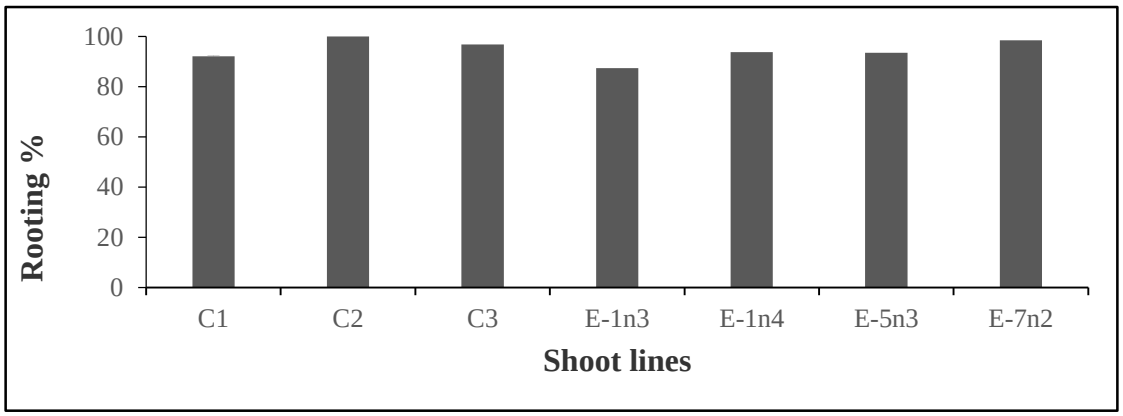


Figure 5*. Spontaneous rooting ability of white birch lines in the proliferation medium after a six-week culture cycle. Values represent means ± standard errors (SE) for three replications.*

Although shoot cultures rooted easily on the proliferation medium, auxin induction of adventitious roots increases the amount of rooted plants, as at least 4-5 rootable shoots are produced at the end of the proliferation stage. A high rooting percentage (98 %) was obtained in IBA-treated shoots. Auxin treatment improved the root system by increasing the root number (from 3.5 to 11) and the root length. The appearance of shoots rooted spontaneously, or after IBA treatment, is shown in Fig. 6. Rooted shoots from the seven lines were successfully acclimatized in greenhouse. The positive effect of auxins promoting the adventitious rooting is well known as they regulate a plethora of genes involved in the root initiation process.

Figure 6*. Root system of white birch shoots rooted spontaneously in the proliferation medium (A) and in IBA-treated shoots (B).*

Conclusions

Seven culture lines established from the crown of a monumental white birch tree were successfully micropropagated. The use of MS medium supplemented with mT was suitable for clonal production of rooted plants thus avoiding additional rooting steps. In vitro performance of lines was influenced by the position of the initial explants on the donor source.

Acknowledgements: This work was partly funded by the Xunta de Galicia through the Contrato Programa 2017-2018 and the project IN607A 2017/6. We thank Alejandro Díaz, Rafael Sánchez and Patricia Val for technical assistance.

References

Almeida JR, Martins CR, Dutra F (2008) Desinfestação de segmentos nodais de *Eucalyptus* dunnii visando estabelecimento in vitro. Revista de FZVA, Uruguaiana, v 15 (n): 54-60

Bonga JM (1987) Clonal propagation of mature trees: problems and solutions. In Cell and Tissue Culture in Forestry. Bonga JM and Durzan DJ (eds), Martinus Nijhoff, Dordrecht, The Netherlands, pp 249-271

Bemahioul B, Dorion N, Kaid-Harche M, Daguin F (2012) Micropropagation and ex vitro rooting of pistachio (*Pistacia vera* L.) Plant Cell Tissue Organ Cult 108: 353-358

Chalupa V (1981) In vitro propagation of birch (*Betula verrucosa* Ehrh.). Biol Plant 23 (6):472-474

Chalupa V (1984) In vitro propagation of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.) Biol Plant 26: 374-377

Clapa D, Fira A, Simu M, Vasu LB, Buduroi D (2014) Improved in vitro propagation of *Paulownia elongate*, *P. fortune* and its interspecific hybrid *P. elongate* x *P. fortune*. Bulletin UASVM Horticulture 71(1): 7-14

Gaidamashvili M, Khurtsidze E, Barblishvili T (2015) Micropropagation of threatened *Betula* species for in vitro conservation. International conference on Plant, marine and Environmental Sciences (PMES-2015)

Girgžde E, Samsone I (2017) Effect of cytoquinins on shoot proliferation of silver birch *(Betula pendula*) in tissue culture. Environ Exp Biol 15: 1-5

Gitonga LN, Gichuki ST, Ngamau K, Muigai AWT, Kahangi EM,, Wasilwa LA, Wepukhulu S, Njogu N.effect of explant type, source and genotype on in vitro shoot regeneration in Macadamia (*Macadamia* spp.) (2010) J Agric Biotech Sustain Dev Vol 2(7):129-135

Hackett WP (1985) Juvenility, maturation and rejuvenation in woody plants. Hortic Rev 7: 109-155

Hackett WP and Murray JR (1993) Maturation and rejuvenation in woody species. *In*: Micropropagation of woody plants. Ahuja MR (ed). Kluwer Academic Pubs, Dordrecht. pp 93-105

Han K-H, Shin D, Keatly D (1997) Tissue culture responses of explants taken from branch sources with different degrees of juvenility in mature black locust (*Robinia pseudoacacia*) trees. Tree Physiol 17: 671-675

Han CR, Wada N, Stockwell V, Reed B: (2016) Node position influences viability and contamination in hazelnut shoot explants. In Vitro Cell Dev Biol.-Plant 52:580-589

Hynynen J, Niemistö P, Viherä-Aarnio A, Bruner A, Hein S, Velling P (2010) Silviculture of birch *(Betula pendula* Roth and *Betula pubescens* Ehrh.) in northern Europe. Forestry 83 (1): 104-119

Jones OP, Welander M, Waller BJ, Ridout MS (1996) Micropropagation of adult birch trees: production and field performance. Tree Physiol 16: 521-525

Kane ME, Phylman NL (1992) Effect of culture vessel on in vitro multiplication of *Pontederia cordata* L. Proc Fla State Hort Soc 105: 213-215

Lin Y, Wagner MR, Heidmann LJ (1991) In vitro formation of axillary buds by inmature shoots of Ponderosa pine. Plant Cell Tissue Organ Cult 26: 161-166

Lloyd G, McCown B (1980) Commercially feasible micropropagation of mountain laurel *Kalmia latifolia* by use of shoot tip culture. Proc Int. Plant Prop Soc. 30:421-427

Magnusson VA, Castillo CM, Dai W (2009) Micropropagation of two elite birch species through shoot proliferation and regeneration. Acta Hortic 812:223-230

Meier-Dinkel A (1992) Micropropagation of birches (*Betula* spp.). *In* Biotechnology in Agriculure and Forestry. Vol. 8. Hightech and Micropropagation II. Bajaj YPS (ed), Springer-Verlag, Berlin, Heidelberg. pp 40-81

Mohamed MA-H, Alsadon AA (2011) Effect of vessel type and growth regulators on micropropagation of *Capsicum annuum*. Biol Plant 55 (2): 370-374

Monette PL (1983) Influence of size of culture vessel on in vitro proliferation of grape in a liquid medium. Plant Cell Tissue Organ Cult 2: 327-332

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plant 15: 437-497

Puddephat IJ, Alderson PG, Wright NA (997) Influence of explant source, plant growth regulators and culture environment on culture initiation and establishment of *Quecus robur* L. in vitro. J Exp Bot 48 (309): 951-962

Perez C, Postigo P (1989) Micropropagation of *Betula celtiberica*. Ann. Bot, 64: 67-69

Reed BM, Sarasa V, Kane M, Bunu E, Pence VC (2011) Biodiversity conservation and conservation biotechnology tools. In Vitro Cell. Dev. Biol.-Plant, 47:1-4

Rathwell R, Shukla MR, Jones AMP, Saxena PK (2016) In vitro propagation of cherry birch (*Betula lenta* L.). Can J Plant Sci 96:571-578

Sanchez MC, San-Jose MC, Ballester A, Vieitez AM (1996) requirements for in vitro rooting of *Quercus robur* and *Q. rubra* shoots derived from mature trees. Tree Pysiol 16: 673-680

Sanchez MC, San-Jose MC, Ferro E, Ballester A, Vieietez AM (1997) Improving micropropagation conditions for adult-phase shoots of chestnut. J Hortic Sci 72 (3): 433-443

Sanchez MC, Vieitez AM (1991) In vitro morphogenetic competence of basal sprouts and crown branches of mature chestnut. Tree Physiol 8: 59-70.

Vieitez AM, San-Jose MC, Vieitez E (1985) *In vitro* plantlet regeneration from juvenile and mature *Quercus robur* L. J Hortic Sci 60:99-106

Viherä-Aarnio A, Welling P (200)1 Micropropagated silver birches (*Betula pendula*) in the field-performance and clonal differences. Silva Fennica 35 (4): 385-401

Welander M (1988) Biochemical and anatomical studies of birch (*Betula pendula* Roth) buds exposed to different conditions in relation to growth in vitro. *In* Genetic manipulation of woody plants. Hanover JW and Keathley DE (eds), Plenum Press, New York, pp. 79-99

Welander M (1993) Micropropagation of birch. *In* Micropropagation of woody plants. Ed. MR Ahuja. Kluwer Academic Pubs, Dordrecht. pp 223-246

Zalewska M, Miler N, Wenda-Piesik A (2010) Effect of in vitro topophysis on the growth, development and rooting of chrysantemun explants (*Chrysanthemum* x *grandiflorum* /Ramat./ Kitam). J of Hortic Sci Biotechnol 85 (4): 362-366

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Improvement of micropropagation by micrografting *in vitro* cultured mature scions of *Castanea sativa* Mill. onto physiologically juvenile rootstocks of the same genotype

Santiago Crecente-Campo[1], Juan L. Fernández-Lorenzo[2], María R. Mosquera-Losada[2], Antonio Rigueiro-Rodríguez[2] Nuria Ferreiro-Domínguez[2], María P. González-Hernández[2] José J. Santiago-Freijanes[2], Rosa Romero-Franco[2]

[1]Centro de Investigacións Agrarias de Mabegondo. A Coruña. Spain
[2] Department of Crop Production and Engineering Projects. University of Santiago de Compostela.
santiago.crecente.campo@xunta.gal , juanluis.fernandez@usc.es

Abstract

Explants of a mature 80-year-old chestnut tree (*Castanea sativa* Mill.) obtained from 'forced' shoots of branch segments of the crown (CR explants), which had been expressing significantly lower in vitro multiplication and rooting capacities than explants from the same tree obtained from forced shoots of branch segments of basal shoots (BS explants) through 28 years of successive subculturing, were serially micrografted in vitro on rooted BS explants. CR explants were re-isolated from the rootstock after 5 micrografting rounds (5µCR explants) and submitted again to successive subculturing. In vitro multiplication rates and rooting percentages of 5µCR explants were compared to those of un-grafted CR explants and BS explants until subculture 25 after re-isolation. Micrografting success percentage was 85-100 %. Multiplication rate of 5µCR explants increased significantly after 6 successive subcultures, and rooting percentage increased significantly after 19 subculture rounds, as compared to CR explants values. Comparing 5µCR explants with BS explants, multiplication rates did not differ significantly from subculture 6 to 25, and rooting percentages did not differ significantly after 19 and 25 subcultures. These results show that serial micrografting of mature scions onto rejuvenated rootstocks of the same genotype as the scion, combined with succesive subculturing after re-isolation from the grafts, permits to achieve a rejuvenation/reinvigoration effect, expressed through improvement of micropropagation performance.

Keywords: chestnut, rejuvenation, multiplication rate, in vitro rooting, succesive subculturing

Introduction

Chestnut (*Castanea sativa* Mill.) is highly recalcitrant to vegetative propagation, and the development of methods to achieve rejuvenation/reinvigoration of selected adult material to improve micropropagation and to permit germplasm conservation is crucial. In several species, (serial) grafting of mature plant material on a juvenile rootstock has been found to improve micropropagation performance (Monteuuis, 2012). In chestnut hybrids, simple grafting on 2-week-old seedlings at the hypocotyl level permitted in vitro establishment, and a slight improvement of rooting ability in one mature clone (Sánchez et al., 1997). Giovannelly and Giannini (2000) achieved improvement of in vitro culture capacity of a recalcitrant mature chestnut tree after four serial grafts on 10-month-old seedlings. Four years were required before shoots could be cultured in proliferation medium. In vitro (Fernández-

Lorenzo and Fernández-López, 2005; Crecente-Campo, 2013) and in vivo (Fernández-Lorenzo and Crecente-Campo, 2010) serial micrografting in short cycles on juvenile rootstocks improved in vitro multiplication rates of mature clones of *Castanea sativa*, but not their rooting ability, which is an essential requirement for effective propagation. Ease of rooting is considered a juvenility trait (Pliego-Alfaro and Murashige, 1987), and restored rooting competence is usually considered the main indicator of rejuvenation (Pliego-Alfaro and Murashige, 1987; Ewald and Kretzschmar, 1996; Huang et al., 1996; Mneney and Mantell, 2001).

On the other hand, in chestnut, as in many other tree species, explants taken from the basal region of the mature tree (e.g. basal sprouts) respond better to micropropagation than those taken from the crown (Sánchez et al., 1991). Juvenile characteristics are retained in ontogenetically young tissues near the trunk at the tree base while maturation is evident in ontogenetically older, but more-recently produced, tissues at the top and periphery of the tree (Bonga, 1982; Wendling, 2014). The differences of in vitro behaviour between materials from both origins can persist for many years, as it was observed in chestnut (Sánchez et al., 1991).

In most experiments, grafting and micrografting for rejuvenation/reinvigoration purposes, is carried out on rootstocks of juvenile origin, namely seedlings (Monteuuis, 2012). In general, genotype proximity between scion and rootstock at the intraspecific level is not taken into consideration as a factor affecting rejuvenation (e.g. Pliego-Alfaro and Murashige, 1987; Detrez, 1994; Giovannelli and Giannini, 2000; Danthu et al., 2002; Ewald, 2007). To our knowledge, to date, genotype identity of rootstock and scion has not yet been considered as a possible factor involved in the rejuvenation effect of micrografting.

In our investigation, micrografting was carried out by using as rootstocks rooted explants from basal sprouts of a mature clone of chestnut, expressing a physiologically juvenile behaviour in vitro (high multiplication rates and high rooting ability). Explants of the same clone, taken from the crown of the tree, and expressing significantly lower in vitro multiplication and rooting rates were used as scions. The aim of this work was to test the in vitro reinvigoration effect of serial micrografting on explants which had expressed poor in vitro performance for more than 28 years of serial subculture, using as rootstock explants of the same genotype expressing juvenile behaviour *in vitro*.

Materials and methods

Plant material

Plant material consisted of two lines of explants, obtained from 'forced' shoots of branch segments of both basal sprouts (BS) and the crown (CR) of the same 80-year-old adult tree (clone P2). Both materials will be referred to as BS and CR explants from this point on. These lines were established in vitro as described by Sánchez & Viéitez (1991), and have been maintained by serial subculturing every 30-40 days for more than 28 years. Multiplication medium (MM) used during the last 5 years consisted of macrosalts, microsalts and vitamins of WPM (Woody Plant Medium) (Lloyd and McCown, 1981) + 3% sucrose + 0,7% Bacto Agar (basal medium), supplemented with 0.1 $mg \cdot l^{-1}$ 6-benzylaminopurine (BAP). The pH was adjusted to 5.5-5.6 prior to autoclaving (Crecente-Campo, 2013).

Micrografting and serial micrografting

Rootstocks for micrografting were in vitro rooted BS explants having three or more roots (see rooting conditions below). Scions were 1-cm-long nodal CR explants. Micrografting was a miniaturized version of cleft grafting (Monteuuis, 1995; Fraga et al., 2002) and was carried out as described by Fernández-Lorenzo and Fernández-López (2005). Micrografts were introduced in glass vessels (750 ml) (three micrografts per vessel) containing 90 ml of MM, and placed in the growth chamber (see growth chamber conditions below). Elongated scions from successful micrografts (Figure 2) were used to obtain either 1) explants, re-isolated from the rootstocks and re-subcultured (see next section), or 2) one-node scions,

which were re-grafted in the conditions described above, initiating a new micrografting round (Figure 1). Both types of materials were collected after 5 successive micrografting rounds. Three repetitions of eight micrografts were done in every micrografting round. Percentage of micrografting success was recorded 35-45 days after every micrografting round.

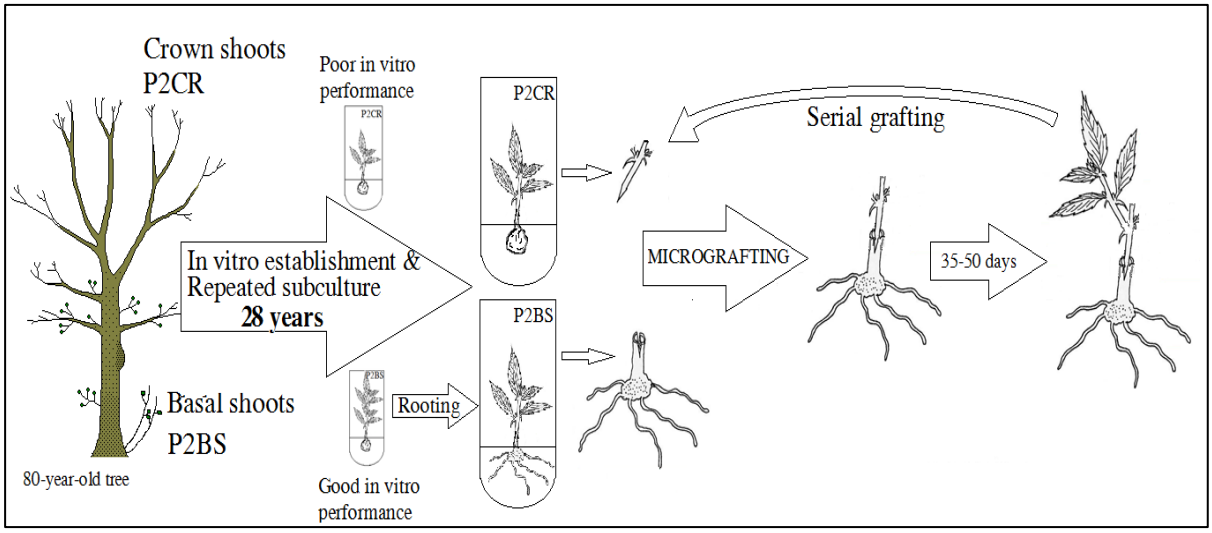


Figure 1. *Serial micrografting of CR explants on in vitro rooted BS explants. The picture of the tree is based on one from Bonga (1982).*

In vitro multiplication rate of explants from micrografts vs. un-grafted material

CR explants (shoot tips and nodes, 1.0-1.5 cm long) obtained from the elongated scion of micrografted plants after five micrografting rounds (from now on, 5µCR explants) were introduced in MM, placed in the growth chamber, and subcultured every 35 days. After re-isolation from the micrografted plant, the multiplication rate (MR) from subculture 1 to 25 was recorded as an average of MR of subculture intervals (subcultures 1-5, 6-10, 11-15, 16-20, 21-25), and compared to the MR of un-grafted CR explants and BS explants, which were subcultured in the same conditions and during the same period as 5µCR explants. For each kind of explant and subculture number, three repetitions of 12 explants (6 shoot tips + 6 nodes) were established.

Figure 2. *Micrografted plant, 40 days after micrografting*

In vitro rooting percentage of explants from micrografts vs. un-grafted material

Explants (shoot tips) obtained from micrografted plants after five successive micrografting rounds (5µCR explants) were used in in vitro rooting tests, which were carried out at different times: immediately after re-isolation from micrografts (this time is refered to as 'subculture 0': SC0), and after subculture 19 and 25. Simultaneously, in vitro rooting tests of un-grafted CR explants as well as BS explants were carried out at the same time under the same conditions. Rooting treatment consisted of 1 min basal dipping in an aqueous solution of indole-3-butyric acid (0.0 mg·ml-1 IBA (control) and 1.0 mg·ml-1 IBA) followed by transfer to hormone-free basal medium. Three repetitions of 12 explants were used per treatment. The rooting percentage was recorded after 35 days.

Growth chamber conditions

All experiments were performed in the growth chamber. Growth chamber conditions were as follows: photosynthetic photon flux density: 30µmol·m^{-2}·s^{-1}, provided by cool white light fluorescent tubes, OSRAM® L. 40W; photo-thermo-period: 16 h light/25°C -8 h darkness/20°C, relative humidity > 70%.

Statistical analysis

Data were analysed by ANOVA and LSD-test, using the IBM SPSS Statistics 20 program. Data in percentages (micrografting success and in vitro rooting) were arcsine-transformed prior to statistical analysis.

Results

Micrografting success

The micrografting success (%) in successive micrografting rounds is shown in Figure 3. Graft-take was between 87.5 and 100%, showing a slight decline at micrografting rounds 4 and 5, as compared to rounds 2 and 3. In a previous work (Fernández-Lorenzo and Fernández-López, 2005), similar percentages of micrografting success were achieved when micrografting two adult clones of chestnut cultivars 'Loura' and 'Parede' onto a rootstock of juvenile origin.

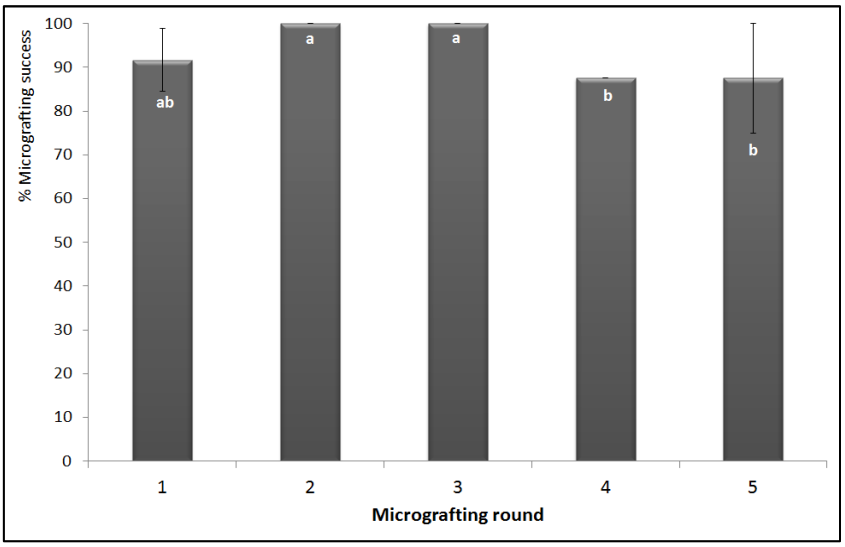


Figure 3. *Micrografting success (%) of 5 micrografting rounds of CR explants on BS rooted explants. Results are means ± standard deviation. Bars with different letters express significant differences according LSD multiple range test at $p \leq 0.05$.*

Multiplication rate

The results (Figures 4, 5) show that serial micrografting significantly increased multiplication rates of 5µCR explants as compared to CR explants after 6 subcultures since re-isolation (Figure 2), reaching values similar to those of BS explants. No significant differences were observed in the first subculture period (SC1 to SC5). The multiplication rate of 5µCR explants remained significantly higher than that of CR explants for at least 25 subcultures (more than 2 years) and was comparable to the multiplication rate of BS explants.

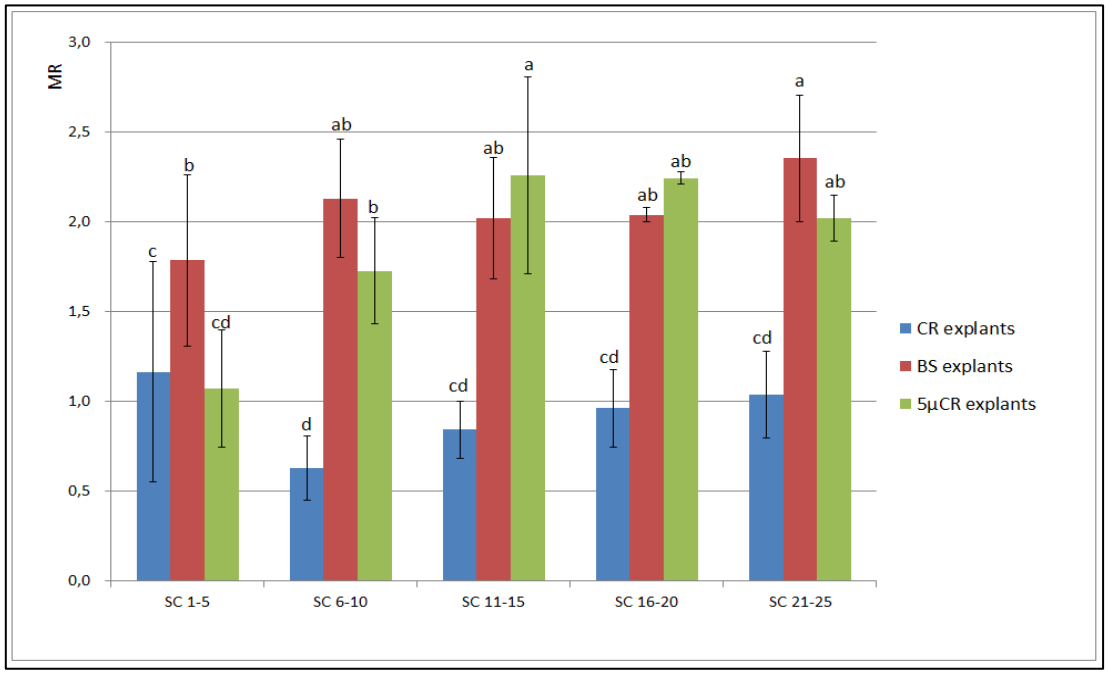


Figure 4. *In vitro multiplication rate (MR) of BS, CR and 5µCR explants in subculture intervals 1-5, 6-10, 11-15, 16-20 and 21-25. Results are means ± standard deviation. Bars with different letters express significant differences according LSD multiple range test at $p \leq 0.05$.*

Figure 5. *BS explants, CR explants and 5µCR explants of clone P2, 35 days after subculture.*

Rooting percentage

Results of rooting tests are shown in Figure 6. All explants required IBA to root. In auxin-treated explants, rooting percentage of BS explants was significantly higher than that of CR and 5µCR explants in rooting tests carried out immediately after re-isolation of 5µCR explants (SC0), while there were no significant differences between CR and 5µCR explants. In contrast, in rooting tests carried out at the

end of subculture 19, rooting percentage of 5µCR explants was significantly higher than that of CR explants, and did not differ from that of BS explants. At the end of subculture 25, rooting percentage decreased significantly in BS and 5µCR explants. Rooting percentage of BS explants continued to differ significantly from that of CR explants, and did not differ from that of 5µCR explants, but no significant differences were observed between 5µCR explants and CR explants.

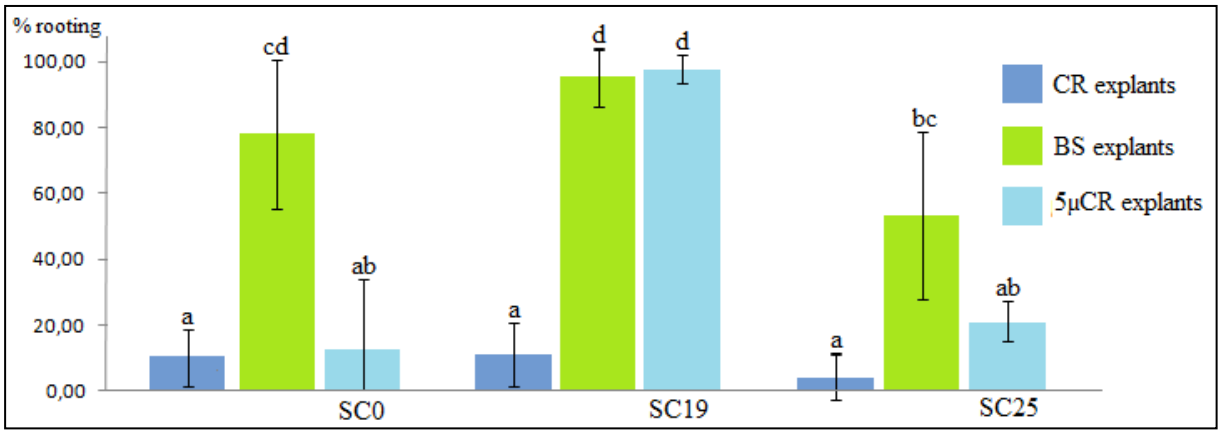


Figure 6. *In vitro rooting (%) of BS, CR and 5µCR explants, immediately after re-isolation of 5µCR explants (SC 0) and after subcultures 19 and 25 (SC19, SC25). Results are means ± standard deviation. Bars with different letters express significant differences according LSD multiple range test ($p \leq 0.01$).*

Discussion

The results show that serial in vitro micrografting of scions of mature chestnut on a rootstock with juvenile behaviour in vitro can improve micropropagation performance of the mature material. Serial conventional grafting on 10-month-old seedlings (Giovannelli & Giannini, 2000) was also effective in improving the micropropagation ability of mature material, but, as compared to serial in vitro micrografting, it was much more time- and space-consuming, as it required about 4 years of previous in vivo manipulation of the donor plant in greenhouse conditions before in vitro establishment. In contrast, in the conditions described in our study, five rounds of in vitro micrografting required 5 to 7 months in in vitro conditions, taking advantage of scion miniaturization being an additional rejuvenation/ reinvigoration factor (Monteuuis, 2012). In general, in vitro micrografting appears more effective for reinvigoration/rejuvenation than conventional nursery grafting. Possible reasons are that the time interval between grafts is shorter, the scions are in closer proximity to the root system, and cytokinins in the culture medium affect plant ageing (Wendling et al., 2014).

In the present work, five micrografting rounds made it possible to observe an increase in both the multiplication rate and in vitro rooting percentage of adult explants. According to Monteuuis (2012), the reason why, in some cases, only one micrografting operation is not enough to achieve rejuvenation could be related to the fact that the scion is not small enough to break the correlative controls that mature tissues of the scion exercise on the scion meristem . In a previous study (Fernández-Lorenzo and Fernández-López, 2005), single in vitro micrografting onto rootstocks of a juvenile clone permitted to improve the multiplication rate of explants of an adult clone of chestnut, but, even after three micrografting rounds, no improvement of in vitro rooting ability was observed. Huang et al. (1992) found that at least four grafting rounds were necessary to observe significant increases in root number of *Sequoia sempervirens* shoots, and complete restoration of the competency for adventitious rooting was achieved after five repeated cycles of micrografting (Huang et al. 1996, Chang et al. 2010). In *Eucalyptus x trabutii*, Siniscalco and Pavolettoni (1988) observed that satisfactory results in rooting rates were reached after six grafting rounds, and Danthu et al. (2002) obtained complete rooting competence restoration in *Faidherbia albida* after three grafting rounds. In contrast, as many as twelve

in vivo micrografting rounds did not permit to recover in vitro rooting competence of explants from a very old *Quercus robur* tree (Crecente-Campo and Fernández-Lorenzo, 2016).

In some cases, the use of a single rejuvenation/reinvigoration method is not sufficient to achieve a rejuvenation effect. In our work, only a combination of micrografting followed by continuous subculturing permitted to achieve a reinvigoration/rejuvenation response in mature CR explants. Interestingly, prior to micrografting experiments, CR explants had not shown any increase either in multiplication rates or in in vitro rooting ability all through 28 years of continuous subculturing. Similarly, Giovannelli & Giannini (2000) combined four rounds of conventional grafting with in vitro successive subculturing, which resulted in gradual reinvigoration/rejuvenation of *Castanea sativa* adult shoots. In other studies, continuous in vitro subculture of shoots in media containing cytokinins resulted in reinvigoration/rejuvenation without the need of combination with other reinvigoration/rejuvenation methods (Kretzschmar and Ewald 1994; von Aderkas and Bonga 2000; Titon et al., 2006; Andrade 2010, Peña-Ramírez et al., 2010). However, serial subculture alone was not sufficient to obtain reinvigoration/rejuvenation in chestnut, especially in terms of recovering of rooting ability (Sánchez and Viéitez, 1991; Crecente-Campo, 2013).

As discussed before, most research aiming to achieve rejuvenation/reinvigoration through micrografting use seedlings as rootstocks. Our results show that physiologically rejuvenated material (rooted BS explants) can also be useful for this purpose.

Concerning the way in which a juvenile rootstock can induce reinvigoration/rejuvenation on a mature scion, the basic hypothesis is that juvenile rootstocks transmit certain compounds through the phloem, which would act as "rejuvenation factors" (Bon, 1988; Monteuuis, 2012). However, the fact that, in our case, the rejuvenation/reinvigoration effect appeared only after a certain number of subcultures, and not just after the re-isolation from the graft, and persisted through many subcultures, cannot be explained by the simple transmission of "rejuvenation factors", as the progressive decrease of their concentration in the tissues through successive subculturing would lead to a loss of the rejuvenation effect (Pliego-Alfaro and Murashige 1987; Padilla and Encina, 2011). There is more and more evidence that such effect would be related to changes at the epigenetical level, caused by molecules transmitted by the rootstock that are able to modulate gene expression (Ruíz-Medrano et al., 1999; Lough and Lucas, 2006; Kudo and Harada, 2007; Harada 2010; Molnar et al., 2010; Kasai et al., 2011; Chen et al., 2013). The fact that, in our case, the genotype of the rejuvenated rootstock was identical to that of the mature scion could have facilitated the recognition of such molecules by the scion.

References

Andrade WF (2010) Indução de rejuvenescimento de teca (*Tectona grandis* L. f) através de enxertia seriada e micropropagação. Thesis, Universidade de São Paulo, São Paulo

Bon M (1988) J 16: An apex protein associated with juvenility of *Sequoiadendron giganteum*. Tree Physiol 4:381-387

Bonga JM (1982) Vegetative propagation in relation to juvenility, maturity, and rejuvenation. In: JM Bonga and DJ Durzan, Eds. Tissue Culture in Forestry. Martinus Nijhoff/W. Junk, Boston, Massachusetts, 387-420

Chang IF, Chen PJ, Shen CH, Hsieh TJ, Hsu YW, Huang BL, Kuo CI, Chen YT, Chu HA, Yeh KW, Huang LC (2010) Proteomic profiling of proteins associated with the rejuvenation of *Sequoia sempervirens* (D. Don). Endl. Protein Sci 8:64

Chen YT, Shen CH, Lin WD, Chu HA, Huang BL, Kuo CI, Yeh KW, Huang LC, Chang IF (2013) Small RNAs of *Sequoia sempervirens* during rejuvenation and phase change. Plant Biol 15:27-36

Crecente-Campo S (2013) Microenxerto e recultivo do calo basal na micropropagación de material adulto de carballo e castiñeiro. PhD Thesis. University of Santiago de Compostela, 227 pp

Crecente-Campo S, Fernández-Lorenzo JL (2016) Production of grafted plants of mature *Quercus robur*

L. 'Fastigiata' through serial grafting on juvenile rootstocks and effect on micropropagation. Propagation of Ornamental Plants 16(2):47-55

Danthu P, Hane B, Sagna P, Gassama IK (2002) Restoration of rooting competence in mature *Faidherbia albida*, a Sahelian leguminous tree, through serial root sucker micrografting. New For 24:239-244

Detrez, C (1994) Shoot production through cutting culture and micrografting from mature tree explants in *Acacia tortilis* (Forsk.) Hayne subsp, *raddiana* (Savi) Brenan. Agrofor Syst 25:171-179

Ewald D, Kretzschmar U (1996) The influence of micrografting in vitro on tissue culture behavior and vegetative propagation of old European larch trees. Plant Cell Tissue Organ Cult 44(3):249-252

Ewald, D (2007) Micropropagation of *Larix* species via organogenesis. In: SM Jain and H Häggman (Eds.), Protocols for Micropropagation of Woody Trees and Fruits:125–136

Fernández-Lorenzo JL, Crecente-Campo S (2010) In vivo serial micrografting of *Castanea sativa* in short cycles. Acta Horticult 866:291-296

Fernández-Lorenzo JL, Fernández-López MJ (2005) Reinvigoration of mature *Castanea sativa* by micrografting onto a juvenile clone. Acta Horticult 693:293-298

Fraga MF, Cacal MJ, Aragonés A, Rodríguez R (2002) Factors involved in *Pinus radiata* D. Don. micrografting. Ann For Sci 59:155-161

Giovannelli A, Giannini R (2000) Reinvigoration of mature chestnut (*Castanea sativa*) by repeated grafting and micropropagation. Tree Physiol 20:1243-1248

Harada T (2010) Grafting and RNA transport via phloem tissue in horticultural plants. Scientia Horticulturae 125:545-550

Huang HJ, Chen Y, Kuo JL, Kuo TT, Tzeng CC, Huang BL, Chen CM, Huang LC (1996) Rejuvenation of *Sequoia sempervirens* in vitro: changes in isoesterases and isoperoxidases. Plant Cell Physiol 37:77–80

Kasai A, Bai S, Li T, Harada T (2011) Graft-transmitted siRNA signal from the root induces visual manifestation of endogenous post-transcriptional gene silencing in the scion. PloS One, 6 (2): e16895. doi:10.1371/journal.pone.0016895

Kretzschmar U, Ewald D (1994) Vegetative propagation of 140-year-old *Larix decidua* trees by different in vitro techniques. Plant Physiol 144:627–630

Kudo H, Harada T (2007) A graft-transmissible RNA from tomato rootstock changes leaf morphology of potato scion. HortScience 42:225-226

Lloyd G, McCown B (1981) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Comb Proceed Intern Plant Propagators Soc 30:421-427

Lough TJ, Lucas WJ (2006) Integrative plant biology: role of phloem long-distance macromolecular trafficking. Annual Rev Plant Biol 57:203-232

Mneney EE, Mantell SH (2001) In vitro micrografting of cashew. Plant, Cell Tissue Organ Cult 66:49-58

Molnar A, Meinyk CW, Bassett A, Hardcastle TJ, Dunn R, Baulcombe DC (2010) Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. Science 328:872-875

Monteuuis O (1995) In vivo grafting and in vitro micrografting of *Acacia mangium*: impact of ortet age. Silvae Genet 44:190-193

Monteuuis O (2012) In vitro grafting of woody species. Propag Ornam Plants 12:11-24

Padilla ING, Encina CL (2011) The use of consecutive micrografting improves micropropagation of cherimoya (*Annona cherimola* Mill.) cultivars. Sci Hortic 129:167-169

Peña-Ramírez YJ, Juárez-Gómez J, Gómez-López L, Jerónimo-Pérez JL, García-Sheseña I, González-Rodríguez JA, Robert ML (2010) Multiple adventitious shoot formation in Spanish Red Cedar (*Cedrela odorata* L.) cultured in vitro using juvenile and mature tissues: an improved micropropagation protocol for a highly valuable tropical tree species. In Vitro Cell Dev Biol Plant 46:149–160

Pliego-Alfaro F, Murashige T (1987) Possible rejuvenation of adult avocado by graftage onto juvenile rootstocks in vitro. HortScience 22:1321-1324

Ruíz-Medrano R, Xoconostle-Cázares B, Lucas WJ (1999) Phloem long-distance transport of CmNACP

mRNA: implications for supracellular regulation in plants. Development 126:4405-4419
Sánchez MC, Ballester A, Viéitez AM (1997) Reinvigoration treatments for the micropropagation of mature chestnut trees. Ann Sci For 54:359-370
Sánchez MC, Viéitez AM (1991) In vitro morphogenetic competence of basal sprouts and crown branches of mature chestnut. Tree Physiol 8:59-70
Siniscalco C, Pavolettoni L (1988) Rejuvenation of *Eucalyptus* × *trabutii* by successive grafting. Acta Hortic 227:98-100
Titon M, Xavier A, Otoni WC (2006) Clonal propagation of *Eucalyptus grandis* using the mini-cutting and micro-cuttings techniques. Sci For 71:109–117
von Aderkas P, Bonga JM (2000) Influencing micropropagation and somatic embryogenesis in mature trees by manipulation of phase change, stress and culture environment. Tree Physiol 20:921–928
Wendling I, Stephen JT, Aloisio X (2014) Maturation and related aspects in clonal forestry. Part II: reinvigoration, rejuvenation and juvenility maintenance. New For 45:473-486

Micropropagation of *Tilia cordata* Mill. and verification of genetic diversity of donor trees

Helena Cvrčková[1], Martina Komárková[1], Olga Trčková[1], Pavlína Máchová[1]

[1]*Forestry and Game Management Research Institute, Strnady, Jiloviste, Czech Republic.*
cvrckova@vulhm.cz, komarkova@vulhm.cz, trckova@vulhm.cz, machova@vulhm.cz

Abstract

The regeneration of linden (*Tilia cordata* M.) *in vitro* together with its genetic diversity was studied. When using dormant buds as primary explants, it was possible to induce shoot growth and multiplication. The best results were obtained by placing shoots on a modified Murashige and Skoog medium containing 0.4 mg l^{-1} BAP and 0.1 mg l^{-1} IBA. Simple Sequence Repeat (SSR) are valuable for genetic research. Microsatellite genotyping of 20 clones of *T. cordata* from a seed orchard in Vranice identified different genotypes between all tested trees. Moreover, analysed linden trees have shown high levels of genetic diversity. Our results suggest that microsatellite markers can be used in plant breeding programs and can help in significant forest improvement.

Keywords: *in vitro* micropropagation, linden, SSR markers

Introduction

Tilia cordata Mill. is historically considered to be a Czech national tree, which is appreciated for its biodiversity and conservation and as a stabilising element in the forest ecosystem. Moreover, thanks to its high adaptability to climatic factors, linden is becoming an essential tree in the forest ecosystem. In the past, this species has been reduced due to the extensive change of forest to agriculture land and as a consequence of artificial reforestation with preferences for conifers (Úradníček et al., 2010).

Nowadays, micropropagation represents the most effective biotechnology enabling the clonal reproduction of parent trees with a high quality of phenotype. Moreover, plant tissue culture can be used for the *ex situ* conservation including, for example, in a gene bank or *In vitro* storage (Oseni et al., 2018). Organogenesis, as a particularly suitable method for clonal propagation of broadleaved trees, was used for the establishment of *in vitro* cultures from selected elite parent linden trees growing in South Bohemia.

In order to obtain knowledge about the level of genetic diversity of donor trees, DNA analyses by nuclear microsatellite markers have been optimised. Simple sequence repeats (SSR) are highly polymorphic and codominant tandem repeats of one to six nucleotide long DNA motifs and can also be used to verify the multiallelic nature, codominant inheritance, clonal identity and for example somaclonal variation in the micropropagated cultures (Kalia et al., 2011). Our research aimed to regenerate linden trees *in vitro* and identify different clones in the seed orchard at Vranice.

Material and methods

The plant material (short twigs with dormant buds) was collected during spring from the selected small-leaved linden trees of the seed orchard in Vranice (Fig. 1), which was established in 1996 from several plus trees of the South Bohemia region. The surfaced sterilized extirpated buds were cultured on modified 6% agar Murashige and Skoog (MS medium, according to Murashige and Skoog, 1962) medium, supplemented with 30 mg l^{-1} of sucrose, 100 mg l^{-1} glutamine, 2 mg l^{-1} glycine and growth regulators 0.4 mg l^{-1} BAP and 0.1 mg l^{-1} IBA, the pH was adjusted to 5.8. Explants were cultivated in constant conditions at 24°C and 16 h photoperiod under white fluorescent light (30 µmol m^{-2} s^{-1}). After 4-5 weeks of growth, the same composition of MS media was used for organogenesis and multiplication. The elongated shoots were subcultured every four weeks and stored in the National Bank of the Explant of Forestry and Game Management Research Institute (Fig. 2).

Total genomic DNA of young leaves collected from 20 linden trees was extracted using a DNeasy Plant Mini Kit (QIAGEN, Germany) according to manufacturer´s protocol. The SSR method based on polymerase chain reaction (PCR) with specific primers was used to analyse the genetic diversity. Linden individuals were screened by eight nuclear polymorphic microsatellite markers Tc4, Tc5, Tc6, Tc7, Tc915, Tc920, Tc937, Tc963 (Phuekvilai and Wolff, 2013). The primers were labelled fluorescently using FAM, VIC, NED, PET dyes and assembled into two multiplexes. PCR products were separated by capillary electrophoresis using a genetic analyser 3500 (Applied Biosystems, USA). Allele calling was performed using GeneMapper® 4.1 software provided by Applied Biosystems. The genetic diversity parameters were calculated with the statistical program GenAlEx 6.5 (Peakall and Smouse, 2012). Finally, Micro-Checker software for identifying and correcting genotyping errors in microsatellite data was used (Van Oosterhout et al., 2004).

Figure 1. *Linden seed orchard Vranice, South Bohemia, Czech Republic*

Figure 2. *In vitro cultures of linden derived from spring buds grown on the nutrient medium MS supplemented with 2 mg l^{-1} glycine, 100 mg l^{-1} glutamine, 0.1 mg l^{-1} IBA and 0.4 mg l^{-1} BAP*

Results and discussion

According to our previous experiments, MS medium was found to be the most suitable medium for the growth and vitality of linden explants, as shown for example also by Üçler and Mollamehmetoğlu (2001). Therefore, nutrient media for establishing cultures as well as for multiplication were based on this basal medium. The highest number of shoots was observed on BAP concentration of 0.4 mg l^{-1}. However, this finding is not consistent with results from Sarvašová and Ďurkovič (2002), who showed that increasing BAP concentration resulted in decreased multiplication activity of *T. x europaea* L. explants. This inconsistency could be explained by different linden species, plant genotype or for example by a composition of nutrient media.

Molecular genetic markers have been used many times among different plant species to identify clones and analyse the genetic diversity in populations. The genotypic data from microsatellite analyses showed different genotypes of all tested linden trees. There were 66 different alleles detected at the 8 loci over 20 individuals, ranging between 5 (loci Tc4, Tc937) and 15 (locus Tc963) alleles. Shannon's information index calculated for genetic diversity ranged from 0.84 at locus Tc937 to 2.48 at locus Tc963. Expected heterozygosity (He) ranged from 0.41 (Tc937) to 0.90 (Tc963). Most of the loci exhibited homozygote excess with positive F values (Table 1). A mean number of different alleles across tested linden trees was 8.25. Mean value of population genetic diversity according to Shannon's information index (I) was 1.65. The mean values of observed and expected heterozygosity were similar (Ho = 0.731, He = 0,728). Analysed linden trees have shown high levels of genetic diversity so that they can be involved *in situ* conservation strategies. Moreover, the use of trees with intraspecific genetic diversity is at low risk of disease attack (Boyd et al., 2013) and do not pose a risk to global biodiversity via biotic homogenization (Polakowski et al., 2011).

Table 1. *Details of 8 nuclear microsatellite markers including their size (bp) and genotypic data. Na – mean number of different alleles, I – Shannon's information index, Ho – observed heterozygosity, He – expected heterozygosity, F - Fixation Index.*

Locus	PCR product size range (bp)	Na	I	Ho	He	F
Tc4	224–236	5	1.28	0.50	0.64	0.219
Tc5	136–176	11	1.97	0.80	0.82	0.024
Tc6	124–140	8	1.72	0.95	0.78	-0.226
Tc7	215–243	7	1.45	1.00	0.71	-0.408
Tc915	149–173	8	1.92	0.80	0.84	0.045
Tc920	220–234	7	1.58	0.55	0.73	0.247
Tc937	146–166	5	0.84	0.40	0.41	0.027
Tc963	245–281	15	2.48	0.85	0.90	0.056

Acknowledgements: This work was supported by the Ministry of Agriculture of the Czech Republic, institutional support MZE-RO0118 and by National Programme of Protection and Reproduction of Forest Tree Gene Resources.

References

Boyd IL, Freer-Smith PH, Gilligan CA, Godfray HCJ (2013) The consequence of tree pests and diseases for ecosystem services. Science 342:1235773

Kalia RK, Rai MK, Kalia S, Singh R, Dhawan AK (2011) Microsatellite markers: an overview of the recent progress in plants. Euphytica 177:309–334

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Oseni OM, Pande V, Nailwal TK (2018) A review on plant tissue culture, a technique for propagation and conservation of endangered plant species. Int J Curr Microbiol Appl Sci 7:3778–3786

Peakall R and Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. Bioinformatics 28:2537–2539

Phuekvilai P and Wolff K (2013) Characterization of microsatellite loci in *Tilia platyphyllos* (Malvaceae) and cross-amplification in related species1. Applications in Plant Sciences, Wiley Online Library

Polakowski NR, Lohr VI, Cerny-Koenig T (2011) Survey of wholesale production nurseries indicates a need for more education on the importance of plant species diversity. Arboriculture and Urban Forestry 37:259

Sarvašová I and Ďurkovič J (2002) *In vitro* regeneration of European linden. Biol Plant 45:149–152

Üçler AÖ and Mollamehmetoğlu N (2001) *In vitro* plantlet regeneration from mature embryos of linden (*Tilia platyphyllos* Scop.) and multiplication of its buds. Turkish Journal of Agriculture and Forestry 25:181–186

Úradníček L, Maděra P, Tichá S, Koblížek J (2010) Woody plants of the Czech Republic. Kostelec nad Černými lesy:Lesnická práce

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4:535–538

Propagation and rooting of *Prunus avium* by temporary and continuous immersion systems

Damián Dasilva[1], Conchi Sánchez[1], Anxela Aldrey[1], Jesús M Vielba[1], Ramiro Martínez[2] Nieves Vidal[1]

[1] Instituto de Investigaciones Agrobiológicas de Galicia. CSIC. Santiago de Compostela, Spain (nieves@iiag.csic.es);[2] Asociación Galega de Froita Autóctona do Eume (agfaeume@gmail.com)

Abstract

The conservation of wild and local varieties of plants contributes to sustainable agricultural production, healthy and diversified diets, healthy ecosystems and sociocultural stability. However, FAO estimates that over the last century about 75 percent of the genetic diversity of agricultural crops has been lost. An increasing number of local varieties of fruit trees are in danger of loss through habitat destruction or socio-economic pressures towards the use of commercial and more uniform varieties. Tissue culture represents a method for conservation and propagation of these genetic resources, promoting their reintroduction in the agricultural sector. The aim of this study was to micropropagate three varieties of cherry growing at local farms in Galicia, Northwestern Spain. Plant material of the varieties named "de Viño", "Negra de San Cristobo" and "Negra de Fene" was provided by a local association of fruit growers (Agfa do Eume). Shoots developed in branches of four-year old trees were used for the initiation of *in vitro* cultures. For proliferation, cherry germplasm was cultured in semisolid medium gelled with agar and in liquid medium by temporary immersion, using RITA® bioreactors. For rooting induction, indole-3-butyric acid was applied at 2 mg L^{-1} for one month or at 25 mg L^{-1} for 24 h. For rooting expression, shoots were cultured in glass jars in semisolid medium or in plantform™ bioreactors with rockwool cubes soaked in liquid medium. One min aeration with CO_2 enriched air was provided 16 times per day to the plantform™ bioreactors. The three varieties of cherry were successfully proliferated in semisolid and liquid medium. Multiplication coefficient and shoot length were affected by genotype in both systems. Rooting ability was also genotype dependent. Shoots rooted in plantform™ bioreactors showed more roots and performed better during the acclimation process than those rooted in semisolid medium.

Keywords: acclimation, bioreactors, cherry, liquid medium, local varieties

Introduction

The conservation of wild and local varieties of plants contributes to sustainable agricultural production, healthy and diversified diets, healthy ecosystems and sociocultural stability (Love and Spaner, 2007). Local varieties, also designed as native or autochthonous, are the consequence both of natural selection and of decades of selection carried out by local farmers (Cleveland and Soleri, 2007). These genotypes are adapted to particular soil and climate conditions, are tolerant to biotic and abiotic stresses and present a high genetic diversity, together with characteristic flavor and taste (Berthaud et al. 2001).

In the last century, large-scale commercialization of food in general, and of fruits in particular, led to the introduction of new varieties, usually bigger and with higher yields, which displaced the traditional crops and its associated cultural methodologies. As a result, in many regions, local varieties of fruits

have nowadays a narrow range of distribution and are linked to self-supply practices. This fact, together with the continuous decrease of the population of rural areas, has provoked a global genetic erosion of agrobiodiversity (FAO 2011). *Ex situ* conservation of the existing genetic resources is one of the strategies recommended to alleviate this situation (Dulloo et al. 2010). Within ex situ conservation approaches, micropropagation is the methodology of choice in the case of species or genotypes that traditionally are propagated vegetatively, by grafting or cuttings (Postman et al. 2006, Pence 2013). Galicia, located at the Northwest of Spain, is one of the regions where the coexistence of forest areas with small but numerous traditional farms favored, during the past centuries, the maintenance of a great forest and agronomic biodiversity (Goded et al. 2018). However, the current and dramatic increase of rural depopulation make it necessary to take urgent actions to prevent the loss of this heritage. The genetic diversity of other fruits, such as apple, has been studied (Pereira-Lorenzo et al. 2007). However, genetic characterization of *Prunus avium* biodiversity in the region was only reported for timber varieties (Fernandez-Cruz et al. 2014), and not for trees cultured for fruit production (Badenes and Zuriaga 2016). In this study we addressed the micropropagation of three Galician varieties of *Prunus avium*: "De Viño", "Negra de San Cristobo" and "Negra de Fene", whose fruits are highly appreciated by local farmers. To the best of our knowledge, these cherries had not been previously cultured *in vitro*. In the present study, we explored the use of alternative methodologies during the multiplication and rooting steps. In order to reduce cost and improve multiplication coefficients, we compared the proliferation of these genotypes in liquid medium in RITA® bioreactors of the temporary immersion system (Etienne and Berthouly 2002) with conventional micropropagation in semisolid medium. Besides, with the aim of increasing the acclimation success, we explored different concentrations and periods of exposure to auxins during the rooting process, both in semisolid medium and in plantform™ bioreactors (Welander et al. 2014).

Materials and Methods

Establishment of shoot multiplication cultures

Shoot cultures of *Prunus avium* were initiated from branches with dormant buds collected in March from 4-year-old trees of the varieties "Negra de San Cristobo", "Negra de Fene" and "De Viño" growing at the orchard of the "Asociacion Galega da Froita Autóctona do Eume", San Sadurniño, Northwestern Spain (Fig 1. A-B). After fungicide treatment, branch segments 25- to 30-cm-long were set upright or horizontally in moistened perlite (Fig. 1C), and forced to flush axillary shoots in a growth cabinet at 25ºC and 80–90% relative humidity under a 16-h photoperiod (90–100 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps). After 2–3 weeks, newly sprouted shoots (3–5 cm in length) were used as the source of explants (Fig. 1D). The shoots were stripped of their leaves and surface sterilized by immersion for 30 s in 70% ethanol and for 10 min in a 6 g L^{-1} solution of sodium hypochlorite containing 2-3 drops of Tween 80®. The shoots were then rinsed three times in sterile distilled water. Nodal segments (10 mm) were cut from the shoots and inoculated in tubes with Murashige and Skoog medium (MS; Murashige and Skoog 1962) supplemented with 0.5 mg L^{-1} of N^6-benzyladenine (BA), 0.5 mg L^{-1} of

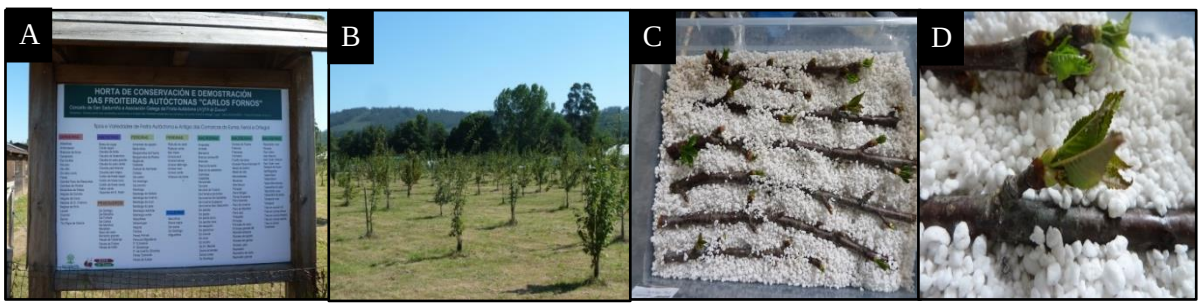


Figure 1. *Plant material for establishment of cherry cultures. A,B) Orchard of the "Asociacion Galega da Froita Autóctona do Eume". C,D) Flushing of shoots in branches of "Negra de Fene" placed horizontally in the phytotron at day 0 (C) and after two weeks (D).*

indole-3-butyric acid (IBA), 3% sucrose and 0.65 % (w/v) agar Vitroagar (Pronadisa, Spain). The medium was adjusted to pH 5.7 before being autoclaved at 120 °C for 20 min. Cultures were incubated under a 16-h photoperiod provided by cool-white fluorescent lamps (50-60 µmol $m^{-2}s^{-1}$) at 25 ˚C light/20 ˚C dark. The explants were transferred every 2 weeks during the first 6 weeks after establishment. Thereafter, the explants were maintained in 50 ml of semisolid medium (SSM) in 300 ml glass jars (7 explants per jar), and were subcultured every 5 weeks. The semisolid proliferation medium (SPM) consisted of MS supplemented with 1 mg L^{-1} of BA, 0.1 mg L^{-1} of IBA, 0.1 mg L^{-1} of Gibberellic acid (GA_3), 3% sucrose and 0.7 % (w/v) agar.

Proliferation in semisolid and liquid medium

Shoots (1.5 cm) were cultured in SSM in jars (Fig. 2A) and also by temporary immersion in liquid medium, using commercial RITA® (www.vitropic.fr) bioreactors (one immersion of 1 min every 8 hours). RITA® bioreactors are cylindrical vessels of volume 1 L (Fig. 2B) comprising two chambers, an upper one housing the explants and a lower one containing the medium. Air (filtered through sterile 0.22 µm hydrophobic (PTFE) membrane filters) enters the containers through an inlet connected to the lower chamber and produces excess pressure that pushes the medium into the upper chamber. During the immersion period, air bubbles through the medium, wetting the tissues and renewing the head space atmosphere inside the vessel, with the excess pressure escaping through an outlet at the top of the apparatus (Etienne and Berthouly 2002). In these experiments, liquid proliferation medium (LPM), of the same composition as SPM but devoid of agar was autoclaved before being added to the containers. Each RITA® contained 7 explants and 150 ml of LPM. Five weeks after the inoculation, the number of shoots, shoot length and leaf length and width were recorded.

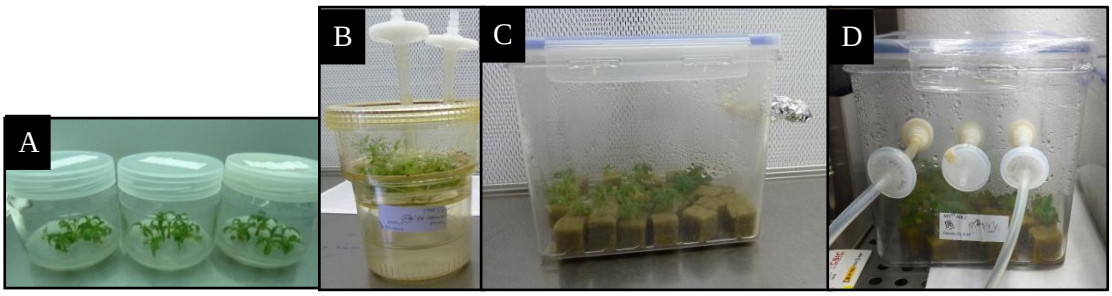


Figure 2*. Containers used for proliferation and rooting of cherry. A) Jars. B) RITA® bioreactors. C,D) Plantform™ bioreactors without inner baskets and with rockwool cubes.*

Elongation and rooting

Vigorous shoots proliferated in semisolid medium, of 1.5 cm or longer, were used for rooting experiments. As shoot length of "Negra de San Cristobo" and "Negra de Fene" averaged only 1.3 cm, a previous elongation step was used with these two genotypes. For elongation, explants cultured in SPM for five weeks were transferred to the same medium or to half strength MS ($MS^{1/2}$) supplemented with 1 mg L^{-1} GA_3, 3 % sucrose and 0.7 % agar for another 5 weeks (EM). Then, various rooting strategies were tested, using jars with semisolid medium or plantform™ bioreactors without the inner baskets (Fig. 2 C,D). In bioreactors, the shoots were inserted in rockwool cubes (2 cm of side) soaked in liquid medium, as described for chestnut (Vidal et al. 2017). Basal medium for rooting experiments consisted in $MS^{1/2}$ with 3% sucrose, supplemented or not with IBA and 0.7 % agar. During rooting, all the shoots were cultured under a 16-h photoperiod provided by white LEDs (150 µmol $m^{-2}s^{-1}$) at 25 ˚C light/20 ˚C dark. Plantform™ bioreactors were aerated for 1 min at a frequency of 16 times every 24 h with CO_2-enriched air (2000 ppm).

The following rooting treatments were tested:

1. Shoots of "Negra de San Cristobo" elongated in SPM or in EM were inoculated in jars with $MS^{1/2}$ 2 mg L^{-1} IBA, 3% sucrose and 0.7 % agar for 6 weeks.

2. Shoots of "Negra de Fene" were treated with MS$^{1/2}$ 25 mg L^{-1} IBA, 3% sucrose and 0.7% agar for 24 h and then transferred a) to jars with the same medium without IBA, or b) to plantform™ bioreactors with the same medium without IBA and agar.
3. Shoots of "De Viño" were treated either as described for "Negra de Fene" or were inoculated in jars or plantform™ with MS$^{1/2}$ 2 mg L^{-1} IBA and 3 % sucrose for 6 weeks. The medium of the jars was gelled with 0.7 % agar.

Six weeks after the beginning of the experiments, roots were counted and rooting percentages were calculated. Rooted shoots were transferred to trays with peat:perlite (3:1) and placed in a phytotron for 2-3 weeks or directly into the greenhouse. Six weeks later, surviving plantlets were transferred to pots and continued growing in the greenhouse. Acclimation percentages were calculated over rooted shoots.

Results and discussion

Proliferation

The Galician varieties "Negra de San Cristobo", "Negra de Fene" and "De Viño" were successfully micropropagated in semisolid and in liquid medium (Fig. 3), although in some cases a certain degree of hyperhydricity was observed in shoots cultured in bioreactors.

***Figure 3**. Proliferation of cherry in semisolid and liquid medium. A) Jars with "De Viño" shoots. B) "Negra de San Cristobo" cultured in RITA®, C) Shoots of "Negra de Fene" cultured in RITA® (left) and jar (right).*

The genotype "De Viño" produced more shoots when cultured in semisolid medium (Fig. 4A), whereas that in "Negra de San Cristobo" and "Negra de Fene", RITA® bioreactors yielded more shoots than jars, and produced larger leaves (Fig. 4B,C). Genotypic differences were also reported by Millar et al. (2017) with cherry rootstocks cultured by temporary immersion in bioreactors of the two-flask system. These authors highlighted the need to adjust immersion and aeration regimes to each genotype. In our study, we applied only one condition to the three genotypes, as a first insight in their ability of being cultured in liquid medium. Future research will be focused in the optimization of proliferation by testing different bioreactors and immersion frequencies.

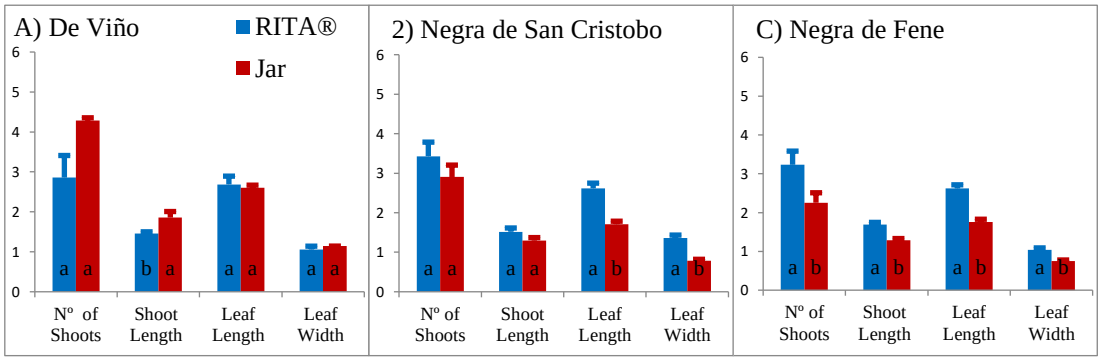


***Figure 4**. Proliferation of shoots of cherry in jars and RITA® bioreactors. A) "De Viño". B) "Negra de San Cristobo". C) "Negra de Fene". Measurements expressed in cm.*

Rooting and acclimation

The appearance of the cherry shoots rooted and acclimated in the different treatments is shown in Fig. 5, and the results of rooting of the three genotypes are shown in Fig. 6.

***Figure 5**. A-D) Cherry shoots rooted in jars (A,B) and bioreactors (C,D). E) Plantlets after 2, 4 and 6 months in the greenhouse.*

The effect of the culture medium used during the elongation step on the subsequent rooting of "Negra de San Cristobo" is shown in Fig. 6A. Lower rooting percentages were observed when the cherry clusters were transferred to the medium with 1 mg L^{-1} of GA_3 instead of being transferred again to the proliferation medium. The use of an elongation medium with only GA_3 as a plant growth regulator was recommended by other authors working with cherry (Iacona and Muleo 2010), but in the case of "Negra

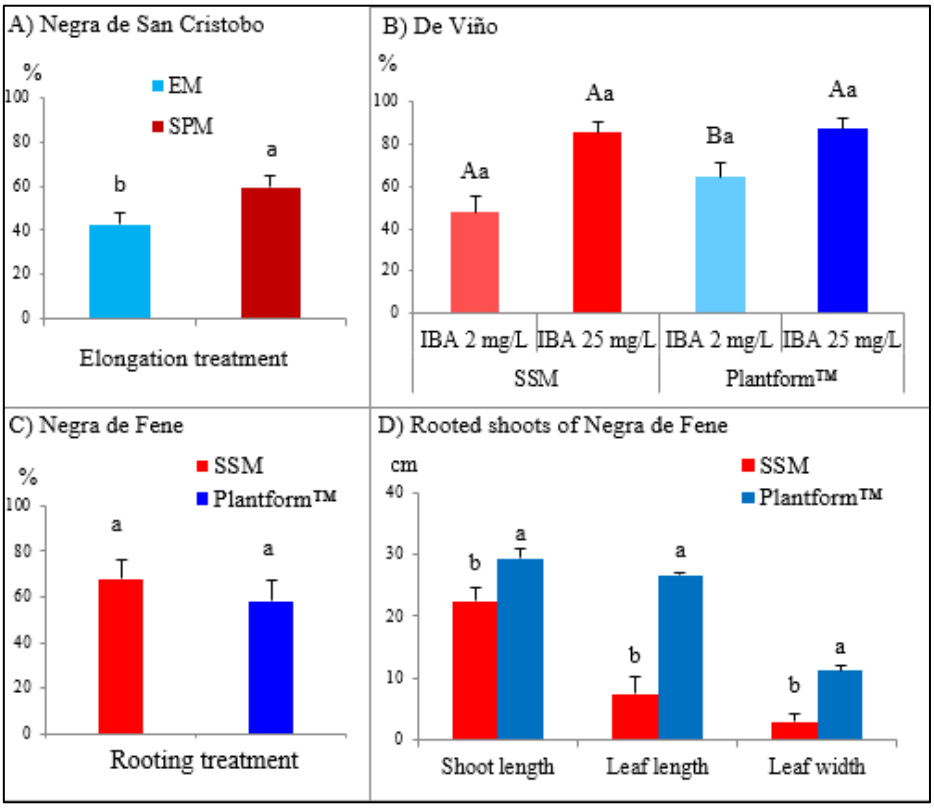


***Figure 6**. Rooting of the three local varieties of cherry in different conditions. A) "Negra de San Cristobo" treated with 2 mg L^{-1} IBA for 6 weeks and previously elongated by transfer to MS ½ 1 mg L^{-1} GA_3 (EM) or to MS 1 mg L^{-1} BA 0.1 mg L^{-1} IBA 0.1 mg L^{-1} GA_3 (SPM). B) "De Viño" treated with 2 mg L^{-1} IBA for 6 weeks or 25 mg L^{-1} IBA for 24 h and cultured in SSM in jars or in plantform™ bioreactors. C) "Negra de Fene" treated with 25 mg L^{-1} IBA for 24 h and cultured in jars or bioreactors. D) Morphological data of rooted shoots of "Negra de Fene" 6 weeks after IBA treatment.*

de San Cristobo" was not beneficial for obtaining higher number of long shoots (data not shown) nor to improve the rooting ability of the elongated shoots. In "De Viño", higher rooting percentages were obtained with 25 mg L^{-1} IBA during 24 h than with 2 mg L^{-1} IBA for six weeks (Fig. 6B), irrespective of the container used (jar or plantform™). In "Negra de Fene", preliminary experiments showed the detrimental effect of using the medium with 1 mg L^{-1} GA_3 during the elongation step (data not shown), and consequently all the shoots were elongated by transfer to the proliferation medium. In this genotype, as reported above for "De Viño", no significant differences were found between rooting percentages of shoots rooted in jars or bioreactors (Fig. 6C), although shoots rooted in plantform™ were longer and with larger leaves (Fig. 6D). Xiao et al. (2011) associated the increase in plant growth observed in bioreactors with forced ventilation and CO_2 supply, which promoted a more photoautotrophic behavior of the explants.

The results of the acclimation of the rooted shoots are shown in Fig. 7. With "Negra de San Cristobo" and "De Viño", acclimation was carried out directly in the greenhouse. Many plants were lost during the first days after transfer to trays, due to fungal contamination. Survival was very low (Fig. 7A,B), especially with the shoots of "De Viño" rooted in jars (Fig. 7B). Antifungal treatments may be necessaries to prevent plant losses in these conditions, as highlighted by Druart (2013). In "Negra de Fene", rooted shoots were transferred to a phytotron for 4 weeks and then placed in the greenhouse. Acclimation was greatly improved, especially with the shoots rooted in plantform™ (Fig. 7C), which caused resumption of growth earlier than in those rooted in jars, increasing the differences in height already observed before transplanting (Fig. 6D, Fig. 7D).

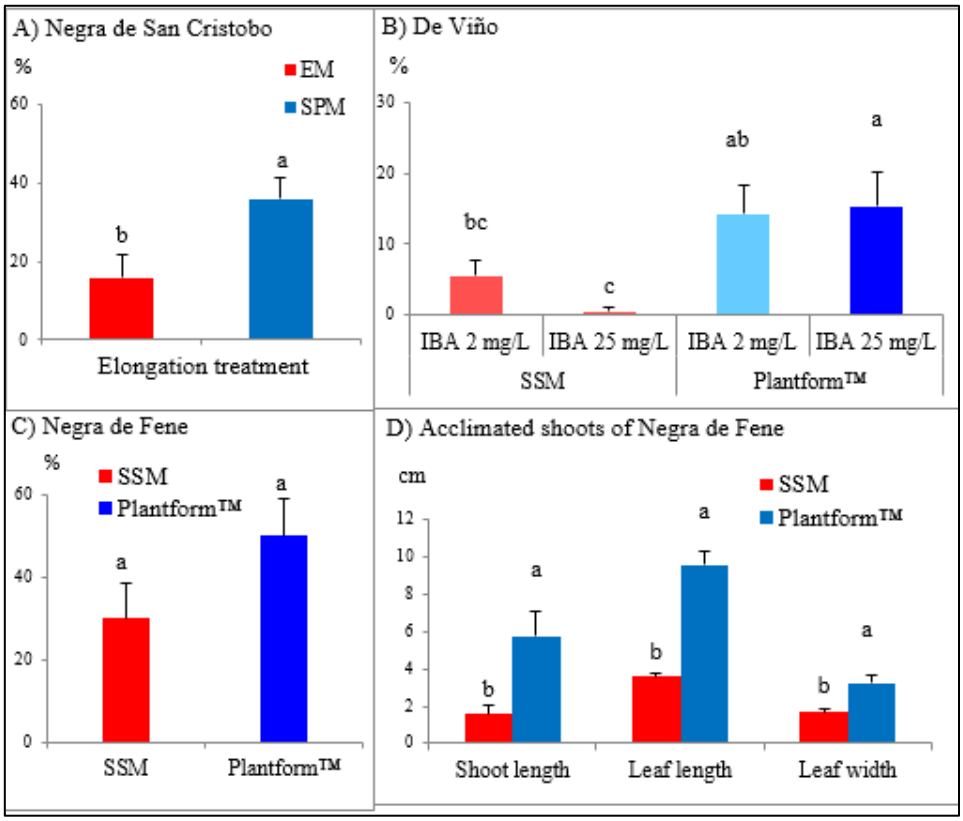


Figure 7*. Acclimation of cherry plantlets. Some shoots were acclimated directly in the greenhouse (A,B) and others had a previous stage in a phytotron (C). A) "Negra de San Cristobo" rooted with 2 mg L^{-1} IBA for 6 weeks and previously elongated by transfer to EM or SPM. B) "De Viño" rooted with 2 mg L^{-1} IBA for 6 weeks or 25 mg L^{-1} IBA for 24 h and cultured in SSM or in plantform™ bioreactors. C) "Negra de Fene" rooted with 25 mg L^{-1} IBA for 24 h in jars or bioreactors. D) Morphological data of "Negra de Fene" four weeks after transfer to the phytotron.*

The rooting percentages observed with these three local genotypes are consistent with other studies with fruit varieties of cherry (Druart 2013). With the best treatments, we obtained rooting percentages of 60% in "Negra de San Cristobo", 68% in "Negra de Fene" and 88% in "De Viño", similar to the 52% of Helshoven and 84% of Schneider reported by Druart (2013). For improving the acclimation success, it seems essential to include a stage in the phytotron for a few weeks and probably an antifungal treatment, as recommended by Druart (2013). Even being low, shoots rooted in bioreactors showed a better response to acclimation than those cultured in jars, with higher survival and more growth (Fig. 7 B-D). Shoots rooted in rockwool cubes were easier to manage without compromising the integrity of the roots (Fig 5D), whereas some roots were broken when the shoots were extracted from the jars (Fig 5B). Besides, explants rooted inside the bioreactors presented more secondary roots, probably due to the porosity of the cubes. These shoots were also longer and more vigorous (Fig 6D). It is possible that the exposure to gas exchange inside the bioreactor could have prepared them to better overcome the stressful step of acclimation to *ex vitro* conditions.

In conclusion, the three Galician local varieties of cherry "de Viño", "Negra de San Cristobo" and "Negra de Fene" were successfully micropropagated and rooted in semisolid and liquid medium. Although acclimation percentages should be improved, rooting in plantform™ bioreactors seems to be a promising method for obtaining cherry plantlets of good quality.

Acknowledgements: This research was partly funded by the Xunta de Galicia (Spain) through the Contrato Programa 2017-2018 and the project IN607A 2017/6. We thank Alejandro Díaz, Rafael Sánchez and Patricia Val for technical assistance and José A. Bellas for plant material collection.

References

Badenes ML, Zuriaga E (2016) Frutales de hueso. In: Las variedades locales en la mejora genética de las plantas. JI Ruíz de Galarreta, J Prohens and R Tierno (eds) pp 239-258. (www.bibliotekak.euskadi.net/WebOpac)

Berthaud J, Clement JC, Emperaire L, Louette D, Pinto F, Sanou J, Second G (2001) The role of local-level gene flow in enhancing and maintaining genetic diversity. In: Cooper HD, Spillane C, and Hodgkin T (eds). Broadening the genetic base of crop production. CABI, Wallingford, Oxon, UK. pp 1-23

Cleveland DA, Soleri, D (2007) Extending Darwin´s analogy: Bridging differences in concepts of selection between farmers, biologists and plant breeders. Econ Bot 61:121-136

Druart P (2013) Micropropagation of *Prunus* species relevant to cherry fruit production. Methods Mol Biol 994:119-36

Dulloo ME, Hunter D, Borelli T (2010) *Ex situ* and *in situ* conservation of agricultural biodiversity: major advances and research needs. Not Bot Hort Agrobot Cluj 38:123-135

Etienne H, Berthouly M (2002) Temporary immersion systems in plant micropropagation. Plant Cell Tissue Organ Cult 69:215–231

FAO 2011 Biodiversity for food and agriculture. Contributing to food security and sustainability in a changing world (www.fao.org/3/a-i1980e.pdf)

Fernandez-Cruz J, Fernandez-Lopez J, Miranda-Fontaiña ME, Diaz R, Toval G (2014) Molecular characterization of Spanish *Prunus avium* plus trees. Forest Systems 23:120-128

Goded S, Ekbroos J, Domínguez J, Guitián JA, Smith HG (2018) Effects of organic farming on bird diversity in North-West Spain. Agric Ecosyst Environ 257:60–67

Iacona C, Muleo R (2010) Light quality affects in vitro adventitious rooting and ex vitro performance of cherry rootstock Colt. Sci Hortic 125:630-636

Love B, Spaner D (2007) Agrobiodiversity: Its value, measurement and conservation in the context of sustainable agriculture. J Sustain Agr 31:53-82

Millar S, Godoy S, Seit P, Catrilaf G, Espinoza D, Prieto H, Tapia E (2017) Scaling-up of Cherry rootstocks in a temporary immersion bioreactor system. In: Bonga JM, Park YS, and Trontin JF (eds.) Proceedings of the 4th International Conference of the IUFRO Unit 2.09.02 on

Development and application of vegetative propagation technologies in plantation forestry to cope with a changing climate and environment. La Plata, Argentina. pp 213-218

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473-497

Pence VC (2013) In vitro methods and the challenge of exceptional species for Target 8 of the Global Strategy for Plant Conservation. Ann Missouri Bot Gard 99:214–220

Pereira-Lorenzo S, Ramos-Cabrer AM, Díaz-Hernández MB (2007) Evaluation of genetic identity and variation of local apple cultivars (*Malus* x *domestica* Borkh.) from Spain using microsatellite markers. Genet Resour Crop Evol 54:405-420

Postman J, Hummer K, Stover E, Krueger R, Forsline P, Grauke Li, Zee F, Ayala-Silva T, Irish B (2006) Fruit and nut genebanks in the US National Plant Germplasm System. HortSci 41:1188-1194

Vidal N, Aldrey A, Blanco B, Correa B, Sánchez C, Cuenca B (2017) Proliferation and rooting of chestnut under photoautotrophic conditions. In: Bonga JM, Park YS, and Trontin JF (eds.) Proceedings of the 4th International Conference of the IUFRO Unit 2.09.02 on Development and application of vegetative propagation technologies in plantation forestry to cope with a changing climate and environment. La Plata, Argentina. pp 119-127

Welander M, Persson J, Asp H, Zhu LH (2014) Evaluation of a new vessel system based on temporary immersion system for micropropagation. Sci Hortic 179:227–232

Xiao Y, Niu G, Kozai T (2011) Development and application of photoautotrophic micropropagation plant system. Plant Cell Tissue Organ Cult 105:149–158

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

A brief review of combining genomic selection and somatic embryogenesis for tree improvement

Chen Ding[1,3], Yill-Sung Park[2], Jan Bonga[2], Benjamin Bartlett[1], Yang Li[4], Fred Raley[1]

[1] Western Gulf Forest Tree Improvement Program, Texas A&M Forest Service, TAMU 2585, College Station, TX, 77843-2585, USA; [2]Scientist Emeritus, Natural Resources Canada - Canadian Wood Fibre Centre, Fredericton, New Brunswick, Canada; [3]Canada Research Chair in Forest Genomics, Institute for Systems and Integrative Biology, Université Laval, Québec, QC, G1V 0A6, Canada; [4] Naveen Jindal School of Management - UT Dallas, 800 W Campbell Rd, Richardson, TX 75080
**Corresponding author: chen.ding@tfs.tamu.edu*

Abstract

Tree breeding needs to overcome the challenges posed by both long testing periods and pre-maturation stages before flowering and seed production. For species with a decades-long rotation time, early selection is one tool for increasing gains by fast-tracking performance of germplasm through a shortened breeding cycle. Successful breeding programs deliver cumulative gains with improved germplasm through continuous efforts with field tests. Genomic selection (GS) became available after the breakthrough development of whole genome sequencing and is a cost-effective tool for breeders to predict the trait performance of untested materials solely based on the genomic markers, which reduce or eliminate the lengthy testing period. In multi-varietal forestry (MVF), along with other horticultural practices, somatic embryogenesis (SE) in tree species creates efficiency in breeding and deployment by mass-producing uniform cultivars or planting stock without gene reshuffling due to meiotic crossing-over. The combination of GS and SE will optimize breeding programs by reducing the time required for testing and increasing the selection intensity for species that have an SE platform, sequencing resources, and existing breeding programs. A highly effective tree improvement program requires developing the SE platform, strengthening models that link phenotypic data with genome-wide data across multiple-breeding populations for both quantitative and categorical traits, and cost-effective sequencing platforms.

Keywords: Genomic selection, GBLUP, HBLUP, somatic embryogenesis, woody plants

Introduction

About two decades ago, genomic selection (GS) was introduced as a new evaluation tool for breeding programs with the aid of whole-genome sequencing data of germplasms and trial phenotypic evaluation data (Meuwissen et al. 2001). In the dairy industry, GS has successfully shortened the breeding generation with an accurate prediction of genomic estimated breeding values (GEBV) even before calving (Pryce and Daetwyler 2012, Zhang et al. 2010), which has lead to the routine use of GS in the commercial dairy industry. With economic efficiency as high as 92%, the cost savings has promoted the rapid deployment of GS in research and development for animal breeding programs (Zhang et al. 2010, Hayes et al. 2009). Moreover, GS now occurs in plant breeding (Sorrells 2015, Jannink et al. 2010) and aquaculture (Sonesson AK and Meuwissen TH 2009). Compared to traditional pedigree-based selection in tree species, GS hastens the breeding cycle and boosts the genetic gain per unit time by shortening generation intervals and omitting a series of progeny tests (Grattapaglia 2017). By abridging the

phenotypic testing period, or assessing the selection materials based on genotypic data, tree breeders can eliminate one generation of testing. The use of GS in *Picea glauca* and *P. abies* in eastern Canada, for example, would reduce the generation interval by about 15 years when compared with the traditional seed orchard breeding. Furthermore, the temporal acceleration provided by GS was projected to be used in multiple forms of research, depending on the current state of the breeding program (Harfouche et al. 2012). For forest species, GS offers a promising future with a moderate to high prediction accuracy compared to that obtained by traditional pedigree-based methods. Most encouragingly, although the early studies (before 2017) used low coverage markers to make predictions in *Eucalyptus*, *Pinus*, and *Picea* species, moderate to high prediction accuracies were reported ranging from 0.5 to 0.8 (Table 2). More recently, improved genotyping technology, such as the EU60K SNP chip, has been applied that can sequence the genome samples with high density markers. To date, GS research has been focused on evaluating the GS efficiency and operational routine with multiple parameters.

Genomics resources for genomic selection

Tree species have important economic, ecological, and social values; of which, many are selected and cultivated in tree improvement or horticulture programs. However, trees are not considered as model species for which rich genomic, physiological, and cytological research resources are available. We summarize the genomic resources available for 40 tree species, including the major commercial conifer species (Pinaceae), that have extensive molecular information, somatic embryogenesis procedures, along with accessible sequencing data, a genotyping platform, and other applications of genomic technology.

There are 14 families of trees for which genome sequencing is available and four families that are more advanced regarding forest tree genomic programs: Pinaceae, Salicaceae, Myrtaceae and Fagaceae (Neale et al. 2013). During the past decade, with the benefits of the next generation sequencing (NGS) for non-model species, tree GS has become promising and feasible. Conifer species (Picea abies (L.) H. Karst., Picea glauca (Moench) Voss, Pinus taeda L., Pinus pinaster Aiton, Pinus radiata D.Don) have large genome sizes (~20 GB), creating a barrier to develop high-density and quality molecular markers (e.g., single nucleotide polymorphisms, a.k.a. SNP) for GS model building.

Before GS, tree breeding programs relied on pedigree structure to track the origin and relatedness of germplasm. Pedigree structure must be maintained and progeny test results evaluated for various mating designs (e.g., structured controlled crosses and open-pollination). With GS, genomic information, provided through constructing high-density markers from large collections of germplasm, is used to evaluate offspring and/or parents, by calculating the genomic estimated breeding (GEBV) and genetic values (GEGV) for commercially important traits without the need for progeny tests. However, without breeding programs it is usually difficult to develop an operational GS program, even when genomic resources are available (Table 1). Recently, genomic development appears to have exceeded the pace of SE development. We foresee more applications in GS and joint research and development of GS-SE (e.g., *Picea* and *Eucalyptus* spp); particularly because tree breeding involves longer breeding cycles due to longer flowering maturity than those of crop species. Currently, two main genotyping methods are employed in GS research, microarray and genotyping-by-sequencing (GBS). In Tables 1 and 2, the most popular microarrays are SNP and DArT arrays (in *Eucalyptus*). SNP arrays also known as SNP chips, are expensive and difficult to develop initially, but could be used continually for that species. GBS provides valuable markers earlier than those provided by SNP arrays. However, genotyping with GBS for another population requires that the process recommence. Meanwhile, the SNP chip could be applied directly to other populations, assuming intraspecific relatedness to the training population is high enough. GS and SE should be integrated to optimize the genetic gains from GS, especially in species where a stable SE protocol has been developed and is used routinely in the production of propagules for planting. In vitro propagation by SE from elite individuals or parents will carry the combination of selected genes, without recombination and Mendelian sampling error, for deployment into plantation forestry. SE provides stable production of high genetic gain material; especially for MVF

or GS-MVF. Besides accelerating a breeding program, multiple traits could be improved simultaneously based on GEBV evaluation. Importantly, many valuable traits are controlled by many genes, which hinders traditional breeding programs that rely solely on parental breeding values to make selections.

Table 1. *Genomic study resources, application examples, and recent somatic embryogenesis progress in tree species*

Family	Species	Common name	Genome size (Mb)	2n	Sequencing technology	Genomic applications	Genotyping platform	Gene and marker discovery	Reference of genomic studies	Somatic embryogenesis (SE) references
Betulaceae	*Betula nana*	Dwarf birch	450	28	Illumina	No GS	-	-	(Wang, *et al.* 2013)	-
	Betula papyrifera	white birch	450	28	Illumina	transcriptome discovery	RNAseq	215,700 transcripts	(Theriault, *et al.* 2016)	-
	Betula pendula Roth	Silver birch or European white birch	-	28	-	-	-	-	-	(Häggman, *et al.* 2007)
Caricaceae	*Carica papaya*	Papaya	372	18	Sanger	Marker-assisted selection (MAS)	-	9,594 genes from 116,453 SSR	(Ming, *et al.* 2008,Vidal, *et al.* 2014)	(Solórzano-Cascante, *et al.* 2018)
Euphorbiaceae	*Hevea brasiliensis*	Rubber tree	~2,150	36	Illumina, Roche 454, and SOLiD, PacBio	Quantitative trait loci (QTL) detection	RNA-seq GAIIx, Illumina	354 SSR and 151 SNP	(Rahman, *et al.* 2013), (Lau, *et al.* 2016), (Tang, *et al.* 2016,Rosa, *et al.* 2018)	(Mignon and Werbrouck 2018)
	Jatropha curcas	Barbados nut	410	22	Sanger, Illumina and Roche 454	GS and gene identification	RNA-seq and qRT-PCR; RNAseq	1,248 DArT PL; MAPK, MAPKK, and MAPKKK genes	(Sato, *et al.* 2011,Azevedo Peixoto, *et al.* 2017); (Wang, *et al.* 2018)	
Fagaceae	*Juglans regia*	Persian walnut	606	32	IlluminaC10: F10B1D10:F10	Genotyping tool	Axiom™ J. regia 700K SNP array	609K SNPs	(Martinez-Garcia, *et al.* 2016,Marrano, *et al.*)	
	Quercus robur	Pedunculate oak	740	24	Illumina, Roche 454, and Sanger	No GS	-	-	(Plomion, *et al.* 2016)	(San-José, *et al.* 2010)
	Quercus lobata	Valley oak	720-730	24		Genome assembly	Illumina	-	(Sork, *et al.* 2016)	
	Quercus ilex L.	holm oak	-	24	-	-	-	-		21-66.7% regeneration rate, 2 genotypes (Martinez, *et al.* 2017)
Malvaceae	*Theobroma cacao*	Cacao	430	20	Illumina, Roche 454, and Sanger	GS and GWAS	Illumina	15K SNP	(Argout, *et al.* 2011,McElroy, *et al.* 2018)	-
Meliaceae	*Azadirachta indica*	Neem tree	~364	30	Illumina, IonTorrent, and Sanger	genomes, transcriptomes and metabolites study	Illumina and Roche 454	32,278 genes expressed	(Krishnan, *et al.* 2012,Kuravadi, *et al.* 2015)	-
Moraceae	*Morus notabilis*	Mulberry	357	14	Illumina	gene expression	-	-	(He, *et al.* 2013,Wen, *et al.* 2017)	-
Myrtaceae	*Eucalyptus globulus*	blue gum	-	22	-	GS	EUChip60K	12,000 SNP	(Durán, *et al.* 2017)	reserve accumulations, <40% (Pinto, *et al.* 2010), (Corredoira, *et al.* 2018)
	Eucalyptus camaldulensis	Red river gum	~650	22	Roche 454 and Sanger	NO GS	-	-	(Hirakawa, *et al.* 2011)	<68% (Prakash and Gurumurthi 2009)
	Eucalyptus grandis	Flooded gum	640	22	Sanger	GS and gene expression	DArT array	2,816 markers	(Myburg, *et al.* 2014,Cappa, *et al.* 2018),(Fan, *et al.* 2018,Du, *et al.* 2016)	
Oleaceae	*Olea europaea*	Olive tree	1,400–1,500	46	Illumina and Roche 454	GBS and genetic mapping	Genotyping by sequencing, Illumina	3,384 expressed markers out of 22,033 transcriptome-based SNP	(Barghini, *et al.* 2014,Ipek, *et al.* 2017)	-
Pinaceae	*Picea abies*	Norway spruce	20,020	24	Illumina	Exome capture and SNP annotation, GS	Illumina Infinium SNP chip	~4,000 SNPs, Exome capture	(Chen, *et al.* 2018,Nystedt, *et al.* 2013,Azaiez, *et al.* 2018)	(Dahrendorf, *et al.* 2018,Varis, *et al.* 2018)
	Picea glauca	White spruce	~20,800	24	Illumina	SNP annotation, GS	Illumina Infinium	~7,000 SNPs, Exome Capture	(Birol, *et al.* 2013, Pavy, *et al.* 2017, Warren, *et al.* 2015, Beaulieu, *et al.* 2014a),	(Varis, *et al.* 2018)
	Picea engelmannii x Picea glauca	Interior spruce	-	24	-	-	Illumina	~50,803 SNPs	(Ratcliffe, *et al.* 2015)	-
	Picea sitchensis	Sitka spruce	-	24	-	Population genomics, evolution study, GS	Illumina GoldenGate array	768 SNPs; 339 polymorphic	(Holliday, *et al.* 2012) (Fuentes-Utrilla, *et al.* 2017)	-
	Pinus lambertiana	Sugar pine	31,000	24	Illumina	Comparative genomics, no GS	Illumina	-	(Stevens, *et al.* 2016,Baker, *et al.* 2018)	-
	Pinus taeda	Loblolly pine	23,200	24	Illumina	GS, association, gene expression	Illumina Infinium SNP chip	-	(Neale, *et al.* 2014), (Wegrzyn, *et al.* 2014), (Resende, *et al.* 2012,Zimin, *et al.* 2014), (Zapata-Valenzuela, *et al.* 2013), (Talbot, *et al.* 2017)	(Klimaszewska, *et al.* 2007)
	Pinus radiata D.Don	Radiata pine	-	24	-	Transcriptome sequencing	RNA 6000 LabChip, Illumia Hiseq 2000	-	(Telfer, *et al.* 2018)	(Klimaszewska, *et al.* 2007) , (Montalbán and Moncaleán 2018),(Laneros, *et al.* 2018)

(Continue to next page)

(Continued from previous page)

Family	Species	Common name	Genome size (Mb)	2n	Sequencing technology	Genomic applications	Genotyping platform	Gene and marker discovery	Reference of genomic studies	Somatic embryogenesis (SE) references
	Pinus flexilis	Limber pine	-	24	-	Transcriptome, genetic mapping	RNAseq	-	(Baker, *et al.* 2018,Lin, *et al.* 2016), (Schoettle, *et al.* 2014)	-
	Pinus pinaster	maritime pine	-	24	Roche 454	genomic gene structures, GS	SNP array	866 transcripts, 2,500-4,332 SNPs	(Seoane-Zonjic, *et al.* 2016), (Isik, *et al.* 2016), (Bartholomé, *et al.* 2016)	(Klimaszewska, *et al.* 2007) , (Trontin, *et al.* 2016)
	Pinus contorta	lodgepole pine	-	24	-	genome-wide association, Linkage map of adaptive trait. No GS	Illumina sequencing of restriction fragment libraries	95,000 SNPs	(Parchman, *et al.* 2012), (Lotterhos, *et al.* 2018)	Poor initiation
	Pseudotsuga menziesii var. menziesii (Mirb.) Franco	Douglas-fir	16,000	26	-	Marker development; GS	Illumina; Exome capture	5,847 SNPs, 69,551	(Howe, *et al.* 2013), (Thistlethwaite, *et al.* 2017)	(Gautier, *et al.* 2018) (Lelu-Walter, *et al.* 2018)
Rosaceae	*Malus × domestica*	Apple	750~632.4	34	Roche 454, Sanger, Illumina HiSeq, PacBio	SNP array development, GS	Illumina 8K SNP array	53,922 coding genes, ~15.8K SNP	(Li, *et al.* 2016,Boocock, *et al.* 2015,Cornille, *et al.* 2012,Velasco, *et al.* 2010,Bianco, *et al.* 2014), (Kumar, *et al.* 2015)	(Ameri, *et al.* 2018)
	Prunus mume	Mei	~280	16	Illumina	Gene interaction, expression analysis	Illumina	-	(Zhang, *et al.* 2012,Zhang, *et al.* 2018,Linlin, *et al.* 2018,Gosik, *et al.* 2018)	
	Prunus persica	Peach	265	16	Sanger	Gene (bioinformatics) analysis, no GS	-	-	(Verde, *et al.* 2013,Cao, *et al.* 2017)	
	Prunus cerasus	Sour cherry	-	32	-	-	-	-		(Ben Mahmoud, *et al.* 2018)
	Pyrus communis	European pear	600	34	Roche 454	GWAS, QTL mapping	GBS	2,036 markers from 148,622 SNPs; 1433 cM, 0.7 cM/marker	(Chagne, *et al.* 2014,Gabay, *et al.* 2018)	
	Pyrus × bretschneideri	Chinese pear	527	34	Illumina	GWAS, QTL mapping	As above	206,971 SNPs; as in *Pyrus communis*	(Wu, *et al.* 2013,Cao, *et al.* 2017,Cao, *et al.* 2016), (Gabay, *et al.* 2018)	
Salicaceae	*Populus euphratica*	Desert poplar	~593	38	Illumina	genome divergence, no GS	Illumina Hiseq 2000 and 2500	-	(Ma, *et al.* 2013,Zhang, *et al.* 2016,Ma, *et al.* 2018)	-
	Populus trichocarpa	Black cot-tonwood	410	38	Sanger	Marker development, linkage disequilibrium (LD), and GS	SNP array	29,213 SNPs	(Tuskan, *et al.* 2006,Slavov, *et al.* 2012),(Kaplan 2016)	-
	Salix suchowensis	Purple willow	429	38	Illumina and Roche 454	No GS	-	-	(Dai, *et al.* 2014)	
	Populus tomentosa Carr.	Chinese white poplar	-		-	No GS	-	-		(Deng, *et al.* 2009)
Thymelaeaceae	*Aquilaria agallocha*	Agarwood	736	16	Illumina	No GS	-	-	(Chen, *et al.* 2014)	-
Lamiaceae	*Tectona grandis* L. f.	Teak	317	36	Illumina and Nanopore	No GS	-	172 protein-coding genes, 16,252 SSR	(Yasodha, *et al.* 2018)	-

Notes, The sequencing technology of trees was reported in a previous study (Neale, et al. 2017). We adopted the idea including family examples to overview the connection between the genomics research and late SE progress. GBS is a method whereby individual genotypes at a large number of single nucleotide polymorphisms (SNPs) are directly detected by re-sequencing the entire or a selected fraction of the genome (Harfouche, et al. 2012); no SNP array is constructed for repeated genotyping. Genome-wide association studies (GWAS): Using genome-wide molecular markers of many individuals of a particular species to identify genetic variations associated with a particular trait at both population and family levels (Harfouche, et al. 2012). ***Please contact the corresponding author (chen.ding@tfs.tamu.edu) for a higher resolution version of this Table.***

SE and other vegetative propagation methods provide the system for delivering the gain for both pedigree-based and marker-based breeding programs (El-Kassaby and Klápště 2015) in uniform traits (e.g., growth and disease resistance). Concisely, GS expedites gain by eliminating progeny tests, while SE provides the deployment pipeline to deliver the gain from GS.

GBLUP and HBLUP

Genomic prediction modeling includes multiple regression methods such as the linear mixed-model (best linear unbiased prediction, a.k.a. BLUP), Bayesian models (including Bayesian ridge regression and LASSO) (Table 2) and other methods, including machine and deep learning (Liu and Wang 2017) (Bellot et al. 2018) (Gonzalez-Camacho et al. 2018). Here we introduce the typical genomic BLUP (GBLUP) and the single step HBLUP.

***Table 2**. List of genomic selection studies based on forest tree species data sets*

Species	Generations	Genotyping Method	Number of markers	Traits analyzed	Models/ Methods	Prediction accuracy	Reference
Eucalyptus hybrids	1	DArT array	3,129	Growth, wood properties	RR-BLUP	0.54–0.6	(Resende, *et al.* 2012a)
	1	DArT array	3,564			0.38–0.55	
Eucalyptus grandis (Hill ex Maiden)	2	DArT array	2,816	Growth and straightness	GBLUP, bivariate	0.77-0.86	(Cappa, *et al.* 2018)
Eucalyptus globulus	1	SNP array (EUChip60K)	12,000	Stem volume	GBLUP, Bayesian lasso regression, Bayes B, and Bayes C models	0.58-0.75	(Durán, *et al.* 2017)
Eucalyptus urophylla × E.grandis	1	SNP array (EuCHIP60K)	60,904	Growth, pulp, density	GBLUP, non-additive	0.02-0.50 (Prediction ability)	(Tan, *et al.* 2018)
Eucalyptus polybractea	1	SNP array	10,000-500,000	terpene traits (Euclyptus oil)	GBLUP, BayesB, BLUP vertical bar GA	0.19-0.73	(Kainer, *et al.* 2018)
Pinus taeda	1	SNP array	4,825	Growth	RR-BLUP	0.63–0.75	(Resende, *et al.* 2012b)
Pinus taeda	1	SNP array	4853	Growth, tree architecture, wood properties, disease resistance	RR-BLUP, Bayes A, Bayes Cπ, B-LASSO, RR-BLUP B	0.17–0.51	(Resende, *et al.* 2012c)
Pinus taeda	1	SNP array	3,406	Growth, wood properties	RR-BLUP	0.30–0.83	(Zapata-Valenzuela, *et al.* 2012)
Pinus taeda	1	SNP array	3,461	Growth	ABLUP, GBLUP	0.37–0.74	(Zapata-Valenzuela, *et al.* 2013)
Pinus pinaster	2	SNP array	2,500	Growth, stem straightness	GBLUP, B-RR, B-LASSO	0.09–0.73	(Isik, *et al.* 2016)
Pinus pinaster	3	SNP array	4,332	Growth, stem straightness	ABLUP, GBLUP, B-LASSO	0.24–0.94	(Bartholomé, *et al.* 2016)
Picea glauca (1)	1	SNP array	6,385	Growth, wood properties	ABLUP, B-RR, B-LASSO	0.50-0.70	(Beaulieu, et al. 2014b)
Picea glauca (2)	1	SNP array	6,932	Growth, wood properties	ABLUP, B-RR, Combined	0.33–0.45	(Beaulieu, *et al.* 2014a)
Picea engelmannii × glauca	1	GBS	34,570–50,803	Growth	RR-BLUP, GRR, Bayes Cπ	0.31–0.55	(Ratcliffe, *et al.* 2015)
Picea abies	1	SNP array	8,000-116,765	Height, pilodyn, velocity, MOE	GBLUP, BayesB, BLUP vertical bar GA	0.40-0.80	(Chen, *et al.* 2018)
Picea mariana	1	SNP array	4,993	Height, DBH, volume, density, MFA	ABLUP, Bayes RR	0.40-0.80	(Lenz, *et al.* 2017)
Pseudotsuga menziesii	1	Exome capture	69,551	Height and wood density	RR-BLUP and GRR	0.80-0.91	(Thistlethwaite, *et al.* 2017)
Pseudotsuga menziesii	2	Exome capture	As above	height	ABLUP, RR-BLUP, GRR, BayesB	0.56-0.92	(Thistlethwaite, *et al.* 2019)
Picea sitchensis (Bong.) Carr)	2	RADseq	8,397	Height and bud burst	ABLUP and GWAS analyses	0.59-0.62	(Fuentes-Utrilla, *et al.* 2017)

Notes, G number of generations included in the study, GBS genotyping-by-sequencing method, RR ridge regression, B-RR Bayes RR, B-LASSO Bayes LASSO regression, GRR generalized ridge regression, GWAS Genome-wide association studies. This table was constructed similar to (Bartholomé, et al. 2016), but latest research were summarized. DArT, Diversity Array Technology to produce markers, 0.1 to 10% of the genome sequenced (Isik 2014). Exome capture is a whole-genome sequencing capture method that extracts only the collection of exons in an individual organism (Hodges, et al. 2007,Suren, et al. 2016), and then marker tool kit (e.g., SNP array) are developed for genotyping more training and validating individuals in GS. RADseq is a whole genome sequencing method discovering the restriction site-associated DNA markers to construct random insert library (Suren, et al. 2016); this method is used for studies such as association mapping and QTL mapping though it may contain contaminant DNA at the scale of complex genomes comparing to the exome capture which is relatively more expensive (Suren, et al. 2016).

In traditional breeding, BLUP-derived breeding values estimates are based on the additive genetic relationships that measure the resemblance or co-ancestry among individuals. Typically, this is accomplished by using the mixed linear model:

$$Y = Xb + Zu + e,$$

where Y is the vector of observed phenotype (trait); X and Z are known design matrices of fixed and random effects, respectively; b is an unknown parameter of fixed values to be estimated; and u is vectors of breeding values to be estimated and e is the residuals, such that $E(u) = E(e) = 0$, $Var(u) = A\,\sigma^2_A$ and $Var(e) = I\,\sigma^2_e$, where A is the numerator relationship matrix based on genes identical by descent, σ^2_A is additive genetic variance, and σ^2_e is the error variance. The BLUP of u, a.k.a. ABLUP, is obtained as an estimator numerator kinship matrix (A) that is constructed by registered pedigree information (e.g., parent-offspring, sibs and cousins, etc). In GS, GBLUP calculates the genetic relationship matrix (G) based on the profile of molecular markers without pedigree information in order to estimate the additive

and non-additive genetic effect. The G matrix can replace the A matrix to upgrade the ABLUP to GBLUP. The G matrix can capture the Mendelian sampling variation, historical co-ancestry relationship (i.e., historical provenance relation), as well as potential pedigree registry errors. The G matrix is estimated using the following formula:

$$G = \frac{(M-P)\cdot(M-P)'}{2\sum_{j=1}^{m} p_j \cdot (1-p_j)}$$

where, M is an m x n matrix with m rows (the number of individuals in the training population) and n columns (the number of the molecular markers used in genotyping). M_{ij} was set to -1 when the genotype of the i_{th} tree for j_{th} marker was homozygous for the rare allele, but was set to zero when heterozygous and 1 when homozygous for the common allele. P is a matrix containing the frequency of the second allele (pj).

When the additive genetic relationship is a mixture of marker-based genomic relationships and the pedigree relationship of individuals, the single-step or blended realized genetic relationship numerator matrix (H) could be constructed to estimate the GEBV. The H matrix can predict both genomic-estimated breeding values (GEBV) of the genotyped individuals and estimated breeding values (EBV) of the non-genotyped individuals from the same model in a single step. The H matrix and its inverse are constructed based on previous studies (Aguilar et al. 2010, Legarra et al. 2009) using the following formulas:

$$H = \begin{bmatrix} A_{11} + A_{12}A_{22}^{-1}(G - A_{22})A_{22}^{-1}A_{21} & A_{12}A_{22}^{-1}G \\ GA_{22}^{-1}A_{21} & G \end{bmatrix}$$

$$H^{-1} = A^{-1} + \begin{bmatrix} 0 & 0 \\ 0 & G^{-1} - A_{22}^{-1} \end{bmatrix}$$

where A_{11} is the block of A for non-genotyped trees with pedigree information; A_{22} is the block of genotyped individuals; A_{12} and A_{21} contain the expected additive genetic relationships between genotyped and non-genotyped individuals. Thus, the HBLUP can use the entire breeding population, including the genotyped and non-genotyped individuals, for GS application (Cappa et al. 2017, Ratcliffe et al. 2017).

Clonal materials are preferred in GS modeling of both additive breeding values and non-additive genetic values, though non-additive genetic effects can be estimated in an open-pollinated population with SNP marker data (Gamal El-Dien et al. 2016). In SE programs, the total genetic values are preferred to determine the rankings within full-sib families. Grattapaglia (2017) proposed using a training population of more than 2,000 clonal individuals, which would not cause a significant burden because of cost savings associated with the refined prediction accuracy and improved handling of genotype x environment (GxE) interactions. HBLUP can be adopted where genotyping is too expensive. High relatedness in the training population will increase GS performance (Chen et al. 2018). Other prediction methods have favorable performance in multiple traits; however, GBLUP and Bayesian methods are more widely reported in previous studies (Table 2). Chen et al. (2018) reported that limited differences exist in the model performance between the GBLUP and other methods (Bayesian ridge, Bayesian, LASSO, Bayes Cπ, etc.). Alternatively, Bayes A and Bayes Cπ outperformed the prediction accuracy of ridge regression in Pinus taeda (Resende et al. 2012) for a specific trait with a major gene effect.

Somatic embryogenesis in breeding programs

Since the 1980s, multiple tree species are documented to utilize SE, although it is not the dominant R&D approach for tree improvement in North America. Currently, only ArborGen Inc. and J.D. Irving operate a conifer SE program for species such as *Picea glauca* (Moench) Voss, *Picea abies* (L.) H. Karst., and *Pinus taeda* L. As a consequence, the level of technological maturation and automation for these species

is somewhat limited for wider adoption. Furthermore, the SE initiation rate in many commercially important trees is insufficient to warrant the cost of clonal propagation, especially if the initiation rate falls below 50% for the best genotypes with superior volume growth (Trontin et al. 2016, Lelu-Walter et al. 2016, Abrahamsson et al. 2017). Another reason is that cell lines are expensive to maintain and propagate at levels needed for commercial operation. While automation appears promising (Adams et al. 2016), the initial costs to establish production lines can be difficult to be absorbed by many breeding programs. Overall, the embryo development, weak propagule germination, poor acclimation success and lack of automation often prevent the large-scale utilization of SE in forest tree breeding programs.

SE as a deployment pipeline in MVF breeding programs

In MVF, the variation within half-sib and full-sib families are exploited with SE lines expressing better performance in growth than open-pollinated seedlings of *Picea glauca* (Moench) Voss (Adams et al. 2016). Gain delivered through SE is realized gain at the plantation site. For quantitative traits that show low genetic control (volume, height, or forking), the selection intensity can be easily manipulated within the SE pipeline at the level of an individual tree or varietal line. Thus, the uniformity of growth traits at rotation age will lead to the successful realized gain potential with proper silviculture management. For traits with dominant or major gene effects, SE provides the platform for both population improvement and intensive varietal line improvement.

When deploying SE material, the cost per seedling could be twice that of the controlled pollinated plantation stock in *Pinus radiata* D.Don (Li and Dungey 2018), where genomic selection and SE could be jointly operated. The GS-SE combined approach needs to be refined to operate in breeding programs. Li and Dungey (2018) proposed multiple pathways to deploy SE in forestry operations with *Pinus radiata* D.Don in New Zealand. Park et al. (2016) proposed a one-generation operation scheme of GS-SE in a Canadian breeding program of *Picea glauca*. In *Pinus taeda* L., Resende et al. (2012) suggested that GS-SE could remarkably increase the realized gain by selecting and deploying the lines directly through massive production of SE for regeneration.

SE benefits future genome editing breeding (CRISPR)

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a powerful tool for producing mutagenesis of favored traits in plant and animal species, including tree species. Current work in CRISPR genome editing could target multiple sites of the genome that reduce the cost and time spent on transgenic development by particle bombardment or Agrobacterium (Jaganathan, et al. 2018). Research involving CRISPR has been reported in crops such as maize, wheat, rice, tomato, soybean, cotton, tobacco, potato, citrus, grape, cocoa, and other plant species including *Arabidopsis thaliana* L. (Jaganathan et al. 2018, Haque et al. 2018, Cabrera-Ponce et al. 2019). Traits controlled by these edited sequences include both quantitative traits (e.g., fruit size) and other traits related to biotic or abiotic stress such as pathogen resistance, stomata, and leaf traits (Jaganathan et al. 2018, Haque et al. 2018, Borrelli et al. 2018).

In tree species, CRISPR provides opportunities to expedite breeding programs by eliminating backcross mating designs, intended to exclude unfavorable alleles, which conventional transgenic/mutagenesis technology rely upon. Thus, stable gene/allele frequency can be deployed with SE for clonal propagation for multiple generations (Bewg et al. 2018). For conservation purposes, functional genes controlling adaptive traits could be tested using CRISPR to evaluate the potential adaptation and performance under the changing environment (Fernandez i Marti and Dodd 2018). Besides the use of CRISPR in Populus spp. (Elorriaga et al. 2018), few cases are reported in forest tree species. CRISPR is not suited to benefit large population improvement schemes where diversity is a primary breeding concern. Additionally, the use of transgenic technology is even more limited than GS for use in tree improvement programs. In GS-SE, the elite varieties can be candidates for CRISPR gene editing.

In animals, emerging applications of CRISPR-based genome editing are tested, but the embryo transfer technique is not as effective as vegetative propagation is in plants. Gene editing applications in tree breeding programs will benefit from SE as a platform to propagate commercially important species. The advent of genome editing applications in forest trees will benefit species such as conifers that readily incorporate SE and other vegetative propagation methods as part of their deployment platform.

Conclusion

SE is a promising tool to commercially produce high quality plantation stock in forest trees, especially for conifers for which organogenesis is not available, although it is not easy for the forestry industry to quickly use GS and SE programs to optimize the gain in forest tree breeding. Only for a few species, such as *Eucalyptus* and *Picea*, has GS and SE been applied commercially and operationally in plantation forestry. Even for species such as *Pinus taeda* L., the use of GS-SE is still in its infancy. By developing more genomic resources and SE platforms, there will be more opportunities to deploy higher gains in commercial trees, especially for conifers. Advancement in both genomic and SE platforms for forest trees will allow further exploration into the use of such technologies as CRISPR to enhance intensive varietal improvement methods.

Acknowledgments: We thank the support from Genome Quebec and Genome Canada.

References

Abrahamsson, M., Valladares, S., Merino, I., Larsson, E. and von Arnold, S. 2017 Degeneration pattern in somatic embryos of *Pinus sylvestris* L. *In vitro cellular & developmental biology. Plant : Journal of the Tissue Culture Association*, **53** (2), 86-96.

Adams, G.W., Kunze, H.A., MCartney, A., Millican, S. and Park, Y.-S. 2016 An industrial perspective on the use of advanced reforestation stock technologies. In *Vegetative Propagation of Forest Trees*. Y.S. Park, J.M. Bonga and H.K. Moon (eds.), National Institute of Forest Science (NiFos), Seoul.

Aguilar, I., Misztal, I., Johnson, D.L., Legarra, A., Tsuruta, S. and Lawlor, T.J. 2010 Hot topic: A unified approach to utilize phenotypic, full pedigree, and genomic information for genetic evaluation of Holstein final score. *Journal of Dairy Science*, **93** (2), 743-752.

Ameri, A., Davarynejad, G.H., Moshtaghi, N. and Tehranifar, A. 2018 Polyethylene glycol and chilling overcome Somatic embryogenesis obstacle in *Pyrus communis*. *Sci Hortic-Amsterdam*, **227**, 57-64.

Argout, X., Salse, J., Aury, J.M., Guiltinan, M.J., Droc, G., Gouzy, J. *et al.* 2011 The genome of *Theobroma cacao*. *Nat Genet*, **43** (2), 101-108.

Azaiez, A., Pavy, N., Gerardi, S., Laroche, J., Boyle, B., Gagnon, F. *et al.* 2018 A catalog of annotated high-confidence SNPs from exome capture and sequencing reveals highly polymorphic genes in Norway spruce (*Picea abies*). *BMC Genomics*, **19** (1), 942.

Azevedo Peixoto, L.d., Laviola, B.G., Alves, A.A., Rosado, T.B. and Bhering, L.L. 2017 Breeding *Jatropha curcas* by genomic selection: A pilot assessment of the accuracy of predictive models. *Plos One*, **12** (3), e0173368.

Baker, E.A.G., Wegrzyn, J.L., Sezen, U.U., Falk, T., Maloney, P.E., Vogler, D.R. *et al.* 2018 Comparative Transcriptomics Among Four White Pine Species. *G3 (Bethesda, Md.)*, **8** (5), 1461-1474.

Barghini, E., Natali, L., Cossu, R.M., Giordani, T., Pindo, M., Cattonaro, F. *et al.* 2014 The peculiar landscape of repetitive sequences in the olive (*Olea europaea* L.) genome. *Genome Biol Evol*, **6** (4), 776-791.

Bartholomé, J., Van Heerwaarden, J., Isik, F., Boury, C., Vidal, M., Plomion, C. *et al.* 2016 Performance of genomic prediction within and across generations in maritime pine. *BMC Genomics*, **17** (1), 604.

Beaulieu, J., Doerksen, T., Clément, S., Mackay, J. and Bousquet, J. 2014a Accuracy of genomic selection models in a large population of open-pollinated families in white spruce. *Heredity*, **113** (4), 343-352.

Beaulieu, J., Doerksen, T.K., MacKay, J., Rainville, A. and Bousquet, J. 2014b Genomic selection accuracies within and between environments and small breeding groups in white spruce. *BMC Genomics*, **15** (1), 1-16.

Bellot, P., de los Campos, G. and Pérez-Enciso, M. 2018 Can Deep Learning Improve Genomic Prediction of Complex Human Traits? *Genetics*, **210** (3), 809-819.

Ben Mahmoud, K., Muhovski, Y., Delporte, F., Jemmali, A. and Druart, P. 2018 Somatic Embryogenesis in Cherry (Prunus sp.). In *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants: Volume II*. S.M. Jain and P. Gupta (eds.), Springer International Publishing, Cham, pp. 113-127.

Bewg, W.P., Ci, D. and Tsai, C.-J. 2018 Genome Editing in Trees: From Multiple Repair Pathways to Long-Term Stability. *Frontiers in plant science*, **9**, 1732-1732.

Bianco, L., Cestaro, A., Sargent, D.J., Banchi, E., Derdak, S., Di Guardo, M. *et al.* 2014 Development and validation of a 20K single nucleotide polymorphism (SNP) whole genome genotyping array for apple (*Malus x domestica* Borkh). *Plos One*, **9** (10), e110377.

Birol, I., Raymond, A., Jackman, S.D., Pleasance, S., Coope, R., Taylor, G.A. *et al.* 2013 Assembling the 20 Gb white spruce (*Picea glauca*) genome from whole-genome shotgun sequencing data. *Bioinformatics*, **29** (12), 1492-1497.

Boocock, J., Chagne, D., Merriman, T.R. and Black, M.A. 2015 The distribution and impact of common copy-number variation in the genome of the domesticated apple, *Malus x domestica* Borkh. *BMC Genomics*, **16**, 848.

Borrelli, V.M.G., Brambilla, V., Rogowsky, P., Marocco, A. and Lanubile, A. 2018 The Enhancement of Plant Disease Resistance Using CRISPR/Cas9 Technology. *Frontiers in plant science*, **9**, 1245-1245.

Cabrera-Ponce, J.L., Valencia-Lozano, E. and Trejo-Saavedra, D.L. 2019 Chapter 3 - Genetic Modifications of Corn. In *Corn (Third Edition)*. S.O. Serna-Saldivar (ed.), AACC International Press, Oxford, pp. 43-85.

Cao, Y., Han, Y., Li, D., Lin, Y. and Cai, Y. 2016 MYB Transcription Factors in Chinese Pear (*Pyrus bretschneideri* Rehd.): Genome-Wide Identification, Classification, and Expression Profiling during Fruit Development. *Front Plant Sci*, **7**, 577.

Cao, Y., Han, Y., Meng, D., Li, D., Jiao, C., Jin, Q. *et al.* 2017 B-BOX genes: genome-wide identification, evolution and their contribution to pollen growth in pear (*Pyrus bretschneideri* Rehd.). *BMC Plant Biol*, **17** (1), 156.

Cao, Y., Han, Y., Meng, D., Li, G., Li, D., Abdullah, M. *et al.* 2017 Genome-Wide Analysis Suggests the Relaxed Purifying Selection Affect the Evolution of WOX Genes in *Pyrus bretschneideri*, *Prunus persica*, *Prunus mume*, and *Fragaria vesca*. *Front. Genet.*, **8** (78).

Cappa, E.P., El-Kassaby, Y.A., Munoz, F., Garcia, M.N., Villalba, P.V., Klapste, J. *et al.* 2017 Improving accuracy of breeding values by incorporating genomic information in spatial-competition mixed models. *Molecular Breeding*, **37** (10).

Cappa, E.P., El-Kassaby, Y.A., Muñoz, F., Garcia, M.N., Villalba, P.V., Klápště, J. *et al.* 2018 Genomic-based multiple-trait evaluation in *Eucalyptus grandis* using dominant DArT markers. *Plant Science*, **271**, 27-33.

Chagne, D., Crowhurst, R.N., Pindo, M., Thrimawithana, A., Deng, C., Ireland, H. *et al.* 2014 The draft genome sequence of European pear (*Pyrus communis* L. 'Bartlett'). *Plos One*, **9** (4), e92644.

Chen, C.H., Kuo, T.C., Yang, M.H., Chien, T.Y., Chu, M.J., Huang, L.C. *et al.* 2014 Identification of cucurbitacins and assembly of a draft genome for *Aquilaria agallocha*. *BMC Genomics*, **15**, 578.

Chen, Z.-Q., Baison, J., Pan, J., Karlsson, B., Andersson, B., Westin, J. *et al.* 2018 Accuracy of genomic selection for growth and wood quality traits in two control-pollinated progeny trials using exome capture as the genotyping platform in Norway spruce. *BMC Genomics*, **19** (1), 946.

Cornille, A., Gladieux, P., Smulders, M.J., Roldan-Ruiz, I., Laurens, F., Le Cam, B. *et al.* 2012 New insight into the history of domesticated apple: secondary contribution of the European wild apple to the genome of cultivated varieties. *PLoS Genet*, **8** (5), e1002703.

Corredoira, E., Vieitez, A.M. and Ballester, A. 2018 Eucalypts (*Eucalyptus globulus* Labill.). In *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants: Volume I.* S.M. Jain and P. Gupta (eds.), Springer International Publishing, Cham, pp. 269-282.

Dahrendorf, J., Clapham, D. and Egertsdotter, U. 2018 Analysis of Nitrogen Utilization Capability during the Proliferation and Maturation Phases of Norway Spruce (*Picea abies* (L.) H. Karst.) Somatic Embryogenesis. *Forests*, **9** (6), 288.

Dai, X., Hu, Q., Cai, Q., Feng, K., Ye, N., Tuskan, G.A. *et al.* 2014 The willow genome and divergent evolution from poplar after the common genome duplication. *Cell Res*, **24** (10), 1274-1277.

Deng, W., Luo, K., Li, Z. and Yang, Y. 2009 A novel method for induction of plant regeneration via somatic embryogenesis. *Plant Science*, **177** (1), 43-48.

Du, P., Kumar, M., Yao, Y., Xie, Q., Wang, J., Zhang, B. *et al.* 2016 Genome-wide analysis of the TPX2 family proteins in *Eucalyptus grandis*. *BMC Genomics*, **17** (1), 967.

Durán, R., Isik, F., Zapata-Valenzuela, J., Balocchi, C. and Valenzuela, S. 2017 Genomic predictions of breeding values in a cloned *Eucalyptus globulus* population in Chile. *Tree Genet. Genomes*, **13** (4), 74.

El-Kassaby, Y. and Klápště, J. Genomic selection and clonal forestry revival. IUFRO Unit 2.09. 02, pp. 98-100.

Elorriaga, E., Klocko, A.L., Ma, C. and Strauss, S.H. 2018 Variation in Mutation Spectra Among CRISPR/Cas9 Mutagenized Poplars. **9** (594).

Fan, C., Yao, H., Qiu, Z., Ma, H. and Zeng, B. 2018 Genome-wide analysis of *Eucalyptus grandis* WRKY genes family and their expression profiling in response to hormone and abiotic stress treatment. *Gene*, **678**, 38-48.

Fernandez i Marti, A. and Dodd, R.S. 2018 Using CRISPR as a Gene Editing Tool for Validating Adaptive Gene Function in Tree Landscape Genomics. *Front Plant Sci*, **6** (76).

Fuentes-Utrilla, P., Goswami, C., Cottrell, J.E., Pong-Wong, R., Law, A., A'Hara, S.W. *et al.* 2017 QTL analysis and genomic selection using RADseq derived markers in Sitka spruce: the potential utility of within family data. *Tree Genet. Genomes*, **13** (2), 33.

Gabay, G., Dahan, Y., Izhaki, Y., Faigenboim, A., Ben-Ari, G., Elkind, Y. *et al.* 2018 High-resolution genetic linkage map of European pear (*Pyrus communis*) and QTL fine-mapping of vegetative budbreak time. *BMC Plant Biol*, **18** (1), 175.

Gamal El-Dien, O., Ratcliffe, B., Klápště, J., Porth, I., Chen, C. and El-Kassaby, Y.A. 2016 Implementation of the Realized Genomic Relationship Matrix to Open-Pollinated White Spruce Family Testing for Disentangling Additive from Non-additive Genetic Effects. *G3: Genes|Genomes|Genetics*.

Gautier, F., Eliášová, K., Leplé, J.-C., Vondráková, Z., Lomenech, A.-M., Le Metté, C. *et al.* 2018 Repetitive somatic embryogenesis induced cytological and proteomic changes in embryogenic lines of *Pseudotsuga menziesii* [Mirb.]. *BMC Plant Biol*, **18** (1), 164.

Gonzalez-Camacho, J.M., Ornella, L., Perez-Rodriguez, P., Gianola, D., Dreisigacker, S. and Crossa, J. 2018 Applications of Machine Learning Methods to Genomic Selection in Breeding Wheat for Rust Resistance. *Plant Genome-Us*, **11** (2).

Gosik, K., Sun, L., Chinchilli, V.M. and Wu, R. 2018 An Ultrahigh-Dimensional Mapping Model of High-order Epistatic Networks for Complex Traits. *Current genomics*, **19** (5), 384-394.

Grattapaglia, D. 2017 Status and Perspectives of Genomic Selection in Forest Tree Breeding. In *Genomic Selection for Crop Improvement: New Molecular Breeding Strategies for Crop Improvement.* R.K. Varshney, M. Roorkiwal and M.E. Sorrells (eds.), Springer International Publishing, Cham, pp. 199-249.

Häggman, H., Sutela, S. and Welander, M. 2007 Micropropagation of *Betula pendula* Roth including Genetically modified Material. In *Protocols for Micropropagation of Woody Trees and Fruits.* S.M. Jain and H. Häggman (eds.), Springer Netherlands, Dordrecht, pp. 153-162.

Haque, E., Taniguchi, H., Hassan, M.M., Bhowmik, P., Karim, M.R., Śmiech, M. *et al.* 2018 Application of CRISPR/Cas9 Genome Editing Technology for the Improvement of Crops Cultivated in Tropical Climates: Recent Progress, Prospects, and Challenges. *Frontiers in plant science*, **9**, 617-617.

Harfouche, A., Meilan, R., Kirst, M., Morgante, M., Boerjan, W., Sabatti, M. *et al.* 2012 Accelerating the domestication of forest trees in a changing world. *Trends in Plant Science*, **17** (2), 64-72.

He, N., Zhang, C., Qi, X., Zhao, S., Tao, Y., Yang, G. *et al.* 2013 Draft genome sequence of the mulberry tree *Morus notabilis*. *Nat Commun*, **4**, 2445.

Hirakawa, H., Nakamura, Y., Kaneko, T., Isobe, S., Sakai, H., Kato, T. *et al.* 2011 Survey of the genetic information carried in the genome of *Eucalyptus camaldulensis*. *Plant Biotechnology*, **28** (5), 471-480.

Hodges, E., Xuan, Z., Balija, V., Kramer, M., Molla, M.N., Smith, S.W. *et al.* 2007 Genome-wide in situ exon capture for selective resequencing. *Nature Genetics*, **39**, 1522.

Holliday, J.A., Suren, H. and Aitken, S.N. 2012 Divergent selection and heterogeneous migration rates across the range of Sitka spruce (*Picea sitchensis*). *Proceedings. Biological sciences*, **279** (1734), 1675-1683.

Howe, G.T., Yu, J., Knaus, B., Cronn, R., Kolpak, S., Dolan, P. *et al.* 2013 A SNP resource for Douglas-fir: de novo transcriptome assembly and SNP detection and validation. *BMC Genomics*, **14**, 137.

Ipek, A., Ipek, M., Ercisli, S. and Tangu, N.A. 2017 Transcriptome-based SNP discovery by GBS and the construction of a genetic map for olive. *Funct Integr Genomics*, **17** (5), 493-501.

Isik, F. 2014 Genomic selection in forest tree breeding: the concept and an outlook to the future. *New Forests*, **45** (3), 379-401.

Isik, F., Bartholomé, J., Farjat, A., Chancerel, E., Raffin, A., Sanchez, L. *et al.* 2016 Genomic selection in maritime pine. *Plant Science*, **242**, 108-119.

Jaganathan, D., Ramasamy, K., Sellamuthu, G., Jayabalan, S. and Venkataraman, G. 2018 CRISPR for Crop Improvement: An Update Review. *Frontiers in plant science*, **9**, 985-985.

Kainer, D., Stone, E.A., Padovan, A., Foley, W.J. and Kulheim, C. 2018 Accuracy of Genomic Prediction for Foliar Terpene Traits in *Eucalyptus polybractea*. *G3-Genes Genom Genet*, **8** (8), 2573-2583.

Kaplan, A. 2016 *Genomic Selection and Genome-Wide Association Study in Populus trichocarpa and Pinus taeda (Master thesis)*, Virginia Tech.

Klimaszewska, K., Trontin, J.-F., Becwar, M.R., Devillard, C., Park, Y.-S. and Lelu-Walter, M.-A. 2007 Recent progress in somatic embryogenesis of four *Pinus* spp. *Tree For Sci Biotechnol*, **1** (1), 11-25.

Krishnan, N.M., Pattnaik, S., Jain, P., Gaur, P., Choudhary, R., Vaidyanathan, S. *et al.* 2012 A draft of the genome and four transcriptomes of a medicinal and pesticidal angiosperm *Azadirachta indica*. *BMC Genomics*, **13**, 464.

Kumar, S., Molloy, C., Munoz, P., Daetwyler, H., Chagne, D. and Volz, R. 2015 Genome-Enabled Estimates of Additive and Nonadditive Genetic Variances and Prediction of Apple Phenotypes Across Environments. *G3 (Bethesda, Md.)*, **5** (12), 2711-2718.

Kuravadi, N.A., Yenagi, V., Rangiah, K., Mahesh, H.B., Rajamani, A., Shirke, M.D. *et al.* 2015 Comprehensive analyses of genomes, transcriptomes and metabolites of neem tree. *PeerJ*, **3**, e1066.

Lau, N.-S., Makita, Y., Kawashima, M., Taylor, T.D., Kondo, S., Othman, A.S. *et al.* 2016 The rubber tree genome shows expansion of gene family associated with rubber biosynthesis. *Sci Rep-Uk*, **6**, 28594.

Legarra, A., Aguilar, I. and Misztal, I. 2009 A relationship matrix including full pedigree and genomic information. *J Dairy Sci*, **92**:4656-63.

Lelu-Walter, M.-A., Gautier, F., Eliášová, K., Sanchez, L., Teyssier, C., Lomenech, A.-M. *et al.* 2018 High gellan gum concentration and secondary somatic embryogenesis: two key factors to improve somatic embryo development in *Pseudotsuga menziesii* [Mirb.]. *Plant Cell, Tissue Organ Culture*, **132** (1), 137-155.

Lelu-Walter, M.-A., Klimaszewska, K., Miguel, C., Aronen, T., Hargreaves, C., Teyssier, C. *et al.* 2016 Somatic embryogenesis for more effective breeding and deployment of improved varieties in *Pinus* spp.: bottlenecks and recent advances. In *Somatic Embryogenesis: Fundamental Aspects and Applications*, Springer, pp. 319-365.

Lenz, P.R.N., Beaulieu, J., Mansfield, S.D., Clement, S., Desponts, M. and Bousquet, J. 2017 Factors affecting the accuracy of genomic selection for growth and wood quality traits in an advanced-breeding population of black spruce (*Picea mariana*). *BMC Genomics*, **18** (1), 335.

Li, X., Kui, L., Zhang, J., Xie, Y., Wang, L., Yan, Y. *et al.* 2016 Improved hybrid de novo genome assembly of domesticated apple (*Malus x domestica*). *Gigascience*, **5** (1), 35.

Li, Y. and Dungey, H.S. 2018 Expected benefit of genomic selection over forward selection in conifer breeding and deployment. *Plos One*, **13** (12), e0208232.

Lineros, Y., Balocchi, C., Muñoz, X., Sánchez, M. and Ríos, D. 2018 Cryopreservation of *Pinus radiata* embryogenic tissue: effects of cryoprotective pretreatments on maturation ability. *Plant Cell, Tissue Organ Culture*, **135** (2), 357-366.

Linlin, X., Xin, Q., Mingyue, Z. and Shaoling, Z. 2018 Genome-Wide analysis of aluminum-activated malate transporter family genes in six *rosaceae* species, and expression analysis and functional characterization on malate accumulation in Chinese white pear. *Plant Science*, **274**, 451-465.

Liu, J.-J., Schoettle, A.W., Sniezko, R.A., Sturrock, R.N., Zamany, A., Williams, H. *et al.* 2016 Genetic mapping of *Pinus flexilis* major gene (Cr4) for resistance to white pine blister rust using transcriptome-based SNP genotyping. *BMC Genomics*, **17** (1), 753.

Liu, Y. and Wang, D. Application of deep learning in genomic selection, 2017 IEEE International Conference on Bioinformatics and Biomedicine (BIBM). pp. 2280-2280.

Lotterhos, K.E., Yeaman, S., Degner, J., Aitken, S. and Hodgins, K.A. 2018 Modularity of genes involved in local adaptation to climate despite physical linkage. *Genome Biol Evol*, **19** (1), 157.

Ma, T., Wang, J., Zhou, G., Yue, Z., Hu, Q., Chen, Y. *et al.* 2013 Genomic insights into salt adaptation in a desert poplar. *Nat Commun*, **4**, 2797.

Ma, T., Wang, K., Hu, Q., Xi, Z., Wan, D., Wang, Q. *et al.* 2018 Ancient polymorphisms and divergence hitchhiking contribute to genomic islands of divergence within a poplar species complex. *Proc Natl Acad Sci U S A*, **115** (2), E236-E243.

Marrano, A., Martínez-García, P.J., Bianco, L., Sideli, G.M., Di Pierro, E.A., Leslie, C.A. *et al.* A new genomic tool for walnut (*Juglans regia* L.): development and validation of the high-density Axiom™ J. regia 700K SNP genotyping array. *Plant Biotechnology*, **0** (0).

Martínez, M.T., San José, M.C., Vieitez, A.M., Cernadas, M.J., Ballester, A. and Corredoira, E. 2017 Propagation of mature *Quercus ilex* L. (holm oak) trees by somatic embryogenesis. *Plant Cell, Tissue, Organ Culture*, **131** (2), 321-333.

Martinez-Garcia, P.J., Crepeau, M.W., Puiu, D., Gonzalez-Ibeas, D., Whalen, J., Stevens, K.A. *et al.* 2016 The walnut (*Juglans regia*) genome sequence reveals diversity in genes coding for the biosynthesis of non-structural polyphenols. *Plant J*, **87** (5), 507-532.

McElroy, M.S., Navarro, A.J.R., Mustiga, G., Stack, C., Gezan, S., Peña, G. *et al.* 2018 Prediction of Cacao (Theobroma cacao) Resistance to spp. Diseases via Genome-Wide Association Analysis and Genomic Selection. *Front Plant Sci*, **9**, 343.

Meuwissen, T.H.E., Hayes, B.J. and Goddard, M.E. 2001 Prediction of total genetic value using genome-wide dense marker maps. *Genetics*, **157** (4), 1819-1829.

Mignon, E. and Werbrouck, S. 2018 Somatic Embryogenesis as Key Technology for Shaping the Rubber Tree of the Future. *Front Plant Sci*, **9** (1804).

Ming, R., Hou, S., Feng, Y., Yu, Q., Dionne-Laporte, A., Saw, J.H. *et al.* 2008 The draft genome of the transgenic tropical fruit tree papaya (*Carica papaya* Linnaeus). *Nature*, **452** (7190), 991-996.

Montalbán, I.A. and Moncaleán, P. 2018 *Pinus radiata* (D. Don) Somatic Embryogenesis. In *Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants: Volume I.* S.M. Jain and P. Gupta (eds.), Springer International Publishing, Cham, pp. 1-11.

Myburg, A.A., Grattapaglia, D., Tuskan, G.A., Hellsten, U., Hayes, R.D., Grimwood, J. *et al.* 2014 The genome of *Eucalyptus grandis*. *Nature*, **510** (7505), 356-362.

Neale, D.B., Langley, C.H., Salzberg, S.L. and Wegrzyn, J.L. 2013 Open access to tree genomes: the path to a better forest. *Genome Biology*, **14** (6), 120.

Neale, D.B., Martínez-García, P.J., Torre, A.R.D.L., Montanari, S. and Wei, X.-X. 2017 Novel Insights into Tree Biology and Genome Evolution as Revealed Through Genomics. *Annu. Rev. Plant Biol.*, **68** (1), 457-483.

Neale, D.B., Wegrzyn, J.L., Stevens, K.A., Zimin, A.V., Puiu, D., Crepeau, M.W. *et al.* 2014 Decoding the massive genome of loblolly pine using haploid DNA and novel assembly strategies. *Genome Biology*, **15** (3), R59-R59.

Nystedt, B., Street, N.R., Wetterbom, A., Zuccolo, A., Lin, Y.C., Scofield, D.G. *et al.* 2013 The Norway spruce genome sequence and conifer genome evolution. *Nature*, **497** (7451), 579-584.

Parchman, T.L., Gompert, Z., Mudge, J., Schilkey, F.D., Benkman, C.W. and Buerkle, C.A. 2012 Genome-wide association genetics of an adaptive trait in lodgepole pine. *Mol Ecol*, **21** (12), 2991-3005.

Park, Y.-S., Beaulieu, J. and Bousquet, J. 2016 Multi-varietal forestry integrating genomic selection and somatic embryogenesis. *Vegetative Propagation of Forest Trees. Seoul: National Institute of Forest Science*, 302-322.

Pavy, N., Lamothe, M., Pelgas, B., Gagnon, F., Birol, I., Bohlmann, J. *et al.* 2017 A high-resolution reference genetic map positioning 8.8K genes for the conifer white spruce: structural genomics implications and correspondence with physical distance. *Plant Journal*, **90** (1), 189-203.

Pinto, G., Silva, S., Neves, L., Araújo, C. and Santos, C. 2010 Histocytological changes and reserve accumulation during somatic embryogenesis in *Eucalyptus globulus. Trees*, **24** (4), 763-769.

Plomion, C., Aury, J.M., Amselem, J., Alaeitabar, T., Barbe, V., Belser, C. *et al.* 2016 Decoding the oak genome: public release of sequence data, assembly, annotation and publication strategies. *Mol Ecol Resour*, **16** (1), 254-265.

Prakash, M.G. and Gurumurthi, K. 2009 Effects of type of explant and age, plant growth regulators and medium strength on somatic embryogenesis and plant regeneration in *Eucalyptus camaldulensis*. *Plant Cell, Tissue Organ Culture*, **100** (1), 13.

Rahman, A.Y., Usharraj, A.O., Misra, B.B., Thottathil, G.P., Jayasekaran, K., Feng, Y. *et al.* 2013 Draft genome sequence of the rubber tree *Hevea brasiliensis*. *BMC Genomics*, **14**, 75.

Ratcliffe, B., El-Dien, O.G., Klápště, J., Porth, I., Chen, C., Jaquish, B. *et al.* 2015 A comparison of genomic selection models across time in interior spruce (*Picea engelmannii* × *glauca)* using unordered SNP imputation methods. *Heredity*, **115** (6), 547-555.

Ratcliffe, B., Gamal El-Dien, O., Cappa, E.P., Porth, I., Klapste, J., Chen, C. *et al.* 2017 Single-Step BLUP with Varying Genotyping Effort in Open-Pollinated *Picea glauca*. *G3: Genes|Genomes|Genetics*.

Resende, M.D.V., Resende, M.F.R., Sansaloni, C.P., Petroli, C.D., Missiaggia, A.A., Aguiar, A.M. *et al.* 2012a Genomic selection for growth and wood quality in *Eucalyptus*: capturing the missing heritability and accelerating breeding for complex traits in forest trees. *New Phytol.*, **194** (1), 116-128.

Resende, M.F.R., Munoz, P., Acosta, J.J., Peter, G.F., Davis, J.M., Grattapaglia, D. *et al.* 2012b Accelerating the domestication of trees using genomic selection: accuracy of prediction models across ages and environments. *New Phytol.*, **193** (3), 617-624.

Resende, M.F.R., Munoz, P., Resende, M.D.V., Garrick, D.J., Fernando, R.L., Davis, J.M. *et al.* 2012c Accuracy of Genomic Selection Methods in a Standard Data Set of Loblolly Pine (*Pinus taeda* L.). *Genetics*, **190** (4), 1503.

Rosa, J.R.B.F., Mantello, C.C., Garcia, D., de Souza, L.M., da Silva, C.C., Gazaffi, R. *et al.* 2018 QTL detection for growth and latex production in a full-sib rubber tree population cultivated under suboptimal climate conditions. *BMC plant biology*, **18** (1), 223-223.

San-José, M.C., Corredoira, E., Martínez, M.T., Vidal, N., Valladares, S., Mallón, R. *et al.* 2010 Shoot apex explants for induction of somatic embryogenesis in mature *Quercus robur* L. trees. *Plant Cell Reports*, **29** (6), 661-671.

Sato, S., Hirakawa, H., Isobe, S., Fukai, E., Watanabe, A., Kato, M. *et al.* 2011 Sequence analysis of the genome of an oil-bearing tree, *Jatropha curcas* L. *DNA research : an international journal for rapid publication of reports on genes and genomes*, **18** (1), 65-76.

Schoettle, A.W., Sniezko, R.A., Kegley, A. and Burns, K.S. 2014 White pine blister rust resistance in limber pine: evidence for a major gene. *Phytopathology*, **104** (2), 163-173.

Seoane-Zonjic, P., Cañas, R.A., Bautista, R., Gómez-Maldonado, J., Arrillaga, I., Fernández-Pozo, N. *et al.* 2016 Establishing gene models from the *Pinus pinaster* genome using gene capture and BAC sequencing. *BMC Genomics*, **17**, 148-148.

Slavov, G.T., DiFazio, S.P., Martin, J., Schackwitz, W., Muchero, W., Rodgers-Melnick, E. *et al.* 2012 Genome resequencing reveals multiscale geographic structure and extensive linkage disequilibrium in the forest tree *Populus trichocarpa*. *New Phytol*, **196** (3), 713-725.

Solórzano-Cascante, P., Sánchez-Chiang, N. and Jiménez, V.M. 2018 Explant Type, Culture System, 6-Benzyladenine, Meta-Topolin and Encapsulation Affect Indirect Somatic Embryogenesis and Regeneration in L. *Front Plant Sci*, **9**, 1769.

Sork, V.L., Fitz-Gibbon, S.T., Puiu, D., Crepeau, M., Gugger, P.F., Sherman, R. *et al.* 2016 First Draft Assembly and Annotation of the Genome of a California Endemic Oak Née (*Fagaceae*). *G3 (Bethesda, Md.)*, **6** (11), 3485-3495.

Stevens, K.A., Wegrzyn, J.L., Zimin, A., Puiu, D., Crepeau, M., Cardeno, C. *et al.* 2016 Sequence of the Sugar Pine Megagenome. *Genetics*, **204** (4), 1613-1626.

Suren, H., Hodgins, K.A., Yeaman, S., Nurkowski, K.A., Smets, P., Rieseberg, L.H. *et al.* 2016 Exome capture from the spruce and pine giga-genomes. *Molecular ecology resources*, **16** (5), 1136-1146.

Talbot, B., Chen, T.W., Zimmerman, S., Joost, S., Eckert, A.J., Crow, T.M. *et al.* 2017 Combining Genotype, Phenotype, and Environment to Infer Potential Candidate Genes. *J Hered*, **108** (2), 207-216.

Tan, B., Grattapaglia, D., Wu, H.X. and Ingvarsson, P.K. 2018 Genomic relationships reveal significant dominance effects for growth in hybrid *Eucalyptus*. *Plant Science*, **267**, 84-93.

Tang, C., Yang, M., Fang, Y., Luo, Y., Gao, S., Xiao, X. *et al.* 2016 The rubber tree genome reveals new insights into rubber production and species adaptation. *Nature plants*, **2** (6), 16073.

Telfer, E., Graham, N., Macdonald, L., Sturrock, S., Wilcox, P. and Stanbra, L. 2018 Approaches to variant discovery for conifer transcriptome sequencing. *Plos One*, **13** (11), e0205835.

Theriault, G., Michael, P. and Nkongolo, K. 2016 Comprehensive Transcriptome Analysis of Response to Nickel Stress in White Birch (*Betula papyrifera*). *Plos One*, **11** (4), e0153762-e0153762.

Thistlethwaite, F.R., Ratcliffe, B., Klapste, J., Porth, I., Chen, C., Stoehr, M.U. *et al.* 2017 Genomic prediction accuracies in space and time for height and wood density of Douglas-fir using exome capture as the genotyping platform. *BMC Genomics*, **18**.

Thistlethwaite, F.R., Ratcliffe, B., Klapste, J., Porth, I., Chen, C., Stoehr, M.U. *et al.* 2019 Genomic selection of juvenile height across a single-generational gap in Douglas-fir. *Heredity*. https://doi.org/10.1038/s41437-018-0172-0

Trontin, J.-F., Aronen, T., Hargreaves, C., Montalbán, I.A., Moncaleán, P., Reeves, C. *et al.* 2016 International effort to induce somatic embryogenesis in adult pine trees. *Vegetative Propagation of Forest Trees. National Institute of Forest Science . Seoul, Korea*, 211-260.

Trontin, J.-F., Teyssier, C., Morel, A., Harvengt, L. and Lelu-Walter, M.-A. 2016 Prospects for new variety deployment through somatic embryogenesis in maritime pine. *Vegetative Propagation of Forest Trees. National Institute of Forest Science . Seoul, Korea*, **1**, 572-606.

Tuskan, G.A., Difazio, S., Jansson, S., Bohlmann, J., Grigoriev, I., Hellsten, U. *et al.* 2006 The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science*, **313** (5793), 1596-1604.

Varis, S., Klimaszewska, K. and Aronen, T. 2018 Somatic Embryogenesis and Plant Regeneration From Primordial Shoot Explants of *Picea abies* (L.) H. Karst. Somatic Trees. *Front Plant Sci*, **9** (1551).

Velasco, R., Zharkikh, A., Affourtit, J., Dhingra, A., Cestaro, A., Kalyanaraman, A. *et al.* 2010 The genome of the domesticated apple (*Malus x domestica* Borkh.). *Nat Genet*, **42** (10), 833-839.

Verde, I., Abbott, A.G., Scalabrin, S., Jung, S., Shu, S., Marroni, F. *et al.* 2013 The high-quality draft genome of peach (*Prunus persica*) identifies unique patterns of genetic diversity, domestication and genome evolution. *Nat Genet*, **45** (5), 487-494.

Vidal, N.M., Grazziotin, A.L., Ramos, H.C.C., Pereira, M.G. and Venancio, T.M. 2014 Development of a Gene-Centered SSR Atlas as a Resource for Papaya (*Carica papaya*) Marker-Assisted Selection and Population Genetic Studies. *Plos One*, **9** (11), e112654.

Wang, H., Gong, M., Guo, J., Xin, H., Gao, Y., Liu, C. *et al.* 2018 Genome-wide Identification of *Jatropha curcas* MAPK, MAPKK, and MAPKKK Gene Families and Their Expression Profile Under Cold Stress. *Sci Rep-Uk*, **8** (1), 16163.

Wang, N., Thomson, M., Bodles, W.J.A., Crawford, R.M.M., Hunt, H.V., Featherstone, A.W. *et al.* 2013 Genome sequence of dwarf birch (Betula nana) and cross-species RAD markers. *Molecular Ecology*, **22** (11), 3098-3111.

Warren, R.L., Keeling, C.I., Yuen, M.M., Raymond, A., Taylor, G.A., Vandervalk, B.P. *et al.* 2015 Improved white spruce (*Picea glauca*) genome assemblies and annotation of large gene families of conifer terpenoid and phenolic defense metabolism. *Plant J*, **83** (2), 189-212.

Wegrzyn, J.L., Liechty, J.D., Stevens, K.A., Wu, L.S., Loopstra, C.A., Vasquez-Gross, H.A. *et al.* 2014 Unique features of the loblolly pine (*Pinus taeda* L.) megagenome revealed through sequence annotation. *Genetics*, **196** (3), 891-909.

Wen, Y., Zou, Z., Li, H., Xiang, Z. and He, N. 2017 Analysis of codon usage patterns in *Morus notabilis* based on genome and transcriptome data. *Genome*, **60** (6), 473-484.

Wu, J., Wang, Z., Shi, Z., Zhang, S., Ming, R., Zhu, S. *et al.* 2013 The genome of the pear (*Pyrus bretschneideri* Rehd.). *Genome Res*, **23** (2), 396-408.

Yasodha, R., Vasudeva, R., Balakrishnan, S., Sakthi, A.R., Abel, N., Binai, N. *et al.* 2018 Draft genome of a high value tropical timber tree, Teak (*Tectona grandis* L. f): insights into SSR diversity, phylogeny and conservation. *DNA research : an international journal for rapid publication of reports on genes and genomes*, **25** (4), 409-419.

Zapata-Valenzuela, J., Isik, F., Maltecca, C., Wegrzyn, J., Neale, D., McKeand, S. *et al.* 2012 SNP markers trace familial linkages in a cloned population of *Pinus taeda*—prospects for genomic selection. **8** (6), 1307-1318.

Zapata-Valenzuela, J., Whetten, R.W., Neale, D., McKeand, S. and Isik, F. 2013 Genomic Estimated Breeding Values Using Genomic Relationship Matrices in a Cloned Population of Loblolly Pine. *G3: Genes|Genomes|Genetics*, **3** (5), 909-916.

Zhang, J., Jia, H., Li, J., Li, Y., Lu, M. and Hu, J. 2016 Molecular evolution and expression divergence of the Populus euphratica Hsf genes provide insight into the stress acclimation of desert poplar. *Sci Rep-Uk*, **6**, 30050.

Zhang, Q., Chen, W., Sun, L., Zhao, F., Huang, B., Yang, W. *et al.* 2012 The genome of *Prunus mume*. *Nat Commun*, **3**, 1318.

Zhang, S., Tong, Y., Li, Y., Cheng, Z.-M. and Zhong, Y. 2018 Genome-wide identification of the HKT genes in five *Rosaceae* species and expression analysis of HKT genes in response to salt-stress in *Fragaria vesca*. *Genes Genomics*.

Zimin, A., Stevens, K.A., Crepeau, M.W., Holtz-Morris, A., Koriabine, M., Marcais, G. *et al.* 2014 Sequencing and assembly of the 22-gb loblolly pine genome. *Genetics*, **196** (3), 875-890.

Induction of embryogenic calli in BC1 zygotic embryos (*Elaeis oleifera* x *Elaeis guineensis*) x *Elaeis guineensis*

[1]Cibelle A. dos Santos, [1]Pamela K. Harada, [1]Ricardo Lopes, [2]Regina C. Quisen

[1]Embrapa Western Amazon, Rodovia AM 010, km 29, 69010-970, Manaus, Amazonas, Brazil
[2]Embrapa Forestry, Estrada da Ribeira, km 111, 83411-000, Colombo, Paraná, Brazil
regina.quisen@embrapa.br

Abstract

The present study aimed to evaluate the induction and proliferation of embryogenic calli in BC1 progenies zygotic embryos ((OxG) x oil palm). Embryogenic calli were induced from zygotic embryos on Murashige and Skoog (MS) and Eeuwens (Y3) medium with 2,4-dichlorophenoxyacetic acid (450 µM) and picloram (450 µM). After 90 days the calli were transferred to callus proliferation medium that consisted of basal MS and Y3 with 40 µM of 2,4-D or picloram combined with 2iP (0 and 10 µM). After cultivating in induction medium, the cultures showed primary calli formation and no significant statistical difference was observed for any of the parameters evaluated. The reduction of the auxin level from 450 to 40 µM was crucial to the differentiation of cells with embryogenic characteristics after 150 days. The highest proliferation rate in terms of primary calli growth was obtained on multiplication medium supplemented with picloram (40 µM) and 2iP (0 and 10 µM) either in MS or Y3 medium. This arrangement also resulted in the formation of potentially embryogenic structures.

Keywords: American oil palm, Oil palm, *in vitro* propagation, somatic embryogenesis.

Introduction

The American oil palm or caiaué (*Elaeis oleifera* Kunth Cortés) species is currently being used in oil palm breeding programs due to some agronomic traits of great interest such as a reduced vertical growth, a high content of unsaturated fatty acids and resistance to fatal yellowing, one the most important diseases affecting oil palm plantations in Central and South America. Therefore, the Oil Palm Genetic Improvement Program belonging to Embrapa Western Amazon has been strongly focused on the development of improved commercial varieties resulting from the hybridization of the African oil palm (*E. guineensis* Jacq.) with the American oil palm. The hybridization via backcrossing of *E. oleifera* and *E. guianeensis* is an important method to combine valuable traits of the parents OxG interspecific hybrid F1 (IH) and backcrossing with *E. guianeensis* (recurrent parent), which generates backcrossing progenies (BC) of high variability (Rios et al. 2015; Sumaryono et al. 2018).

For the genus *Elaeis*, as for most palm species, somatic embryogenesis (SE) holds promise as a technique for clonal propagation, being considered in the breeding program as an important biotechnological tool that allows the clonal propagation of BC progenies to be used in the amplification of the genetic base tested in the field crossings. However, oil palm SE is slow, inefficient and genotypically dependent, which makes optimization of the regeneration system essential. This requires the establishment and refinement of specific protocols that consider such factors as basal medium, plant growth regulators, explant source and genotype. In this present study, the zygotic embryos of BC1

progenies of IH (F1) x oil palm were cultured on MS and Y3 media containing 2,4-D and picloram and the proliferation of embryogenic calli was evaluated.

Material and methods

Mature seeds were obtained from Embrapa Western Amazon, Rio Preto da Eva, Amazonas, Brazil. Zygotic embryos (ZE) were excised from mature seeds of BC1 progenies of HIE (F1) x oil palm. The seeds were surface-sterilizated in 50% (v/v) commercial bleach (2.0-2.5% sodium hypochlorite) for 15 min, washed and the endocarp was then removed. The nuts were sterilizated in 70% alcohol for 3 min, the embryos were excised under aseptic conditions with a scalpel and surface-sterilizated in 5% (v/v) commercial bleach (2,0-2,5% sodium hypochlorite) for 10 min and rinsed three times in sterilized water and then cultured on callus induction media.

The callus induction was performed in 9-cm diameter Petri dishes sealed with transparent plastic film and contained 25 mL induction medium that consisted of salts and vitamins of Murashige and Skoog (1962) or Y3 (Eeuwens 1976) supplemented by 500 mg L^{-1} hydrolyzed casein, 100 mg L^{-1} glutamine, 2.5 g L^{-1} activated charcoal, 30 g L^{-1} sucrose and 450 µM 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram. The pH of media was adjusted to 5.8 and 7.0% agar (Sigma®) was added before autoclaving for 15 min at 121°C. The cultures were subcultured every month and after 90 days, the calli were transferred to callus proliferation/differentiation medium of the same basal composition but containing either 2,4-D (40 µM) or picloram (40 µM) combined with 2-isopenteniladenine (2iP) (0 and 10 µM). The cultures were maintained in the dark at 26±2ºC and at a relative humidity of 60-70%. A completely randomized experimental design was used in a 2 x 2 factorial arrangement with 5-6 replications (5 explants per Petri dish). There were two levels of the first factor (basal medium) and two levels of the second factor (growth regulator).

The cultures were subcultured every 30 days in fresh culture medium and evaluated at the end of 120 days considering the following parameters: explants with presence/absence of callus and typology (visual evaluation), being Type I - primary calli, Type II - calli with embryogenic characteristics (nodular / globular structures), and Type III - calli with non-embryogenic characteristics (compact or abnormal structures). Data were arcsin-square transformed to achieve homogeneity of variances and then submitted to analysis of variance (ANOVA). Means were compared by Tukey's test ($P \leq 0.05$).

Results and discussion

After 10 days of culture on the callus induction medium, it was observed that the zygotic embryos started to swell. After four weeks, the first primary calli were visible, mainly at the distal region of the explant, corresponding to the cotyledonary region of the ZE of the genus *Elaeis*. The calli were small cellular masses, opaque white in color, most of the time after expansion and rupture and opening of the region of the (proximal) base of the explant.

At the end of the multiplication and differentiation phase, it was possible to characterize the calli masses according to the color and three types of cell structures were visualized. The primary calli (Type I) presented a clear and translucent color, they consisted of small, apparently more organized, cellular clusters. Calli with embryogenic characteristics (Type II) showed beige and light white coloration, with nodular or globular cell structures. The non-embryogenic calli (Type III), presented compact white or yellow, bright and transparent structures of elongated shape, not always cohesive.

At this stage of somatic embryogenesis, where the differentiation of cells with embryogenic characteristics occurs, picloram was superior to 2,4-D for all embryogenic typologies, except for non-embryogenic calli (Table 1), promoting a greater induction of primary nodular and globular calli. This

superiority of picloram in the embryogenic processes has also been described in studies dealing with some Arecaceae palms, such as *Areca catechu* (Karun et al. 2004), *Bactris gasipaes* (Steinmacher et al. 2007) and *Acrocomia aculeata* (Moura et al. 2008). According to Fitch and Moore (1990), this behavior may be associated with an effective absorption and mobilization of this auxin, with rapid metabolization in the target cells.

Table 1. *Calli typology induced by mature zygotic embryos obtained from BC1 progenies of IH (F1) x oil palm after 150 days in vitro, in two different basal media (MS and Y3) supplemented with 2,4-D or Picloram (Pic). Manaus, Brazil, 2017.*

	Type I (%)			Type II (%)			Type III (%)		
	2,4-D	Pic	mean	2,4-D	Pic	mean	2,4-D	Pic	mean
MS	36,0 Aa	40,0 Ab	38,0 a	8,0 Ba	32,0 Aa	20,0 a	24,0 a	28,0 a	26,0 a
Y3	20,0 Ba	64,0 Aa	42,0 a	8,0 Ba	37,6 Aa	22,8 a	24,0 a	36,8 a	30,4 a
mean	28,0 B	52,0 A		8,0 B	34,8 A		24,0 A	32,4 A	

Means followed by the same uppercase letter in a column and lowercase in rows, do not differ statistically amongst themselves (P≤ 0.05) by Tukey's test. Type I - primary callus, II - embryogenic (nodular/globular), III - non-embryogenic.

Despite the wide variation in the percentage of embryogenic calli of Type II induced, i.e., between 8.0 and 37.6%, the alteration in the hormonal balance in the culture medium, the reduction of the auxins and the supplementation with 2iP at 10 μM stimulated the growth and differentiation of globular and/or nodular calli. Steinmacher et al. (2007) and other authors agree that in palm trees the transfer of cultures to a lower auxin concentration is a key factor for the differentiation and later development of somatic embryos (Karun et al. 2004).

Regarding the basal medium, there was no significant difference between these with respect to calli formation, despite quite variable percentages (Table 1). Among the averages obtained, it was observed that the Y3 medium had higher percentages than MS, except for globular callus. In the interaction between the factors basal medium and growth regulators in the formation of Type I and II calli, a significant difference was observed between the means, reaching higher values for both (MS and Y3) in the presence of the Picloram, with the Y3 means in the primary callogenesis (64%) being the highest. For non-embryogenic calli, there was no significant difference in the interaction, with varying percentages between 24 and 36.8% (Table 1).

Conclusions

The results obtained by the conditions used in the present study allow us to conclude that:

1. The basal medium MS and Y3 presented similar performance throughout the evaluated phases, while the auxins 2,4-D and Picloram differentially influenced the proliferation and differentiation of potentially embryogenic structures;
2. The basal medium MS and Y3 supplemented with 2,4-D or Picloram satisfactorily induced primary calli formation after 90 days of culture; and
3. The basal medium supplemented with 2iP and the auxin Picloram at 40 μM favored the multiplication of primary calli, as well as the formation of potentially embryogenic structures, either in MS or Y3 medium.

References

Eeuwens CJ (1976) Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palms (*Cocos nucifera* L.) and culture in vitro. Physiol Plant 36:23-28

Fitch MMM, Moore PH (1990) Comparison of 2,4-D and picloram for selection of long-term totipotent green callus of sugarcane. Plant Cell Tissue Organ Cult 20:157-163

Karun A, Siril EA, Radham E, Parthasarathy VA (2004) Somatic embryogenesis and plantlet regeneration from leaf and inflorescence explants of areca nut (*Areca catechu* L.). Curr Sci 86:1623-1628

Moura, EF, Ventrella MC, Motoike SY, Sá Júnior AQ, Carvalho M, Manfio CE (2008) Histological study of somatic embryogenesis induction on zygotic embryos of macaw palm (*Acrocomia aculeate* (Jacq.) Lodd. ex Martius). Plant Cell Tissue Organ Cult 95:175–184

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497

Rios SA, Cunha RNV Lopes R, Barcelos E, Lima WAA, Rodrigues MRL, Krug C, Bittencourt DMC, Quisen RC, Gomes Junior RA, Rocha RNC (2015) Caiaué. Lopes R, Oliveira M do SP de, Cavallari MM Conceição LDHCS da (Eds). Palmeira Nativas do Brasil. Embrapa. Brasília, Brazil. pp 211-267

Steinmacher DA, Cangahuala-Inocente GC, Clement CR, Guerra MP (2007) Somatic embryogenesis from peach palm zygotic embryos. In Vitro Cell Dev Biol – Plant 43:124–132

Sumaryono, Riyadi I, Saptari RT, Rahmadi HY, Ernayunita (2018) Embryogenic callus initiation from leaf explants of Elaeis oleifera x Elaeis guineensis (OxG) hybrids. IOP Conf. Ser.: Earth Environ. Sci. 183 012009

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Effect of sucrose supplementation and culture system on growth and stress status of *Prunus domestica* and *Castanea sativa* x *C. crenata* shoots

Diego Gago[1,2], Mª Ángeles Bernal[2], Conchi Sánchez[1], Anxela Aldrey[1], Beatriz Cuenca[3], Nieves Vidal[1]

[1] Instituto de Investigaciones Agrobiológicas de Galicia. CSIC. Santiago de Compostela, Spain (nieves@iiag.csic.es); [2] Universidade de Coruña, A Coruña, Spain; [3] Viveiro de Maceda, TRAGSA, Ourense, Spain.

Abstract

Micropropagated plants are frequently cultured in photomixotrophic conditions: under low light intensity, with limited CO_2 availability, and using simple sugars (as sucrose) as a carbon source. The supplementation of sucrose does not only affect growth, but it is also involved in oxidative stress regulation and can influence acclimation and ex vitro performance. The aim of this study was to propagate two woody plants (plum and chestnut) with different concentrations of sucrose to gain a first insight in the ability of this sugar to induce changes in the level of phenolic and antioxidant compounds. After culturing the shoots in liquid medium in photomixotrophic or photoautotrophic conditions growth parameters were recorded and phenolics and total antioxidants were quantified. In the first experiments, the initial explants were obtained from shoots grown in semisolid medium (SSM) with 3% sucrose (w/v) for both species. Then, plum shoots were cultured in liquid medium (LM) by temporary immersion, using RITA® bioreactors, whereas chestnut shoots were propagated in LM by temporary or continuous immersion, using planform™ and 6 L vessels adapted from food containers, respectively. Three concentrations of sucrose were evaluated (0, 1 and 3% in plum and 0.5, 1 and 3% in chestnut). The bioreactors were placed either under conventional lights providing a photosynthetic photon flux density (PPF) of 50 µmol m^{-2} s^{-1} or under white LEDs to increase PPF to 150 µmol m^{-2} s^{-1}. In this latter case, CO_2-enriched air was provided. In subsequent experiments, the explants were obtained from shoots cultured in SSM with 3% sucrose or from shoots cultured in LM with different sucrose concentrations. The results obtained in plum suggest a direct relationship between the sucrose supplementation and the content of phenolic compounds, the antioxidant activity and the morphological development reached for the explants. The explants that performed better were those cultured with 3% sucrose under high PPF, followed by those cultured with 1% sucrose in the same conditions. In chestnut, the preliminary results indicate that phenolic compounds and antioxidant activities decreased with sucrose increment, showing the opposite trend than the one observed previously in plum. New experiments are in progress in order to investigate the discrepancy found between the two species.

Keywords: antioxidants, bioreactors, chestnut, phenolics, plum

Introduction

The acclimation step remains a critical stage for the large-scale application of micropropagation of woody plants. Micropropagated plants are frequently cultured in photomixotrophic conditions: low light intensity, with limited CO_2 availability, and using sugars as a carbon source. During acclimation, plants

have to attain a proper stomatal functionality as well as to adapt their metabolism to the ex vitro photoautotrophic conditions.
Sucrose is used as a generic energy source in the *in vitro* culture systems (Yaseen et al. 2013), but it is also considered as the main substrate for bacteria and fungi, which tend to invade the cultures. For this reason, the reduction of sucrose content in the culture medium decreases the contamination rate, improving the production (Martin, 2004). High sucrose concentrations can cause hyperhydricity and other disorders (González et al. 2011). It has been claimed that the elimination of sugar from nutritive media during micropropagation promotes photosynthetic activity, produces a healthier physiological state and increases the adaptation of the plantlets to greenhouse conditions (Xiao et al. 2011). However, lowering sucrose supplementation alone usually reduces the growth of the explants, and for this reason most of the laboratories still add sucrose to the culture medium, as a source of carbon and energy. To counterbalance the effects of the decrease of sucrose supply on the metabolism of the plants, it is necessary to provide the factors that enhance the photosynthetic activity inside the flasks: high light intensity and CO_2. In photoautotrophic systems, this gas is provided mainly by applying forced ventilation to the containers (Nguyen et al., 2016, Saldanha et al., 2014; Xiao et al., 2011).

In normal conditions, free radicals are produced in small numbers by the plant metabolism. These free radicals are highly reactive and potentially harmful compounds. Under stress conditions, these compounds increase, producing an unbalance between the free radicals and the enzymes that are responsible for their control, damaging lipidic walls, proteins and DNA, and disturbing the normal functioning of the plant (Demidchik, 2015).

Antioxidants play an important role in the defensive system of the plant during oxidative stress situations, acting as an effective barrier against free radicals. For this reason, their levels in the plant can indicate the physiological state of the cultures. Total soluble phenolic compounds have been investigated in a large number of species and tissues, due to their involvement in signaling and their relevant role in the control of the oxidative stress in plants. These compounds have both antioxidant and antimutagenic properties (Kubola and Siriamornpun, 2008).

The aim of this study was to explore the possible benefits of decreasing sucrose supplementation during the micropropagation of woody plants. For that, we used shoots from a *Prunus domestica* tree, cv. "Claudia Blanca País", and from two clones of hybrid chestnut (*Castanea sativa* x *C. crenata*). These explants were cultured in different conditions of light, ventilation and sucrose supplementation, in order to evaluate their growth response by morphological parameters and their stress status in terms of the total phenolic content and the antioxidant activity.

Materials and Methods

Plant material and standard culture conditions

Stock cultures of plum "Claudia Blanca País" (CBP) were maintained in 50 ml of semisolid medium (SSM) in 300 ml glass jars with Murashige and Skoog medium (MS; Murashige and Skoog 1962) supplemented with 0.5 mg/L of N^6-benzyladenine (BA), 0.5 mg/L of indole-3-butyric acid (IBA), 3% sucrose and 0.7 % (w/v) agar Vitroagar (Pronadisa, Spain). For the propagation of chestnut two genotypes resistant to ink disease were used. The clones "P042" and "C053" were selected in Galicia (Spain) by TRAGSA, and were characterized as natural hybrids of *Castanea sativa* x *C. crenata*. These shoots were maintained in SSM in test tubes containing Gresshoff and Doy (GD) medium (Gresshoff and Doy 1972) supplemented with 0.1 mg/L BA and 3% sucrose. All media were adjusted to pH 5.7 before being autoclaved at 120 °C for 20 min. Cultures were incubated under a 16-h photoperiod provided by cool-white fluorescent lamps (50-60 µmol $m^{-2}s^{-1}$) at 25 ˚C light/20 ˚C dark (standard conditions). The explants were subcultured every four-five weeks.

Culture in photoautotrophic or photomixotrophic conditions

For the experiments performed with plum, shoots cultured in jars (1.5 cm) were inoculated in RITA® bioreactors (www.vitropic.fr). Shoots were cultured by temporary immersion with liquid medium of the same composition as the medium used for maintenance of stock cultures but devoid of agar. Photoautotrophic or photomixotrophic (standard) conditions are specified in Table 1. These conditions were set by using a prototype described in Vidal et al. (2017), which provide different levels of light, sucrose supplementation and immersions. After a 5-week period, shoots were evaluated and used as explants to initiate a new cycle of culture in bioreactors under the same conditions. Shoots cultured in SSM in standard conditions were used as control.

***Table 1**. Culture conditions used in the experiments with plum.*

Bioreactor	Claudia Blanca País (CBP) System: Temporary immersion Conditions: Standard or Photoautotrophic Micropropagation (PAM)			
RITA®	Conditions	Standard		
	Air quality	Standard		
	Light	PPF: 50-60 µmol m^{-2} s^{-1}	Sucrose	0 %
	Nº immersions/day; duration	16; 1 min		1%
	Nº aerations/day; duration	0		3%
	Conditions	Photoautotrophic Micropropagation (PAM)		
	Air quality	CO_2-enriched air (2000 ppm)		
	Light	PPF: 100-150 µmol m^{-2} s^{-1}	Sucrose	0 %
	Nº immersions/day; duration	16; 1 min		1%
	Nº aerations/day; duration	0		3%

For the experiments performed with chestnut, shoots maintained in tubes were cultured in liquid medium by two approaches. Clone P042 was cultured by temporary immersion in commercial plantform™ bioreactors (Welander et al. 2014) as described in Vidal et al. (2015), whereas the clone C053 was cultured by continuous immersion in in-house 6 L bioreactors prepared as previously described (Cuenca et al. 2017). In both cases MS containing half strength nitrates (MSN½) and supplemented with 0.05 mg/L BA was used. Rockwool cubes (1 cm^3) were used as support material. Photoautotrophic conditions were set by using high light intensity, with photosynthetic photon flux densities (PPF) around 100-150 µmol m^{-2} s^{-1} and CO_2-enriched air (2000 ppm). Sucrose supplementation of each treatment, as well as the number and duration of immersions and additional aeration are specified in Table 2. After a 6-week period, shoots were harvested and evaluated.

The following variables were used for evaluation: 1) nº number of shoots/explant, 2) the length of the longest shoot per explant, 3) the length and width of the largest leaf per explant,4) total soluble phenolic compounds, and 5) antioxidant compounds.

Determination of soluble phenolic compounds and antioxidants

Extraction was performed following the method described by Díaz et al. (2001). Briefly, apical leaves of plum or chestnut shoots were weighted (50-100 mg) and homogenized with 5 ml of methanol 80%. The mixture was centrifuged at 10000 g for 5 min and the supernatant was used for the analysis of phenolic compounds and antioxidants. Phenolic compounds were quantified by the Folic-Ciocalteu method (Singleton and Rossi, 1965). Results were expressed as mg of equivalents of gallic acid per g of plant material, on a fresh weight basis. Antioxidants were determined by the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay (Blois 1958). Results were expressed as µmoles of Trolox equivalents per g of sample on a fresh weight basis.

***Table 2**. Culture conditions used in the experiments with chestnut*

Bioreactor	Chestnut System: Temporary or Continuous immersion Conditions: Photoautotrophic Micropropagation (PAM)			
Plantform™	System	Temporary immersion		
	Air quality	CO_2-enriched air (2000 ppm)		
	Light	PPF: 100-150 µmol m^{-2} s^{-1}	Sucrose	0.5 %
	Nº immersions/day;	16; 1 min		1%
	Nº aerations/day; duration	16; 1 min		3%
C-6	System	Continuous immersion (basal sections of		
	Air quality	CO_2-enriched air (2000 ppm)		
	Light	PPF: 100-150 µmol m^{-2} s^{-1}	Sucrose	0.5 %
	Nº immersions/day;	Continuous immersion		1%
	Nº aerations/day; duration	16; 1 min		3%

Results and discussion

Plum

The results of culturing CBP shoots in the conditions described in Table 1 are shown in Fig. 1 and Fig. 2. Healthy shoots were obtained in jars (Fig. 1A, B) and in RITA® containers (Fig. 1C) in most of the treatments (Fig. 1D-I). In RITA®, CBP shoots could be successfully cultured with 1% or 3% of sucrose in both standard and photoautotrophic conditions (Fig. 1E, F, H, I). However, the complete elimination of sugar prevented the growth under standard conditions (Fig. 1G), and limited growth was observed when high light and CO_2-enriched air were supplied (Fig. 1D). Only a small number of short shoots were obtained under these treatments (Fig. 2). Most of the explants that survived without sucrose developed hyperhydricity, which made them unsuitable for re-culturing. A certain degree of hyperhydricity was also shown by the shoots cultured in the other treatments. This disorder had not been detected in previous experiments with CBP immersed three times a day for 1 min (data not published). In this study, with the aim of increasing the CO_2 availability, the frequency of immersions was set at 16 times per day, but probably this frequency resulted too high. The relationship of hyperhydricity with high immersion frequencies was reported in other woody plants as pistachio (Akdemir et al. 2014).

Although there are reports of plants successfully propagated without sugar supplementation (Xiao et al. 2011), CBP shoots could not be cultured in medium devoid of sucrose. The limited growth shown by

explants cultured in PAM conditions without sugar could be partly related with the size of the explants (1.5 cm). In chestnut, it was necessary to increase the size of explants and the number of leaves to obtain good results without sucrose supplementation in PAM conditions (Vidal et al. 2017). In that study, the authors also stressed the beneficial effect of lowering sucrose supplementation in a progressive manner on the response of chestnut shoots to photoautotrophic conditions. It may be that this habituation is also necessary in plum.

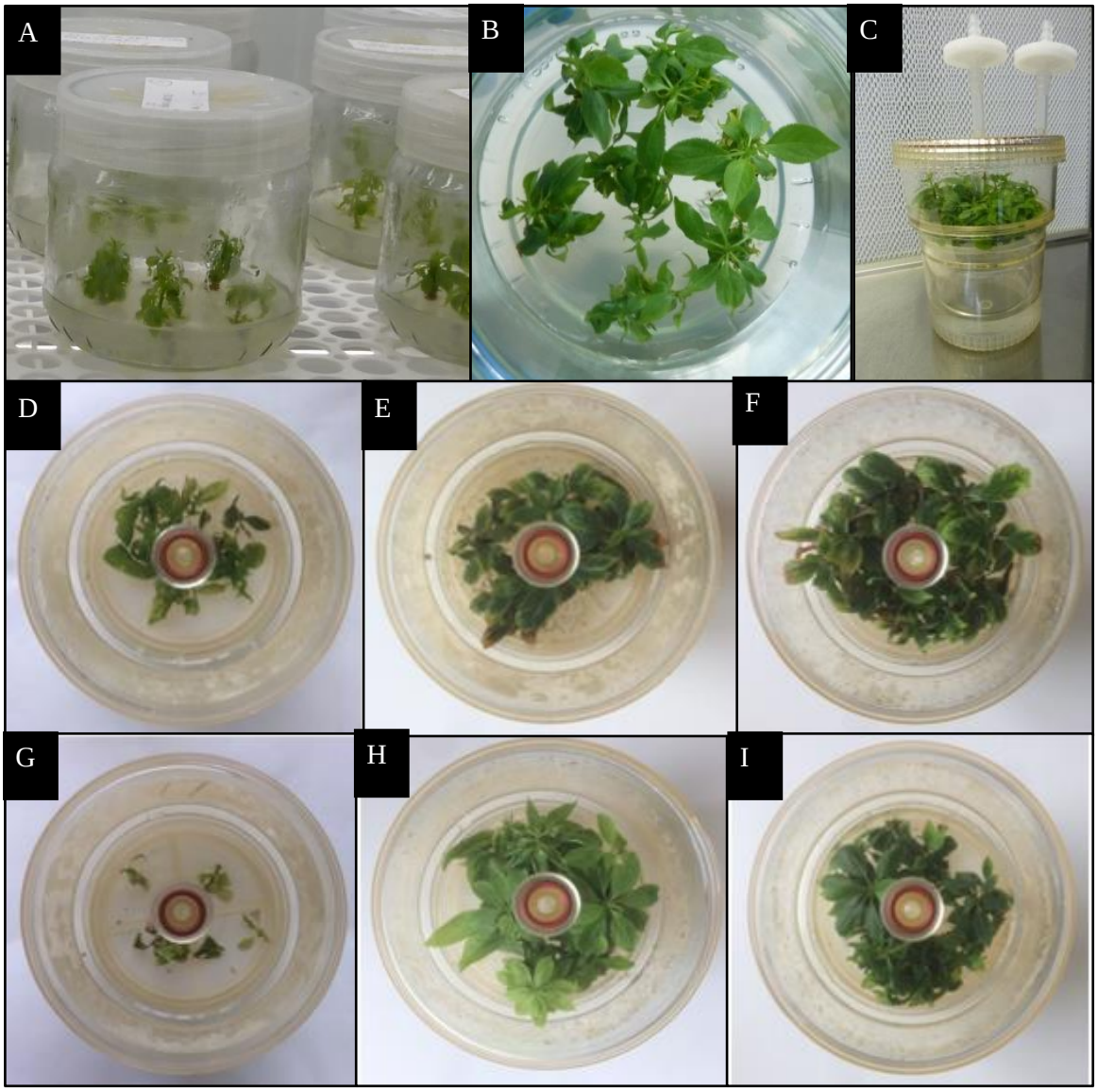


***Figure 1**. Plum shoots cultured in different conditions. A, B) Control shoots cultured in jars with 3% sucrose. C) RITA® container. D-F) Shoots cultured in RITA® under photoautotrophic conditions (high light intensity and with CO_2-enriched air) in medium without sucrose (D), with 1% sucrose (E) or with 3% sucrose (F). G-I) Shoots cultured in RITA® under standard conditions (low light intensity and without CO_2-enriched air) in medium without sucrose (G), with 1% sucrose (H) and with 3% sucrose (I).*

In general, the use of RITA® bioreactors with 16 immersions per day did not present advantages compared with control treatment in jars, with the exception of leaf parameters. Leaves were longer and wider under temporary immersion than when cultured in semisolid medium (Fig. 2). In RITA® the use of PAM conditions increased the proliferation of CBP. More shoots were obtained under PAM than under standard conditions, and cultures supplemented with 3% sucrose under PAM presented more

shoots than the control in jars (Fig. 2). The longest shoots were also observed in the 1º cycle of culture under PAM.

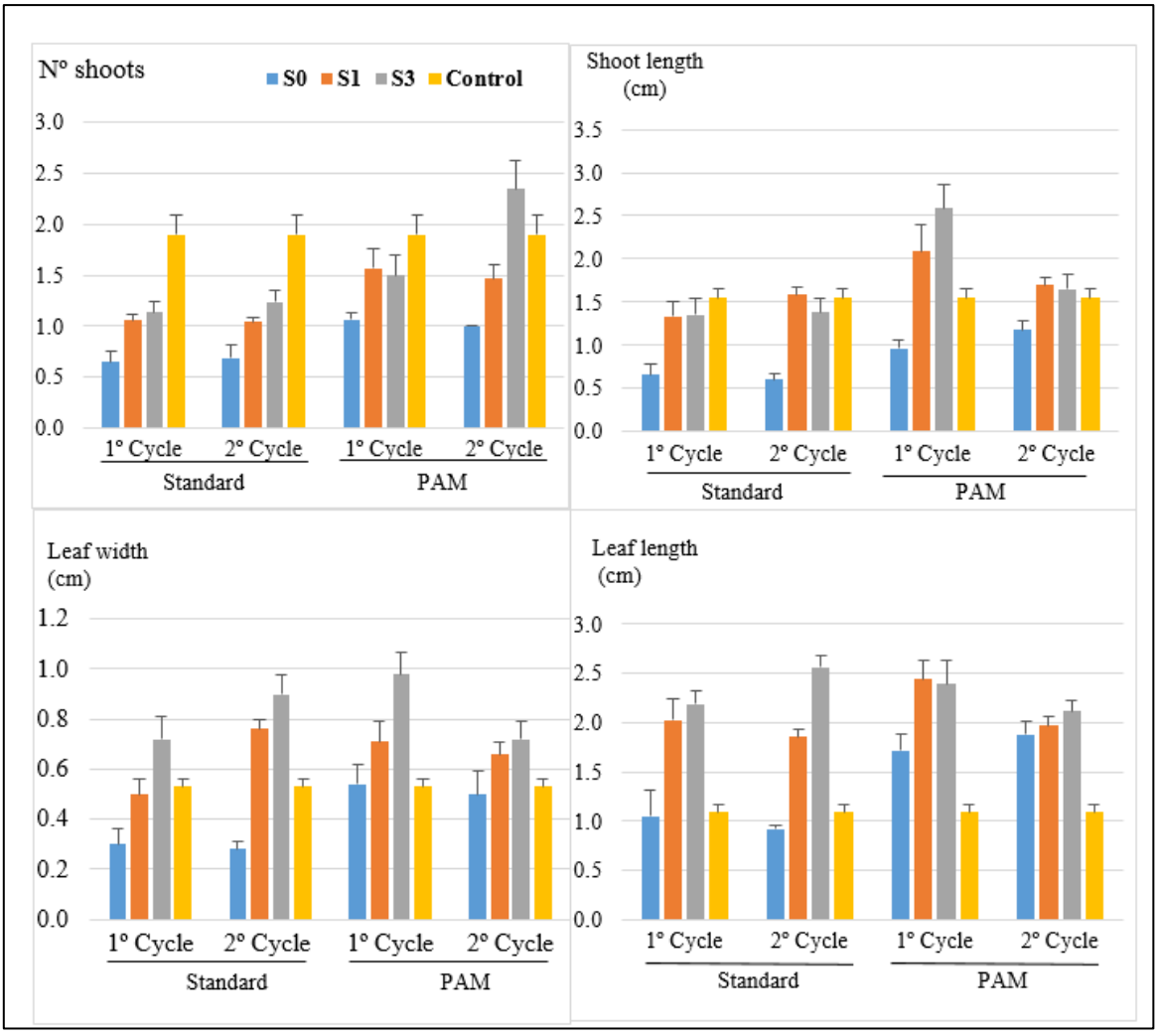


***Figure 2**. Effect of the sucrose supplementation on the growth of shoots of plum cultured in RITA® under standard conditions or under PAM conditions. Standard conditions: low light intensity and no CO_2-enriched air. PAM conditions: high light intensity and CO_2-enriched air. S0: medium without sucrose. S1, S3: medium with 1% or 3% sucrose. Control: explants cultured in semisolid medium under standard conditions with 3% sucrose. In the 1º cycle the explants originated from control shoots. In the 2º cycle, the explants were cultured again in the same conditions as in the 1º cycle.*

The effect of culture conditions on the total amount of soluble phenolics of CBP is shown in Fig. 3. The results indicate that soluble phenolic compounds content is strongly influenced by the sucrose presence in the medium. Higher values are obtained with sucrose increase, and the highest content of phenolics was observed in control shoots in both cycles and under the two growth conditions (standard and PAM). Similar trends were found regarding antioxidant content (Fig. 4), indicating the significant role of

phenolic compounds in the antioxidant capacity of the explants (Sharma et al. 2012). As reported for phenolics, the highest values of antioxidants were obtained in the control in the two cycles. In the first cycle, the more sucrose supplemented to the medium, the higher the antioxidant level found in the explants. However, in the second cycle and irrespective of growing conditions, shoots cultured in RITA® with 1% sucrose exhibited higher antioxidant levels than those cultured in RITA® with 3% sucrose.

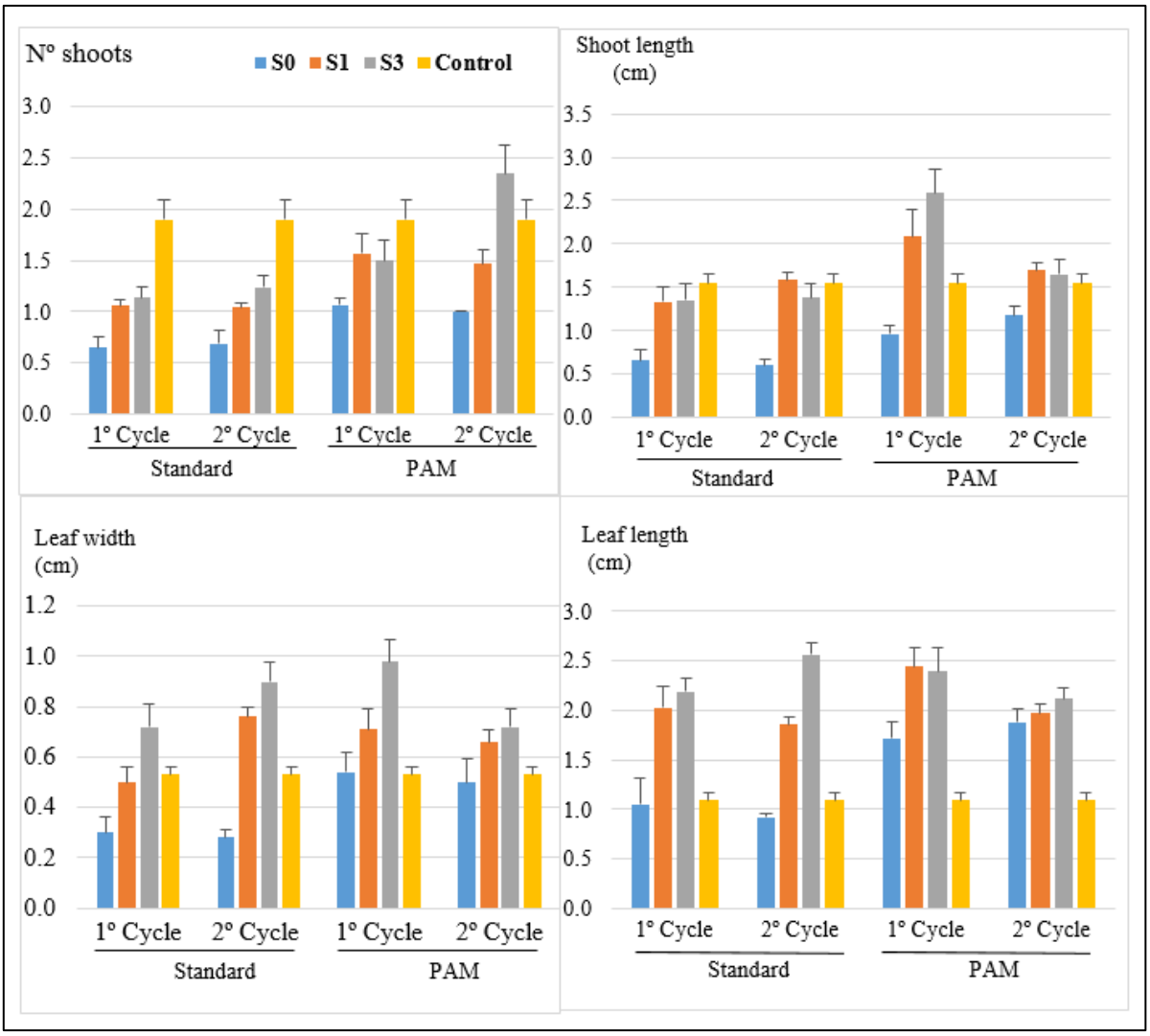


***Figure 3**. Effect of the sucrose supplementation on the soluble phenolics of shoots of plum cultured in RITA® under standard conditions or under PAM conditions. Standard conditions: low light intensity and no CO_2-enriched air. PAM conditions: high light intensity and CO_2-enriched air. S0: medium without sucrose. S1, S3: medium with 1% or 3% sucrose. Control: explants cultured in semisolid medium under standard conditions with 3% sucrose. In the 1º cycle the explants originated from control shoots. In the 2º cycle, the explants were cultured again in the same conditions as in the 1º cycle.*

Under conventional micropropagation, plants are exposed to several environmental stresses, especially when they have to face a change of conditions. This stress produces the formation of a wide number of reactive oxygen species (ROS), which cause tissue damage, making the explants vulnerable (Dias et al. 2011). As a method of reducing the oxidative stress, the explants produce antioxidant and phenolic compounds. In the case of plum cultures, stress exposure probably increased with the change of culture conditions (liquid medium, light, sucrose…). However, the capacity to start the production of phenolics and other compounds with antioxidant activity needs energy consumption by the explants, which are in

a debilitated state after been cut at the beginning of the culture cycles. The presence of a minimal sucrose concentration may be necessary to achieve a progressive recuperation and reduce the oxidative stress, and this could explain the increment in antioxidant and phenolic compounds observed with the sucrose increase in the medium.

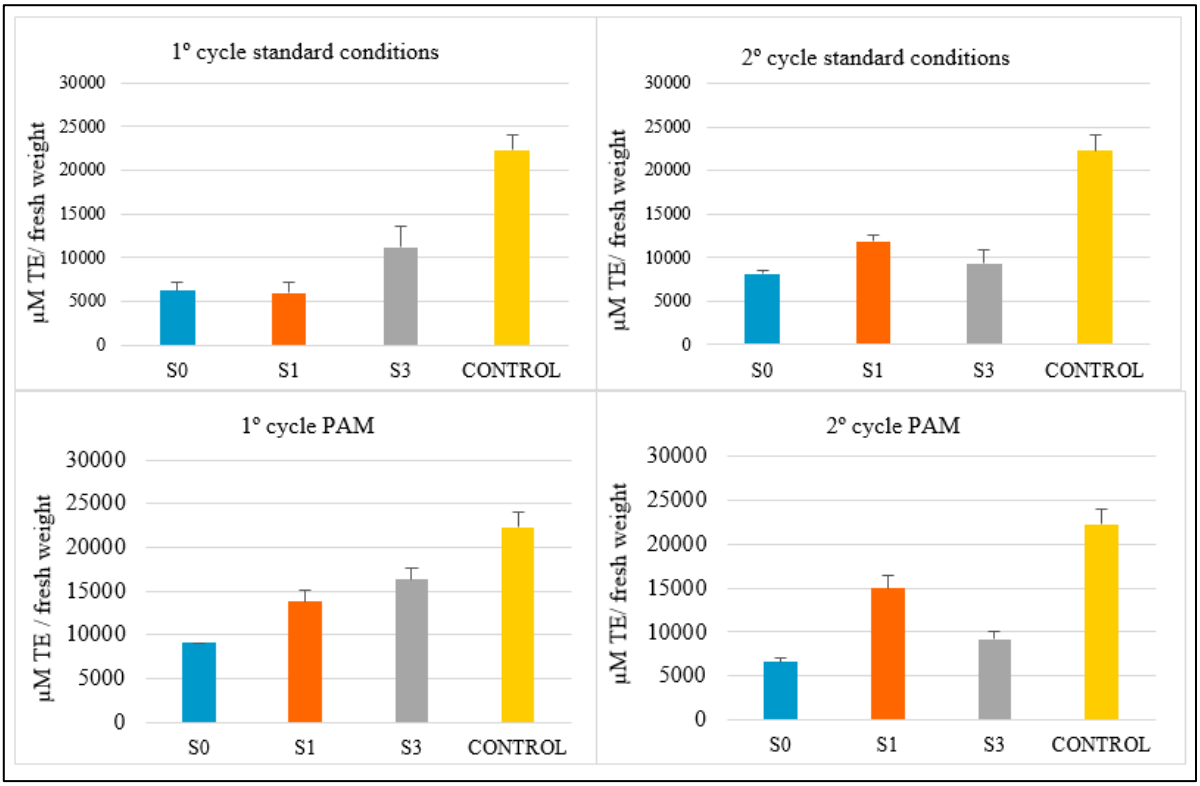


***Figure 4**. Effect of the sucrose supplementation on the antioxidant activity of shoots of plum cultured RITA® under standard conditions or under PAM conditions. Standard conditions: low light intensity and no CO_2-enriched air. PAM conditions: high light intensity and CO_2-enriched air. S0: medium without sucrose. S1, S3: medium with 1% or 3% sucrose. Control: explants cultured in semisolid medium under standard conditions with 3% sucrose. In the 1º cycle the explants originated from control shoots. In the 2º cycle, the explants were cultured again in the same conditions as in the 1º cycle.*

Chestnut

Chestnut shoots were cultured in PAM conditions with the treatments described in Table 2. Shoots originated in tubes (Fig. 5A) were used as a source of explants for experiments developed in plantform™ bioreactors by temporary immersion (Fig. 5B) or in 6 liter in-house bioreactors by continuous immersion (Fig. 5C). The results of the first insight regarding the phenolic content and antioxidant activity of clones P042 and C053 are shown in Fig. 6.

In both clones, phenolic compounds and antioxidant activities decreased with sucrose increment, showing a trend opposite to the one observed previously in plum (Fig. 3-4). The diminution of phenolic compounds was very slight (less than 0.2 units between each treatment, compared with 2-3 units observed in plum (Fig. 3, Fig. 6A)). However, the decrease in the antioxidant activity was more pronounced, and the differences between treatments were of a similar range as the one observed in plum (Fig. 4, Fig. 6B). As mentioned above, the data shown in chestnut correspond to preliminary experiments. Currently, new experiments are in progress in order to investigate the discrepancy found between the two species.

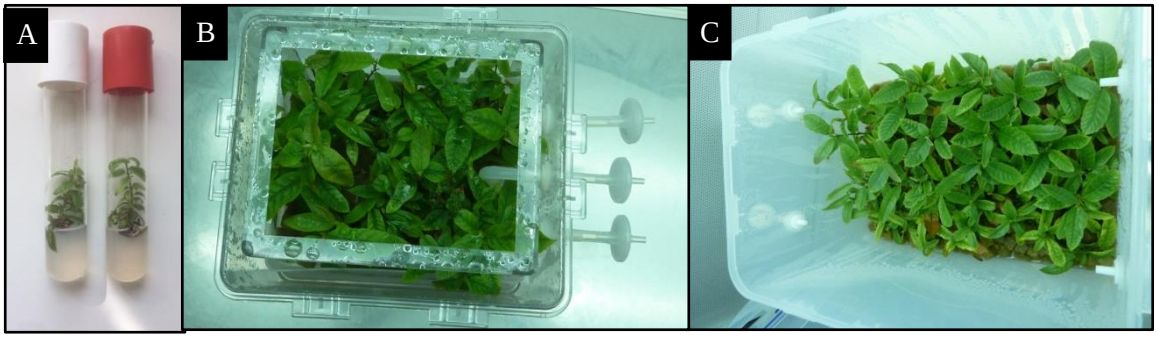


***Figure 5**. Chestnut shoots of clones P042 and C053 growing in standard and photoautotrophic conditions. A) Shoots cultured in tubes in standard conditions. B-C) Shoots cultured in PAM conditions. B) P042 shoots cultured in plantform™ bioreactors by temporary immersion. C) C053 shoots cultured in 6 liter in-house bioreactors by continuous immersion.*

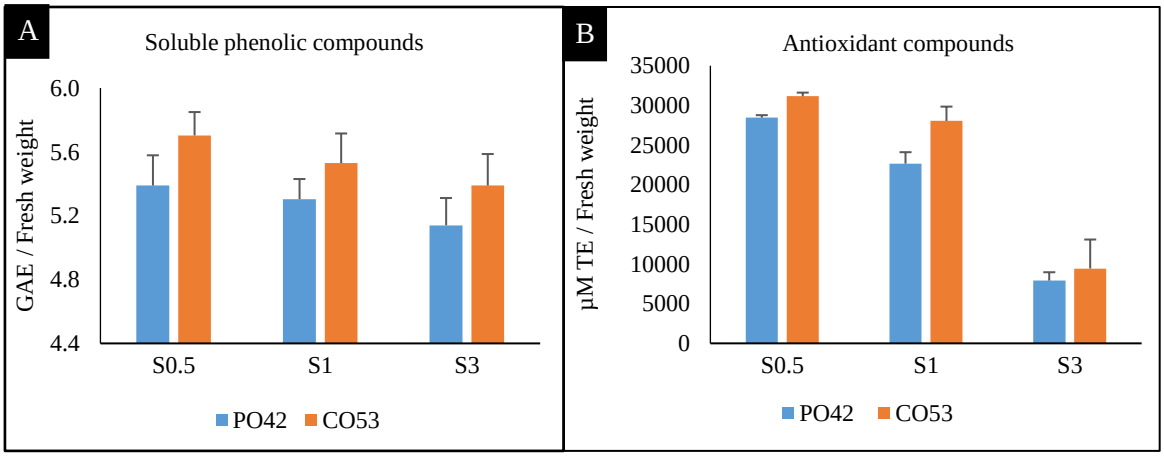


***Figure 6**. Soluble phenolic content and antioxidant activities of chestnut clones P042 and C053 cultured in PAM conditions: high light intensity and CO_2-enriched air. S0.5, S1, S3: medium with 0.5%, 1% or 3% sucrose.*

Acknowledgements: This research was partly funded by the Xunta de Galicia (Spain) through the Contrato Programa 2017-2018 and the project IN607A 2017/6. We thank Alejandro Díaz, Rafael Sánchez, and Patricia Val for technical assistance.

References

Akdemir H, Süzerer V, Onay A, Tilkat E, Ersali Y, Çiftçi YO (2014) Micropropagation of the pistachio and its rootstocks by temporary immersion system. Plant Cell Tissue Organ Cult 117:65–76

Blois MS (1958) Antioxidant determinations by the use of a stable free radical. Nature. 181:1199–1200

Cuenca B, Sánchez C, Aldrey A, Bogo B, Blanco B, Correa B, Vidal N (2017) Micropropagation of axillary shoots of hybrid chestnut (*Castanea sativa* x *C. crenata*) in liquid medium in a continuous immersion system. Plant Cell Tissue Organ Cult 131:307-320

Demidchik V (2015) Mechanisms of oxidative stress in plants: From classical chemistry to cell biology. Environ Exp Bot 109:212–28

Dias MC, Pinto G, Santos C (2011) Acclimatization of micropropagated plantlets induces an antioxidative burst: a case study with *Ulmus minor* Mill. Photosynthetica 49:259-266

Díaz J, Bernal A, Pomar F, Merino F (2001) Induction of shikimate dehydrogenase and peroxidase in pepper (*Capsicum annuum* L.) seedlings in response to copper stress and its relation to lignification. Plant Sci 161:179-88

González R, Ríos D, Avilés F, Sánchez-Olate M (2011) In vitro multiplication of *Eucalyptus globulus* by temporary immersion system. Bosque 32:147-154

Gresshoff PM, Doy CH (1972) Development and differentiation of haploid *Lycopersicon esculentum*. Planta 107:161-170

Kubola J, Siriamornpun S (2008) Phenolic contents and antioxidant activities of bitter gourd (*Momordica charantia* L.) leaf , stem and fruit fraction extracts *in vitro*. Food Chem 110:881–90

Martin KP (2004) In vitro propagation of the herbal spice *Eryngium foetidum* L. on sucrose-added and sucrose-free medium without growth regulators and CO_2 enrichment. Sci Hortic 102:277-282

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473-497

Nguyen QT, Xiao Y, Kozai T (2016) Photoautotrophic Micropropagation. In: Kozai T, Niu G, Takagaki M (eds). Plant Factory: An Indoor Vertical Farming System for Efficient Quality Food Production. Burlington: Academic Press, 2016, pp. 271-283

Saldanha CW, Otoni CG, Rocha DI, Cavatte PC, Detmann K da SC, Tanaka FAO, Dias LLC, DaMatta FM, Otoni WC (2014) CO_2-enriched atmosphere and supporting material impact the growth, morphophysiology and ultrastructure of *in vitro* Brazilian-ginseng [*Pfaffia glomerata* (Spreng.) Pedersen] plantlets. Plant Cell Tissue Organ Cult 118:87-99

Singleton VL, Rossi JA (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Amer J Enol Viticult 16:144-58

Sharma P, Jha AB, Dubey RS, Pessarakli M (2012) Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. J Bot. Article ID 217037, 26 pages

Vidal N, Aldrey A, Blanco B, Correa B, Sánchez C, Cuenca B (2017) Proliferation and rooting of chestnut under photoautotrophic conditions. In: Bonga JM, Park YS, and Trontin JF (eds.) Proceedings of the 4th International Conference of the IUFRO Unit 2.09.02 on Development and application of vegetative propagation technologies in plantation forestry to cope with a changing climate and environment. La Plata, Argentina. pp 119-127

Vidal N, Blanco B, Cuenca B (2015) A Temporary Immersion System for Micropropagation of Axillary Shoots of Hybrid Chestnut. Plant Cell Tissue Organ Cult 123:229-243

Welander M, Persson J, Asp H, Zhu LH (2014) Evaluation of a new vessel system based on temporary immersion system for micropropagation. Sci Hortic 179:227-232

Xiao Y, Niu G, Kozai T (2011) Development and application of photoautotrophic micropropagation plant system. Plant Cell Tissue Organ Cult 105:149-158

Yaseen M, Ahmad T, Sablok G, Standardi A, Hafiz IA (2013). Review : role of carbon sources for *in vitro* plant growth and development. Mol Biol Rep 40:2837-2849

Conservation and vegetative propagation of forest genetic resources from Talares and Monte Blanco ecosystems in argentina

Sebastián P. Galarco[1], Maite Romero Alves[1], Patricia Boeri[2], Luciano Roussy[1], Marina Adema[1], Blanca Villarreal[1], María V. Briones[3], María de los A. Basiglio Cordal[1,4], Tatiana Cinquetti[1], Diego I. Ramilo[1] and Sandra E. Sharry[1,2,3]

[1]*Wood Research Laboratory, School of Agriculture and Forestry Sciences, National University of La Plata, Buenos Aires, Argentina.*

[2]*Integrated Unit for the Innovation of the Agri-Food System of Northern Patagonia, National University of Rio Negro, Río Negro, Argentina.*

[3]*Biodiversity and Biotechnology Research Institute, National Council of Scientific and Technical Research, Buenos Aires, Argentina*

[4]*In vitro culture laboratory – CEAMSE (Ecological Coordination Metropolitan Area, State Society) . Villa Domínico, Buenos Aires, Argentina.*

[▯] *Corresponding Author Email: ssharry@gmail.com*

Abstract

Deforestation and forest degradation have led to widespread biodiversity and environmental losses in Argentina. The Talares and Monte Blanco forests are a relict vegetation type throughout Northeast of Buenos Aires province. These forests have suffered extensive deforestation and fragmentation. It is demonstrated that there is a low capacity of regeneration of the indigenous species after clearing, given that the growth of other previous species causes water deficit and lack of light (Arturi, 1997). That is why selection and successful propagation of native tree species are important for improving ecological restoration of these forests.

Buenos Aires province forests status

The native forests of Buenos Aires province are strictly confined to the coastal strip of Río de la Plata (coastal forests, locally "Talares" and "Monte Blanco") and to the western region (locally Caldén forests) (Parodi 1940). The use of forests for firewood was so intensive that the resources were exhausted at the beginning of the 19th century.

Currently there are only remnants or relicts of these forests, locally called "Talares," "Caldenal" and "Monte Blanco," as mentioned before. The native dry forest, dominated by *Celtis erhenbergiana* (Ulmaceae) and *Scutia buxifolia* (Rhamnaceae), constitutes the main woodland community of the eastern plain called Pampa in Buenos Aires province (Parodi 1940; Cabrera 1976).

The Talares forests area is an environmental mosaic where thorny forests and woodland patches combine with humid grasslands and coastal communities of Río de La Plata. It is a xerophyte forest type structured by few tree species surrounded by a lower and moister soil matrix. The forest grows on highly calcareous parent materials, derived from sea transgressions and regressions in the Quaternary (Cabello and Arambarri 2002). The current status of conservation shows that the protection of these forests is considered a priority due to their high biodiversity and fragility. The unique characteristics of the Talares

led to the creation, in 1984, of the Biosphere Reserve "Parque Costero del Sur" (MAB-UNESCO), now considered a natural and cultural heritage site. This reserve consists of a group of natural interphases, with exceptionally rich flora and fauna. Forests dominated by *Celtis ehrenbergiana* are becoming susceptible to invasion by exotic species such as *Ligustrum lucidum* (privet). The tala tree is usually common in the riparian zone, although human activities limit its propagation. It is a valuable species for the survival of native animals as it serves as shelter, food source and bird nesting sites. Ribichich and Potomastro (1998) found that the dominant trees are *Scutia buxifolia* and *Celtis ehrenbergiana*. There is also present *Schinus polygamus*, which presents the bell-shaped structure of the pioneer species. Over 1200 m distant from the river, this species is co-dominated by *S. buxifolia* and by *C. ehrenbergiana* trees regenerated from stumps. The differences between the old-growth stands seem to be related to the gradients of soil texture and nutrient concentration, raising edaphic stress towards the river. The stress tolerance of *S. buxifolia*, and the aptitude of *S. polygamus* to survive in disturbed habitats seem to have prevented the post-logging survival of *C. ehrenbergiana*. Tala regenerated possibly due to a better competitive performance in a more favourable site. Ribichich and Potomastro (1998) recommend the restoration of the qualitative features and the control of privet. Goya et al. (1992) and Arturi and Goya (2004) have conducted several studies in order to protect the species. The reproductive system varies among the different species: *C. ehrenbergiana* is anemophilous and selfcompatible. *S. buxifolia* is entomophilous and floral visitor's dependant. *J. rhombifolia* is entomophylous, although spontaneous autogamy could favour reproduction in the absence of pollinators. *S. longifolia* could be an ambophilous species (pollinated both by insects and by the wind). This dual system may be the result of a system flexibility mechanism or an evolutionary transition. (Torreta and Basilio 2013).

Another relict forest ecosystem in Buenos Aires is the locally named Monte Blanco. This forest, that originally occupied the elevated border areas of the Low Delta islands of the Río Paraná and some areas of Río de La Plata river coast, has almost been eliminated as a consequence of unsustainable timber harvesting carried out in the region during the past century. At present, only relict patches with scarce regional representation may be found along the north-eastern part of the Río de La Plata shore. Many of these forests have been abandoned, resulting in secondary forest formation that is subject to numerous invasive exotic species, such as *Ligustrum lucidum, Morus alba, Rubus ulmifolius, Gleditsia triacanthos, Fraxinus pennsylvanica* and other species. A priori observations suggest that successional trends do not lead to recovery of the original forest. Because of the low density of native tree species, it is difficult to predict their future persistence. In conclusion, ecological restoration strategies will be needed in order to increase native tree species richness and forest biodiversity in the Lower Delta of the Paraná River, the original forest, locally referred to as the Monte Blanco forest (Kalesnik et al. 2008).

Figure 1. *Location of natural forests (Talares and Monte Blanco) in Buenos Aires Province, Argentina.*

Methods for conservation and propagation of native forest species from Talares and Monte Blanco

At the Facultad de Ciencias Agrarias y Forestales (FCAyF), Universidad Nacional de La Plata (UNLP), we carried out an investigation about the propagation requirements of indigenous tree species from Buenos Aires Talares and Monte Blanco forests. We adjusted the system of propagation by seeds, cuttings and/or *in vitro* morphogenesis in some of these trees species:

- *Celtis ehrenbergiana Gill. ex Planch "tala",* Ramilo and Abedini (2007) achieved rooting of cuttings by using 50 ppm NAA. After 5 months, higher survival (71%) was recorded for NAA treatment (50 ppm) versus a mean survival of 2,5% when IBA was used (Figure 1).

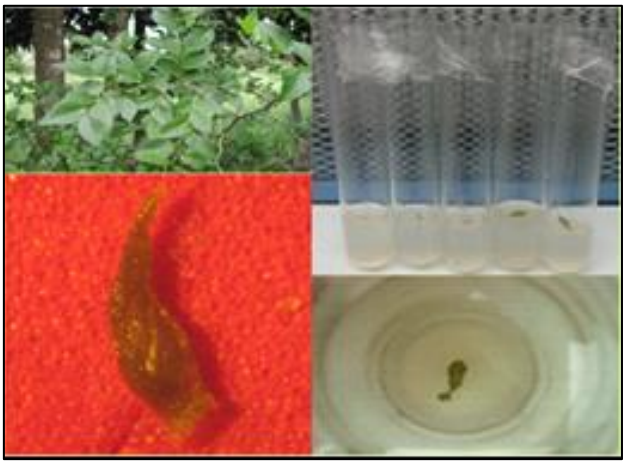


Figure 1. *Micropropagation Celtis ehrenbergiana*

- *Salix humboldtiana "South American Native Willow"* We improved the macro and micropropagation of this species (Adema et al. 2010; 2014). Macro propagation was achieved by rooting of cuttings (30 cm. long and 0.8 - 1.5 cm in diameter), immersed in IBA during 24 hours. *In vitro* rooting occurs on WPM supplemented with 0.1 mg. L-1 of IBA (Figure 2).

Figure 2. *Macropropagation of Salix humboldtiana*

- *Parkinsonia aculeata "Cina-cina",* micropropagation was achieved from nodal segments in BTM medium (Chalupa 1983) with KIN. The macropropagation can be obtained from juvenile cuttings collected during springtime.
- *Erythrina crista-galli L. "Ceibo",* the *in vitro* protocol for Ceibo regeneration was adjusted from nodal sections of seedlings, which were obtained from seeds, germinated *in vitro.* The shoots formed roots in WPM medium with 0.1 mg. L-1 of NAA. (Ruscitti and Abedini 1996).

- *Terminalia australis "Palo Amarillo", m*acropropagation was achieved by using cuttings (200 mm long and 8 mm in diameter on average) immersed in NAA solutions (Aquila et al 2001). *Acacia caven Molina "Espinillo",* a micropropagation system was developed using somatic embryogenesis. The explants were cotyledons obtained from mature seeds (Marinucci et al. 2003; 2005).
- *Phytolacca tetramera "Ombusillo",* micropropagation was possible via organogenesis from nodal and internodal sections cultivated on MS culture medium, supplemented with sucrose and IBA and BAP (Basiglio Cordal and Sharry 2012; Basiglio Cordal et al. 2014).
- *Phyllantus sellowianus "Sarandí blanco"* Rivas and Abedini (1996) improved the micropropagation through direct organogenesis induced from nodal and internodal segments.
- *Citharexylum montevidense "Espina de bañado",* macropropagation was possible by using cuttings from 7 mm. in diameter and with material less than 1 year old after 110 days (Roussy et al. 2011) (Figure 3).

Figure 3. *Cuttings of Citharexylum montevidense*

- *Scutia buxifolia "coronillo"* stem cuttings collected in winter were submerged for a 24 hr period in 5 concentrations of IBA and 5 of NAA resulting in 47 % survival with IBA (Ramilo and Abedini, unpublished).

We describe evidence of protocols through the major methods being used, developed and applied to propagate and conserve native species.

Conclusion

We describe challenges associated with the restoration of threatened trees in the Talares and Monte Blanco forests of Northeast of Buenos Aires and analyse the effectiveness of methods used to define target species. We identified seed sources and generated information on the biology of rare or threatened tree species. Despite the many challenges associated with collection of seeds and to adjust propagation systems from rare and threatened native species, our data represent a significant first step towards improving the genetic diversity and species richness of the seedlings produced in the Buenos Aires forests. The methods described here may be of relevance to ecological restoration programmes elsewhere where the challenges of including rare and threatened tree species in a germplasm bank, seedling production and planting operations are likely to be similar.

References

Adema M., Basiglio Cordal M., Briones V., Villareal B., Ciocchini G., Abedini W., Sharry S. (2014). Macro and micropropagation of *Salix humboldtiana* and *Salix babilónica*. Evaluation of the removal capacity of Cu. Jornadas de Salicaceae 2014- IV International Congress of Salicaceae, Argentina.

Adema M., Curuchet G., Abedini W., Sharry S., Briones V., Villareal B., Basiglio Cordal MA.,

Ciocchini G. (2010). Preliminary study of copper phytoremediation using *Salix humboldtiana*, Acts VII Latin American and Caribbean Encounter on Biotechnology.

Aquila S. V., Bonicelli V., Carparo S., Rossi J., Castro M. T., Spegazzini E., Abedini W. (2001) Vegetative Propagation of *Terminalia Australis* "Palo Amarillo," *Combretaceae*, Proceedings IV International Congress of Medicinal Plants.

Arturi M (1997) Regeneration of *Celtis tala* Gill ex Planch in the NW of the Province of Buenos Aires. (Ph.D. thesis) School of Agriculture and Forestry Sciences, National University of La Plata, Buenos Aires. SEDICI Web http://sedici.unlp.edu.ar/handle/10915/4658.

Arturi M. F., Goya J. F. (2004) Structure, dynamics and management of the talares of the NE of Buenos Aires. In: Arturi M., Frangi J., Goya J. (eds.), Ecology and Management of Argentina's forests EDULP, Argentina, 10:1-23

Basiglio Cordal M., Sharry S. (2012) Conservation of medicinal species from the Province of Buenos Aires, the case of Ombusillo. Bioethics Notebooks. Ed. Ad Hoc, Argentina 17: 45 – 47.

Basiglio Cordal M., Adema M., Briones V., Villarreal B., Panarisi M., Abedini W., Sharry S. (2014) Induction of somatic embryogenesis in *Phytolacca tetramera*, medicinal species of Argentina. Emirates J Food Agric 26(6): 552 - 557.

Cabello M., Arambarri A. (2002) Diversity in soil fungi from undisturbed and disturbed *Celtis tala* and *Scutia buxifolia* forests in the eastern Buenos Aires province (Argentina). Microbiol Res 157:115–125.

Cabrera A (1976) Argentine phytogeographic regions. Encyclopedia of agriculture and gardening Argentina. ACME S.A.I.C. Buenos Aires.

Chalupa V. (1983) Micropropagation of Conifer and Broadleaved Forest Trees. Communicationes Instituti Forestalis. Cechosloveniae 13: 7-39.

Goya J., Placi L., Arturi M., Brown A. (1992) Distribution and structural characteristics of the talares of the Biosphere Reserve "Parque Costero del Sur". Agronomics Faculty Journal. La Plata 68: 53-64.

Kalesnik F., Valles L., Quintana R., Aceñolaza P. (2008) Relictual Patches of Monte Blanco Forest en Delta del Río Paraná region Serie Misc. INSUGEO-CONICET 17:169 – 193.

Parodi L. R. (1940) Native forests of Buenos Aires province. Proc Natl Sci Acad 7: 97-90.

Ramilo D.I., Abedini W.I. (2007). Vegetative propagation of *Celtis tala* Gill. Ex Planch by rooting wooden stakes semilignified. XXI Jornadas Forestales de Entre Ríos. Argentina.

Ramilo D.I., Abedini W.I (unpublished). Vegetative propagation of *Scutia buxifolia* Reissek by rooting wooden stakes semilignified, internal report. CEPROVE. School of Agriculture and Forestry Sciences.

Ribichich A., Protomastro J. (1998) Woody vegetation structure of xeric forest stands under different edaphic site conditions and disturbance histories in the Biosphere Reserve Parque Costero del Sur, Argentina Plant Ecol 139 (2):189–201.

Rivas M.C., Abedini W. (1996) Rapid clonal propagation of *Phyllantus sellowianus* (Sarandí blanco). Third International Symposium on in vitro culture and Horticultural Breeding. Jerusalem, Israel.

Roussy L., Pinciroli L., Briones V., Sharry S., Ciocchini G., Abedini W. (2011) Rooting of cuttings of different diameter of *Citharexylum montevidense* (Spreng) Moldenke, forest species native to the flora of Buenos Aires. V Latinamerican Forestry Congress, Lima, Peru. CONFLTA. La Molina University.

Ruscitti M, Abedini W (1996) In vitro cloning of *Erythrina crista-galli* ecotypes resistant to environmental contamination. XVI Congress of Phytogenetics. Mexican Society of Phytogenetics (SOMEFI). Institute of Genetic Resources and Productivity of the Postgraduated School of Montecillo, Mexico.

Torretta J., Basilio A. (2013) Pollen dispersion and reproductive success of four tree species of a xerophytic forest from Argentina. Rev de Biología. http://dx.doi.org/10.15517/rbt.v57i1-2.11321.

Genetic characterisation of oak trees with limited natural recovery potential and their effective reproduction by the organogenesis

Martina Komárková[1], Eva Pokorná[1], Martin Fulín[1], Radek Lefnar[1], Pavlína Máchová[1]

[1]*Forestry and Game Management Research Institute, Strnady, Jiloviste, Czech Republic.*
komarkova@vulhm.cz, pokorna@vulhm.cz, fulin@vulhm.cz, lefnar@vulhm.cz, machova@vulhm.cz

Abstract
Oak species represent an essential component of the European forests with remarkable genetic variability and adaptation to different ecological niches. To reveal the genetic diversity of selected oak trees, 60 samples from 2 localities were analysed with 11 nuclear microsatellite markers (SSR). Microsatellite analysis presented a relatively high level of genetic variability, and in all 60 samples, unique genotypes were found. To standardise the *in vitro* micropropagation method of oak species, different nutrient media were investigated. Bud explants and elongated shoots showed good growth performance and vitality on Woody Plant Medium 2 (WPM2) supplemented with 2 mg l^{-1} glycine, 200 mg l^{-1} glutamine, 200 mg l^{-1} casein, 0.1 mg l^{-1} IBA and 0.2 mg l^{-1} BAP.

Keywords: oak micropropagation, *Quercus* species, SSR markers

Introduction

Quercus L. (Oak) belongs to a large genus in the family *Fagaceae* with a wide range of habitats from Spain to Russia. The landscape matrix, climate conditions, biotic and abiotic stresses profoundly impact the above ground vegetation and reproduction potential of several oak trees in the Czech Republic. Nowadays, the sensitive and outstanding individuals may be vegetatively propagated, which may be crucial for many oak species, in which seed production or growth opportunities are limited.

Our present work aims to determine genetic variability of selected oak trees using nuclear microsatellite markers (SSRs) and to develop an appropriate *in vitro* propagation method of oak species, in which conventional vegetative propagation by cuttings is problematic. To reveal potential selective effects of local environment on the variability, 60 oak individuals collected from a rocky (dry area with a low-nutrient availability) and forest area (good moisture conditions with enriched soil) in the Czech Republic will be analysed. For evaluation of genetic diversity among the selected oaks, 11 polymorphic SSR markers will be used to calculate genetic diversity parameters using the statistical program GenAlEx 6.501. The next step will be the development of micropropagation method to multiply selected oak clones. For this purpose, collected oak bud explants were sterilised and transferred into the agar media with different cytokinin concentrations (BAP, 6-benzylaminopurine). After obtaining the results, we will be able to establish a suitable micropropagation method for active shoot growth and proliferation.

Material and methods

Oak (*Quercus* spp.) young leaves were collected at two localities in the Czech Republic differing in long-term forest conditions: 1) a drought area with a low-nutrient availability at Petrov (A_DB); 2) a control area (optimum conditions) at Cholupice (B_DB). From all 60 samples, total DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer´s protocol. Oak individuals were screened by 11 polymorphic nuclear microsatellite markers QrZAG87, MsQ13, QrZAG96, QrZAG65, QpZAG104, QrZAG112, QpZAG110, QpZAG15, GOT066, QrZAG20 and QrZAG11 (Table 1) previously characterised by Barreneche et al. (2004) and Durand et al. (2010). Fluorescently labelled primers were sorted into the two multiplexes and used for polymerase chain reaction (PCR). The size of fragments was analysed with capillary electrophoresis using a genetic analyser 3500 (Applied Biosystems, USA). Allele calling was performed using GeneMapper® 4.1 software provided by Applied Biosystems. Allele binning was done manually after plotting of fragment size distribution for each locus (Guichoux et al., 2011). The genetic diversity parameters were calculated using the statistical program GenAlEx 6.5 (Peakall and Smouse, 2012).

Table 1. *Details of 11 nuclear microsatellite primers including their repeat motif and allele size*

	Nuclear microsatellite	Motif	Allele size
Multiplex 1	QrZAG87	(TC)20	101-141
	MsQ13	(GA)14	191-233
	QrZAG96	(TC)20	137-191
	QrZAG65	(TC)21(TA)10	249-306
	QpZAG104	(AG)16AT(GA)3	183-249
	QrZAG112	(GA)32	71-139
Multiplex 2	QpZAG110	(AG)15	192-266
	QpZAG15	(AG)23	108-152
	GOT066	(GAA)10	210-250
	QrZAG20	(TC)18	160-200
	QrZAG11	(TC)22	227-288

For vegetative organogenesis, early spring buds were sterilized (rinsed with tap water for 30 min, disinfected with 1% sodium hypochlorite (v/v) for 20 min, followed by 50% Korsolex® (v/v) for 20 min, then by sterile distilled water for 20 min, 1% $HgCl_2$ (w/v) for 20 min and finally three rinses with sterile distilled water for 15 min). The buds were cultured on Woody Plant Medium (WPM, according to Mccown and Sellmer, 1987) supplemented with 30 g l^{-1} sucrose, 2 mg l^{-1} glycine, 200 mg l^{-1} glutamine, 200 mg l^{-1} casein, 0.1 mg l^{-1} IBA and two different concentrations of BAP (0.4 and 0.6 mg l^{-1}). In each case the pH of the medium was adjusted to 5.7 with 1 N NaOH or 1 N HCl and supplied with 0.7% agar before autoclaving at 120 °C for 20 minutes. Explants were grown under controlled conditions at a photon flux density of 30 μmol m^{-2} s^{-1} (16/8 day/night period) at 21 °C temperature for 30 days. Once shoots had elongated from axillary buds (approximately 3 weeks), they were excised and transferred into the shoot induction media with different composition of glutamine, casein and BAP (Table 2).

Table 2. *Basal nutrient media composition used for the shoot oak induction*

	Glutamine (mg l^{-1})	Casein (mg l^{-1})	BAP (mg l^{-1})
WPM1	200	200	0.4
WPM2	200	200	0.2
WPM3	400	400	0.4
WPM4	400	400	0.2

Results and discussion

Molecular markers developed for *Quercus* species can help us to study the genetic architecture and genetic diversity. We determined relatively high polymorphic patterns of the two tested oak populations. The eleven SSR markers that were analysed detected a total of 137 alleles in population A_DB and 126 alleles in population B_DB. Allele frequencies were entirely different in each locus between studied populations, as shown in Fig. 1, e.g. for locus QrZAG96. The level of genetic diversity presented by F_{ST} values reached 0.099, which indicates an average level of genetic differentiation (Wright, 1965) between two tested localities. The multilocus matches of microsatellite analysis revealed unique genotypes in all 60 individuals of selected oak species.

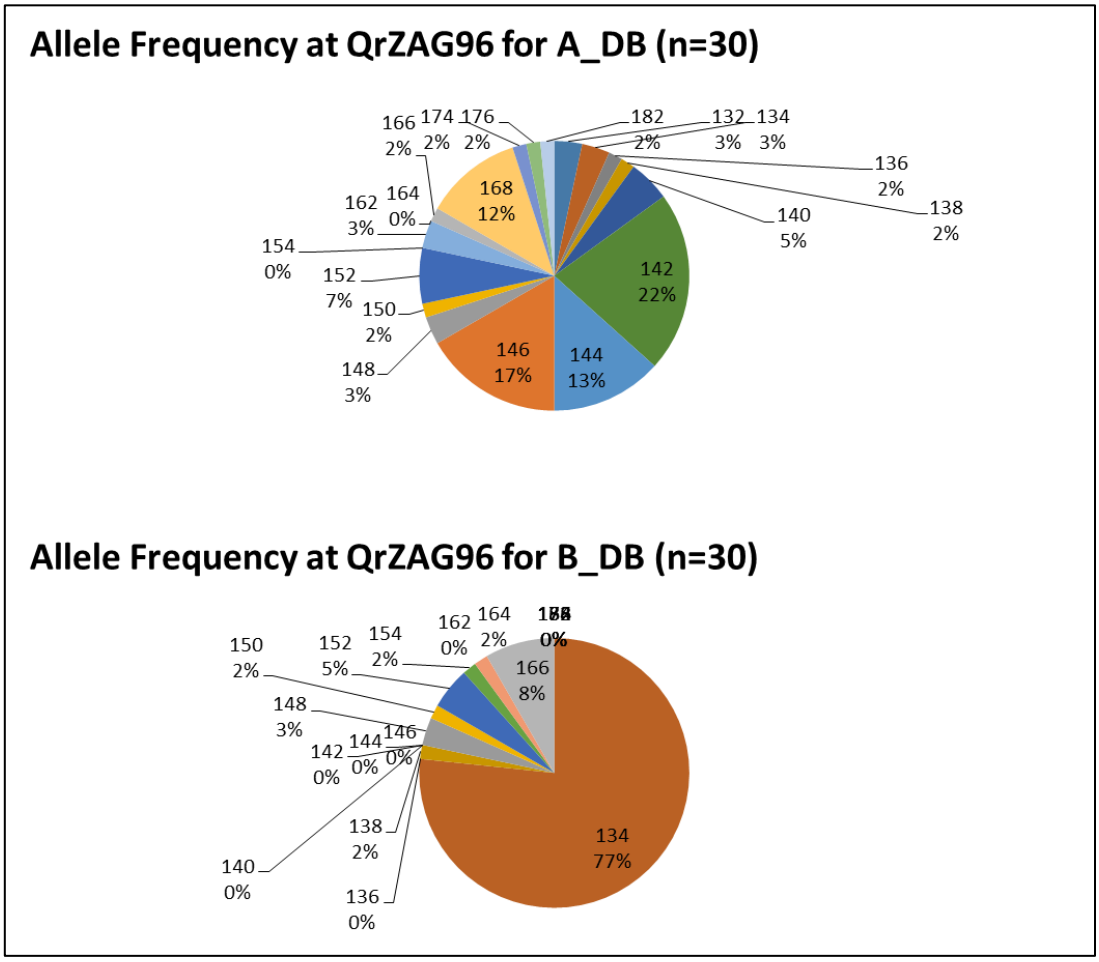


Figure 1. *Allele frequency at QrZAG96 for two tested oak populations, A_DB (drought area with low nutrient availability at Petrov) and B_DB (control area with optimum conditions at Cholupice)*

In our earlier experiments, WPM was found as the most suitable medium to support the growth and vitality of *Quercus* explants, as also reported by Vieitez et al. (1993) or by Vengadesan and Pijut (2009). Therefore, nutrient agar media for establishing cultures as well as for multiplication was based on this basal medium. Induction of organogenesis was observed at both concentrations of BAP 0.2 and 0.4 mg l^{-1}, the ability of proliferation was rather clonal specific. Genotype is known to affect *in vitro* somatic embryogenesis or regeneration (Toribio et al., 2004; Vieitez et al., 2009) and a proper genotype may therefore also be essential for induction of bud proliferation in explants from oak trees. Explants were able to elongate and produce new shoots, mainly on the medium WPM2 (Fig. 2) while the use of WPM3

or WPM4 medium led to necrosis of the material (Fig. 3). Micropropagation approach using WPM2 medium was established as the most suitable for selected oak clones.

Figure 2. *In vitro culture of oak derived from a spring bud grown on the nutrient medium WPM2 supplemented with 2 mg l^{-1} glycine, 200 mg l^{-1} glutamine, 200 mg l^{-1} casein, 0.1 mg l^{-1} IBA and 0.2 mg l^{-1} BAP*

Figure 3. *Shoot in vitro culture of oak turned brown after growing on an improper medium composition*

Acknowledgements: This work was supported by the Ministry of Agriculture of the Czech Republic, institutional support MZE-RO0118 and by the project TACR No. TJ01000385.

References

Barreneche T, Casasoli M, Russell K, Akkak A, Meddour H, Plomion C, Villani F, Kremer A (2004) Comparative mapping between *quercus* and *castanea* using simple-sequence repeats (SSRs). TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik 108:558–566

Durand J, Bodénès C, Chancerel E, Frigerio J-M, Vendramin G, Sebastiani F, Buonamici A, Gailing O, Koelewijn H-P, Villani F, Mattioni C, Cherubini M, Goicoechea PG, Herrán A, Ikaran Z, Cabané C, Ueno S, Alberto F, Dumoulin P-Y, Guichoux E, de Daruvar A, Kremer A, Plomion C (2010) A fast and cost-effective approach to develop and map EST-SSR markers: oak as a case study. BMC Genomics 11:570

Guichoux E, Lagache L, Wagner S, Chaumeil P, Léger P, Lepais O, Lepoittevin C, Malausa T, Revardel E, Salin F, Petit R (2011) Current trends in microsatellite genotyping. Molecular Ecology Resources 11:591–611

Mccown BH and Sellmer JC (1987) General media and vessels suitable for woody plant culture. In cell and tissue culture in forestry, forestry sciences, 4–16, Springer, Dordrecht

Peakall R and Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. Bioinformatics 28:2537–2539

Toribio M, Fernández C, Celestino C, Martínez MT, San-José MC, Vieitez AM (2004) Somatic embryogenesis in mature *Quercus robur* trees. Plant Cell, tissue and organ Culture 76:283–287

Vengadesan G and Pijut PM (2009) *In vitro* propagation of northern red oak (*Quercus rubra* L.). In Vitro Cellular & Developmental Biology - Plant 45:474–482

Vieitez AM, Corredoira E, Ballester A, Muñoz F, Durán J, Ibarra M (2009) *In vitro* regeneration of the important North American oak species *Quercus alba*, *Quercus bicolor* and *Quercus rubra*. Plant Cell, Tissue and Organ Culture (PCTOC) 98:135–145

Vieitez AM, Pintos F, San-José MC, Ballester A (1993) *In vitro* shoot proliferation determined by explant orientation of juvenile and mature *Quercus rubra* L. Tree Physiology 12:107–117

Wright S (1965) The interpretation of population structure by F-statistics with special regard to systems of mating. Evolution 19:395–420

Regeneration of a unique grey poplar population by organogenesis and characterisation of individuals by SSR analysis

Pavlína Máchová[1], Eva Pokorná[1], Martina Komárková[1], Helena Cvrčková[1]

[1]*Forestry and Game Management Research Institute, Strnady, Jiloviste, Czech Republic.*
machova@vulhm.cz, pokorna@vulhm.cz, komarkova@vulhm.cz, cvrckova@vulhm.cz

Abstract

Vegetative propagation of adult grey poplar (*Populus* × *canescens* Aiton Sm.) by cuttings is not very effective and is a limiting factor for active multiplication. Therefore, methods for vegetative cutting propagation based on *in vitro* organogenesis were tested. Bud explants were cultured on the modified MS medium supplemented with 0.2 mg l^{-1} BAP and 0.1 mg l^{-1} IBA. The same composition of MS media was used for the multiplication phase. Formation of roots was observed in 1/4 MS salt combined with 0.5 mg l^{-1} IBA. To characterise the genetic structure of the 155 grey poplar individuals, 14 polymorphic SSRs loci were tested. In all tested samples, only 33 different genotypes were detected. Among 120 female individuals only 6 different genotypes were found while among 35 tested male genotypes 69% were different individuals. Our data suggest that SSR markers could be useful in genetic identification and registration of clones as well as for genetic resource management in *Populus*.

Keywords : *Populus canescens*, clonal micropropagation, SSR markers

Introduction

Grey poplar (*Populus x canescens*) is a a hybrid between its parents, white poplar (*P. alba*) and aspen (*P. tremula*) (Lexer et al., 2005). Like all other species of the genus *Populus*, male and female flowers occur on separate trees (dioecy). The natural population of grey poplar located in the flood plain forest at Dyjákovice is characterised by unique and valuable phenotype traits. However, vegetative propagation of these old individuals by cuttings showed us to be a limiting factor for active multiplication. Based on this finding, micropropagation *in vitro* methods to multiply selected material were developed.

To acquire more detailed knowledge about the genetic diversity and clonal identification of selected trees of grey poplar, the Simple Sequence Repeats (SSR) method was used. Simple sequence repeats are sequences with tandemly repeated bases, ranging from the 1-6 base pair (bp) long units. Variation in SSR regions frequently originates from DNA Polymerase slippage, thus resulting in insertions or deletions (Iyer et al., 2000). The abundance and amount of information derived from SSR markers make them ideal for genetic population studies. For this purpose, we screened 155 grey poplar trees from a unique population with 14 selected SSR markers.

Material and methods

Plant material, in vitro culture establishment, rooting and acclimatisation

Dormant buds were collected from the selected grey poplar trees growing in the forest of Dyjákovice (South Moravia of the Czech Republic) during spring and autumn. Surface sterilized buds were cultured on a modified MS medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 10 mg l^{-1}

glutamine, 2 mg l^{-1} glycine, 0.2 mg l^{-1} 6-benzylaminopurine (BAP), 0.1 mg l^{-1} indole-3-butyric acid (IBA) and 6 g l^{-1} agar, pH was adjusted to 5.8. After 6 weeks of growth, the same composition of MS media was used for the multiplication phase. The cultures were transferred every 4 weeks to fresh multiplication medium. Cultures were maintained in an air-conditioned room at 24 °C, with white fluorescent light (30 µmol m^{-2} s^{-1}) under a 16-hour photoperiod. A high number of adventitious shoots (20–30) was formed in one multi-topped culture.

For rooting, shoots 2-2.5 cm long were excised and cultured on the modified 1/4 MS medium supplemented with 10 g l^{-1} of sucrose and 0.5 mg l^{-1} IBA for one month. Plantlets with well-developed roots were transferred from rooting medium into pots (Quick Pot T 35) with agroperlit and watered with basal 1/10 MS medium without phytohormones and sucrose. Acclimatisation proceeded in an air-conditioned room at 20°C, with white fluorescent light (30 µmol m^{-2} s^{-1}), and under 24-hours photoperiod at the 90% of relative air humidity. After 3 weeks, the plantlets were transferred into pots (Quick Pot T 60) with substrate (mixture of garden soil, peat and perlite in a ratio 2:1:1), and placed in a glasshouse for 3–4 weeks under 70% of relative air humidity. Several healthy plantlets were transferred to an outside bed of the experimental nursery and then to plots at Dyjákovice.

Microsatellite analysis

Total genomic DNA was extracted with the DNA Plant Mini Kit (QIAGEN) from buds or leaves taken from sampled grey poplar trees following instructions of the manufacturer. For analysis, 155 grey poplar trees from a unique population were selected (35 males and 120 females). DNA was quantified using a NanoPhotometr (Implen). Polymerase chain reactions (PCR) with 14 nuclear microsatellite primers used in the previous studies of van der Schoot et al. (2000), Smulders et al. (2001), Tuskan et al. (2004) and Politov et al. (2015) were optimised. The PCR products were analysed with the capillary electrophoresis using a genetic analyser 3500 (Applied Biosystems, USA). Allele calling was performed using the program GeneMapper 4.1 (Applied Biosystems). The obtained data were analysed by means of the statistical programs CERVUS (Kalinowski et al., 2007) and GenAlEx 6.501 (Peakall and Smouse, 2012).

Results and discussion

In some previous studies, Woody Plant Medium (WPM) was described as a suitable medium to support the growth and vitality of *Populus* explants (Bittsánszky et al., 2009; Di Lonardo et al., 2011). However, according to our experience, MS medium was found to be the best medium for micropropagation of Poplar (Fig. 1A). Bud, shoot vegetative propagation, and root induction was successful on the MS medium with the low concentrations of BAP (0.2 mg l^{-1}) (Fig. 1A, B).

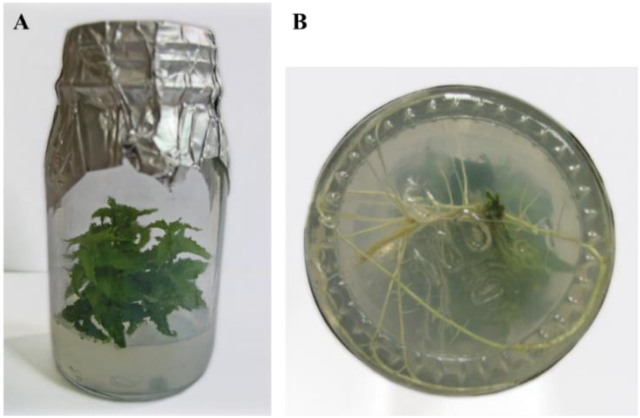


Figure 1. *In vitro cultures of grey poplar. A – shoots induced from a tree-derived bud explant in MS medium containing 0.2 mg l^{-1} BAP and 0.1 mg l^{-1} IBA; B – root induction in 1/4 MS medium containing 0.5 mg l^{-1} IBA*

The same BAP concentration was also used in the work of Di Lonadro et al. (2011). For rooting, adventitious shoots propagated during cultivation were used. The losses of plantlets during rooting and acclimatisation were minimal, only around 2 % (Fig. 2). Our results, therefore, suggest that *in vitro* micropropagation can improve rooting efficiency and thus clonal propagation of adult grey poplar trees (Fig. 3A, B).

Figure 2. *Acclimatisation of grey poplar plantlets*

Figure 3. *Plantlets of grey poplar after 1 (A) and 4 (B) weeks of acclimatisation in the glasshouse*

To characterise the genetic structure of the 155 grey poplar individuals, the 14 SSRs loci were tested. According to analysis resultes, 100 different alleles at 14 loci were found, i.e. 7.1 alleles per locus on average. The most polymorphic locus over our set of samples was locus ORPM30 with 13 different alleles. Some alleles at loci ranged from 3 – 13. The levels of expected heterozygosities of each locus were between 0.231–0.699, observed heterozygosities ranged from 0.135 to 0.974. The results of Polymorphism Information Content (PIC) value of 155 tested samples showed that PIC ranged from 0.044 to 0.647. The mean PIC value for 14 selected loci was 0.4168. By applying of the 14 suitable markers to the 155 grey poplar individuals, we obtained multilocus genotypes (MLG) shown in Table 1. Based on data evaluation, we detected only 33 different genotypes in all samples tested. Among 120 female individuals were found only 6 different genotypes while of the 35 male trees 69 % belonged to different genotypes. To date, the genetic diversity and population structure has been investigated also in other poplar species, for example in *P. tomentosa* (Du et al., 2012), *P. nigra* (Gaudet et al., 2008), *P. tremuloides* (Dayanandan et al., 1998) or *P. deltoides* (Rahman and Rajora, 2002). According to our results, the identified genetic loci have been verified as highly polymorphic and could be further used for clonal identification also in *Populus* × *canescens* Aiton Sm.

Table 1. *Multilocus genotypes (MLG) of selected grey poplar trees (ramets)*

Ramets	sex	ORPM1	WPMS5	WPMS1	ORPM2	ORPM1	ORPM3	ORPM3	ORPM6	WPMS1	ORPM1	WPMS2	WPMS1	WPMS1	ORPM2
TPE 1 – 90, TPE 109, 111, 113, 120, 122, 124 133 135 137 155 157 159 163	*f*	223/22	298/29	189/19	180/19	189/18	221/22	195/19	200/20	165/17	175/19	178/17	222/22	195/20	217/22
TPE 107	*f*	217/22	290/31	186/19	209/20	189/18	209/21	195/19	206/20	177/18	190/19	174/17	219/22	186/21	217/22
TPE 108	*f*	223/23	290/29	186/19	188/19	189/18	209/21	195/19	206/20	177/18	190/19	174/17	219/22	186/21	217/22
TPE 151, 179, 201	*f*	223/22	276/29	189/19	188/20	189/18	207/21	183/18	203/20	171/17	175/19	174/17	219/22	192/20	217/22
TPE 199	*f*	223/22	310/31	189/19	189/20	189/18	215/21	195/19	206/20	171/17	180/19	178/17	225/22	186/18	217/21
TPE 200	*f*	223/22	276/31	189/19	197/20	189/18	207/21	195/19	206/20	165/17	175/19	178/17	219/22	186/18	217/21
TPE 101	*m*	223/23	304/30	183/18	196/20	189/18	207/21	183/18	206/20	165/17	185/19	178/17	222/22	195/21	217/22
TPE 102	*m*	223/22	304/30	183/18	196/20	189/18	207/21	183/18	206/20	156/17	175/19	178/17	228/23	192/21	217/22
TPE 103	*m*	223/23	298/29	192/19	205/20	189/18	219/22	186/18	206/20	150/17	190/19	178/17	222/22	192/25	217/22
TPE 116	*m*	217/22	300/30	189/19	201/20	189/18	217/23	189/19	206/20	144/17	175/19	178/17	222/22	195/19	217/21
TPE 117	*m*	223/23	310/31	186/18	188/20	189/18	217/23	189/20	203/20	144/17	190/19	178/17	222/22	195/19	217/21
TPE 119	*m*	223/23	298/29	180/18	196/20	189/18	213/21	192/19	206/20	171/17	185/19	178/17	228/22	192/21	217/21
TPE 123	*m*	211/22	298/31	189/19	188/20	189/18	223/22	225/22	203/20	171/17	190/19	178/17	225/22	186/19	217/21
TPE 125, 126, 130	*m*	223/22	292/29	180/19	188/19	189/18	217/22	195/19	206/20	171/18	175/19	170/17	219/22	192/21	217/21
TPE 127	*m*	205/20	300/30	189/19	188/20	217/21	217/21	198/19	206/20	168/17	185/19	178/17	228/22	192/21	217/21
TPE 128	*m*	205/20	300/30	189/19	188/20	191/19	217/21	198/19	206/20	168/17	185/19	178/17	228/22	192/21	217/21
TPE 131	*m*	205/20	300/30	189/19	188/20	191/19	217/21	198/19	206/20	168/17	185/19	178/17	228/22	192/21	217/21
TPE 132	*m*	223/23	290/29	186/19	205/20	189/18	209/22	183/18	206/21	171/18	190/19	170/17	225/22	186/25	217/22
TPE 134	*m*	223/23	290/31	186/19	205/20	189/18	209/22	183/18	206/21	171/18	190/19	170/17	225/22	186/25	217/22
TPE 139, 140, 149, 150, 162, 191	*m*	223/23	298/29	180/18	196/20	189/18	213/21	192/19	206/20	153/17	185/19	174/17	222/22	192/22	217/22
TPE 141	*m*	223/23	298/31	186/19	188/20	189/18	217/22	201/22	203/20	171/17	190/19	178/17	222/22	171/19	217/21
TPE 144	*m*	223/23	276/31	198/19	193/19	189/18	217/22	189/18	203/20	168/17	185/19	178/17	219/22	174/21	217/22
TPE 164, 192	*m*	223/23	300/31	186/19	188/20	189/18	217/21	195/19	206/20	168/17	175/19	178/17	219/22	186/21	217/22
TPE 167	*m*	223/23	298/30	186/19	180/19	189/18	217/22	195/19	200/20	165/16	175/19	170/17	222/22	195/19	217/22
TPE 175	*m*	217/22	290/30	189/18	209/20	189/18	207/20	183/18	206/20	168/17	185/19	178/17	219/22	192/21	217/22
TPE 180	*m*	217/22	308/31	186/18	188/19	189/18	217/22	195/19	206/20	171/17	190/19	178/17	222/22	186/19	217/21
TPE 184	*m*	223/22	276/31	186/19	196/20	189/18	217/22	195/19	206/20	165/17	185/19	178/17	222/22	195/21	217/22
TPE 185	*m*	211/21	298/29	189/18	188/18	189/18	217/22	189/19	206/20	171/17	190/19	178/17	222/22	195/19	217/21
TPE 186	*m*	223/23	302/30	192/19	180/19	189/18	219/22	183/18	206/20	165/17	185/19	178/17	222/22	195/21	217/22
TPE 188	*m*	223/23	300/31	192/19	196/20	189/18	217/22	192/19	206/20	156/17	215/21	178/17	213/22	174/21	217/22
TPE 189	*m*	217/21	300/30	189/19	188/20	189/20	217/21	198/19	206/20	168/17	185/19	178/17	228/22	192/21	217/21
TPE 194	*m*	217/22	300/30	186/19	188/20	189/18	217/21	192/19	203/20	165/17	180/19	170/17	219/22	192/21	217/22
TPE 193	*m*	223/22	292/29	180/19	188/19	189/18	217/22	195/19	206/20	183/19	175/19	170/17	219/22	192/21	217/21

Acknowledgements: This work was supported by the Ministry of Agriculture of the Czech Republic, institutional support MZE-RO0118 and by the project No. QJ1520297.

References

Bittsánszky A, Gyulai G, Gullner G, Kiss J, Szabó Z, Kátay G, Heszky L, Kömíves T (2009) *In vitro* breeding of grey poplar (*Populus* × *canescens*) for phytoremediation purposes. J Chem Technol & Biotechnol 84:890–894

Dayanandan S, Rajora OP, Bawa KS (1998) Isolation and characterization of microsatellites in trembling aspen (*Populus tremuloides*). Theor Appl Genet 96:950–956

Di Lonardo S, Capuana M, Arnetoli M, Gabbrielli R, Gonnelli C (2011) Exploring the metal phytoremediation potential of three *Populus alba* L. clones using an *in vitro* screening. Environ Sci Pollution Res 18:82–90

Du Q, Wang B, Wei Z, Zhang D, Li B (2012) Genetic diversity and population structure of chinese white poplar (*Populus tomentosa*) revealed by SSR markers. J Heredity 103:853–862

Gaudet M, Jorge V, Paolucci I, Beritognolo I, Mugnozza GS, Sabatti M (2008) Genetic linkage maps of *Populus nigra* L. including AFLPs, SSRs, SNPs, and sex trait. Tree Genet & Genomes 4:25–36

Iyer RR, Pluciennik A, Rosche WA, Sinden RR, Wells RD (2000) DNA polymerase III proofreading mutants enhance the expansion and deletion of triplet repeat sequences in *Escherichia coli.* J Biol Chem 275:2174–2184

Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Mol Ecol 16:1099–1106

Lexer C, Fay MF, Joseph JA, Nica M-S, Heinze B (2005) Barrier to gene flow between two ecologically divergent Populus species, *P. alba* (white poplar) and *P. tremula* (European aspen): the role of ecology and life history in gene introgression. Mol Ecol 14:1045–1057

Murashige T and Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Peakall R and Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. Bioinformatics 28:2537–2539

Politov DV, Belokon MM, Belokon YS, Polyakova TA, Shatokhina AV, Mudrik EA, Azarova AB, Filippov MV, Shestibratov KA (2015) Application of microsatellite loci for molecular identification of elite genotypes, analysis of clonality, and genetic diversity in Aspen *Populus tremula* L. (Salicaceae). Intern J Plant Genom 2015

Rahman MH and Rajora OP (2002) Microsatellite DNA fingerprinting, differentiation, and genetic relationships of clones, cultivars, and varieties of six poplar species from three sections of the genus *Populus*. Genome 45:1083–1094

van der Schoot J, Pospíšková M, Vosman B, Smulders MJM (2000) Development and characterization of microsatellite markers in black poplar (*Populus nigra* L.). Theor Appl Genet 101:317–322

Smulders MJM, Schoot JVD, Arens P, Vosman B (2001) Trinucleotide repeat microsatellite markers for black poplar (*Populus nigra* L.). Mol Ecol Notes 1:188–190

Tuskan GA, Gunter LE, Yang ZK, Yin T, Sewell MM, DiFazio SP (2004) Characterization of microsatellites revealed by genomic sequencing of *Populus trichocarpa*. Can J For Res 34:85–93

Putative rejuvenation through tissue culture in the success of *Eucalyptus globulus* vegetative propagation

Cristina M. P. Marques[1], António R. Teixeira[1], Ana F. Rodrigues[1], Joana S. P. N. Costa[1], Nuno M. G. Borralho[2], Ana M. Pires[3]

[1] RAIZ, Instituto de Investigação da Floresta e do Papel, Herdade de Espirra, 2985-270, Pegões-Gare, Portugal (cristina.marques@thenavigatorcompany.com)
[2] RAIZ, Instituto de Investigação da Floresta e do Papel, Apt 15, 3801-501 Aveiro, Portugal
[3] CEMAT-IST, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

Abstract
Clonal propagation of selected genotypes allows for genetic gains to be captured, resulting in improved productivity, adaptation and wood properties. However, adventitious rooting is a complex process, often limiting the success of tree cloning. In eucalypts, the ability of cuttings to form roots varies with species. *Eucalyptus globulus*, the major plantation species in Portugal, has a particularly irregular adventitious rooting behavior. This constitutes a bottleneck in the production of elite genotypes. In the last 30 years RAIZ has been investigating the role of different factors affecting the productivity of mother plants and the subsequent rooting success of its cuttings, including the role of nutrition, substrate, hormones and pruning strategies as well as possible rejuvenation effects. Some of these factors have been associated with greater propagation success, but results are irregular across time, and genotype dependent. The putative rejuvenation of mother plants, through tissue culture, on vegetative propagation success will be presented, as a case study. The experiments include mother plants that had originated from macro and micropropagated clones, studied over three consecutive years with up to 5 harvesting dates per year. Despite the influence of year, harvesting date and genotype, it was possible to detect significant effects of the origin of mother plant, with tissue cultured mother plants producing more cuttings, with higher rooting success. The impact of mother plant origin was greater in *E. globulus* than in the hybrid and appeared to drop with time. Increasing the value and productivity of Portuguese eucalypt forests, taking into account current regulatory, social and climate change issues, requires enhancing eucalypt productivity and adaptability. Improvements in vegetative propagation protocols for *E. globulus* would allow increments in the availability (number and diversity) of improved clones for replanting. Innovative research directions to improve propagation success will be discussed.

Keywords: *Eucalyptus globulus*, future, micropropagation, rejuvenation, vegetative propagation

Introduction

Eucalyptus globulus is the main eucalypt species planted in Portugal with around 800,000 hectares, grown mainly for pulpwood. The species is considered of excellent quality for pulp and paper production due to its high basic density and pulp yield (henceforth very low wood consumptions even by eucalypt standards), and excellent physical and chemical fiber properties, especially suitable for high quality printing papers. Several breeding programs have been established for this species, in many parts of the world. In Portugal, RAIZ Forest and Paper Research Institute has been responsible since the early 90s for one such program. It is now entering in its fourth generation of breeding.

RAIZ - Forest and Paper Research Institute is a private non-profit organization, recognized as a Research and Technology Organization of the Portuguese Scientific and Technological System, an Interface Center - Valorization and Technology Transfer Center (raiz-iifp.pt) and a Business Innovation Centre (BIC) of the European Business Network (EBN). Its associates are The Navigator Company (http://www.thenavigatorcompany.com/), the University of Coimbra, the University of Aveiro and the University of Lisbon (School of Agriculture). RAIZ activities aim to produce and transform knowledge into products, technology, and services in the forest, pulp, paper and forest-based biorefinery areas

RAIZ elite genetic materials have been mostly deployed by clonal plantations, since the late 90s. Clonal propagation of *Eucalyptus globulus* from adult material is feasible, although the rooting success is low (e.g. Eldridge et al 1994; Alfenas et al., 2009). RAIZ works in this area since the 90's, in proximity with The Navigator Company's nursery, Viveiros Aliança (VA). VA is the largest producer of certified forest plants in Europe, essentially *E. globulus* clonal plants, which are used in the company plantations and for sale to third parties. Macropropagation is the main cloning system. Despite the screening step of superior clones according to their rooting ability, there is much variability in the rooting response. This unpredictability limits the ability to produce improved material, for which demand is higher than supply in the national market. The putative rejuvenation of mother plants, through tissue culture, on vegetative propagation success will be presented, as a case study.

The last 30 years of *E. globulus* macropropagation

Before 1960, the only genera of forest trees mass propagated for wood production by making rooted cuttings of superior individuals were *Populus* and *Salix* in temperate areas and *Cryptomeria* and *Cunninghamia* in subtropical areas (Eldridge 1994). Advances in cloning techniques in Aracruz (Brazil) and Pointe Noire (Congo) allowed mass production of eucalypt hybrids since 1975: *E. tereticornis* x *E. grandis* and *E. grandis* x *E. urophylla*, respectively in Congo and Brazil (Dellwaulle 1983, Brandão et al 1984). In Portugal, CELBI reported the first results with *E. globulus*, considered a difficult to root species, around 1982.

The first clonal plantations of Portucel and Soporcel (now merged in The Navigator Company), date from the 90's. Despite continuous protocol improvements, the traditional system remains in place: outstanding trees are felled and the physiologically juvenile coppice shoots are used to make rooted cuttings. New mother plants are usually taken from previously rooted cuttings. In *E. globulus*, around 95% of selected clones do not form adventitious roots at an interesting rate (above 60%) and are not cloned operationally. Cuttings of the good rooting clones are used as mother plants, cultivated in the open air and pruned for large scale cuttings production from May to August (usually once a month). Cuttings are transferred to a shade house and it takes on average 120 days to produce and acclimate a cloned eucalypt plant. At this time, the resulting plants are evaluated for quality through screening: A - plants suitable for planting; B - plants almost ready for planting (need a few days more to develop); C - plants with reduced stem and root development (may or may not develop); D - dead plants. Success (defined as the number of A + B plants), varies significantly with the clone, but also with the year and cuttings harvesting date.

According to Borralho and Wilson (1994), estimates of the genetic control of *E. globulus* adventitious rooting ability are modest ($h^2 \approx 0.4$). There is room for the manipulation of physiological and environmental factors, in order to enhance the success of the vegetative propagation. The existing variability of vegetative propagation in open air operations makes it difficult to standardize environmental variation. Given the fluctuating scale of production of different clones, it is also difficult to implement specific protocol requirements for different genetic materials (e.g. custom nutrition, water). As a result, many of the clones (*E. globulus* or alternative species/hybrids) produced by the

genetic improvement program, with gains and / or superior adaptive potential, do not, at the moment, reach the operational production by macropropagation. In addition, the architecture of the adventitious rooting system also varies with the clone, influencing the survival at field establishment of clonal plantations.

In fact, despite much technical progress, the mechanisms by which adventitious roots are formed in woody plants are still not well understood. Adventitious root formation can be influenced by many factors, both endogenous and exogenous to the plant (da Costa et al 2013). Rooting inhibitors (Paton et al. 1970), peroxidases (Phytoud and Buchala 1989), rooting co-factors (Wilson and Staden 1990), phenolic substances (Curir et al. 1990), growth regulators (Liu and Reid 1992), polyamines (Tepfer et al. 1994) and thiamine (Chee 1995), among others, have been reported to influence this process. The large array of biochemical, physiological and anatomical processes involved suggests an underlying complexity in adventitious root formation (Hartmann et al 1997). Some of these factors have been associated with greater propagation success, but results have been irregular across time, and are genotype dependent.

RAIZ's vegetative propagation protocol was developed through experiments over the years with many different clones, and incorporates improvements in factors we can control. It is worth mentioning the aspects that have been studied, i.e., the management of mother plants (e.g. coppice shoot location in the original plant, soil preparation, fertility correction, planting density, pruning, watering periodicity, putative rejuvenation through micropropagation) and cuttings production (e.g. size, cut position/instrument, substrate, hormones/rooting stimulators, irrigation management, mineral fertilization, containers, spontaneous vegetation, biotic stress control, shoot harvesting method).

Putative rejuvenation through tissue culture – a case study

Soon after the first success in producing rooted cuttings of eucalypts (Fielding 1948), Pryor & Willing (1963) observed the decline in rooting capacity with ageing of the mother plant. In fact, adult tissues of many eucalypt species are known to be difficult to propagate by cuttings (Eldridge 1994). The existence of dormant bud strands near the base of the trunk in eucalypts, allows the production of coppice shoots, after cutting down the tree. Since the tissue at the outer end of the bud strands remains meristematic, these shoots are "juvenile" and can be used to produce cuttings. Further rejuvenation by serial production of cuttings over several generations (Franclet 1956), was trialed, as well as rejuvenation by repeated grafting (Franclet 1970). In difficult to root species, the success of vegetative propagation is directly related to the juvenility of plant propagules, in terms of root formation and quality of the root system (Alfenas et al 2009). Therefore, when new mother plants are systematically taken from previously rooted cuttings there is the risk that the plants lose their juvenility.

In *E. globulus*, like in other trees, one or more axillary buds are located between the leaves and the stem, and will develop to new shoots, once apical dominance is removed. During micropropagation subcultures, the development of these buds is stimulated, after removal of the apical meristem of the explants. The literature reports that this procedure promotes a reversal in the maturation stage of the adult original material (Eldridge 1995). In eucalypts, a minimum of 1 year of *in vitro* culture has been reported necessary to rejuvenate tissues, with the resulting positive effect in improving adventitious rooting (Alfenas et al 2009).

The next sections summarize results from experiments followed up for several years in two mother plant areas, aiming to investigate the putative effect of mother plant rejuvenation, through tissue culture, on vegetative propagation success of several *E. globulus* clones and one eucalypt hybrid.

Materials and Methods

Mother Plant Area 1 (MPA1)

The first series of experiments was based on 180 mother plants established in Abril 2011 at Viveiros Aliança Nursery, in Herdade de Espirra (Pegões, Central Portugal). A set of 90 mother plants were obtained from macropropagated cuttings (taken from the 2010 propagation campaign) and a set of 90 mother plants were obtained from micro-propagated epicormic shoots (on *in vitro* culture for at least one year). Each set included three *E. globulus* clones (H1, S7 and G4). The identity of all plants was certified at the molecular level.

These 180 mother plants were arranged in three consecutive rows, each one having a different clone (viz H1, S7 and G4). In each row (with a unique clone), the two mother plant origins (i.e macro and micropropagated) were randomly assigned in six repetitions (of five mother plants each). Experiments taken from MPA1 were carried out in three consecutive years (2012, 2013 and 2014). The MPA1 is illustrated in Figure 1.

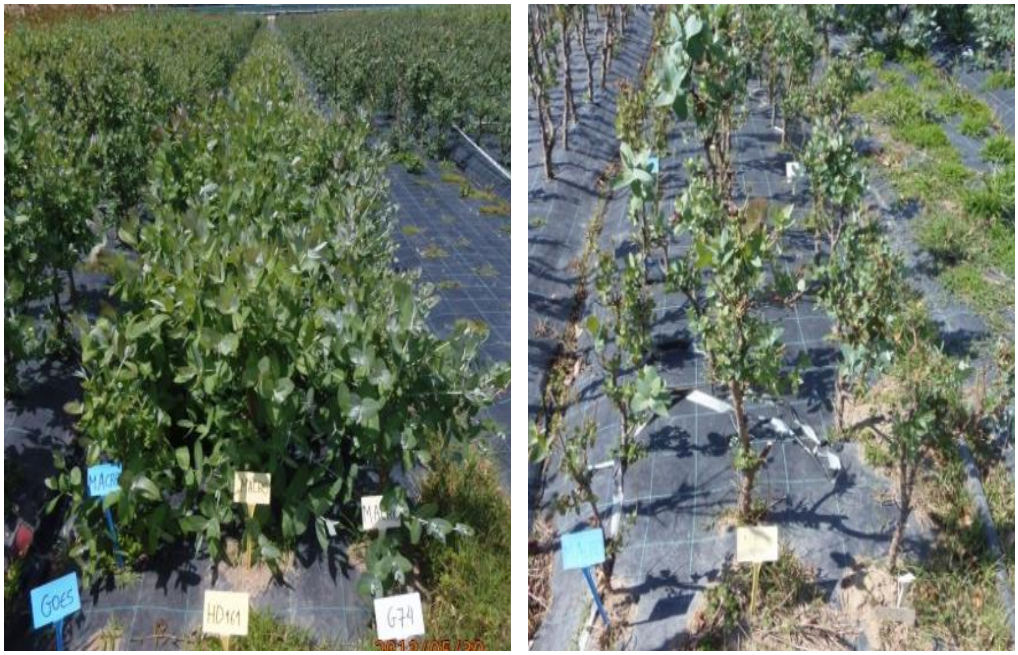


Figure 1. *Mother plants at Viveiros Aliança (MPA1) before (left) and after (right) cuttings harvest for vegetative propagation (macropropagation).*

Mother Plant Area 2(MPA2)

The second set of experiments was based on mother plants established in March 2014 at RAIZ nursery facilities, also in the Herdade de Espirra (Pegões). Half of the mother plants were obtained by macropropagation (from the 2013 campaign) and another half from micropropagation (at least one year old in vitro cultures). This experiment included two E. globulus clones (12 and G4) and one hybrid clone 77 (E. grandis x E. globulus). The identity of all plants was certified at the molecular level.

The 280 mother plants in MPA2 were arranged in six lines, each one with a different clone (12, G4 and 77), each one with five repetitions of 10 mother plants, either macro or micropropagated (for the two E. globulus clones) and four blocks of 10 plants for the hybrid clone. Experiments taken from MPA2 were carried out in three consecutive years (2014, 2015 and 2016). The MPA2 is illustrated in Figure 2.

Figure 2. *Mother plants at RAIZ R&D nursery (MPA2) before (left) and after (right) cuttings harvest for vegetative propagation (macropropagation).*

Propagation trials

One clone (G4) was present both in MPA1 and MPA2. Management of the two mother plant areas, including irrigation, fertilization and weed control, followed the company's standard procedures. In 2012 (MPA1), the mortality rate in the mother plants was 25%, and in 2013 it was 28%, with a predominance of the S7 clone (22% of the 25% in 2012 and 25% of the 28% in 2013), less developed than the other clones throughout the trial, due to competition of neighboring materials. In MPA2, a sprinkler irrigation system was installed, which caused flooding problems in some areas and resulted in the mortality of some mother plants over time (about 23% in 2016), affecting some experimental blocks (especially block 3 and clone 12).

The propagation trials with cuttings collected in MPA1 MPA2 were carried out over three years and different harvesting dates within each year (Table 1). Harvesting dates were defined according to shoot development status, but tended to occur once a month, between May and September. The total number of cuttings harvested per treatment (macro or micropropagated mother plants) varied between 132 and 1122 (clone.year.date).

Table 1. *Number of rooting experiments performed in MPA1 and MPA2, per year and clone.*

Mother plant area (MPA)	Year	Time since establishment (years)	Number of harvests	Clones
1	2011	1	4	G4, H1 and S7
1	2012	2	5	G4, H1 and S7
1	2013	3	4	G4, H1 and S7
2	2014	1	4	G4, 12 and 77
2	2015	2	3	G4, 12 and 77
2	2016	3	4	G4, 12 and 77

All rooting experiments followed the same basic design. Cuttings were set to root in rigid polyethylene 200 cm^3 containers with a substrate composed of 70% peat and 30% styrofoam in supporting trays with 6 x 10 alveoli. The base of each cutting was immersed in liquid IBA rooting hormone solution (at 5000

ppm concentration for five seconds). The cuttings were placed in a shade house, with no controlled environment other than irrigation. Phytosanitary controls were done once or twice a week, depending on the need. After approximately 60 days the plants were transferred to an acclimatization zone. Intermediate assay surveys were performed at 30 days and final surveys at 120 days (Figure 3).

Figure 3. *Shade house facility used for rooting cuttings and close up of the cuttings after set.*

Measurements and analysis

Measurements reported focus on the average number of cuttings collected per mother plant and rooting success at 120 days (number of plants suitable or almost ready for planting, Class A + B plants). A detailed report of other variables assessment can be found in Marques et al 2012, Costa et al 2014, Costa et al 2015.

Statistical analyses included analysis of variance (ANOVA) and Tukey HSD *post hoc* tests ($p < 0.05$). Data analysis models were fitted using the lm function of the R Software (R Core team 2016). Given the variation in this type of experimental data, it was necessary to take into account the influence of other factors (clone, cuttings harvesting date, block and year), that was investigated separately.

Results and Discussion

The main objective of MPA1 (2011 to 2013) and MPA2 (2014 to 2016) trials was to compare the productivity and performance of cuttings taken from micropropagated mother plants (expected to have been rejuvenated) and conventionally macropropagated mother plants. Since the experiment was repeated in three consecutive years, this also allowed investigating the effects of ageing in productivity and rooting ability.

Despite the possibility of performing many repetitions of the experiment (n equals the number of cuttings induced to root), over time, the variability of this type of data is enormous, reflecting the strong influence of the environment on trait expression. This includes variation on the number of cuttings harvesting dates that were possible in different years (three in 2015, four in 2011-2013-2014-2016, five in 2012). Moreover, for different reasons, after three years, mortality in mother plants in the operational and R&D nurseries, limited the possibility of monitoring effects for a longer period.

In practical terms the observed data variation in different years and harvesting dates, cannot be eluded in the current propagation system. Despite the difficulties in generalizing the results, the decision of implementing rejuvenation strategies for E. globulus macropropagation mother plants in the open air, has to be based on overall results that accommodate the cumulative effect of the environment in different years. These two sets of three year-long experiments are informative in that respect.

Mother plant productivity

Overall mother plant productivity, across the two MPA and ages was around 18 cuttings per harvesting date. In this respect, a common pattern emerges in both MPA1 and MPA2 (Figures 4 and 5, respectively): productivity differs significantly with the age of the mother plants, being lower in the 1st year (with an average of 10 cuttings per harvest per mother plant), increased in the 2nd year (with an average of 25 cuttings), and dropped on the 3rd year to an average of 19 cuttings. The low productivity in the year of establishment is expected since mother plants are still small in size. The drop at the 3rd year, however is surprising and suggests some ageing effects might already occur.

Clone effects were also only significant in MPA1 (Figure 4 and 5), with differences between clones remaining consistent across ages (i.e. there was no clone by age interactions).

Differences between micro and macropropagated mother plants were only statistically significant in MPA2 and of small magnitude.

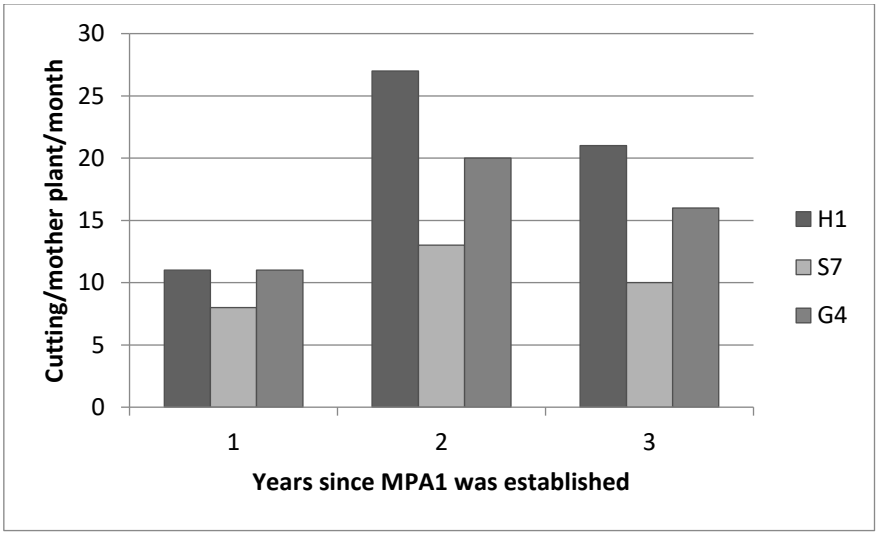


Figure 4. *Average number of cuttings collected per mother plant in MPA1 (at 1 to 3 years since being established), for three E. globulus clones (H1, S7 and G4).*

On the other hand, in MPA2, clones did not have a significant effect in cuttings collected per mother plant (Figure 5). Therefore, a linear model with the other significant factors was first fitted to the data (year x date + block). This model explained 61% of the variance of the cuttings/mother plant data. After this, another linear model was fitted to the residuals of the first model, with factors clone and mother plant origin, both showing a statistically significant effect with p-values of 0.007 and 0.019 respectively.

On average, one-year old micropropagated mother plants produced two more cuttings/mother plant each month than macropropagated mother plants of the same age. In two-year old mother plants, this difference was four and at three-year old mother plants it was zero. Across the three years, the average overall difference was two more cuttings/micropropagated per mother plants, relatively to macropropagated mother plants.

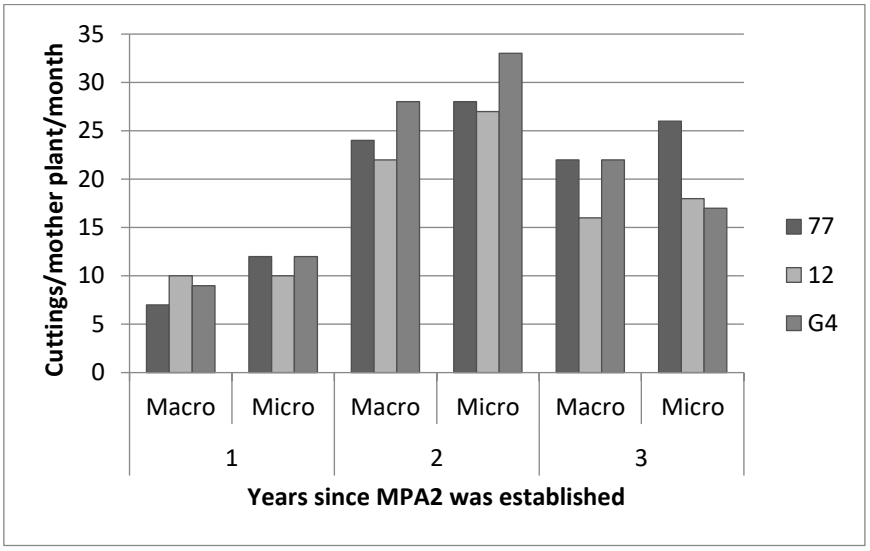


Figure 5. *Average number of cuttings collected per mother plant in MPA2 (at 1 to 3 years since being established), for two E. globulus clones (12 and G4) and one hybrid clone 77.*

Rooting percentage at 120 days

Rooting tests from cuttings collected in MPA1 (conducted between one to three years after the mother plant yard was established), indicate a significant effect for clone, mother plant origin and date (Figure 6). On average (in 2011), cuttings from one-year old micropropagated mother plants rooted better (12% more) than macropropagated mother plants. In two-year old mother plants, the difference dropped to 6 percentage points and in three-year old mother plants it dropped further to 4 percentage points. Over the three years, mother plants originated from tissue culture had on average 7% better rooting.

The effects of micropropagation were generally consistent across clones. However, it is interesting to point out that the clone which rooted best (*E. globulus* H1), was less responsive to the putative mother plant rejuvenation treatment (Figure 6), with an improvement only apparent in one-year old mother plants.

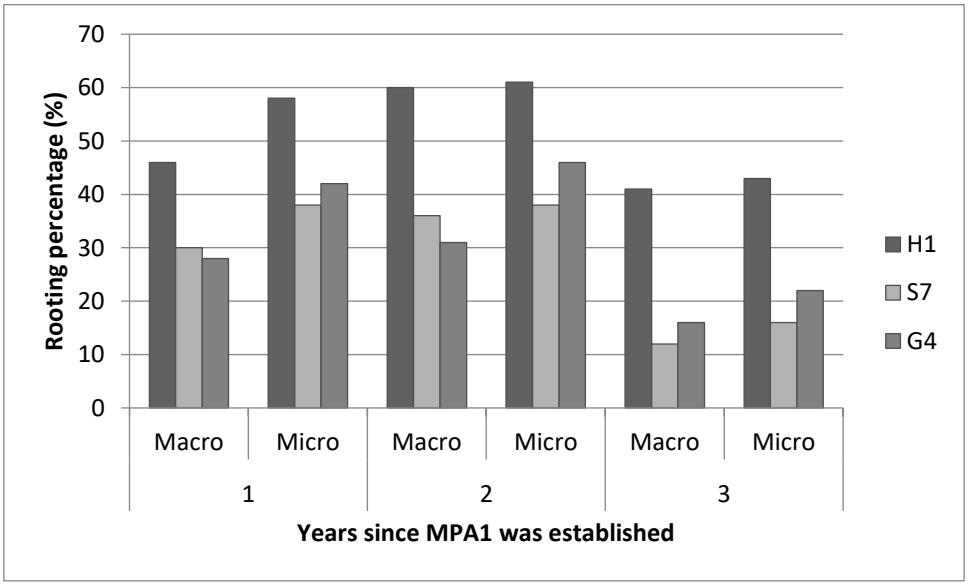


Figure 6. *Percent rooting for cuttings harvested in MPA1 (at 1 to 3 years since being established) and for three E. globulus clones (H1, S7 and G4).*

Results from the rooting experiments in MPA2 were generally consistent with MPA1 (Figure 7). The clone effects were again highly significant, accounting for 10% of the trait's variation. On average, one-year old micropropagated mother plants had a rooting success 8 percentage points above macropropagated mother plants, although the effect was mainly only observed for the two *E. globulus* clones. In fact, it is interesting to note that the hybrid clone 77 (an *E. grandis x E. globulus*), was less responsive to the mother plant putative rejuvenation treatment compared with all the other *E. globulus* clones.

In two and three-years old mother plants, differences in rooting due to mother plant origin were small or null (0% and 4% respectively. The average overall difference in rooting from micropropagated mother plants across the three years was 4 percentage points.

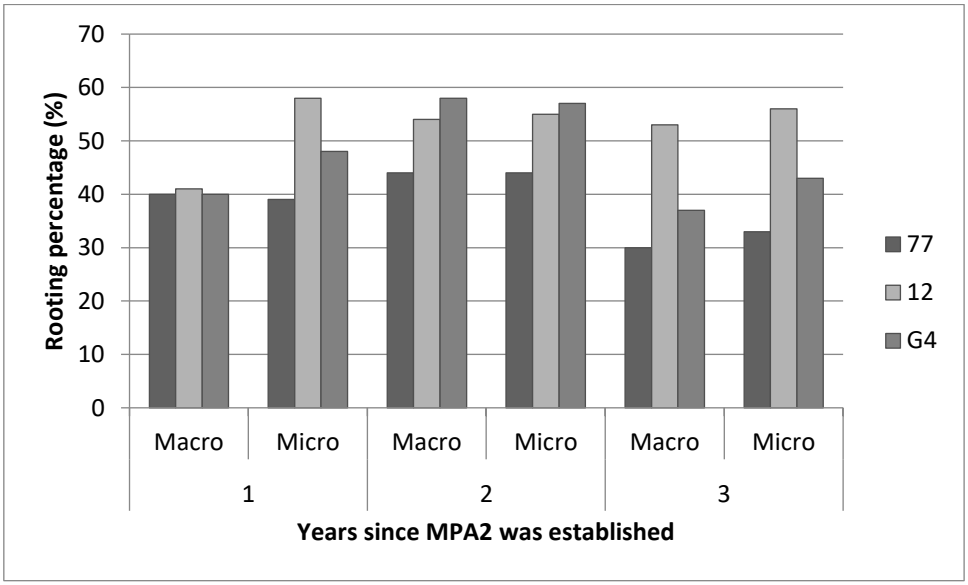


Figure 7. *Percent rooting for cuttings harvested in MPA2 (at 1 to 3 years since being established), for two E. globulus clones (12 and G4) and one hybrid clone 77.*

Across the two series of trials (MPA1 and MPA2) micropropagated mother plants improved rooting by 5.5% relatively to macropropagated mother plants, over a three year period. However, the impact of tissue culture occurred mostly in the first year after establishing the multiplication yard. After that, differences dropped significantly.

Conclusions and future research directions

Vegetative propagation data is known to be highly variable, as a result of a complex array of genetics and environmental factors. Traditional vegetative propagation schemes, which rely on open air multiplication yards, make it even harder to understand what triggers adventitious rooting success and how to best manipulate them.

The propagation experiments reported here were based on two separate mother plant areas (MPA), five different clones, collected in three consecutive years with up to 5 harvesting dates per year. The overall results indicate a small, but significant, improvement in mother plant productivity (on average two more cuttings/micropropagated mother plant, only in MPA2) and better rooting of micropropagated mother plants (in both MPAs). The mean improvement in rooting success over the three years was around 5.5%. Albeit small, this difference would be important from the point of view of the economics of any clonal nursey, and could be justified if the cost of micropropagation was compatible.

The results indicate that mother plant mortality after three years is on average 26% and the positive effects due to micropropagation may decrease after the first year. Three years after the MPA were established, overall differences in productivity and rooting between mother plant origins were null or small (0% and 4%, respectively). This would suggest a mother plant turnover of two to three years, henceforth the ability to micropropagate *E. globulus* on a large scale and at reasonable costs.

Mini cuttings production systems, as the ones successfully implemented in *Eucalyptus* ssp. clonal propagation in Brazil, have improved the rooting and root quality of most clones significantly (Assis 1998). Mini mother plants, growing in controlled stable environments, are expected to be more juvenile and performance consistent. However, propagation protocols need to be adjusted to recalcitrant species, such as *E. globulus*, and for more seasonal climates such as that in Portugal. New strategies are needed to overcome these challenges. Good results in certain environmental conditions reassure us that genetically, improvements are possible, in many clones. Preliminary results point out the largely unexplored effects of light quality and intensity in adventitious rooting success (Hoad & Leakey 1996; Ruedell 2013). In addition, the use of rhizobacteria strains to promote rooting in eucalypts appears to be another promising approach. Its potential has been studied in several woody species, including *E. nitens* x *E. globulus* mini-cuttings (Gonzalez et al 2017).

The ability to improve cloning of elite forest tree genotypes will be central to promote intelligent land-use planning, prizing the role of, and enhancing resilience and adaptive capacity of ecosystems to climate change. This will result in social, environmental and economic benefits. Wood will be increasingly sought after as raw material and eucalypts are admirable biomills.

Acknowledgments: To the late Mr Pedro Queiróz Pereira, Chairman of the The Navigator Company, for his love of Portugal. To staff of The Navigator Company operational nursery, with special mention to José Carvalho and Vasco Paiva, for assistance during the trials and discussions. Lúcia Fernandes, Alexandra Nunes, Ana Rodrigues, Daniela Ferreira and Gabriel Dehon are also greatly acknowledged for their assistance at various point of the project.

References

Alfenas AC, Zauza EAV, Mafia RG, Assis TF (2009) Clonagem e doenças do eucalipto. Universidade federal de Viçosa

Assis TF, Teixeira SL (1998) Enraizamento de plantas lenhosas. In: Torres AC, Caldas LS, Buso JÁ. Cultura de tecidos e transformação genética de plantas. Brasília, Embrapa

Brandão LG, Campinhos E & YK Ikemori (1984) Brazil's new forest soars to success. Pulp and Paper International 26 (12): 38-40

Borralho NMG, Wilson PJ (1994) Inheritance of initial survival and rooting ability in *Eucalyptus globulus* Labill. stem cuttings. Silvae Genet 43:238–242

Chee PP (1995) Stimulation of adventitious rooting of *Taxus* species by thiamine. Plant Cell Rep 14: 753-757

Costa J, Nunes, A, Ferreira D, Carvalho JC, Paiva V, Marques C (2014) Avaliar o efeito de plantas micropropagadas como pés-mãe, na capacidade de produção de estacas- ensaio realizado na campanha 2012. Relatório técnico interno RPJ09012, junho

Costa J, Nunes, A, Ferreira D, Carvalho JC, Paiva V, Marques C (2015) Avaliar o efeito de plantas micropropagadas como pés-mãe, na capacidade de produção de estacas- ensaio realizado na campanha 2013. Relatório técnico interno RPJ09012, março

Curir P, VanSumere CF, Termini A, Barthe P, Marchesini A, Dolci M (1990) Flavonoid accumulation is correlated with adventitious roots formation in *Eucalyptus gunnii* Hook micropropagated through axillary but stimulation. Plant Physiol 92: 1148-1153

da Costa CT, de Almeida MR, Ruedell CM, Schwambach J, Maraschin FS, Fett-Neto AG (2013) When stress and development go hand in hand: main hormonal controls of adventitious rooting in cuttings. Front Plant sci 4:133

Delwaulle JC (1983) Creation et multiplication vegetative par boutourage d'*Eucalyptus* hybrids en

République Populaire du Congo. Silvicultura, São Paulo 32, 775-778.
Eldridge K, Davidson J, Harwood C, van Wyk G (1994) Eucalypt domestication and breeding. Oxford
Fielding JM (1948) *Eucalyptu*s cuttings. Wildlife 10, 153
Franclet A (1956) Premiers travaux d'amélioration génétique des *Eucalyptus*. Annales de la Recherche Forestiére au Maroc 4, 63-85
Franclet A (1970) Techniques de bouturage des *Eucalyptus camaldulensis*. Note technique 12. FAO/IRT
Gonzalez P, Sossa K, Rodríguez F, Sanfuentes E (2017) Rhizobacteria strains as promoters of rooting in hybrids of *Eucalyptus nitens* x *Eucalyptus globulus*. Chilean F Agricl Res 78 (1): 3-12
Hartmann HT, Kester DE, Davies JR, Geneve RL (1997) Plant propagation – Principles and practices. NJ, Prentice Hall International
Hoad SP, Leakey RRB (1996) Effects of pre-severance light quality on the vegetative propagation of *Eucalyptus grandis* W. Hill ex Maiden. Trees 10: 317-324
Liu J-H, Reid DM (1992) Auxin and ethylene-stimulated adventitious rooting in relation to tissue sensitivity to auxin and ethylene production in sunflower hypocotyls. J Exp Bot 43(254): 1191-1198
Marques C, Fernandes L, Dehon G (2012) Avaliar o efeito de plantas micropropagadas como pés-mãe, na capacidade de produção de estacas- desempenho dos pés-mãe macro/micro em 2010 e 2011. Relatório técnico interno RPJ09012, junho
Paton DM, Willing RR, Nicholls W, Pryor LD (1970) Rooting of stem cuttings of *Eucalyptus*: a rooting inhibitor in adult tissue. Aust J Bot 18: 175-183
Phytoud F, Buchala AJ (1989) Peroxidase activity and adventitious rooting in cuttings of *Populus tremula*. Plant Physiol Biochem 27(4): 503-510
R Core Team (2016) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/
Pryor LD, Willing RR (1963) The vegetative propagation of *Eucalyptus* – an account of progress. Australian For 27, 52-62
Ruedell CM, de Almeida MR, Schwambach J, Posenato CF, Fett-Neto AG (2013) pre and post-severance effects of light quality on carbohydrate dynamics and microcutting adventitious rooting of two *Eucalyptus* species of contrasting recalcitrance. Plant Growth Regul 69: 235-245
Tepfer D, Damon J-P, Ben-Hayyim G, Pellegrineschi A, Burtin D, Martin-Tanguy J (1994) Control of root system architecture through chemical and genetic alterations of polyamine metabolism. In:
Davis TD, Haissig BE (eds) Biology of adventitious root formation. Plenum Press, New York, pp 181-189
Wilson PJ, Van Staden J (1990) Rhizocaline, rooting co-factors, and the concept of promoters and inhibitors of adventitious rooting - a review. Ann Bot 66: 479-490

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Epigenetics in trees: a source of plasticity and adaptation in the context of climate change

Stéphane Maury[1*], Anne-Laure Le Gac[1], Clément Lafon-Placette[1], Mamadou Dia Sow[1], Régis Fichot[1], Alain Delaunay[1], Isabelle Le Jan[1], Marie-Anne Lelu-Walter[2], Vincent Segura[2], Odile Rogier[2], Jean-François Trontin[3], Christophe Plomion[4], Grégoire Le Provost[4], François Ehrenmann[4], Jérome Salse[5], Christophe Ambroise[6], Svetlana Gribkova[7], Marie Mirouze[8], Cristoph Grunau[9], Cristian Chaparro[9], Steeven H. Strauss[10], Daniel Conde[11], Isabel Allona[11], Jörg Tost[12]

[1] Laboratoire De Biologie Des Ligneux Et Des Grandes Cultures, LBLGC, 1207 USC1328, INRA-Université Orléans, Orléans France ; [2] Biologie Intégrée Pour La Valorisation De La Diversité Des Arbres Et De La Forêt, BIOFORA, UMR 0588, INRA-ONF, Orléans France ; [3] FCBA, Pôle Biotechnologies Et Sylviculture Avancée, Cestas, France ; [4] Biodiversité, Gènes Et Communautés, BIOGECO, UMR 1202, INRA-Université Bordeaux, Bordeaux France; [5] Génétique, Diversité Et Écophysiologie Des Céréales, GDEC, UMR 1095, INRA-Université Clermont Auvergne, Clermont-Ferrand France; [6] Laboratoire De Mathématiques Et Modélisation d'Évry, LAMME, UMR 8071, CNRS-Université d'Evry Val d'Essonne, Evry France; [7] Laboratoire De Probabilités Et Modèles Aléatoires, LPMA, UMR 8001 CNRS-Université Paris Diderot, Paris France; [8]Laboratoire Génome Et Développement Des Plantes, LGDP, UMR 5096 – CNRS/UPVD, IRD Perpignan, France ; [9] Laboratoire des Interactions Hôtes-Pathogènes-Environnements, IHPE, UMR 5244 Université De Perpignan Via Domitia, Perpignan France ; [10] Department Of Forest Ecosystems and Society, Oregon State University, Corvallis, OR 97331, USA; [11] Centro De Biotecnología Y Genómica De Plantas, Universidad Politécnica De Madrid (UPM)–Instituto Nacional De Investigación Y Tecnología Agraria Y Alimentaria (INIA), 28223 Madrid, Spain ; [12] Laboratoire Épigénétique Et Environnement, LEE, CNRGH CEA, Evry France
**Corresponding author: stephane.maury@univ-orleans.fr*

Abstract
Ongoing global climate changes will impact forest productivity notably through reduced water availability and heat periods. One possibility to adapt to this situation is via phenotypic plasticity for which we propose epigenetic mechanisms to be the main source of flexibility. Indeed, trees that are e sessile organisms with a long-life span and playing major ecological roles represent valuable models to study the role of epigenetics in plasticity. Epigenetics can be defined as any non-genetic molecular modification (no DNA sequence change) of the genome that alters gene expression and the phenotype. While many papers have been published over the last few years dealing with annual plants such as Arabidopsis, tomato and maize, only few recent reports, but increasing, are actually available on trees. Here, we present some of these studies, encouraging new efforts in order to clarify the specific roles of epigenetics in trees. These studies will help to evaluate potential recommendations for tree breeding, genetic resources conservation and forest management.

Keywords: Shoot apical meristem, somatic embryo, DNA methylation, poplar, maritime pine, stress

Introduction, discussion and conclusion

Global changes affect the productivity and survival of most living organisms in all ecosystems (Chapin et al., 2000). Plants, as sessile organisms, have developed a high sensitivity to their environment and a strong potential for adaptation based on a high level of standing genetic variation (Chen et al., 2017) and remarkable phenotypic plasticity (Nicotra et al., 2010). Trees are outstanding organisms in terms of their extreme size, long lifespan, complex life cycles and wood production (Neale et al 2017). Trees and forests also play a major role in Earth's ecology, by supplying a range of ecosystem services. In the past decades, widespread forest die-off due to drought and/or heat constraints has been observed around the world for all forest biomes, and is predicted to increase with ongoing climate change (Allen et al., 2010; Intergovernmental Panel on Climate Change, IPCC, 2014). Climate change models predict further increases in mean temperature, and increases in the frequency and severity of extreme droughts. There is, therefore, an urgent need to improve our understanding of the mechanisms of adaptation in trees, to facilitate the design of new genetic material through breeding and/or biotechnological approaches, to improve the management of genetic resources, to prevent forest loss and to guarantee that these ecosystems continue to play their economic and ecological roles.

Studies of the genetic bases of plant adaptation to date have focused mostly on the contribution of standing structural variation (mostly single nucleotide polymorphisms - SNPs) to local adaptation (Lascoux et al., 2016). Surprisingly, epigenetic mechanisms (DNA methylation and histone modifications) have remained largely unstudied, despite their great importance in long-lived organisms, in which they allow rapid phenotypic modifications in response to changing environmental conditions ('priming effect'). It has been suggested that sessile organisms persisting at the same site for long periods of time make particular use of DNA methylation to facilitate rapid adaptation through phenotypic plasticity (Nicotra et al., 2010). A single genotype may produce different epigenomes during its lifespan, to cope with varying environmental cues. Alternatively, individual trees may, during the course of their lifespan, produce large numbers of generations of offspring with different epigenomes. A number of highly significant abiotic and biotic environmental factors vary over the long lifespan of trees, so each generation of seeds can receive a different epigenetic 'priming' that is then exposed to natural selection. Trees, therefore, build up seed banks that "store" these different epigenetic 'primings', thereby generating an "ecological epigenetic memory" that exposes epigenetic responses to a broad range of environmental cues.

Most epigenomics studies to date have focused on DNA methylation, because it is relatively easy to analyze at the genomic scale. Indeed, DNA methylation is a single chemical modification, rather than multiple histone modifications that can be easily detected with appropriate next-generation sequencing methods. In addition, the changes remain stable through cell division (mitosis and meiosis) and play an integral role in relation to RNA-directed DNA methylation, mobilize transposable elements (TE) and its complex relationships with gene expression (Meyer, 2015; Seymour and Becker, 2017). However, the relationship between DNA methylation and gene expression remains complex, and the relative contribution of DNA methylation to phenotypic variation, therefore, requires further clarification. It also remains difficult to differentiate between changes directly mediated by DNA methylation and secondary effects. DNA methylation has been extensively studied in the model plant *Arabidopsis thaliana* and in several crop species, and has been shown to have effects extending from gene expression to phenotypes (Pecinka et al., 2013; Schmitz and Ecker, 2012), including adaptive traits (Cortijo et al., 2014; Johannes et al., 2009; Kooke et al., 2015; Schmid et al., 2018), and to be strongly correlated with climate of origin (Kawakatsu et al., 2016).

A few studies have already confirmed that epigenomic approaches are useful for improving our understanding of development and adaptive responses to environmental constraints in forest trees (see reviews Bräutigam et al., 2013; Plomion et al., 2016; Sow et al., 2018a). Poplar (Populus spp.) is a model tree with sequenced genomes, genetic diversity, fast juvenile growth, and large water requirements and thus relevant for investigating the ecophysiological and molecular determinants of tolerance to water

deficit (Tuskan et al., 2006; Jansson and Douglas, 2007). Accordingly, poplar has been used to investigate the role of epigenetics in the response of trees to environmental variations (Gourcilleau et al., 2010; Raj et al., 2011; Vining et al., 2012; Lafon-Placette et al., 2013; Zhu et al., 2013; Liang et al., 2014; Guarino et al., 2015; Schönberger et al., 2016; Conde et al., 2017; Lafon-Placette et al., 2018; Le Gac et al., 2018; Sow et al., 2018b). Several studies tested whether signs of epigenetic reprogramming in response to environmental variations could be observed in the shoot apical meristem (SAM). The SAM-specific epigenetic component of geographic site-dependent growth performances (Guarino et al., 2015; Schönberger et al., 2016), environment-induced bud break (Conde et al., 2017) and response to water availability (Gourcilleau et al., 2010; Raj et al., 2011; Liang et al., 2014; Lafon-Placette et al., 2018; Le Gac et al., 2018; Sow et al., 2018b) have been reported. One major point is that changes in the epigenome and transcriptome of the poplar shoot apical meristem in response to water availability were shown to affect preferentially hormone pathways and could participate in the developmental plasticity (Lafon-Placette et al., 2018). In addition, an investigation on field grown poplar clones or natural populations revealed that the winter-dormant shoot apical meristem shows environmental epigenetic memory of changes that have occurred during the vegetative period (Le Gac et al., 2018; Sow et al., 2018b). These data suggested a possible mitotic transmission in the SAM from the induction time to the winter.

There is evidence to suggest that genetic variation is a major source of epigenetic variation, and that DNA methylation directly influences gene expression at only a small subset of specific loci, other than through its control of TE activity. The EPITREE project (ANR-17-CE32-0009-01; https://www6.inra.fr/epitree-project/) will study the impact of epigenetic marks (DNA methylation) together with gene expression, and allelic variation on forest tree adaptation to local environments and phenotypic plasticity. In particular, we will explore the benefits of considering epigenetic marks in addition to genetic polymorphisms and phenotypes in tree breeding and the characterization of genetic resources, to provide proof-of-concept in two major forest tree species (poplar and oak).

Another interest of epigenetics for forest trees is the possibility to use it for 'priming'. Thus, priming by biotic or abiotic stress exposure is associated to epigenetic remodeling and increased tolerance to stress that could be transmitted by clonal propagation (Springer and Schmitz, 2017). For example, Yakovlev et al. (2011; 2016) have identified a temperature-dependent epigenetic memory affecting the timing of bud burst and bud set in trees generated by temperature changes during somatic embryogenesis (Carneros et al. 2017; Yakovlev & Fossdal, 2017). Various clonal propagation systems (cuttings, somatic embryogenesis) are available for some tree species, and the mass production of a few elite genotypes. The success of clonally propagated trees is conditioned by both genetic and non-genetic effects as epigenetics. However, the role of DNA methylation in tree somatic embryogenesis and environmental memory is still a challenging question (Teyssier et al., 2013; Sow et al, 2018a).
Altogether, the role of epigenetics in forest trees is an ambitious and still open question that will need fundamental studies by public/private international consortiums with a view to establishing ambitious objectives for tree breeders and the managers of genetic resources as we recently reported (Sow et al., 2018a).

Acknowledgements: The authors thank the Conseil Régional Centre-Val de Loire (IMTEMPERIES project / 2014-00094511) and the French National Research Agency (EPITREE, ANR-17-CE32-0009-01).

References

Allen CD, Macalady AK, Chenchouni H, Bachelet D, McDowell N, Vennetier M, Kitzberger T, Rigling A, Breshears DD, Hogg EH, et al. (2010) A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests. Forest Ecology and Management 259: 660–684

Bräutigam K, Vining K, Lafon-Placette C, Fossdal CG, Mirouze M, Gutiérrez MJ, Fluch S, Fernández

Fraga M, Guevara MÁ, Abarca D, Johnsen Ø, Maury S, Strauss SH, Campbell M, Rohde A, Díaz-Sala C, Cervera MT (2013) Epigenetic regulation of adaptive response of forest tree species to the environment. Ecology and Evolution 3: 399-415

Carneros E, Yakovlev I, Viejo M, Olsen JE, Fossdal CG (2017) The epigenetic memory of temperature during embryogenesis modifies the expression of bud burst-related genes in Norway spruce epitypes. Planta 246: 553–566

Chapin FS, Zavaleta ES, Eviner VT, Naylor RL, Vitousek PM, Reynolds HL, Hooper DU, Lavorel S, Sala OE, Hobbie SE, Mack MC, Díaz S (2000) Consequences of changing biodiversity. Nature 405: 234-242

Chen J, Glémin S, Lascoux M (2017) Genetic Diversity and the Efficacy of Purifying Selection across Plant and Animal Species. Mol Biol Evol 34: 1417-1428

Conde D, Le Gac AL, Perales M, Dervinis C, Kirst M, Maury S, González-Melendi P, Allona I (2017) Chilling-responsive DEMETER-LIKE DNA demethylase mediates in poplar bud break: role of active DNA demethylase in trees' bud break. Plant Cell and Environment 40: 2236-2249

Cortijo S, Wardenaar R, Colome-Tatche M, Gilly A, Etcheverry M, Labadie K, Caillieux E, Hospital F, Aury JM, Wincker P, et al. (2014) Mapping the epigenetic basis of complex traits. Science 343: 1145–1148

Gourcilleau D, Bogeat-Triboulot M-B, Thiec D, Lafon-Placette C, Delaunay A, El-Soud WA, Brignolas F, Maury S. 2010. DNA methylation and histone acetylation: genotypic variations in hybrid poplars, impact of water deficit and relationships with productivity. Annals of Forest Science 67: 208–208

Guarino F, Cicatelli A, Brundu G, Heinze B, Castiglione S (2015) Epigenetic Diversity of Clonal White Poplar (*Populus alba* L.) Populations: Could Methylation Support the Success of Vegetative Reproduction Strategy? PLOS ONE 10 e0131480

Intergovernmental Panel on Climate Change (2014) Field CB, Barros VR, (eds). Climate change: impacts, adaptation, and vulnerability: Working Group II contribution to the fifth assessment report of the Intergovernmental Panel on Climate Change. New York, NY: Cambridge University Press

Jansson S, and Douglas CJ (2007) *Populus*: a model system for plant biology. Annual Review Plant Biology 58: 435–458

Johannes F, Porcher E, Teixeira FK, Saliba-Colombani V, Simon M, Agier N, Bulski A, Albuisson J, Heredia F, Audigier P, et al. (2009) Assessing the impact of transgenerational epigenetic variation on complex traits. PLoS Genetics 5, e1000530

Kawakatsu T, Huang SC, Jupe F, Sasaki E, Schmitz RJ, Urich MA, Castanon R, Nery JR, Barragan C, He Y, et al. (2016) Epigenomic diversity in a global collection of Arabidopsis thaliana accessions. Cell 166: 492–505

Kooke, R., Johannes, F., Wardenaar, R., Becker, F., Etcheverry, M., Colot, V., Vreugdenhil, D., and Keurentjes, J.J. (2015). Epigenetic basis of morphological variation and phenotypic plasticity in Arabidopsis thaliana. Plant Cell 27: 337–348

Lafon-Placette C, Faivre-Rampant P, Delaunay A, Street N, Brignolas F, Maury S (2013) Methylome of DNase I sensitive chromatin in Populus trichocarpa shoot apical meristematic cells: a simplified approach revealing characteristics of gene-body DNA methylation in open chromatin state. New Phytologist 197:416-430

Lafon-Placette C, Le Gac A-L, Chauveau D, Segura V, Delaunay A, Lesage-Descauses MC, Hummel I, Cohen D, Jesson B, Le Thiec D, Bogeat-Triboulot MB, Brignolas F, Maury S (2018) Changes in the epigenome and transcriptome of the poplar shoot apical meristem in response to water availability affect preferentially hormone pathways. Journal of Experimental Botany 69: 537-551

Lascoux M, Glémin S, Savolainen O (2016) Local Adaptation in Plants. Encyclopedia of Life Sciences. Chichester, UK: John Wiley & Sons, Ltd https://doi.org/ 10.1002/9780470015902. a00252701–7

Le Gac A-L, Lafon-Placette C, Chauveau D, Segura V, Delaunay A, Fichot R, Marron N, Le Jan

I, Berthelot A, Bodineau G, Bastien J-C, Brignolas F, Maury S (2018) Winter-dormant shoot apical meristem in poplar trees shows environmental epigenetic memory. Journal of Experimental Botany 69: 4821-4837

Liang D, Zhang Z, Wu H, Huang C, Shuai P, Ye CY, Tang S, Wang Y, Yang L, Wang J (2014) Single-base-resolution methylomes of Populus trichocarpa reveal the association between DNA methylation and drought stress. BMC Genetics. BioMed Central, p. S9

Meyer P (2015) Epigenetic variation and environmental change. Journal of Experimental Botany 66: 3541–3548

Neale DB, Martínez-García PJ, De La Torre AR, Montanari S, Wei XX (2017) Novel Insights into Tree Biology and Genome Evolution as Revealed Through Genomics. Annual Review of Plant Biology 68: 457-483

Nicotra AB, Atkin OK, Bonser SP, Davidson AM, Finnegan EJ, Mathesius U, Poot P, Purugganan MD, Richards CL, Valladares F, et al. (2010) Plant phenotypic plasticity in a changing climate. Trends in Plant Science 15: 684–692

Pecinka A, Abdelsamad A, Vu GT (2013) Hidden genetic nature of epigenetic natural variation in plants.Trends in Plant Science 18: 625–632

Plomion C, Bastien C, Bogeat-Triboulot MB, Bouffier L, Déjardin A, Duplessis S, Fady B, Heuertz M, Le Gac AL, Le Provost G, Legué V, Lelu-Walter MA, Leplé JC, Maury S, Morel A, Oddou-Muratorio S, Pilate G, Sanchez L, Scotti I, Scotti-Saintagne C, Segura V, Trontin JF, Vacher C (2016) Forest tree genomics: 10 achievements from the past 10 years and future prospects. Ann For Sci 73:77–103

Raj S, Bräutigam K, Hamanishi ET, Wilkins O, Thomas BR, Schroeder W, Mansfield SD, Plant AL, Campbell MM. 2011. Clone history shapes Populus drought responses. Proceedings of the National Academy of Sciences, USA 108, 12521–12526

Schönberger B, Chen X, Mager S, Ludewig U (2016) Site-Dependent Differences in DNA Methylation and Their Impact on Plant Establishment and Phosphorus Nutrition in *Populus trichocarpa*. PLOS ONE 11, e016862

Schmid MW, Heichinger C, Schmid DC, Guthörl D, Gagliardini V, Bruggmann R, Aluri S Aquino C, Schmid B, Turnbull LA, Grossniklaus U (2018) Contribution of epigenetic variation to adaptation in Arabidopsis. Nature Communication 9:4446 DOI: 10.1038/s41467-018-06932-5

Schmitz RJ, Ecker JR (2012). Epigenetic and epigenomic variation in Arabidopsis thaliana. Trends in Plant Science 17: 149–154

Seymour DK and Becker C (2017) The causes and consequences of DNA methylome variation in plants. Current Opinion in Plant Biology 36:56–63

Sow, M. D. Allona I, Ambroise C, Conde D, Fichot R, Gribkova S, Jorge V, Le-Provost G, Pâques L, Plomion C, Salse J, Sanchez-Rodriguez L, Segura V, Tost J, Maury S (2018a) Epigenetics in forest trees: state of the art and potential implications for breeding and management in a context of climate change. Advances in Botanical Research 88, 387-453

Sow MD, Segura V, Chamaillard S, Jorge V, Delaunay A, Lafon-Placette C, Fichot R, Faivre-RampantP, Villar M, Brignolas F, Maury S (2018b) Narrow-sense heritability and PST estimates of DNA methylation in three *Populus nigra* L. populations under contrasting water availability. Tree Genetics & Genomes 14:78

Springer NM and Schmitz RJ (2017) Exploiting induced and natural epigenetic variation for crop improvement. Nature Reviews Genetics 18:563-575

Teyssier C, Maury S, Beaufour M, Grondin C, Delaunay A, Le Metté C, Ader K, Cadene M, Label P, Lelu-Walter MA (2014) In search of markers for somatic embryo maturation in hybrid larch (*Larix×eurolepis*): global DNA methylation and proteomic analyses. Physiologia Plantarum 150: 271–291

Tuskan GA, DiFazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, RombautsS, Salamov A, Schein J, Sterck L, AertsA, Bhalerao RR, Bhalerao RP, Blaudez D, BoerjanW, Brun A, Brunner A, Busov V, CampbellM, Carlson J, ChalotM, Chapman J, Chen GL, Cooper D, Coutinho PM, Couturier J, Covert S, Cronk Q, Cunningham R, Davis J, Degroeve S, Dejardin A, dePamphilis C, Detter J, Dirks B, Dubchak I, Duplessis S, Ehlting J, Ellis B, Gendler K, Goodstein D, Gribskov M, Grimwood J, Groover A, Gunter L, Hamberger

B, Heinze B, Helariutta Y, Henrissat B, Holligan D, Holt R, HuangW, Islam-Faridi N, Jones S, Jones-Rhoades M, Jorgensen R, Joshi C, Kangasjarvi J, Karlsson J, Kelleher C, Kirkpatrick R, KirstM, Kohler A, Kalluri U, Larimer F, Leebens-Mack J, Leple JC, Locascio P, Lou Y, Lucas S, Martin F, Montanini B, Napoli C, Nelson DR, Nelson C, Nieminen K, Nilsson O, Pereda V, Peter G, Philippe R, Pilate G, Poliakov A, Razumovskaya J, Richardson P, Rinaldi C, Ritland K, Rouze P, Ryaboy D, Schmutz J, Schrader J, Segerman B, Shin H, Siddiqui A, Sterky F, Terry A, Tsai CJ, Uberbacher E, Unneberg P, Vahala J, Wall K, Wessler S, Yang G, Yin T, Douglas C,Marra M, Sandberg G, Van de Peer Y, Rokhsar D (2006) The genome of Black Cottonwood, Populus trichocarpa (Torr. & Gray). Science 313:1596–1604

Vining KJ, Pomraning KR, Wilhelm LJ, Priest HD, Pellegrini M, Mockler TC, Freitag M, Strauss SH (2012) Dynamic DNA cytosine methylation in the Populus trichocarpa genome: tissue-level variation and relationship to gene expression. BMC Genomics 13:27

Yakovlev IA, Asante DK, Fossdal CG, Junttila O, Johnsen Ø (2011) Differential gene expression related to an epigenetic memory affecting climatic adaptation in Norway spruce. Plant Science 180: 132–139

Yakovlev IA, Carneros E, Lee Y, Olsen JE, Fossdal CG (2016) Transcriptional profiling of epigenetic regulators in somatic embryos during temperature induced formation of an epigenetic memory in Norway spruce. Planta 243: 1237–1249

Yakovlev IA, Fossdal CG (2017) In silico analysis of small RNAs suggest roles for novel and conserved miRNAs in the formation of epigenetic memory in somatic embryos of Norway spruce. Frontiers in Physiology 8: 674

Zhu R, Shevchenko O, Ma C, Maury S, Freitag M, Strauss SH (2013) Poplars with a PtDDM1-RNAi transgene have reduced DNA methylation and show aberrant post-dormancy morphology. Planta 237: 1483–1493

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Hybrid pine (*Pinus attenuata* x *Pinus radiata*) seeds: what do you prefer, mother or nurse?

Itziar A. Montalbán[1], Cathy L. Hargreaves [2], Keiko Gough[2], Cathie B. Reeves[2], Shaf van Ballekom[3], Tomás Goicoa[4], María Dolores Ugarte[4], Paloma Moncaleán[1*]

1. Neiker-Tecnalia, Centro de Arkaute, Apdo 46, 01080 Vitoria-Gasteiz, Spain.
2. Scion, Private Bag 3020, Rotorua 3046, New Zealand.
3. Proseed, 177 Beach Road, RD1 Amberley, New Zealand.
4. Dpto. Estadística e Investigación Operativa, Universidad Pública de Navarra. 31006 Pamplona, Spain.
**Corresponding author: pmoncalean@neiker.net*

Abstract

Development of hybrid pines, with *Pinus radiata* D. Don being one parent, for commercial forestry presents an opportunity to diversify the current resource. Challenges which include the effects of climate change and different land uses make necessary alternative species to guarantee wood and non-wood products in the future. *Pinus radiata* var. cedrosensis X *Pinus attenuata* hybrid possess different attributes, such as tolerance to drought conditions, better growth and resistance to frost damage at higher altitudes, and importantly, different wood quality characteristics. Embryogenic cell lines were successfully initiated from first generation reciprocal hybrids using *P. radiata* protocols. This was the first report where *P. radiata* somatic embryogenic tissue was used as a nurse to facilitate somatic embryogenesis initiation from hybrid pine excised zygotic embryos. It was clear that the excised embryo was better for somatic embryogenesis cell line initiation than the whole megagametophyte. But, the question is: Does the presence of the megagametophyte at initiation worsen or enhance the maturation success months later? In the present work we analysed the maturation rate, number of somatic embryos, germination success, *ex vitro* survival and growth in cell lines derived from embryonal masses initiated from excised zygotic embryos or intact megagametophytes. No differences were observed in *in vitro* parameters such as maturation and germination; in *ex vitro* parameters such as growth, no differences were observed in relation to the treatments but significant differences were observed due to the species, with hybrid somatic embryos being smaller than those from *P. attenuatta* mothers.

Keywords: embryonal masses, embryogenic cell lines, germination, Knobcone pine, maturation, propagation, radiata pine, somatic embryogenesis.

Abbreviations: EM, Embryonal mass

Introduction

Pinus spp. are frequently used in reforestation programs and, specifically, *Pinus radiata* D. Don is one of the most cultivated species in New Zealand and Spain because of its fast growth and wood production. According to the predictions (IPCC 2007), climatic change will increase the incidence and severity of droughts in ecosystems worldwide (Sheffield and Wood, 2008). Different pine species, in comparison with radiata pine (*Pinus radiata* D. Don), possess different attributes, such as tolerance to subtropical climatic conditions, better growth at higher altitudes, lower susceptibility to disease, and, importantly, different wood quality characteristics (Dungey et al., 2003). Therefore, hybrid pines have been known as an opportunity for the future (Hargreaves et al. 2008). *P. attenuata*, for instance, has potential to

contribute drought resistance, cold resistance, and resistance to damage from wet snow when compared to *P. radiata*, which does not have those attributes (Dungey et al. 2003). In view of this information about *P. attenuata* pure species, some years ago we started studying the drought stress tolerance mechanism of the hybrid *P. attenuata* X *P. radiata*, trying to understand the mechanisms involved in this process (De Diego et al. 2012). This hybrid showed higher drought tolerance than breeds from the two pure species growing in different geographical and climatological growth areas (De Diego et al. 2012, 2013a, 2013b, 2015).
Conifer somatic embryogenesis facilitates amplification and storage of embryogenic tissue while field testing takes place. This technology is well established for *P. radiata* (Hargreaves et al. 2009; Montalbán et al. 2010, 2012); but this propagation method has not yet been developed for the *Pinus radiata* × *Pinus attenuata* hybrid. For this reason, SCION (New Zealand) and Neiker (Spain) focused on testing and modifying existing *P.radiata* protocols to produce hybrid plants for field trials.

Some previous studies carried out in radiata pine have shown the effect of the initial conditions (temperature and water availability) of somatic embryogenesis in the subsequent stages of the process (García-Mendiguren et al., 2016). For this reason, and taking into account our previous studies about this effect, we were interested in answering to the following questions: Does the presence of the megagametophyte at initiation inhibit or enhance the maturation success months later?; What is the effect of the mother (i.e. *P. radiata* or *P. attenuata* as the female parent) on maturation rates?; What if the same parents are used in reciprocal crosses?.

Materials and methods

Plant Material
Proseed (North Canterbury, New Zealand) provided all the test material for this investigation. Green cones were collected from two hybrid crosses *Pinus attenuata* x *P. radiata* var. cedrosensis and *Pinus radiata* var. cedrosensis x *P. attenuata*. All the information about these both hybrid crosses are described in Hargreaves et al. (2017). Green cones were stored at 4ºC for 1-3 days depending on when each cross was put into culture (Montalbán et al. 2014).

Experimental design
Following staging, immature megagametophytes were aseptically removed from the seed coat and given one of the following three treatments:

- Treatment A: Megagametophyte + Glitz medium (Litvay et al. 1985; Hargreaves et al. 2009);
- Treatment B: Excised zygotic embryo + Glitz medium (Litvay et al. 1985; Hargreaves et al.
- Treatment C: Excised zygotic embryo + Glitz medium (Litvay et al. 1985; Hargreaves et al. 2009) + Nurse

The pH of the medium was adjusted to 5.7 prior to autoclaving. Amino acids were filter-sterilised, and were added to the autoclaved medium. The medium was then dispensed into gamma-irradiated polyethylene Petri dishes (90 x 25 mm).

Nurse
The nurse tissue used consisted of a vigorously growing *P. radiata* cell line maintained routinely in Glitz media for approximately 10 years (Hargreaves et al. 2002); this line does not form mature somatic embryos, which is important because it avoids hybrid and *P. radiata* somatic embryos getting accidentally mixed together.. Cultures were incubated in low light (5 μmol $m^{-2}s^{-1}$) at 24±1 ºC. All responding explants were transferred to Glitz medium and sub-cultured at 14 day intervals. Following 8 -10 weeks of subculture a final assessment was made to determine cell line establishment as evidenced by EM proliferation, cell lines had to have in excess of 50 mg of tissue to be scored as established.

Maturation and germination
A subset of cell lines (37) 19 with *P. attenuata* as the mother tree, 18 with *P. radiata* var. cedrosensis as the mother tree were used in the initiation treatments (A, B, C) and crosses were screened for somatic embryo maturation and germination ability following Montalbán et al. (2010).

Acclimatization
After 15 weeks, plantlets showing roots and an aerial part of more than 15 mm were considered suitable to be transplanted following Montalbán and Moncaleán (2018).

Results and discussion

Figure 1 shows the somatic embryo yield after the maturation stage. The number of somatic embyos produced depends on the mother tree and the corresponding cross, but the highest and the lowest yield was found in those embryogenic lines coming from crosses in which *P. attenuatta* was the mother tree. It's generally known that the embryogenic cell line has an effect in maturation success and the present results are in agreement with those of others obtained in conifers, particularly in *Pinus* spp. (Montalbán et al. 2012).

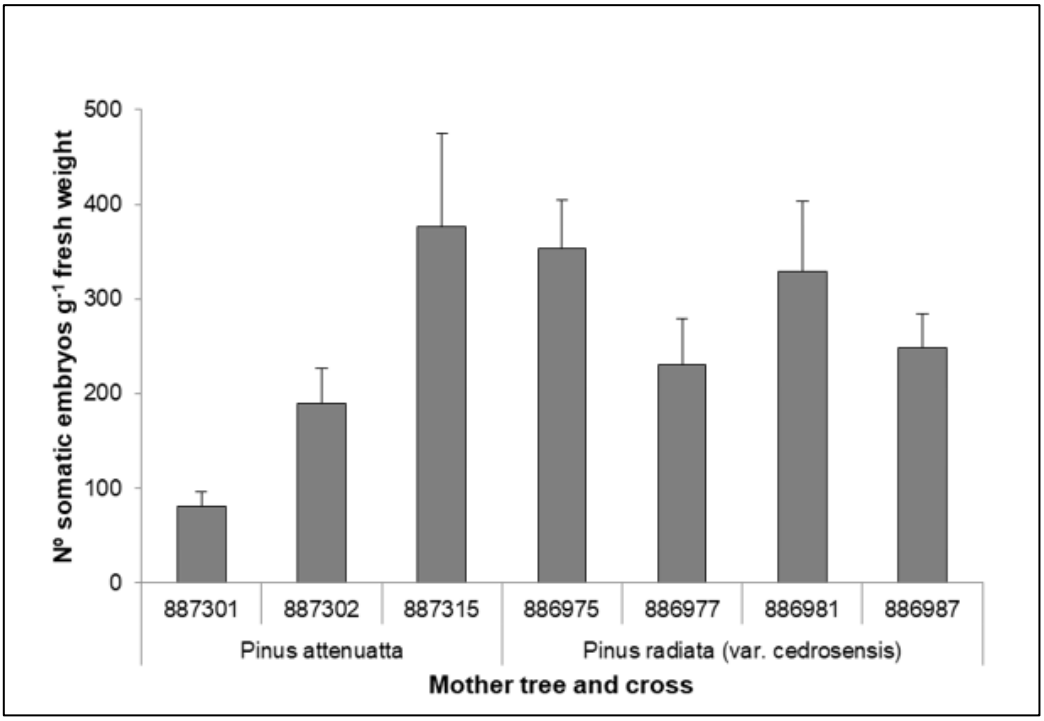


Figure 1. *Somatic embryo yield (number per g^{-1} fresh weight) in several embryogenic cell lines coming from different mother trees and crosses.*

When different treatments were evaluated, independently of the mother tree, the best results regarding yield of somatic embryos was obtained in treatment B (Excised zygotic embryo + Glitz medium); Poor results for each mother tree group were obtained in embryogenic cell lines initiated on nurse (Figure 2). Our results are opposite to those obtained by Hargreaves et al. (2002) in which the use of nurse increased the efficiency of recovery in cryopreserved cell lines.

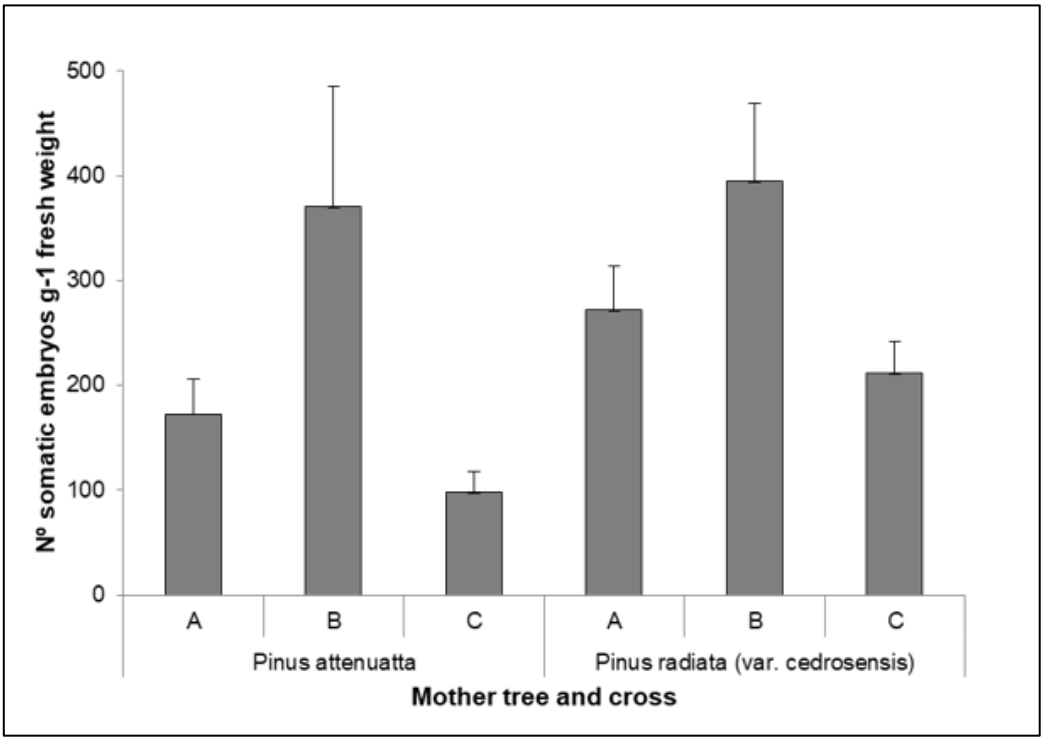


Figure 2. *Somatic embryo yield (number per g^{-1} fresh weight) obtained in embryogenic cell lines coming from different mother trees and crosses cultured under different treatments (Treatment A: Megagametophyte + Glitz medium; Treatment B: Excised zygotic embryo + Glitz medium; Treatment C: Excised zygotic embryo + Glitz medium + Nurse.*

Figure 3 shows germination rate (%) and no differences were detected neither in relation with mother trees nor with crosses. Similarly, no differences were obtained in germination according to mother trees and crosses of different embryogenic cell lines (Figure 4).

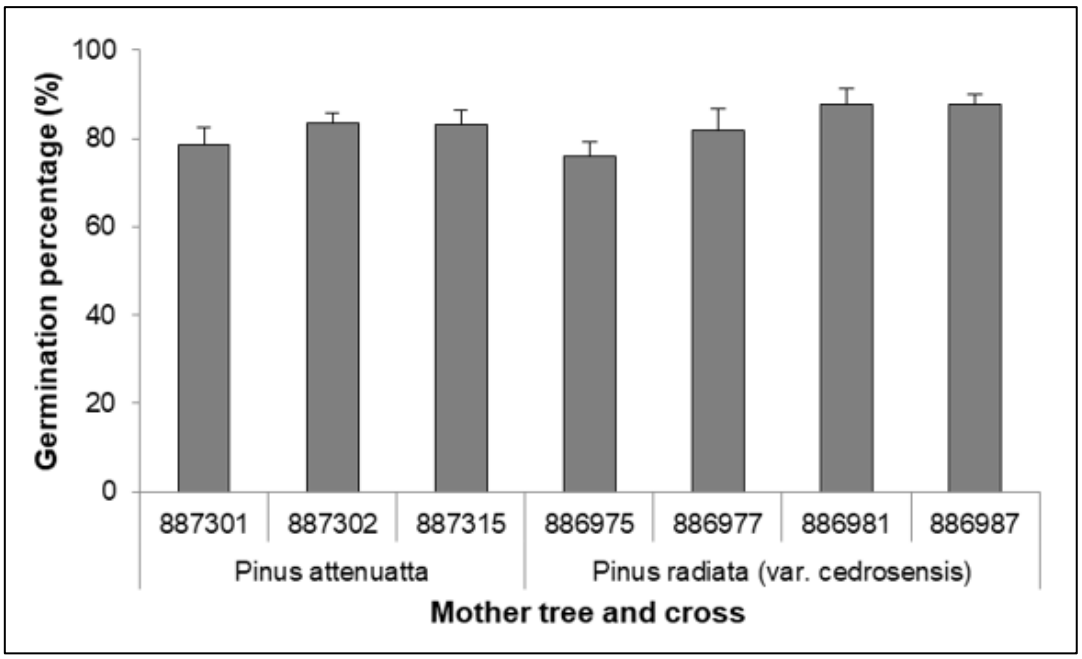


Figure 3. *Somatic embryos derived from several embryogenic cell lines, different mother trees and crosses.*

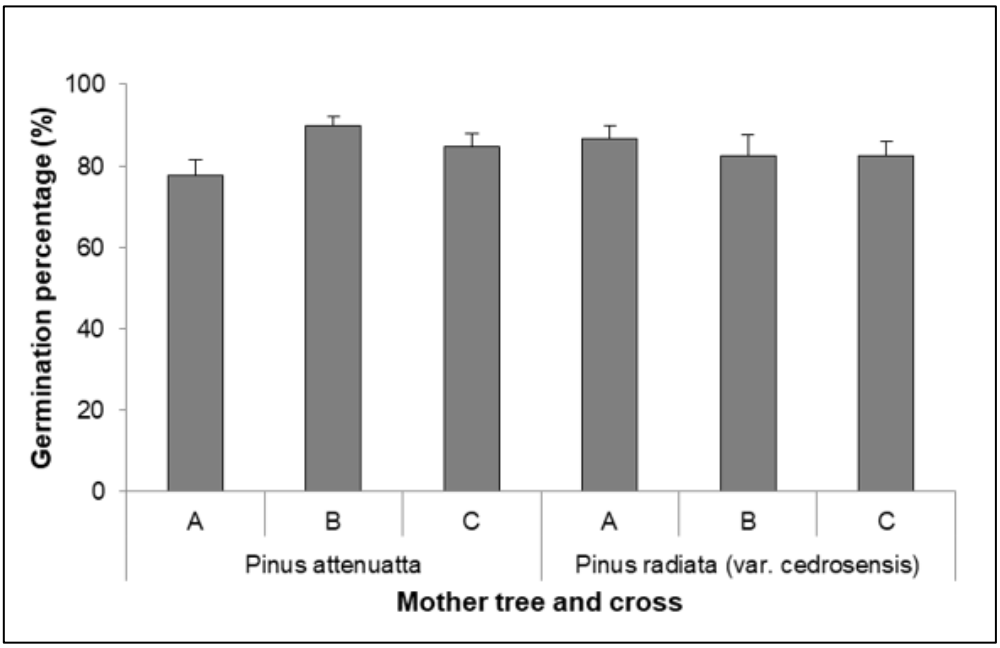


Figure 4. *Germination rate (%) obtained in somatic embryos derived from different mother trees and crosses cultured under different treatments (Treatment A: Megagametophyte + Glitz medium; Treatment B: Excised zygotic embryo + Glitz medium; Treatment C: Excised zygotic embryo + Glitz medium + Nurse).*

After 15 weeks growing in the greenhouse under *ex vitro* conditions, somatic plants (Figure 5) coming from crosses in which *P. radiata* was the mother tree showed higher height than the other group of plants evaluated (Table 1). In this sense, our results are in agreement with previous studies which showed the low growth rate of *P. attenuatta* plants (De Diego et al. 2012).

Figure 5. *Hybrid somatic plant after 15 weeks growing in the greenhouse under ex vitro conditions.*

Table 1. *Height (cm) after 15 weeks in ex vitro conditions of hybrids of P. attenuatta with P. radiata in different crosses and treatments (Treatment A: Megagametophyte + Glitz medium; Treatment B: Excised zygotic embryo + Glitz medium; Treatment C: Excised zygotic embryo + Glitz medium + Nurse).*

Mother Tree	Treatment	Height (cm)
P. Attenuatta	A	8.89
P. Attenuatta	B	9.38
P. Attenuatta	C	9.23
P. radiata	A	13.99
P. radiata	B	13.29
P. radiata	C	12.18

Based on the results obtained, we conclude that no differences were observed in *in vitro* parameters such as maturation and germination in relation to mother tree and cross. In *ex vitro* parameters such as growth, no differences were observed in relation to the treatments but significant differences were observed due to the species with somatic plants from *P. attenuatta* mothers being the smallest.

Funding: We thank the OECD Co-operative Research Programme Fellowship (Biological Resource Management for Sustainable Agricultural Systems, 2013) for supporting the visit of Paloma Moncaleán to Scion and Scion Core Funding for supporting the hybrid pine initiative.

Acknowledgements: Shaf van Ballekom of Proseed Ltd New Zealand is thanked for the provision of seed and warm support of this research.

References

De Diego N, Pérez-Alfocea F, Cantero E, Lacuesta M, Moncaleán P (2012) Physiological response to drough in radiata pine: phytohormone implication at leaf level. Tree Physiol 32: 485-449

De Diego N, Sampedro MC, Barrio RJ, Saiz-Fernández I, Moncaleán P, Lacuesta M (2013a) Solute accumulation and elastic modulus changes in six radiata pine breeds exposed to drought. Tree Physiol 33: 69-80

De Diego N, Rodríguez JL, Dodd IC, Pérez-Alfocea F, Moncaleán P, Lacuesta M (2013b) Immunolocalization of IAA and ABA in roots and needles of radiata pine (*Pinus radiata*) during drought and rewatering. Tree Physiol 33: 537-549

De Diego N, Saiz-Fernandez I, Rodriguez JL, Perez-Alfocea P, Sampedro MC, Barrio RJ, Lacuesta M, Moncaleán P (2015) Metabolites and hormones are involved in the intraspecific variability of drought hardening in radiata pine. J plant physiol 188: 64-71

Dungey HS, Carson MJ, Low CB, King NG (2003) Potential and niches for inter-specific hybrids with *Pinus radiata* in New Zealand. NZ J For Sci 33(3): 295-318

García-Mendiguren O, Montalbán IA, Goicoa T, Ugarte MD, Moncaleán P (2016) Environmental conditions at the initial stages of *Pinus radiata* somatic embryogenesis affect the production of somatic embryos. Trees-Struct Funct 30 (3): 949-958

Hargreaves CL, Grace LJ, Holden DG (2002) Nurse culture for efficient recovery of cryopreserved *Pinus radiata* D. Don embryogenic cell lines. Plant Cell Rep 21:40–45

Hargreaves C, Sigley M, Menzies M, Dungey H (2008) Hybrid pines: Opportunities for Life on the Edge. Proc Int Plant Propagators' Soc 58: 162-167

Hargreaves CL, Reeves CB, Find JI, Gough K, Josekutty P, Skudder DB, van der Maas SA, Sigley MR, Menzies MI, Low CB, Mullin TJ (2009) Improving initiation, genotype capture, and family representation in somatic embryogenesis of *Pinus radiata* by a combination of zygotic embryo maturity, media, and explant preparation. Can J For Res 39 (8): 1566-1574

Hargreaves C, Reeves C, Gough K, Montalbán IA, Low C, van Ballekom S, Dungey HS, Moncaleán P

(2017) Nurse tissue for embryo rescue: testing new conifer somatic embryogenesis methods in a F1 hybrid pine. Trees-Struct Funct 31 (1): 273-283

IPCC (2007) Climate change 2007. Mitigation of climate change. Contribution of working Group III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, UK, 862 pp.

Litvay JD, Verma DC, John MA (1985) Influence of loblolly pine (*Pinus taeda* L.) culture medium and its components on growth and somatic embryogenesis of the wild carrot (*Daucua carota* L.). Plant Cell Rep 4:325–328

Montalbán IA, De Diego N, Moncaleán P (2010) Bottlenecks in *Pinus radiata* somatic embryogenesis: Improving maturation and germination. Trees-Struct Funct 24 (6): 1061-1071

Montalbán IA, de Diego N, Moncaleán P (2012) Enhancing initiation and proliferation in radiata pine (*Pinus radiata* D. Don) somatic embryogenesis through seed family screening, zygotic embryo staging and media adjustments. Acta Physiol Plant 34 (2): 451-460

Montalbán IA, García-Mendiguren O, Goicoa T, Ugarte MD, Moncaleán P (2014) Cold storage of initial plant material affects positively somatic embryogenesis in *Pinus radiata*. New For 46 (2): 309-317

Montalbán IA, Moncaleán P (2018) Rooting of *Pinus radiata* somatic embryos: factors involved in the success of the process. J For Res: 1-7

Sheffield J, Wood EF (2008) Global trends and variability in soil moisture and drought characteristics, 1950-2000, from observation-driven simulations of the terrestrial hydrologic cycle. J Climate 21: 432-458

Comparison of rooting rates of cuttings from different mother stocks of *Eucalyptus pellita* ID30 clone growing in different conditions

Heung-Kyu Moon[1], Nelly S. Aggangan[2]

[1]R&D Department, PT. Korintiga Hutani, Camp Pellita, Ds. Nanga Mua, Kab. Kotawaringin Barat 74152, Kalimantan Tengah, Indonesia. Email: hkmoon@korindo.co.id
[2]National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Baños, College, Laguna 4031, Philippines. E-mail: nsaggangan@up.edu.ph

Abstract
In order to develop an effective cutting method, *Eucalyptus pellita* ID 30 clone was examined by means of Oasis cell cuttings. Cuttings were taken from four places of mother stocks (MS, Ms sarlon, HO and SA), which is a similar age hedge orchard. Rooting rates varied according to mother stocks (92% for MS sarlon, 84% for MS, 80% for SA and 36% for HO, respectively). On the other hand, the average number of roots was about 2.5 for all stocks. The rooting rate of mother stocks seemed to be related to N, Mg and Ca concentrations in the stocks. The lowest rooting rate of HO cuttings (out door hedge orchard) showed relatively a low Mg and Ca content and a high N content. The results showed that maintaining the juvenility of mother stocks and proper management by fertilization were very important factors for the rooting of *E. pellita*.

Keywords: cutting propagation, mother stocks, rooting, nutrient analysis

Introduction

Eucalyptus pellita is a native of Iriyan Jaya, Indonesia, and are now commercially planted in Kalimantan and Sumatra, Indonesia. Wood of the tree is known as red mahogany and is used as building, pulp and pellet materials, for charcoal, and others (http://tropical.theferns.info/viewtropical.php?id=Eucalyptus+pellita). Increasing wood productivity through commercial planting, requires the development of good clones as well as efficient propagation techniques. Traditionally, *E. pellita* have been mostly propagated through seed. However, in commercial plantations, the use of cuttings of selected clones is preferred to increase productivity. Moreover, vegetative propagation by means of cuttings or tissue culture is dependent on the clone and the rooting capability. Thus, proper cutting technology should be developed according to the target clone for large-scale application.

KTH (PT. Korintiga Hutani) has been developing clones of *E. pellita* for the last 20 years and has been planting different clones on a large scale since 2003. Clonal productivity at the rotation stage is closely related to the survival rate and individual tree growth. In particular, cutting's propagules, which have poor root development, usually suffer from wind damage in the course of their subsequent growth. Thus, production of good quality cutting propagules with a good root system has become an important task. In general, various factors such as mother plant aging (juvenility), mother plant nutrient condition, cutting's environmental (temperature, humidity, lighting etc.) conditions, rooting media (soil), rooting hormone as well as genetic factors are affecting rooting capacity (Fett-Neto et al. 2001; Nakhooda et al. 2012; Venkataramanan et al. 2015). The clone that is planted intensively here is *E. pellita* clone ID30.

This study is to compare the rooting rate of the same ID30 clone taken from different mother stocks (hedge orchards).

Material and methods

Cuttings (5-7.5 cm long from the shoot tips) were collected from mother stocks of *E. pellita* ID30 clones growing in different conditions. The cuttings (stecks) were collected from four mother stocks coded as MS, MS (sarlon), HO and SA. Table 1 summarizes growing conditions of the the mother stock where cuttings were collected.

Table 1. *Mother stocks' growing conditions as source of E. pellita ID30 clone cuttings.*

Mother stocks	Remarks
MS	Mother stocks hedge orchard made of brick in outdoor (1~1.5 year old)
MS (sarlon)	MS with shading net (1~1.5 year old)
HO	Outdoor hedge orchard (1.5~3.5 years old)
SA	Indoor hedge orchard with shading net (0.8~1 year old)

The oasis (floral form) cell was used for the cuttings, and the rooting hormone was IBA at 1,000 ppm concentration (Fig. 2). One hundred cuttings per treatment and 2 repetitions were made. After treatment of the cuttings, these were incubated in a propagation house with periodic misting and irrigation. The rooting rate of the cuttings was examined after 2 weeks. In addition, leaves of the mother stocks were sampled and analyzed for nutrients.

Results

The rooting rate of cuttings obtained from the outdoor hedge orchard (HO) gave the lowest (36%) (Fig. 1) rooting rate. The rooting rates of cuttings from the other mother stocks were: MS (sarlon) gave the highest rooting rate followed by MS, and then SA. The MS (sarlon) rooting rate is 2.9 on average, but comparable with the other mother stocks due too high standard deviation value.

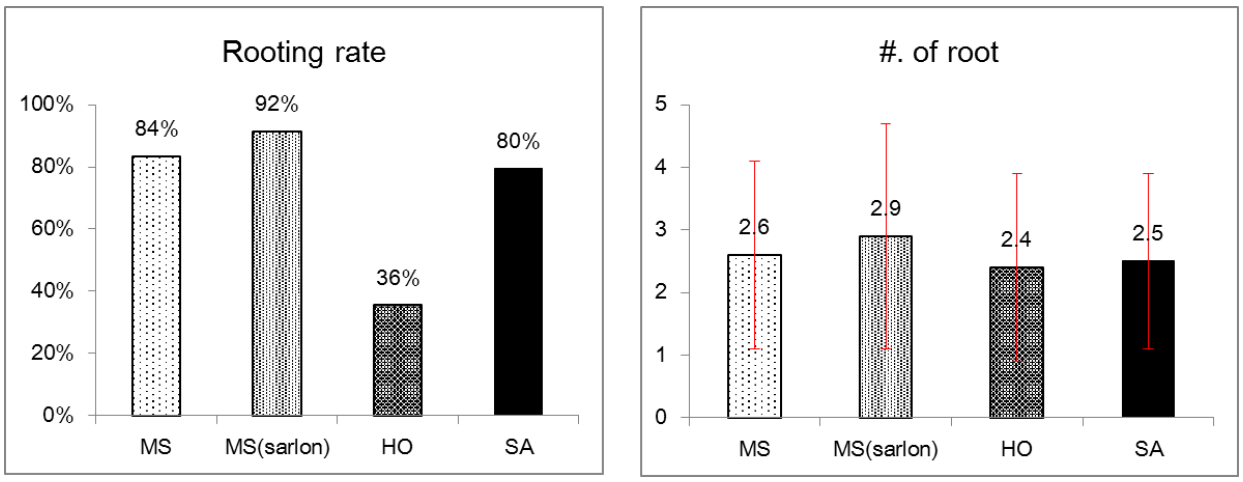


Figure 1. *Rooting rate and root number in cuttings collected from different mother stocks growing in different conditions.*

Figure 2. *Oasis cell cutting and rooted plantlets. (1,2- cell preparation; 3,4- IBA application and insertion; 5,6 – rooted plantlets)*

Leaves of mother stocks were also collected for nutrient analysis. Results showed that the leaf Ca and Mg contents of HO, were lower than those of the other mother stocks but the nitrogen content was the highest in the HO (Table 2, Fig. 3) It should be noted that cuttings from HO mother stock had the lowest rooting rate. Thus, we assumed that the low rooting rate of HO is caused by the excessive nitrogen fertilization and the relative lack of Ca and Mg along with the increase of mother tree's age. In conclusion, the data showed that proper fertilization and juvenility maintenance of the mother stocks are important to efficient cutting propagation of the clone. In the near future, we will make a sand bed in the greenhouse and intensively manage the mother stocks. Using the mother stocks, we can expect high rooting rate and good root development of the clone.

Table 2. *Comparison of leaf nutrient contents of the mother stocks growing in different conditions.*

Location	% on dry matter					ppm on dry matter				
	N	P	K	Mg	Ca	B	Cu	Zn	Fe	Mn
MS	2.12	0.22	1.15	0.18	0.88	17.9	9.6	45.8	513.4	83.4
MS (sarbn)	2.08	0.20	1.30	0.19	0.89	20.2	8.6	41.3	501.8	98.0
HO	2.37	0.22	1.45	0.11	0.53	20.3	7.4	30.8	321.0	90.4
SA	2.04	0.23	1.79	0.29	0.81	23.3	8.2	42.7	382.1	86.5

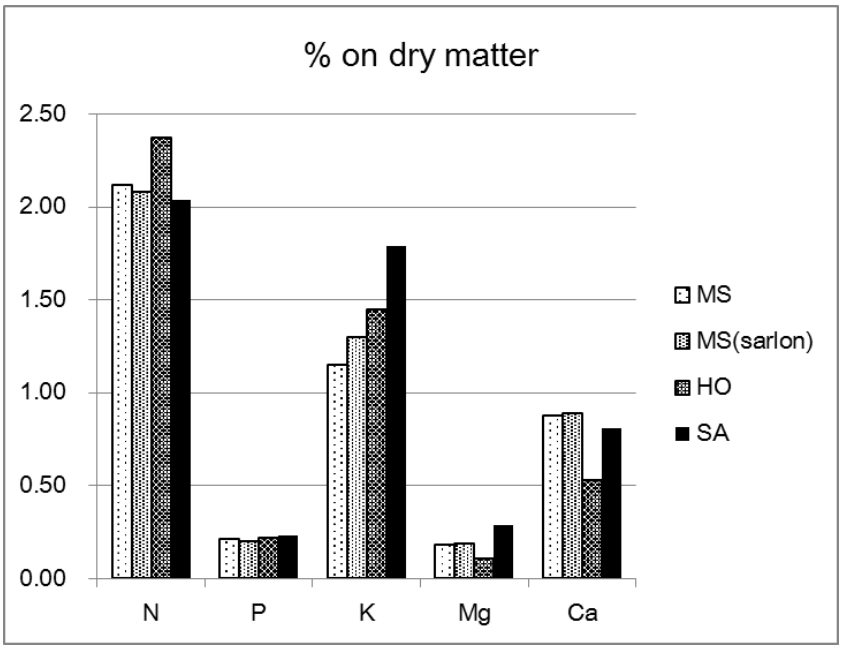


Figure 3. *Nutrient contents in the leaves of mother trees growing in different conditions.*

References

Eucalyptus pellita. http://tropical.theferns.info/viewtropical.php?id=Eucalyptus+pellita

Fett-Neto AG, Fett JP, Vieira Goulart LW, Pasquali G, Termignoni RR, Ferreira AG. 2001. Distinct effects of auxin and light on adventitious root development in *Eucalyptus saligna* and *Eucalyptus globulus*. Tree Physiol 21: 457–464

Nakhooda M, Watt MP, Mycock D (2012). The properties and interaction of auxins and cytokinins influence rooting of shoot cultures of Eucalyptus. Afr J Biotech 11(100): 16568-16578

Venkataramanan KS, Palanisamy M, Selvaraj P, Vellaichamy P, Senthamil Selvan S, Divya G (2015) Vegetative propagation of Eucalyptus hybrids through water culture method. Int Res J Biol Sci 4(5): 15-18

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Growth and mineral uptake of *Eucalyptus pellita* in response to inoculation with different mycorrhizal inoculants

Heung-Kyu Moon[1], Nelly S. Aggangan[2]

[1]R&D Department, PT. Korintiga Hutani, Camp Pellita, Ds. Nanga Mua, Kab. Kotawaringin Barat 74152, Kalimantan Tengah, Indonesia; Email: hkmoon@korindo.co.id
[2]National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Baños, College, Laguna 4031; E-mail: nsaggangan@up.edu.ph

Abstract
This study determined the response of Eucalyptus pellita cuttings to different mycorrhizal inoculants from the Philippines as compared with ectomycorrhizal fungi collected under E. pellita plantations in Kalimantan, Indonesia. Shoot tips (5-7.5 cm) of eucalypts were collected from the seedlings orchard, dipped in rooting hormone, inserted in rooting materials and incubated under a mist system. After two weeks, the rooted cuttings were transferred into containers filled with soil. During seedling transfer to individual containers, they were inoculated with mycorrhizal inoculants: KTH (contains spores of Scleroderma and Pisolithus sp. native in Kalimantan, Indonesia), MYKOVAM®, MYKORICH®, MYKOCAP®, Mycogroe, Mycogroe+MYKORICH® and MYKORICH®+MYKOCAP® from the Philippines, following the recommended dosages as stated on the label. Mycogroe contains spores of Scleroderma and Pisolithus while the others contain spores, mycorrhiza, colonized chopped roots and other infective propagules of arbuscular mycorrhizal fungi. MYKOCAP® and Mycogroe+MYKORICH® inoculated plants were 51% and 49%, respectively, taller than the control (8.86 cm). In terms of mineral components, highest total plant N, K, Mg, Fe, and Mn uptakes were observed in plants inoculated with Mycogroe+MYKORICH®. MYKORICH® alone gave the highest percent increase in total plant uptakes of B (86%), Cu (76%) and Zn (104%). The results clearly showed that growth and mineral composition of E. pellita were greatly improved by inoculation with mycorrhizal fungi particularly Mycogroe+MYKORICH®.

Keywords: arbuscular mycorrhizal fungi, ectomycorrhizal fungi, mineral elements, nitrogen, phosphorus

Introduction

Eucalyptus is a genus under the large family of Myrtaceae. This genus presents many desirable characteristics that include rapid growth, high cellulose production and resistance to disease and adverse environmental conditions (Santos et al. 2001). It can also produce oil for cleaning, functions as a natural insecticide, or have an ability to drain swamps (Hiwale, 2015). Eucalyptus species have an important role for wood production in plantations in Asia (Midgley & Pinyopusarerk, 1996). The Food and Agriculture Organization of the United Nations (1996) stated that some species of eucalyptus are planted for developing the timber estate in Indonesia. It includes *E. deglupta, E. alba, E. urophylla, E. pellita, E. saligna*, and *E. grandis*. *Eucalyptus pellita*, specifically, is a native of Iriyan Jaya, Indonesia, and is now commercially planted in Kalimantan and Sumatra, Indonesia. The wood of the tree is known as red mahogany and is used to construct buildings, making pulp and pellets for charcoal, and others.
Nowadays, regular plantation of *Eucalyptus* are very common due to its demands mainly in construction work and also in the paper industry" (Hiwale, 2015). In this case, due to the high volume requirement

of raw materials by KTH, Indonesia, the company continually establishes commercial plantations to keep the company profitable and to maintain sustained operations. Commercial plantation establishments have used cuttings of selected clones to increase productivity. However, selected clones produced from cuttings do not have a tap root, and thus, can be toppled easily by wind.

Mycorrhizal technology is well known due to its application in forest plantations and land restoration; it can also be applied in agricultural and horticultural crops for better nutrient utilization and more effective land use (Chen et al. 2014). Moreover, it can also play an important role in succession and maintenance of plant community diversity (Brundrett, 1991). According to van der Heijden et al. (2015), estimates suggest that approximately 50,000 species of fungi form mycorrhizal associations with about 250,000 species of plants. Since mycorrhizae consist of partnerships between fungi and the roots of plants and their main role is to enhance plant nutrient uptake, it can promote root development, can facilitate faster growth, high seedlings survival and greater wood yield, but more importantly, can help minimize the toppling down of field plants.

Eucalyptus can form both ectomycorrhiza and vesicular-arbuscular mycorrhiza in the same root systems both in plantation soils and under controlled conditions (Lapeyrie & Chilvers 1985; Vozzo & Hacskaylo 1974; Brundrett et al., 1996; Oliveira et al., 1997; Chen et al. 1998, 2000; Lodge 2000). Thus, the purpose of this study is to present information on the effect of inoculation of different arbuscular mycorrhizal fungi on eucalypt growth; the response of *Eucalyptus pellita* cuttings to different mycorrhizal inoculants from the Philippines as compared with ectomycorrhizal fungi collected under *E. pellita* plantations in Kalimantan, Indonesia.

Methodology

Experimental design
A nursery experiment was conducted following a Randomized Complete Block Design with five blocks (20 seedlings per treatment per block). There were 8 treatments including the control. Destructive sampling was done for ten seedlings from each treatment.

Production of planting materials
Shoot tips (5-7.5 cm) of E. pellita were collected from the hedge orchard (Fig. 1), dipped in rooting hormone, inserted in rooting materials and incubated under a mist system. After two weeks, the rooted cuttings were transferred into containers filled with cooked potting soil (Fig. 2).

***Figure 1**. Eucalypt seedlings orchard for shoot tip production.*

Figure 2*. Production of rooted cuttings and transfer (inset) into individual containers filled with sterilized (cooked) potting soil.*

Mycorrhizal inoculation

Rooted cuttings were transferred into root trainers (Fig. 2). Mycorrhizal inoculants MYKOVAM®, MYKORICH®, MYKOCAP® and Mycogroe™ from the Philippines and KTH containing ectomycorrhizal fungi (Fig. 3a) were placed beneath the roots during this stage. The first three inoculants contain spores of twelve species of AMF (Fig. 3b) while the last two contains spores of ectomycorrhizal fungi. Mycorrhizae were inoculated with one capsule per pot consisting of 1 g of powder per capsule.

Figure 3a. *Mycorrhizal inoculants from the Philippines: MYKOVAM, MYKOCAP, MYKORICH, MYCOGROE and KTH from E. pellita in Kalimantan, Indonesia used in this study.*

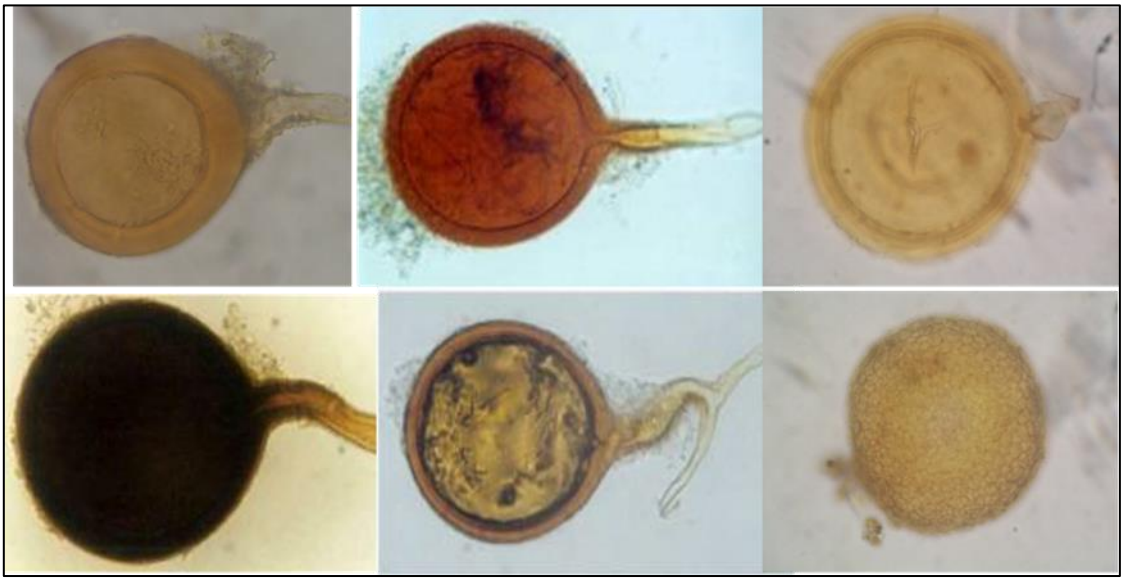


Figure 3b. *Example of spores of arbuscular mycorrhizal fungi in the mycorrhizal inoculants produced by the National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Banos*

Parameters gathered
Height, survival rate and dry weight were measured. Seedling height was regularly measured 3 weeks after the treatment. One month later, liquid fertilizer (NPK 4g + urea 2g/L) was applied. Mineral contents of treated plants were analyzed at the Analytical Service Laboratory, Kalimantan, Indonesia.

Statistical analyses
All data gathered were analyzed using ANOVA in RCBD and treatment means were compared using Tukey's test at $p<0.05$. Analyses were done using the MSTAC computer program and treatment means were compared using Tukeys test if $p<0.05$ (MSU 1999).

RESULTS

Plant growth
The general appearance of uninoculated and mycorrhiza inoculated (KTH and Mykorich+Mycogroe) *E. pellita* is shown in Fig. 4. Eucalypts inoculated with Mykorich+Mycogroe grew healthy with broader leaves than those inoculated with other mycorrhizal inoculants (Fig. 4). On the other hand, growth of the control counterpart was stunted with prominent yellowing of leaves and small and narrow leaves.

Figure. 4. *General appearance of un-inoculated (A), inoculated with KTH mycorrhiza (B) and Mykorich+Mycogroe (C) of one month old E. pellita in response to different mycorrhizal inoculation treatments*

Height of the control plants consistently was the shortest throughout the two months (data not shown) growth in the nursery (prior to field planting). Seedlings inoculated with Mykorich or Mycogroe+Mykorich outgrew the plants with other mycorrhizae (Fig. 5a). Mykogroe+Mykorich consistently promoted ($p<0.001$) the highest height increment after five weeks (data not shown). After five weeks, the Mykocap and Mycogroe+Mykorich inoculated plants were 51% and 49%, respectively, taller than the control which is 8.86 cm (Fig. 5b).

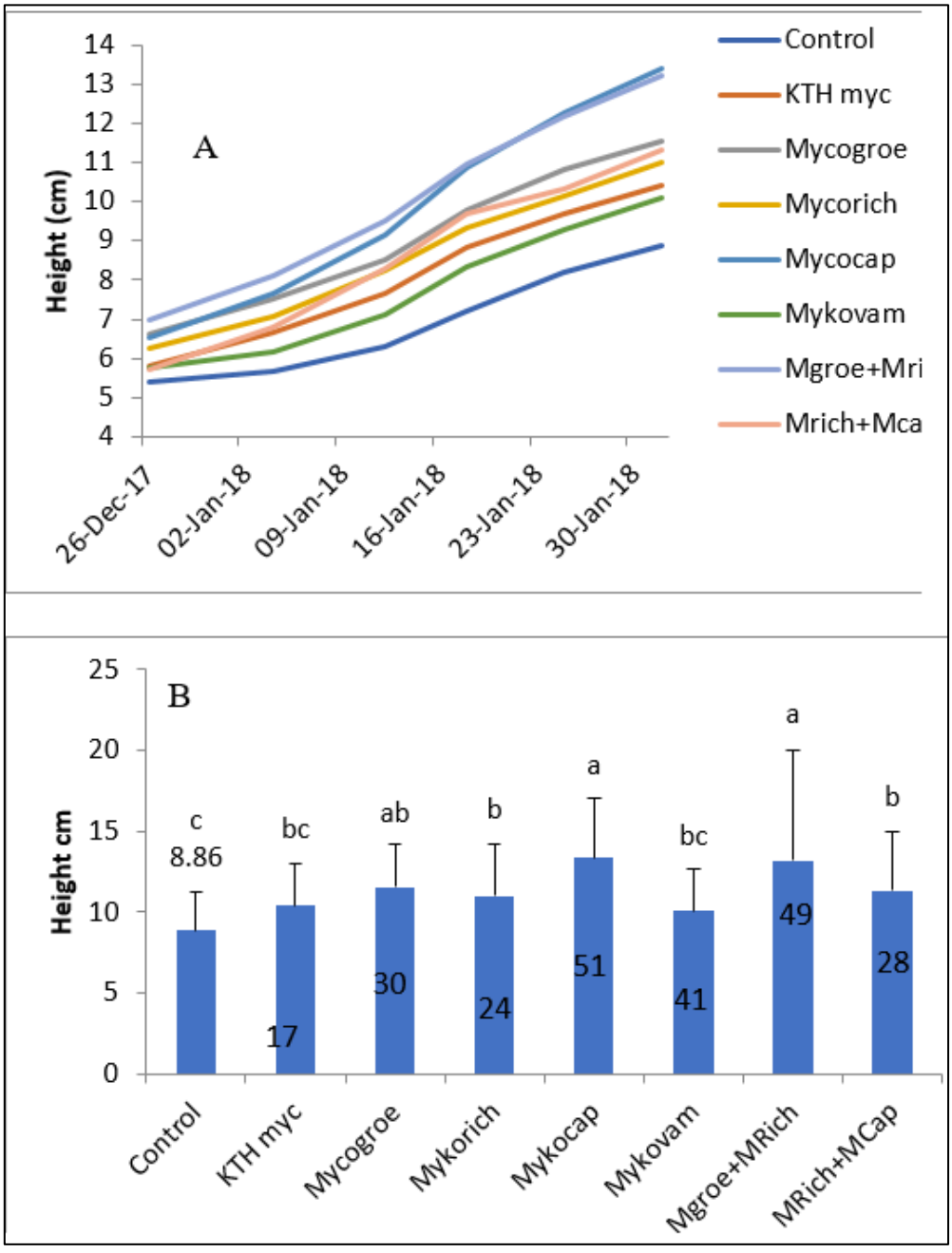


Figure 5. *Periodic height (A) and height after five weeks (B) of E. pellita in response to different mycorrhizal inoculation treatments. Bars with the same letters are not significantly different using Tukey's test. Values inside the bars are percentage increase relative to the control. N=10*

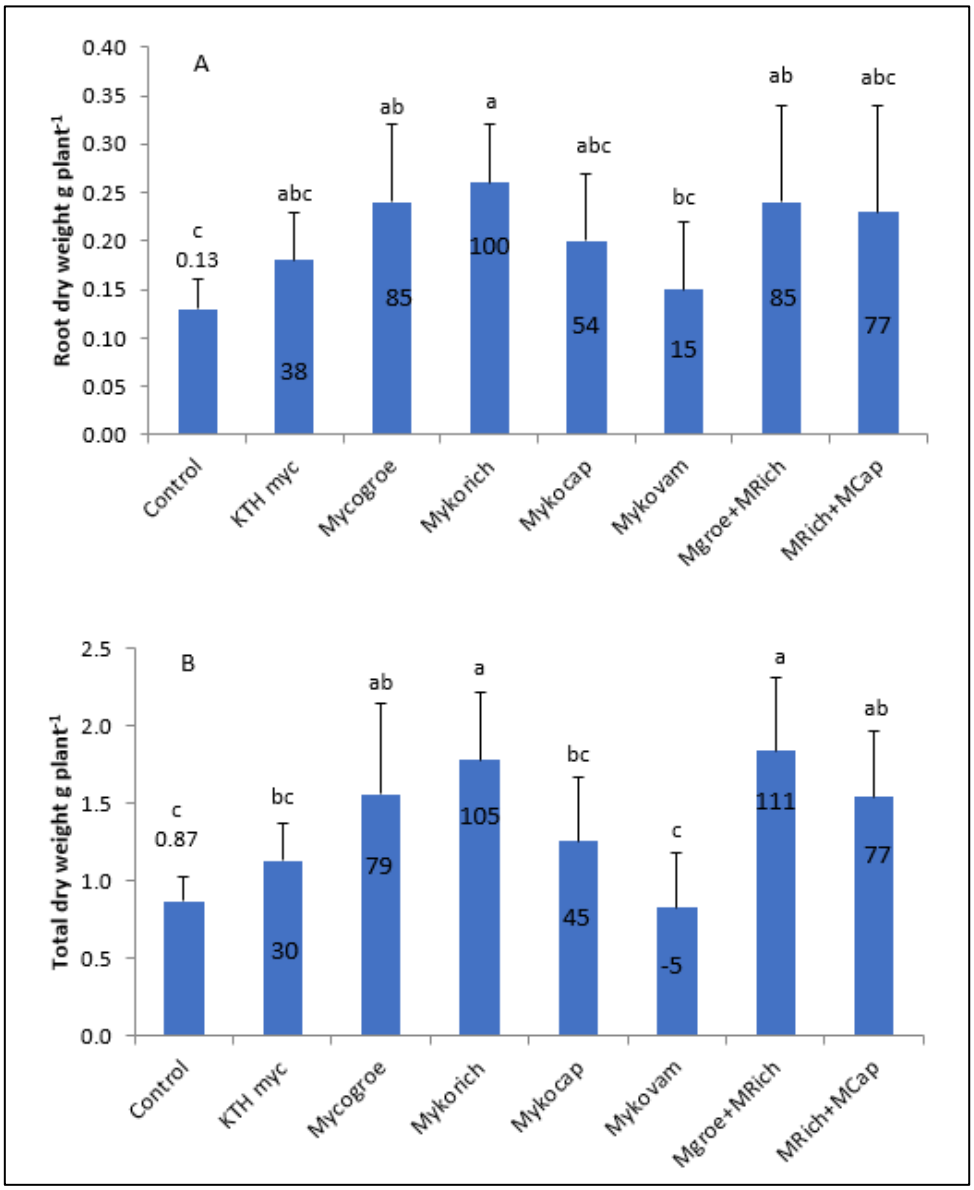


Figure 6. *Root (A) and total plant dry weight of five weeks (B) old E. pellita in response to different mycorrhizal inoculation treatments. Values inside the bars are percentage increase relative to the control. N=10*

Plant dry weight

Mykovam promoted the highest (100%) increase in root dry weight (Fig. 6A) while Mycogroe+Mykorich gave the highest (111%) increase in plant dry weight (Fig. 6B). The control plants had the lowest root (0.13 g plant^{-1}) followed by those inoculated with KTH (0.18 g plant^{-1}). Control plants also had the lowest total dry (0.87 g plant^{-1}) weight. Among the mycorrhiza inoculated plants, those inoculated with Mykovam had the lowest total dry weight (0.94 g plant^{-1}). Mykorich increased root dry weight by 100% while Mycogroe and Mycogroe+Mykorich increased root dry weight by 85%.

Nutrient uptake

In terms of mineral components, the highest increases in total plant uptake, N (131%), K (111%), Mg (106%), Fe (92%), and Mn (86%), were observed in plants inoculated with Mycogroe+Mykorich (Tables 1 and 3). Mykorich alone gave the highest percent increase in total plant uptake of Ca (102%), B (86%), Cu (76%) and Zn (104%). Root P uptake was highest (75%) in plants inoculated with

Mykorich (Table 2) but similar to those inoculated with Mycogroe+Mykorich (72%) relative to the control (0.035 mg g^{-1}). Increase in micronutrients B, Cu and Zn was highest in plants inoculated with Mykorich (Table 4). Mykorich increased root uptake of B by 84%, Cu by 64%, and Zn by 106% relative to the control.Except for Fe and Mn, the lowest root uptake of the other micronutrients was observed in the control plants (Table 4).

Table 1. *Total plant macronutrient uptake (mg plant^{-1}) and percent increase relative to the control in E. pellita in response to different mycorrhizal inoculation treatments.*

Treatment	Total N uptake	% inc relative to control	Total P uptake	% inc relative to control	Total K uptake	% inc relative to control	Total Mg uptake	% inc relative to control	Total Ca uptake	% inc relative to control
Control	0.226		0.035		0.169		0.034		0.185	
KTH	0.283	25	0.041	17	0.228	35	0.043	27	0.237	28
Mycogroe	0.407	80	0.050	44	0.307	82	0.061	79	0.365	97
Mykorich	0.441	95	0.061	**75**	0.347	**106**	0.062	84	0.374	**102**
Mykocap	0.290	28	0.040	16	0.243	44	0.048	41	0.277	50
Mykovam	0.189	-16	0.030	-13	0.165	-2	0.033	-2	0.184	-1
Mycogroe+Mykorich	0.523	**131**	0.060	72	0.357	**111**	0.070	**106**	0.364	97
Mykorich+Mykocap	0.373	65	0.045	28	0.277	64	0.059	72	0.343	85

Table 2*. Root macronutrient uptake (mg plant^{-1}) and percent increase relative to the control in E. pellita in response to different mycorrhizal inoculation treatments.*

Treatment	Root N uptake	% inc relative to control	Root P uptake	% inc relative to control	Root K uptake	% inc relative to control	Root Mg uptake	% inc relative to control	Root Ca uptake	% inc relative to control
Control	0.012		0.0022		0.0104		0.0020		0.014	
KTH	0.017	40	0.0024	5	0.0140	35	0.0025	29	0.018	27
Mycogroe	0.023	85	0.0026	18	0.0187	80	0.0034	**72**	0.027	**88**
Mykorich	0.022	81	0.0033	**48**	0.0213	105	0.0029	47	0.025	77
Mykocap	0.017	34	0.0023	4	0.0170	63	0.0026	33	0.022	54
Mykovam	0.011	-9	0.0022	0	0.0119	14	0.0023	15	0.018	24
Mycogroe+Mykorich	0.024	**96**	0.0027	20	0.0197	**89**	0.0029	48	0.023	56
Mykorich+Mykocap	0.021	73	0.0023	1	0.0175	68	0.0028	42	0.023	56

Table 3. *Total plant micronutrient uptake (µg plant^{-1}) and percent increase relative to the control in E. pellita in response to different mycorrhizal inoculation treatments.*

Treatment	Total B uptake	% inc relative to control	Total Cu uptake	% inc relative to control	Total Zn uptake	% inc relative to control	Total Fe uptake	% inc relative to control	Total Mn uptake	% inc relative to control
Control	2.05		1.39		7.59		73.94		58.74	
KTH	2.11	3	1.83	32	10.88	43	92.22	25	73.96	26
Mycogroe	3.63	77	2.06	48	11.84	56	111.63	51	103.58	76
Mykorich	3.81	**86**	2.46	**76**	15.49	**104**	118.99	61	93.90	60
Mykocap	2.68	31	1.89	36	8.93	18	85.96	16	83.13	42
Mykovam	1.98	-4	1.34	-4	6.57	-13	72.99	-1	57.83	-2
Mycogroe+Mykorich	3.11	51	2.28	64	13.21	74	141.97	**92**	109.13	**86**
Mykorich+Mykocap	3.14	53	2.08	49	9.99	32	117.86	59	97.64	66

Table 4. *Root micronutrient uptake (µg plant^{-1}) and percent increase relative to the control in E. pellita in response to different mycorrhizal inoculation treatments*

Treatment	Root B uptake	% inc relative to control	Root Cu uptake	% inc relative to control	Root Zn uptake	% inc relative to control	Root Fe uptake	% inc relative to control	Root Mn uptake	% inc relative to control
Control	0.096		0.11		0.60		3.67		2.70	
KTH	0.097	1	0.14	32	0.91	52	4.65	27	3.71	37
Mycogroe	0.190	97	0.15	41	1.00	66	4.07	11	5.39	**99**
Mykorich	0.177	**84**	0.18	**64**	1.24	**106**	4.08	11	2.61	-3
Mykocap	0.124	29	0.13	21	0.72	19	3.41	-7	4.04	49
Mykovam	0.140	45	0.12	7	0.63	5	3.56	-3	3.19	18
Mycogroe+Mykorich	0.122	27	0.15	38	0.84	39	5.55	**51**	3.86	43
Mykorich+Mykocap	0.129	34	0.14	31	0.72	19	3.67	0	4.16	54

Discussion

The presence of mycorrhiza in eucalypts was suspected early in 1910s (van der Bijl, 1917 in Keane et al. 2000); the first synthesis in controlled conditions happened in the 1980's **(**Malajczuk et al. 1981 in Carrenho et al. 2008). Different species of *Eucalyptus* demonstrate distinct nutrient requirements and growth rhythm that have a different level of dependence on this association. There is evidence that this association can improve plant growth in some countries (Bowen 1980; Janos 1983; Marx 1991). The results from the experiment revealed that *E. pellita* was greatly improved in terms of its growth and mineral composition. The mycorrhizal inoculants contained AMF and ECM fungi. The occurrence of both fungi in the root system was verified in the root system of inoculated *E. pellita*. This supports earlier observations on *Eucalyptus* by Boudarga et al., 1990; Chilvers et al., 1987; and Lapeyrie & Chilvers, 1985.

The dominance of mycorrhizas under the conditions of the *E. pellita* experiment shows that the mixture of AMF and ECM (Mycogroe+Mykorich) has initiated more primary infections, and consequently healthier growth and taller height, than when inoculated singly with either ECM, AMF, and the control alone (Figure 7). According to Chen et al. (2000), when both types of fungi were present in the same pot, ECM replaced VAM as the dominant mycorrhiza type in *Eucalyptus* roots after several months of plant growth. However, in the study of Chilvers et al. (1987), it has been described that early vesicular-arbuscular mycorrhizas dominate ectomycorrhizas. Chilvers et al. (1987) also mentioned that ECM fungi colonize roots more slowly than AMF unless there are nearby trees supplying carbon to the ectomycorrhizal fungi (Fleming 1983). This order of succession is believed to be a consequence of differing abilities of the two types of fungi. These abilities could fit well with their distribution among host species. Several studies have been made on stimulation of growth and nutrient uptake of *Eucalyptus* spp. by both ECM (Aggangan et al., 2013, Malajczuk et al. 1975, Heinrich & Patrick 1986) and AMF associations (Lapeyrie & Chilvers 1985, Adjoud et al. 1996).

Marschner & Dell (1994) stated that mycorrhizal fungi can improve plant growth by promoting nutrient uptake. Research has also been carried out to determine whether fungi promote better plant growth (Aggangan et al. 2011, Wang et al. 2018, Zhao et al. 2015). In this study, Mycogroe+Mykorich and Mykocap were better plant growth promoters than all other treatments. According to Lapeyrie and Chilvers (1985) as cited by Egerton-Warburton & Allen (2001), the presence of two mycorrhizal types may increase the uptake of nutrients because both fungi affect the soil resources differentially. Also, the typical lifespan of arbuscules is 8.5 days and has been shown to depend on their ability to deliver nutrients to the host and is regulated by the host plant demand (Bücking et al. 2012).

Eucalyptus, being classified as a broad leafed species, ECM may cover 40-50 percent of the root apical zones by enclosure with a fungal sheath. In contrast, VAM infected apical root zones are free of fungal

structures. Since vesicular-arbuscular mycorrhiza do not form a fungal sheath, it can use both pathways for nutrient uptake (Bücking et al. 2012). Vesicular arbuscular mycorrhizal (VAM) hyphae also manifest larger absorbing areas because they are thinner and have a relative larger fungal network compared to ECM fungi (Viertelhauzen 2013). Therefore, the root P uptake was highest (75%) in plants inoculated with Mykorich (AMF) alone and the highest increases in total plant N, K, Mg, Fe, Mn uptake were observed in Mycogroe+Mykorich (mixture of ECM and AMF, respectively) inoculated plants. This validates the statement of Smith and Read (2008) that different plant–fungus combinations would show different symbiotic compatibility as reflected by the plant growth responsiveness.

Conclusion and recommendation

the results clearly showed that growth and mineral composition of *E. pellita* were greatly improved by inoculation with mycorrhizal fungi particularly mycogroe+mykorich from the philippines, a combination of ecto and vesicular arbuscular mycorrhizal fungi. the species *e. pellita* when inoculated can be valuable for reforestation in degraded lands. also, it can be of importance for afforestation of other natural habitats such as coastal lands. it is highly recommended that survival and persistence of these fungi should be determined under field trials to validate these results under controlled conditions.

References

Adjoud D, Plenchette C, Halli-Hagas R, and Lapeyrie F (1996) Response of 11 eucalyptus species to inoculation with three arbuscular mycorrhizal fungi. Mycorrhiza 6: 129–135 https://doi.org/10.1007/s005720050117

Aggangan N, Yong-Wook K, Han SM, Paek K, and Moon HK (2011*)* Selection of arbuscular mycorrhizal inoculants for improved growth of somatic embryo-derived *Liriodendron tulipifera* (Magnoliaceae) plantlets. Philippine J Sci 140(1): 56-67

Aggangan N, Moon HK and Han SH (2013) Growth and nutrient accumulation of *Eucalyptus pellita* F. Muell. in response to inoculation with edible ectomycorrhizal mushrooms. Asia Life Sci 22(1): 95-112

Boudarga K, Lapeyrie F and Dexheimer J (1990) A technique for dual vesicular-arbuscular endomycorhizae/ectomycorhizal infection of Eucalyptus in vitro. New Phytol 114:73-76

Bowen GD (1965) Mycorrhiza inoculation in forestry practices. Australian For 29: 231-237 doi: 10.1080/00049158.1965.10675964

Brundrett M (1991) Mycorrhizas in natural ecosystems. Adv Ecol Res 21:171-271 http://dx.doi.org/10.1016/S0065-2504(08)60099-9

Brundrett M, Bougher N, Dell B, Grove T and Malajczuk N (1996) Working with mycorrhizas in forestry and agriculture. ACIAR Monograph 32. Canberra, Australia: Austalian Centre for International Agricultural Research.

Bücking H, Liepold E and Ambilwade P (2012) The Role of the Mycorrhizal Symbiosis in Nutrient Uptake of Plants and the Regulatory Mechanisms Underlying These Transport Processes. Plant Sci 4:107-138 doi:10.5772/52570

Carrenho R, Barbosa FF, Araújo CVM, Alves LJ and Santos OM (2008) Mycorrhizal Associations in Eucalyptus spp.: Status and Needs. Tree and Forestry Science and Biotechnology. Global Science Books. 2(1): 57-67

Chen YL, Gong MQ, Wang FZ and Chen Y (1998) Study on mycorrhizal physiology of *Eucalyptus urophylla* inoculated with dual ECM and VAM fungi. For Res 11:237–242

Chen YL, Brundrett MC and Dell B (2000) Effects of ectomycorrhizas and vesicular–arbuscular mycorrhizas, alone or in competition, on root colonization and growth of *Eucalyptus globulus* and *E. urophylla*. New Phytol 146:545-556 doi: 10.1046/j.1469-8137.2000.00663.x

Chen Y, Run JL, Yin LB and Gu F (2014) Use of Mycorrhizal Fungi for Forest Plantations and Minesite Rehabilitation. 325−355. doi: 10.1007/978-3-662-45370-4_21

Chilvers GA, Lapeyrie FF and Horan DP (1987) Ectomycorrhizal vs endomycorrhizal fungi within the same root system. New Phytol 107:441-448. https://doi.org/10.1111/j.14698137.1987.tb00195.x

Egerton-Warburton L and Allen MF (2001) Endo- and ectomycorrhizas in *Quercus agrifolia* Nee. (Fagaceae): patterns of root colonization and effects on seedling growth. Mycorrhiza 11(6): 283-290 https://doi.org/10.1007/s005720100134

Fleming LV (1983) Succession of mycorrhizal fungi on birch: infection of seedlings planted around mature trees. Plant & Soil 71: 263–267 doi: 10.1007/BF02182661

Food and Agriculture Organization of the United Nations (1996) Research Experiences on Eucalyptus in Indonesia - Irfan Budi Pramono and Ag. Pudjiharta. Retrieved from http://www.fao.org/docrep/005/AC772E/ac772e09.htm

Heinrich P A and Patrick J W (1986) Phosphorus acquisition in the soil–root system of *Eucalyptus pilularis* Smith seedlings. II. The effect of ectomycorrhizas on seedling phosphorus and dry weight acquisition. Australian J Bot 34: 445–454. doi: 10.1071/BT9860445

Hiwale S (2015*)* Eucalyptus (*Eucalyptus sp.*). In: Sustainable Horticulture in Semiarid Dry Lands. Springer, New Delhi. https://doi.org/10.1007/978-81-322-2244-6_23

Janos DP 1983 Tropical mycorrhizas, nutrient cycles and plant growth. In: Sutton SL, Whitmore TC and Chadwick AC (eds) Tropical rain forest: ecology and management. Blackwell Scientific Publications, London, 327-345

Keane PJ, Kile GA, Podger FD and Brown BN (2000) Disease and Pathogens of Eucalpyts. Australia, CSIRO Publishing.

Lapeyrie FF and Chilvers GA (1985) An endomycorrhiza– ectomycorrhiza succession associated with enhanced growth of *Eucalyptus dumosa* seedlings planted in a calcareous soil. New Phytol 100: 93–104 https://doi.org/10.1111/j.1469-8137.1985.tb02761.x

Lodge DJ (2000) Ecto- or arbuscular mycorrhizas, which are best? New Phytol 146:353–354

Malajczuk N, McComb AJ and Loneragan JF (1975) Phosphorus uptake and growth of mycorrhizal and uninfected seedlings of *Eucalyptus calophylla*. R.Br. Australian J Bot 23: 231–238 https://doi.org/10.1071/BT9750231

Marschner H and Dell B (1994) Nutrient uptake in mycorrhizal symbiosis. Plant and Soil 159(1): 89–102 doi: 10.1007/bf00000098

Marx DH 1991 The practical significance of ectomycorrhizae in forest establishment. In: Ecophysiology of ectomycorrhizae of forest trees. The Marcus Wallenberg Foundation Symposia Proceedings, Stockholm, Sweden, 54-90

Midgley SJ and Pinyopusarerk K (1996) The role of eucalypts in local development in the emerging economies of China, Vietnam and Thailand. In: Eldrige KG, Crowe MP and Old KM (eds) Environmental Management : the role of Eucalypts and other fast-growing species. Canberra, Australia: CSIRO Forestry and Forest Products, 4–10

[MSU] Michigan State University. 1996. User's guide to MSTAT-C. Design, Management and Statistical Research Tool. East Lansing, Michigan: Michigan State University.

Oliveira VL, Schmidt VDB and Bellei MM (1997) Patterns of arbuscular- and ecto- mycorrhizal colonization of *Eucalyptus dunnii* in Southern Brazil. Annales des Sciences Forestieres 54: 473–481

Santos VL, Muchovej RM, Borges AC, Neves JCL and Kasuya MCM (2001) Vesicular-Arbuscular/Ecto-Mycorrhiza Succession in Seedlings of Eucalyptus Spp. Brazilian J Microbiol Vol. 32 No.2. http://dx.doi.org/10.1590/S1517-83822001000200002

Smith SE and Read DJ (2008) Arbuscular mycorrhizas. Mycorrhizal Symbiosis. Academic Press, New York, 31-134. Retrieved from https://books.google.com.ph

van der Heijden MGA, Martin FM, Selosse MA and Sanders IR (2015) Mycorrhizal ecology and evolution: the past, the present, and the future. New Phytol 205: 1406–1423 doi: 10.1111/nph.13288

Viertelhauzen LM (2013) Arbuscular mycorrhizae vs. Ectomycorrhizae: limiting or stimulating tree growth with N deposition? Masterthesis GEO4-2321 45 ECTS Utrecht

Vozzo J and Hacskaylo E (1974) Endo- and ectomycorrhizal associations in five Populus species. Bull Torrey Bot Club. 101(4): 182-186 doi: 10. 2307/2484641

Wang Y, Wang M, Li Y, WuA and Huang J (2018) Effects of arbuscular mycorrhizal fungi on growth and nitrogen uptake of *Chrysanthemum morifolium* under salt stress. PLOS ONE, 13(4), e0196408. https://doi.org/10.1371/journal.pone.0196408

Zhao R, Guo W, Bi N, Guo J, Wang L, Zhao J and Zhang J (2015) Arbuscular mycorrhizal fungi affect the growth, nutrient uptake and water status of maize *(Zea mays L.)* grown in two types of coal mine spoils under drought stress. Appl Soil Ecol 88: 41-49 doi: 10.1016/j.apsoil.2014.11.016

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Use of liquid medium and biofortificants for improving micropropagation and acclimation of *Musa* AAA cv. Williams

Alexander Moreno[1], Ángeles Bernal[2], Francisco Ugarte[1], Kevin Lima[1], Mónica Coig [3], Conchi Sánchez[4], Anxela Aldrey[4], Nieves Vidal[4]

[1] *Universidad Técnica de Machala, Ecuador.*
[2] *Universidade de Coruña, A Coruña, Spain.*
[3] *CULTESA. Tacoronte, Santa Cruz de Tenerife, Spain.*
[4] *Instituto de Investigaciones Agrobiológicas de Galicia. CSIC. Santiago de Compostela, Spain.*
nieves@iiag.csic.es.

Abstract
In Ecuador, banana production is being threatened by the age of plantations and diseases like *Mycosphaerella fijiensis* Morelet. Although bananas are traditionally propagated by suckers, by using tissue-cultured plants it is possible to produce larger quantities of healthy plantlets for both small- and large-scale farmers. The aim of this study was to develop alternative methods for the micropropagation of banana cv. Williams, as well as for the acclimation of the obtained plantlets. We present three sets of experiments i) *in vitro* establishment of genotypes of banana growing at "El Oro" (Ecuador), ii) micropropagation of banana in liquid medium by temporary immersion, and iii) use of extracts of *Moringa oleifera* Lam as a biofortificant during the acclimation of banana vitroplants. The obtained results indicate: i) the need to follow extremely careful sterilization protocols for establishing banana cultures, in order to counteract the presence of fungal and bacterial contaminants due to the climate conditions of "El Oro" (Ecuador), ii) the potential of temporary immersion systems for propagation of banana, and iii) the beneficial effects of extracts of Moringa leaves on vitroplants of banana during the acclimation step.

Keywords: banana, bioreactors, *Moringa oleifera*, sterilization

Introduction

Banana tree crops play a determining role in the local economy of South American countries. This is especially true about coastal districts of Ecuador, such as El Oro, where banana tree plantations are currently the main source of sustenance for the population. Conventional systems of production can be found within the available growing systems. In these, monoculture persists for over fifty years, plans for plantation renewal are lacking and chemical fertilizers and pesticides are used on the crops. This model of production increases risks of plant damage regarding biotic and abiotic factors, which abound in the production regions.

Particularly, in the El Oro region, banana production is being threatened by the age of plantations and diseases like *Mycosphaerella fijiensis* Morelet. Although bananas are traditionally propagated by suckers, by using tissue-cultured plants it is possible to produce larger quantities of healthy plantlets for both small- and large-scale farmers. In micropropagation systems, the use of liquid medium presents some advantages over the use of gelled medium, as it causes a reduction in costs due to agar consumption and the possibility of automation (Etienne and Berthouly, 2002). Moreover, the atmosphere in temporary immersion systems (TIS) can be renewed, thus preventing disorders such as asphyxia and hyperhydricity (Etienne and Berthouly, 2002). The use of TIS to improve plant propagation has been reported for some species of economic relevance such as eucalyptus, apple, teak, pistachio, chestnut, yerba mate and

willow (McAlister et al. 2005, Chakrabarty et al. 2003, Zhu et al. 2005, Quiala et al. 2012, Akdemir et al. 2014, Vidal et al. 2015, Luna et al. 2017, Regueira et al. 2018). In banana (*Musa* AAA) and plantain (*Musa* AAB), some attempts of using bioreactors have been reported (Alvard et al. 1993, Roels et al. 2005, Aragon et al. 2014), with promising results.

One of the objectives of this study was to apply the TIS technology to the banana genotypes growing in the El Oro region, comparing the efficiency of commercial and in-house bioreactors. The other main objective was to improve the acclimation and transfer to field conditions of plantlets obtained by micropropagation, using extracts of *Moringa oleifera* Lam as biofortificant. *Moringa oleifera* is a tree with high biotechnology potential as growth enhancer and plant defense promoter. This plant, originally from Asia, shows excellent adaptation to a great variety of climates, having a very effective growth rate and an elevated nutritional potential in its leaves. Its use as a biostimulating plant has improved the efficiency of wheat (Yasmeen et al., 2012) and corn (Basra et al., 2011) crops.

The use of natural fortificants such as Moringa extract could offer an alternative to the use of chemical fertilizers and pesticides in order to boost banana production systems, with the goal of achieving a sustainable and eco-friendly production. In this work, we aim to determine for the first time the possible beneficial effects of watery extracts from Moringa leaves on the growth and development of banana vitroplants. Two variables have been studied as a first approach to the use of the said extracts: i) starting material, comparing Moringa plant leaves in different states of development (young plants and adult trees), ii) concentration of pulverized leaves in each extract.

Materials and Methods

In vitro establishment of genotypes of banana growing at El Oro.
Banana trees were selected in commercial plantations of "El Oro" (Ecuador) by its yield and other productive parameters (**Fig. 1A**). Sword suckers of eight genotypes of *Musa* AAA cv. Williams were

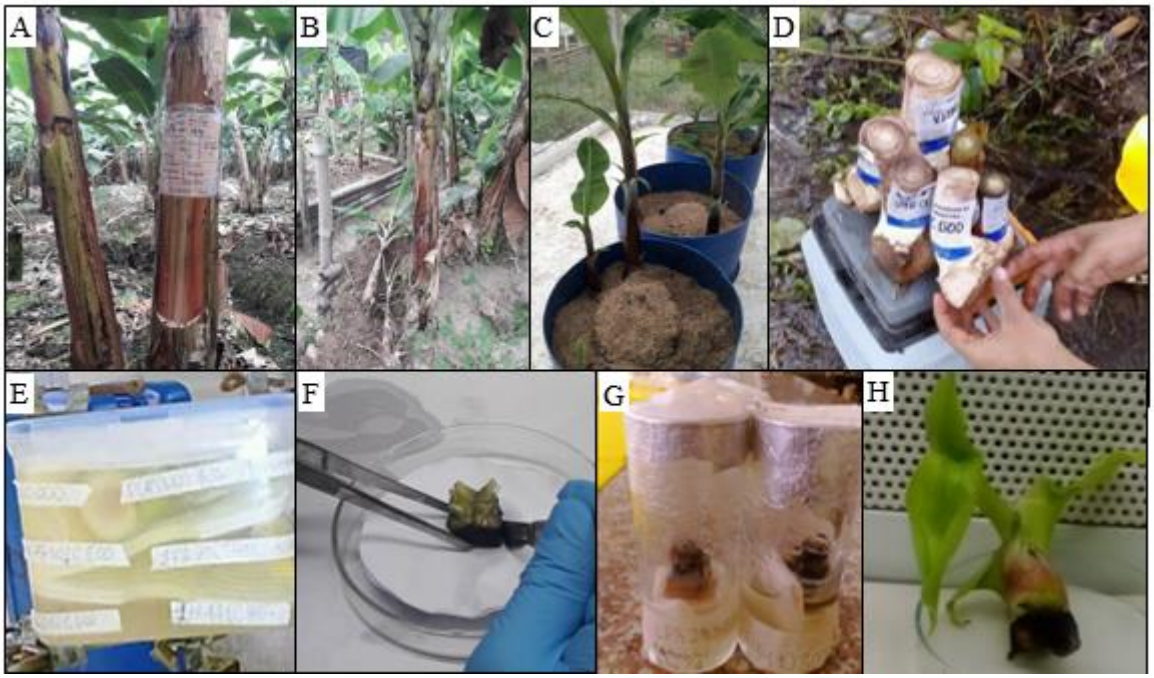


***Figure 1**. In vitro establishment of banana genotypes growing at El Oro**.** A) Mother plants growing at commercial plantations. B) Selected genotypes established in UTMACH experimental fields. C) Bananas growing in pots in the greenhouse. D) Explants for in vitro establishment. E) Pre-sterilization step in a commercial shaker. F-G) Explant two weeks after inoculation, cross-sectioned to detect latent contaminants. H) Healthy explant used for initiating an aseptic culture line.*

removed from vigorous growing plants and used for conventional propagation in the experimental field at the UTMACH (Universidad Técnica de Machala, Ecuador). Before planting, these materials were peeled and disinfected by 20 min immersion in 1 ml/L DISS SEKTOR and 1 ml/L DISS 4X4 (UNGUERER S.A., Ecuador), with insecticidal and fungicidal activities. Suckers obtained from these plants growing in the field (Fig. 1B) were planted in pots in the greenhouse (Fig. 1C), in order to provide the explants for *in vitro* establishment (Fig. 1D).

In the first experiments, the prevalence of fungal and bacterial contaminants hampered the establishment of aseptic cultures. In subsequent experiments, the following protocol was applied: 1) The shoots were peeled and washed thoroughly with water and industrial detergents; 2) the pseudostems were peeled again to a size of 4 cm diameter and 10 cm height, introduced in a plastic container with sodium hypoclorite (3%) and vigorously shaken using a commercial device for 20 min (Fig. 1E); 3) the explants were introduced in the laminar flow cabinet and peeled to reach 1.5-2 cm diameter x 1.5-2 cm height, and disinfected again with sodium hypoclorite for 20 min (manual shaking); 4) the explants were washed 3 times with sterile water before being treated with cystein (100 mg L^{-1}) for elimination of phenolics; 5) the explants were peeled again to a size of 8 mm diameter and 10 mm height and were inoculated on paper bridges over liquid medium MS (Murashige and Skoog 1962) with 2 mg L^{-1} N^6-benziladenine (BA), 0.023 mg L^{-1} indole-3-butyric-acid (IBA), and sucrose (3%); 6) two weeks after planting, the explants were cross sectioned for detecting endogenous contaminants (Fig 1F-G), and 7) surviving explants with healthy appearance were used to initiate aseptic cultures (Fig 1H).

Micropropagation of banana in liquid medium by temporary immersion

For the micropropagation of banana in liquid medium we used plant material of cv. Williams previously established *in vitro* by CULTESA in Spain as well as genotypes established *in vitro* in Ecuador. Explants were propagated in liquid MS with 2 mg L^{-1} BA, 0.65 mg L^{-1} indole-3-acetic-acid (IAA), and sucrose (3%), whereas control explants were cultured in jars with the same medium supplemented with 1.9 g L^{-1} of Gellam gum. Containers used for temporary immersion systems (TIS) were commercial bioreactors RITA® and plantform™, as well as bioreactors of the two-flask system adapted from glass bottles (in-house TIS). RITA® and plantform™ were used as previously described for willow (Regueira et al. 2018). Bioreactors were programmed for 8 immersions per day (4 min each). The volume of medium and number of explants per container is detailed in Table 1.

Table 1. *Volume of medium and number of explants per container in the experiments of temporary immersion*

	Container			
	Jar	RITA®	plantform™	In-house TIS
Volume (ml)	200	200	500	1000
Nº of explants	4	4	10	20

Use of extracts of Moringa oleifera during the acclimation of banana vitroplants

For acclimation experiments, we used banana vitroplants produced by Orangelab (Quito, Ecuador), and acclimated for 3 weeks in a greenhouse. These plantlets were removed from the substrate, carefully cleaned and planted in pots with fiberglass soaked in nutritive medium (Fig 2A). After 7 days, the leaves of banana plantlets were sprayed with extracts obtained from juvenile and mature leaves of a Moringa tree (Fig 2B-C). Moringa leaves were dried (70ºC, 24 h), ground, dissolved in water and filtered to obtain extracts ranging from 0.06 to 0.2 mg L^{-1} on a dry weight basis (Fig 2D). Ten plantlets were used for each treatment. After 3 weeks growing in the greenhouse (Fig 2E), banana plantlets were characterized.

For the morphological characterization of the plantlets the following parameters were recorded: number of leaves, length and width of the third leaf, and height and diameter of the pseudostem. For mass determination whole plantlets were cleaned with plenty of water and dried with filter paper. Then, fresh and dry weight, as well as ash free dry weight were recorded. Dry weight was obtained after 72 h in an oven at 100 ºC, and ass free dry weight after 24 h in a muffle furnace at 600 ºC. Electrolytic leakage was determined in leaf discs (9 mm diameter) by electrical conductivity (Lafuente et al. 1991). Briefly, 10 discs per treatment were washed thrice with deionized water and maintained in water for 20 h at room temperature. The conductivity was recorded (C1) and then discs were heated to 50 ºC for 15 min to record the new conductivity value (C2). Electrolytic leakage was calculated as 100*C1/C2.

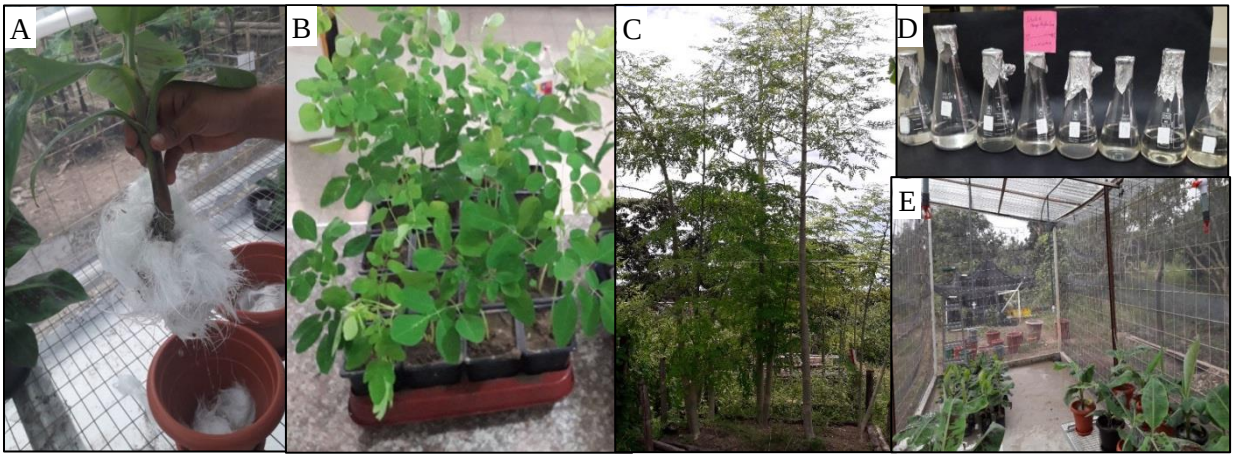


Figure 2. *Acclimation of banana vitroplants with extracts of Moringa oleifera Lam. A) Banana vitroplant planted in a pot with fiberglass as sustrate. B) Juvenile Moringa plants. C) Mature Moringa trees. D) Water extracts of Moringa. E) Banana plantlets treated with Moringa extracts during acclimation in the greenhouse*.

Results and discussion

The prevalence of fungal and bacterial contamination represented the main limitation for the successful establishment of aseptic cultures of banana growing at El Oro (Ecuador). The high contamination levels can be related to the climate conditions, with high humidity and temperatures, as well as to the deficient sanitary state and susceptibility of the plantations of the region (Cedeño García et al. 2017). In this study, we developed a protocol to counteract these difficulties. In first place, banana plants previously disinfected were cultured in a controlled area in the University facilities, and then in the greenhouse, as a way of obtaining healthy plant material for *in vitro* establishment. Besides, two modifications were incorporated to the conventional sterilization method, in order to decrease exogenous and endogenous contaminants: 1) mechanical shaking of the explants using a commercial device during the first sterilization step outside the laminar flow cabinet, and 2) wounding of the explants that had survived the sterilization process, in order to detect latent contamination. In this way, only healthy explants were selected for initiation of cultures.

The results of culturing banana in semisolid and liquid medium in TIS are shown in Fig 3-4. Banana explants could be cultured in semisolid medium (Fig 3A), but better results were observed in liquid medium in the three bioreactors tested (Fig 3B-F). Similarly, more growth of *Musa* vitroplants cultured by temporary immersion were reported by Alvard et al. (1993) with the banana cultivar ‘Grande Naine’, as well as by Roels et al. (2005) and Aragon et al. (2014) with plantain.

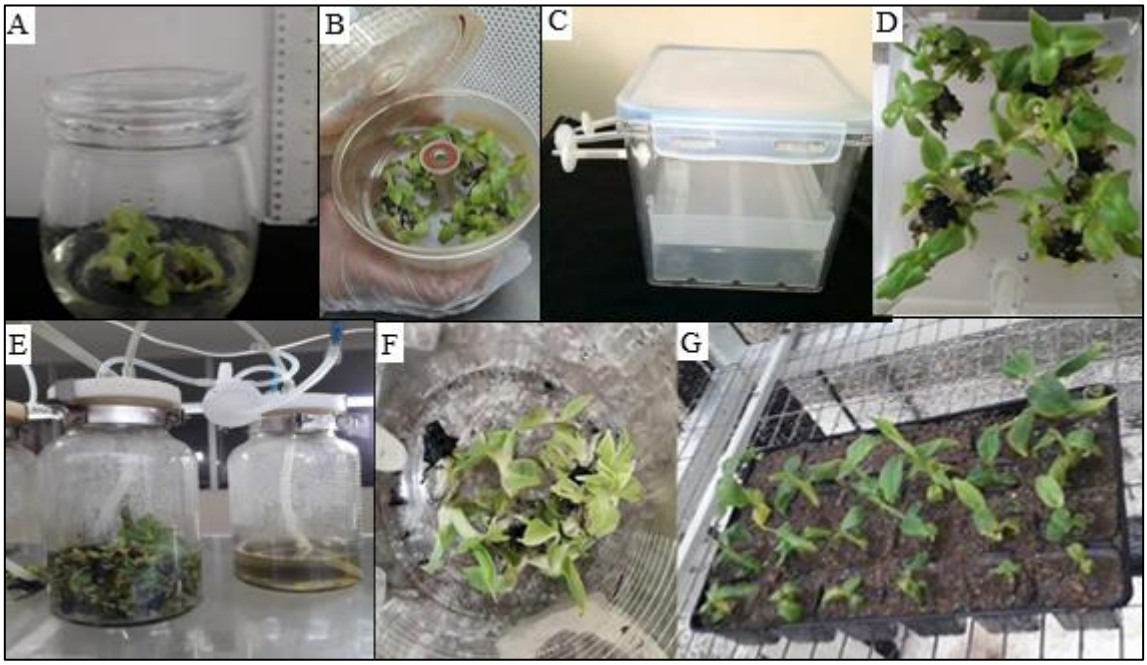


Figure 3*. Shoots of banana growing in different containers. (A) Control explants cultured in jars. (B) Explants cultured by TIS in bioreactors RITA®. (C, D) Explants cultured in plantform™. (E, F) Explants growing in the in-house TIS. (G) Rooted plantlets obtained in TIS during the acclimation process.*

The highest proliferation and the most robust pseudostems were obtained in RITA® containers (Fig 4). Besides, RITA® and Plantform™ produced spontaneously rooted explants in proliferation medium, and these rooted plants were successfully acclimated (Fig 3G).

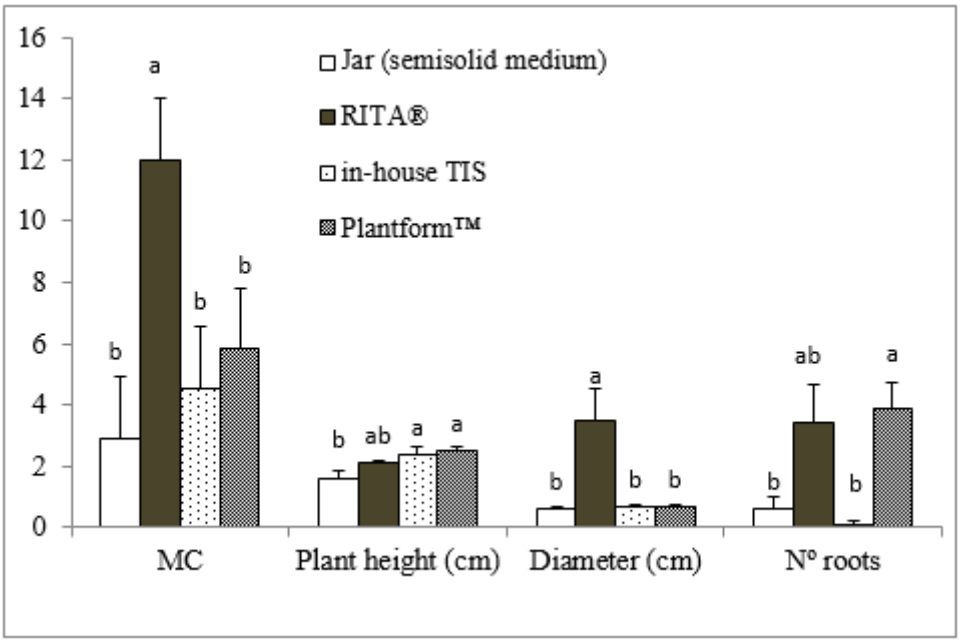


Figure 4*. Effect of the culture system on the proliferation of banana shoots. MC: Multiplication Coefficient. For each variable, different letters indicate significant differences (P < 0.05).*

Similar results were obtained by using Plantform™ and in-house TIS, except for the number of roots. Although proliferation in in-house TIS did not reach the values obtained in RITA®, these bioreactors

made in the laboratory were not as expensive as commercial bioreactors, and its components were easier to acquire in non-specialized stores. These advantages could justify its use and improvement.

The results of applying water extracts made from Moringa leaves to banana plantlets during acclimation are shown in Fig 5. Although a single treatment, affecting positively all the parameters tested, was not identified in these experiments, the extracts with 0.06 mg L^{-1} of leaves from juvenile plants and 0.18 mg L^{-1} of leaves from mature plants improved the acclimation of banana vitroplants based on the number and quality of leaves obtained and on the size of the pseudostem. Similar results were reported by Basra et al. (2011) applying 0.06 mg L^{-1} of Moringa to hybrid corn. The use of 0.18 mg L^{-1} of leaves from mature plants increased the dry weight and the ass-free dry weight of the banana plantlets.

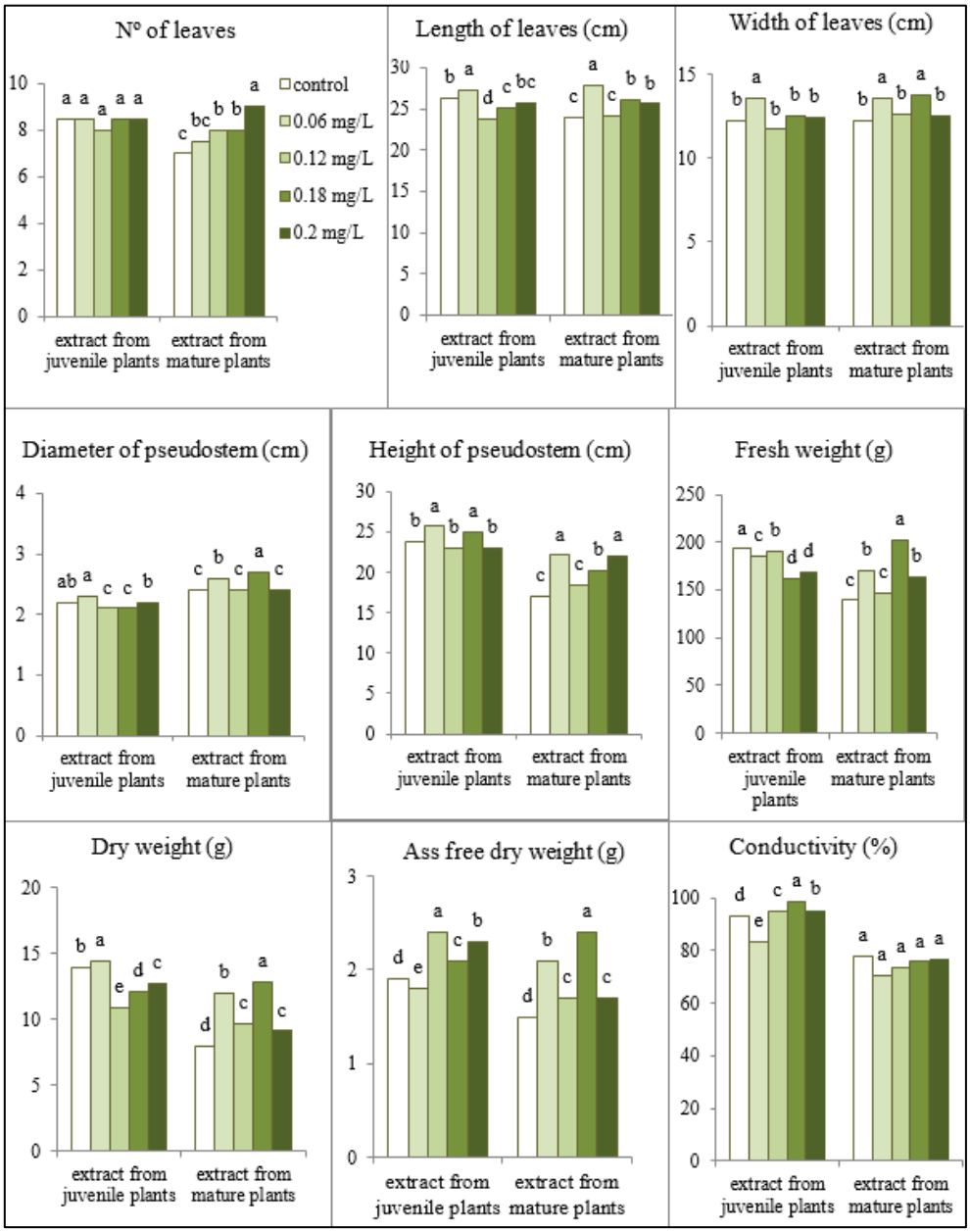


Figure 5*. Effect of the concentration of extracts obtained from juvenile and mature Moringa plants on acclimation of banana vitroplants. Data were recorded 21 days after treatment. For each parameter and origin of extract (juvenile or mature plants) different letters indicate significant differences (P < 0.05).*

The electrolytic leakage of leaf disks indicates the behavior of plant cells exposed to stress conditions (Shanahan et al. 1990). The application of Moringa extracts (0.06 mg L^{-1}) decreased the electrolytic leakage (expressed here as the change of conductivity after a thermal shock), indicating a proper functioning of the cellular membranes and a good capacity of water and solute retention. Taken all these results together, Moringa extracts had a beneficial effect on the growth and development of banana vitroplants. In these preliminary experiments we detected that juvenile and mature Moringa plants caused improvements in the growth of banana vitroplants. However, in order to recommend the best concentration of each type of extract, two approaches should be taken: i) to carry out more experiments with homogeneous material and ii) to select the most relevant parameters affecting acclimation of banana, taking into account not only the plant performance during the first days in the greenhouse but also the global acclimation process (greenhouse and field conditions).

In conclusion, several genotypes of banana growing at "El Oro" (Ecuador) were established *in vitro*, propagated and acclimated. The use of careful sterilization protocols was essential for establishing banana cultures, due to the climate conditions in the region. Once established, the use of different bioreactors improved the micropropagation rate of banana cultures, and Moringa extracts were tested as biofortificant during the acclimation step with promising results.

Acknowledgements: This research was partly funded by the Xunta de Galicia (Spain) through the Contrato Programa 2017-2018, by the Univ. Técnica de Machala (Ecuador) and by CYTED through the BIOALI net (P117RT0522). We thank Alejandro Díaz, Rafael Sánchez and Patricia Val for technical assistance.

References

Akdemir H, Süzerer V, Onay A, Tilkat E, Ersali Y, Çiftçi YO (2014) Micropropagation of the pistachio and its rootstocks by temporary immersion system. Plant Cell Tissue Organ Cult 117:65-76

Alvard D, Cote F, Teisson C (1993) Comparison of methods of liquid medium culture for banana micropropagation. Plant Cell Tissue Org Cult 32:55–60

Aragón CE, Sánchez C, Gonzalez-Olmedo J, Escalona M, Carvalho L, Amancio S (2014) Comparison of plantain plantlets propagated in temporary immersion bioreactors and gelled medium during in vitro growth and acclimatization. Biol Plant 58:29-38

Basra SMA, Iftikhar MN, Afzal I (2011) Potential of Moringa (*Moringa oleifera*) leaf extract as priming agent for hybrid maize seeds. Int J Agric Biol 13:1006-1010

Cedeño García G, Suarez Capello C, Vera Coello D, Fadda C, Jarvis D, de Santis P (2017). Detección temprana de resistencia a *Mycosphaerella fijiensis* en genotipos locales de Musáceas en Ecuador. Scientia Agropecuaria 8:29-42

Chakrabarty D, Hahn EJ, Yoon YS, Paek KY (2003) Micropropagation of apple root stock 'M9 EMLA' using bioreactor. J Hortic Sci Biotechnol 78:605-609

Lafuente MT, Belver A, Guye MG, Salveit ME (1991) Effect of temperature conditioning on chilling injury of cucumber cotyledons-posible role of abscisic acid and heat shock proteins. Plant Physiol 5:443-449

Luna CV, Gonzalez AM, Mroginski LA, Sansberro PA (2017) Anatomical and histological features of *Ilex paraguariensis* leaves under different in vitro shoot culture systems. Plant Cell Tissue Organ Cult 129:457-467

McAlister B, Finnie J, Watt MP, Blakeway F (2005) Use of temporary immersion system (RITA®) for production of commercial *Eucalyptus* clones in Mondi Forests (SA). Plant Cell Tissue Organ Cult 81:347-358

Mahmood KT, Mugal T, Haq IU (2010) *Moringa oleifera*: A natural gift-a review. J Pharm Sci Res 2:775-781

Mona MA (2013) The potential of *Moringa oleifera* extract as a biostimulant in enhancing the growth, biochemical and hormonal contents in rocket (*Eruca vesicaria* subsp. *sativa*) plants. Int J Plant Physiol Biochem 5:42-49

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473-497

Quiala E, Cañal MJ, Meijón M, Rodriguez R, Chavéz M, Valledon L, Feria M, Barbón R (2012) Morphological and physiological responses of proliferating shoots of teak to temporary immersion and BA treatments. Plant Cell Tissue Organ Cult 109:223-234

Regueira M, Rial E, Blanco B, Bogo B, Aldrey A, Correa B, Varas E, Sánchez C, Vidal N (2018) Micropropagation of axillary shoots of *Salix viminalis* using a temporary immersion system. Trees 32:61–71

Roels S, Escalona M, Cejas I, Noceda C, Rodriguez R, Canal MJ, Debergh P (2005). Optimization of plantain (*Musa* AAB) micropropagation by temporary immersion system. Plant Cell, Tissue Organ Cult 82: 57-66

Shanahan JF, Edwards IB, Quick JS, Fenwick JR (1990) Membrane thermostability and heat tolerance of spring wheat. Crop Sci 30:247-251

Vidal N, Blanco B, Cuenca B (2015) A temporary immersion system for micropropagation of axillary shoots of hybrid chestnut. Plant Cell Tissue Organ Cult 123:229-243

Yasmeen A, Basra SMA, Ahmad R, Wahid A (2012) Performance of late sown wheat in response to foliar application of *Moringa oleifera* Lam. leaf extract. Chilean J Agric Res 72:92-97

Zhu LH, Li XY, Welander M (2005) Optimisation of growing conditions for the apple rootstock M26 grown in RITA® containers using temporary immersion principle. Plant Cell Tissue Organ Cult 81:313-318

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Using *in vitro* culture for conservation of genetic resources: micropropagation of a monumental *Prunus dulcis* tree

Conchi Sánchez[1], Saleta Rico[1], Anxela Aldrey[1], Damián Dasilva[1], Jose María Rey-Benayas[2], Nieves Vidal[1]

[1] Instituto de Investigaciones Agrobiológicas de Galicia. CSIC. Avda. de Vigo s/n. Apdo 122. 15780 Santiago de Compostela, Spain (conchi@iiag.csic.es).
[2] Fundación Internacional para la Restauración de Ecosistemas – FIRE (www.fundacionfire.org). C/ Princesa 3 dpdo., Apto 703. 28008, Madrid, Spain.

Abstract
In this study we present the micropropagation of an ancient almond tree, probably 300- years-old, together with juvenile material proceeding from its seeds. Three types of material were used: 1) shoots flushed on the tree at the beginning of spring, 2) shoots forced to flush in a phytotron from branch segments collected in late winter, and 3) seeds collected in autumn and stored at a cool place for six months before being germinated *in vitro*. Murashige and Skoog medium supplemented with 0.5 mg L^{-1} N^6-benzyladenine and 0.05 mg L^{-1} indole-3-butyric acid was used for culture establishment and stabilization. Different combinations of plant growth regulators were evaluated for shoot proliferation and elongation. Roots formed spontaneously in the multiplication medium. Rooted shoots from cultures obtained from the mother tree and from lines that had been obtained from small seedlings were successfully acclimatized in the phytotron and the greenhouse.

Keywords: almond, biodiversity, education, divulgation, mature tree

Introduction

The need to conserve biodiversity has been granted increasing political and social attention in the last years (FAO 2011). Monumental or emblematic trees, both "wild" trees living in forests and century-old, "domesticated" agricultural trees, should be preserved *in situ* for their intrinsic value, their cultural legacy, the rich diversity of microhabitats they generate, and the quantity of organisms that depend on them for life (Moya and Moya 2013). Also, they should be preserved *ex situ* for the study and the conservation of their genetic resources, for educational issues and for reintroducing plant material of high quality in their natural areas, most of them currently degraded or threatened (Dulloo et al. 2010). Within *ex situ* conservation methods, *in vitro* culture presents special advantages in the case of emblematic trees, such as the small quantity of plant material needed to begin the micropropagation procedure and the possibility of implementing such long-term conservation techniques as cryopreservation (Postman et al. 2006, Pence 2013).

The aim of this study was to micropropagate mature material from an ancient almond tree, named "Gladiador" (Fig. 1A), located in Membrilla (Central Spain), together with juvenile material proceeding from its seeds. This monumental tree, probably 300-years-old, dominates a landscape formed by hundreds of olive trees, and has a special emblematic meaning for the population of the area, as has been recognized by the International Foundation for Ecosystem Restoration (FIRE).

Materials and Methods

For establishment of axillary shoot cultures, plant material was provided by the FIRE foundation. Three types of material were used: 1) shoots flushed on the tree at the beginning of spring (Fig. 1B), 2) shoots forced to flush in a phytotron from branch segments collected in late winter (Fig. 1C,D), and 3) seeds collected in autumn and stored at a cool place for six months before being germinated *in vitro* (Fig. 1E).

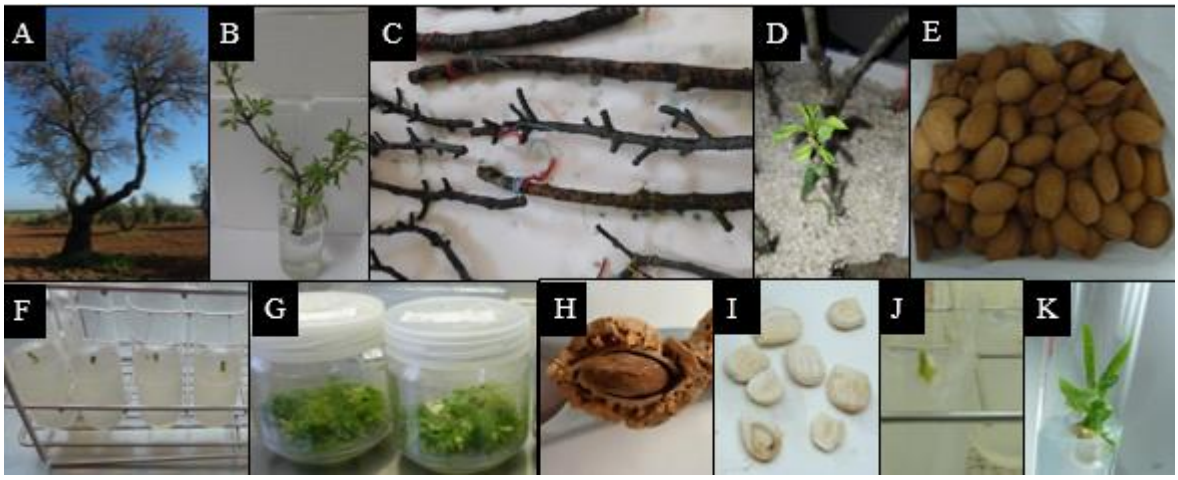


***Figure 1**. Plant material used for in vitro establishment. A) "Gladiador" almond tree. B) Shoots flushed on the tree at the beginning of spring. C,D) Shoots forced to flush in a phytotron from branch segments collected in late winter. E) Seeds. F) Nodal segments from the mother tree after sterilization. G) Shoots of mature origin cultured in jars. H) Cracked seed. I) Seeds before sterilization and embryo isolation. J) Embryo inoculated in a test tube. K) Germinating embryo used for the initiation of a juvenile line.*

For establishment of mature material originated from branches of the mother tree, the shoots were stripped of their leaves and surface sterilized by immersion for 40-50 s in 70% ethanol and for 15 min in a 10 g L^{-1} solution of sodium hypochlorite containing 2-3 drops of Tween 80®. The shoots were then rinsed three times in sterile distilled water. Nodal segments (10 mm) were cut from the shoots and inoculated in tubes with Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0.5 mg L^{-1} N^6-benzyladenine (BA) and 0.05 mg L^{-1} indole-3-butyric acid (IBA), 3% sucrose and 0.7 % (w/v) Bacto agar (Fig. 1F). The medium was adjusted to pH 5.7 before being autoclaved at 121 °C for 20 min. Cultures were incubated under a 16-h photoperiod provided by cool-white fluorescent lamps (50-60 µmol $m^{-2}s^{-1}$) at 25 ˚C light/20 ˚C dark. The explants were transferred every 2 weeks during the first 6 weeks after establishment and were then maintained in 50 ml of the above mentioned medium (multiplication medium; MM) in 300 ml glass jars (Fig. 1G) and were subcultured every 4-5 weeks.

For establishment of juvenile material, seeds collected in autumn under the canopy of the tree were stored in a cool chamber (8-12 ºC) for six months. Then, the shells were cracked (Fig. 1H) and the seeds (Fig. 1I) were washed with tap water before being sterilized following the same protocol as described for shoots, but with 60 s of ethanol treatment and 20-40 min in a 10-15 g L^{-1} solution of sodium hypochlorite. Embryos were isolated and inoculated in tubes with MS medium with 0.5 or 1 mg L^{-1} BA for *in vitro* germination (Fig. 1J). Shoots obtained from germinated embryos (Fig. 1K) were used to initiate separate juvenile lines, which were maintained in jars as described for mature material.
After 7-9 months of culture in MM for culture stabilization, the effect of different combinations of plant growth regulators (BA, IBA and Gibberellic acid (GA_3)), were evaluated for shoot proliferation, elongation and adventitious root formation. These treatments are described in Table 1. The explants (15 mm) were cultured in the proliferation medium (1 month), and the obtained clusters were transferred to elongation medium (1 month).

Rooted shoots from cultures obtained from the Gladiador mother tree and from lines originated from seeds were planted in pots with a commercial peat:perlite mixture (2:1) and acclimatized in a phytotron before being transferred to the greenhouse. For mid-term conservation, shoots from mature and juvenile origins were submitted to cold storage (4-6 ºC) under dim light.

Results and discussion

Seventeen lines of almond were successfully established, 12 corresponding to mature material originated from different branches of the mother tree (Fig. 2A,B) and 5 obtained from seeds (Fig. 2C).

***Figure 2**. Shoots of almond regenerated from mature material obtained from the mother tree (A, B) and from juvenile material obtained from seeds germinated in vitro (C).*

As the micropropagation of plant material derived from mature zones of ancient tree frequently presents more problems than the culture of juvenile material derived from seeds (Sánchez and Vieitez, 1991; Hackett and Murray, 1993), our first efforts were focused on the development of a reliable protocol for proliferation and rooting of the mature lines. Four treatments, combining two proliferation media and three elongation media, were tested. Briefly, the explants were cultured in MS BA 1 IBA 0.1 GA_3 0.1 (mg L^{-1}) or in MS BA 0.5 AIB 0.05 (mg L^{-1}) for one month, and then transferred to the same medium or to a half strength MS with GA_3 1 mg L^{-1} for another month (Table 1).

***Table 1**. Proliferation and elongation media used for culturing almond shoots. The concentration of plant growth regulators is expressed in mg L^{-1}.*

Treatments	Proliferation medium	Elongation medium
T1	MS BA 1 IBA 0.1 GA_3 0.1	MS BA 1 IBA 0.1 GA_3 0.1
T2	MS BA 1 IBA 0.1 GA_3 0.1	MS ½ GA_3 1
T3	MS BA 0.5 IBA 0.05	MS BA 0.5 IBA 0.05
T4	MS BA 0.5 IBA 0.05	MS ½ GA_3 1

The explants cultured in MS BA 1 IBA 0.1 GA_3 0.1 and transferred to the same medium (T1) produced more and longer shoots than the other treatments (Fig. 3A,B). However, T3 was overall more efficient, as 50% of the clusters developed spontaneous roots during the elongation step. The transfer of the cultures to half strength MS with GA_3 1 mg L^{-1} as the only plant regulator decreased the length of the shoots compared to the length of the shoots of cultures transfered to the original medium. This reduction was especially evident between T1 and T2 treatments. A sharp decrease in the number or rooted shoots was also observed, and T2 did not produce rooted shoots. The medium with GA_3 1 mg L^{-1} was used for elongation of other *Prunus* (Iacona and Muleo 2010) but it did not improve the propagation of our almond material.

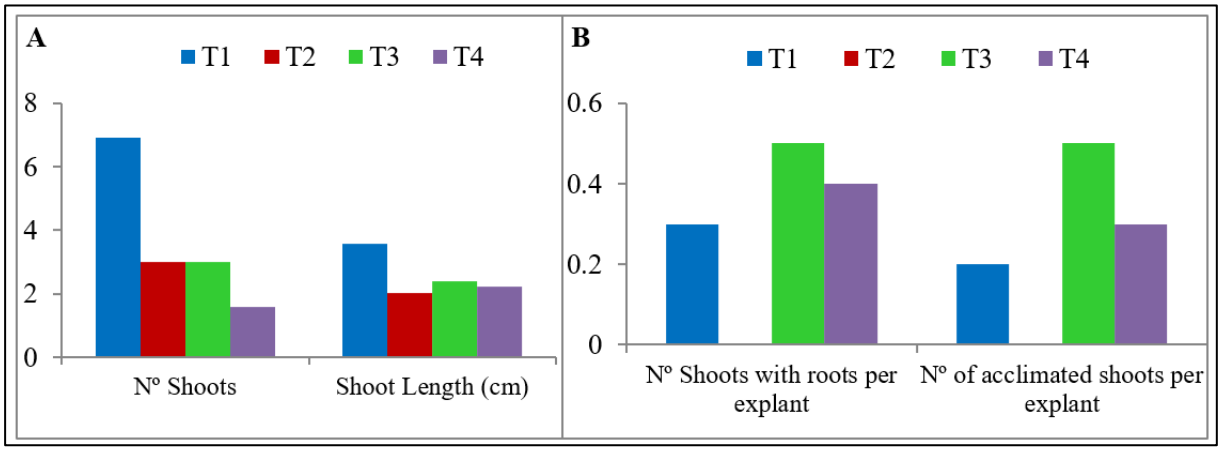


***Figure 3**. Effect of the combination of different proliferation and elongation media on the erformance of mature almond shoots. T1 to T4 treatments are described in **Table 1**.*

Rooted shoots which were at least 25 mm high were chosen for transfer to pots (Fig. 4A,B). Almost all these plantlets were successfully acclimated (Fig. 3B, Fig. 4C). T3 treatment was chosen for subsequent experiments, as it provided a good number of relatively long shoots that easily formed adventitious shoots spontaneously. The protocol was applied to juvenile material with similar results (Fig. 4D).

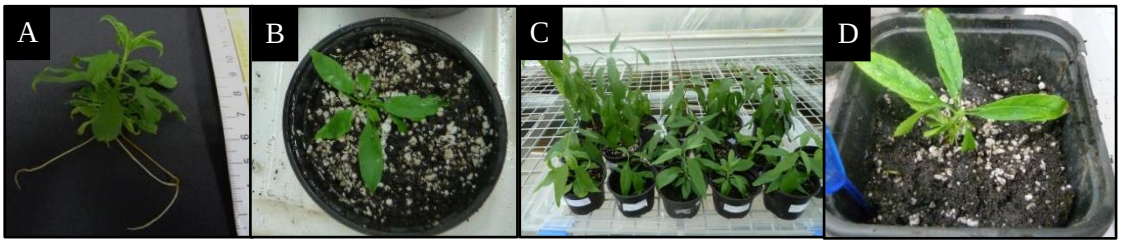


***Figure 4**. A-C) Rooted shoots of mature origin. (A,B) Rooted plant before (A) and after being transferred to a pot (B). C) Acclimated plantlets in the greenhouse. D) Juvenile plant during the acclimation step.*

So far, we have obtained 128 acclimated plantlets, 122 corresponding to clonal material from the mother tree and 6 corresponding to clonal material from seeds. Experiments are in progress to propagate more plants of juvenile origin, and both shoots from mature and juvenile origin were submitted to cold-storage at 4-6 ºC for mid-term conservation.

Acknowledgements: This research was partly funded through the contract FIRE-CSIC Ref. 20175906. We thank Alejandro Díaz, Rafael Sánchez and Patricia Val for technical assistance and Carlos Torres for plant material collection.

References

Dulloo ME, Hunter D, Borelli T (2010) *Ex situ* and *in situ* conservation of agricultural biodiversity: major advances and research needs. Not Bot Hort Agrobot Cluj 38:123-135

FAO (2011) Biodiversity for food and agriculture. Contributing to food security and sustainability in a changing world (www.fao.org/3/a-i1980e.pdf)

Hackett WP, Murray JR (1993) Maturation and rejuvenation in woody species. In: Ahuja MR (ed), Micropropagation of woody plants. Kluwer Academics Publishers, The Netherlands, pp. 93–105

Iacona C, Muleo R (2010) Light quality affects *in vitro* adventitious rooting and *ex vitro* performance of cherry rootstock Colt. Sci Hortic 125:630-636

Moya B, Moya J (2013). Monumental trees and mature forests threatened in the Mediterranean landscapes. Imelsa. Valencia (Spain)

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473-497

Pence VC (2013) *In vitro* methods and the challenge of exceptional species for Target 8 of the Global Strategy for Plant Conservation. Ann Missouri Bot Gard 99:214–220

Postman J, Hummer K, Stover E, Krueger R, Forsline P, Grauke Li, Zee F, Ayala-Silva T, Irish B (2006) Fruit and nut genebanks in the US National Plant Germplasm System. HortScience 41:1188-1194

Sánchez MC, Vieitez AM (1991) *In vitro* morphogenetic competence of basal sprouts and crown branches of mature chestnut. Tree Physiol 8:59-70

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Micropropagation of willow shoots under photomixotrophic and photoautotrophic conditions: proliferation in liquid medium and acclimation in different soils

Conchi Sánchez[1], Carmen Trasar[1], Mar Casalderrey[1], Diana Bello[1], Anxela Aldrey[1], Nieves Vidal[1]

[1] *Instituto de Investigaciones Agrobiológicas de Galicia. CSIC. Avda. de Vigo s/n. Apdo 122. 15780 Santiago de Compostela, Spain (nieves@iiag.csic.es)*

Abstract
Salix viminalis is a species with a high capacity for micropropagation and acclimation, and could therefore be used to develop emergent techniques in the field of plant propagation. The aims of this study were to propagate willow in liquid medium, both by temporary and continuous systems, to explore the application of photoautotrophic conditions and to investigate the acclimation of willow plantlets in different types of soils that could be used as alternatives for commercial peat. The results indicate that liquid medium under photomixotrophic and under photoautotrophic conditions can be satisfactorily used to culture willow axillary shoots. Regarding acclimation, although there were some differences in aerial growth, the willow plants were successfully acclimated in all soils.

Keywords: bioreactors, continuous immersion, enzymes, rhizosphere, temporary immersion

Introduction

Salix viminalis L., also known as basket willow, is a promising candidate for bioenergy and phytoremediation applications (Mleczek et al. 2010; Touceda-González et al. 2017). Some micropropagation protocols in semisolid medium have been developed for *Salix* species (Bhojwani 1980; Bergman et al. 1985; Read et al. 1989; Amo-Marco and Lledo 1996; Park et al. 2008; Mashkina et al. 2010; Skálová et al. 2012; Palomo-Ríos et al. 2015). Its feasibility for propagation and acclimation make it a useful material to develop emergent techniques in the field of plant propagation. Recently, a protocol for culture in liquid medium by temporary immersion was reported (Regueira et al. 2018). Micropropagation in liquid medium, providing uniform and better controlled environmental conditions, may complement or represent an alternative to the hydroponic culture systems frequently used to test the ability of willow to absorb and accumulate heavy metals and other contaminants.

The objectives of the present study were: i) to investigate the propagation of willow shoots in liquid medium by temporary and continuous immersion systems, ii) to propagate willow under photomixotrophic and photoautotrophic conditions, iii) to acclimate willow plantlets in different types of soils that could be used as alternatives to commercial peat.

Materials and methods

Willow shoots were previously established in vitro from actively growing branches collected in midsummer from a mature tree (Regueira et al. 2018).

Propagation of willow was carried out in jars (Fig. 1A) in semisolid medium (SSM) in standard conditions and in liquid medium (LM) both in standard and in photoautotrophic conditions (see below). The medium consisted of MS (Murashige and Skoog) salt and vitamin mixture (Murashige and Skoog, 1962) with half strength nitrates (MS½N) and supplemented with 0.22 µM of BA and 3% sucrose. In SSM, 0.7 % (w/v) Bacto agar was used. In some experiments with LM, sucrose was not supplemented. In standard conditions, cultures were incubated under a 16-h photoperiod provided by cool-white fluorescent lamps (photosynthetic photon flux density (PPF) of 50-60 µmol $m^{-2}s^{-1}$) at 25 ˚C light/20 ˚C dark, whereas in photoautotrophic conditions a higher PPF (150 µmol m^{-2} s^{-1}) was provided by white LEDs and CO_2-enriched air was supplied.

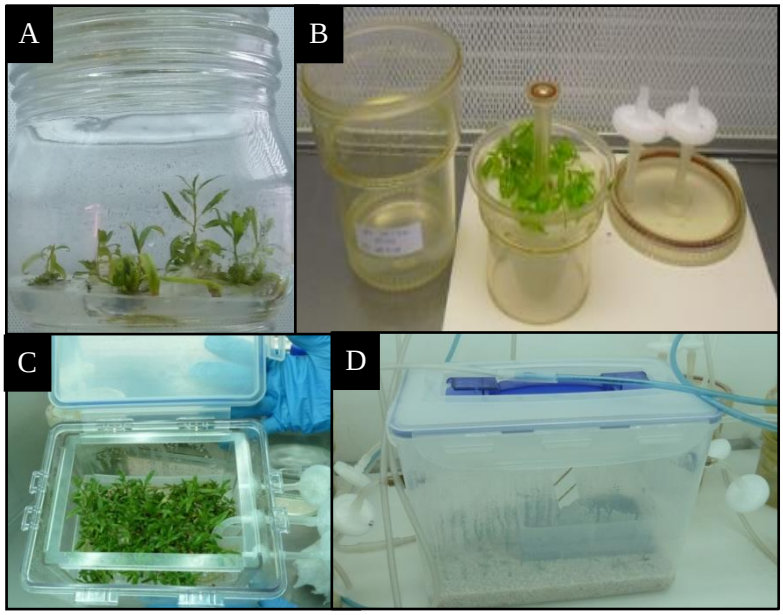


Figure 1*. Vessels used for propagation of willow. A) Glass jars. B-C) Commercial bioreactors RITA® (B) and plantform™ (C). D) In-house bioreactors made from food storage containers.*

Proliferation by temporary immersion

Shoots were cultured in liquid medium by temporary immersion (TIS) under standard conditions (low PPF and 3% sucrose). Apical and basal segments of willow were cultured in RITA® (www.vitropic.fr) and plantform™ (www.plantform.se; Welander et al. 2014) bioreactors (Fig. 1B, C) as described in Regueira et al. (2018). Three immersions of 1 min per day were applied, and in the case of plantform, additional aerations of 1 min/h were supplied.

Proliferation by continuous immersion

For continuous immersion (CIS), basal sections of willow were placed in rockwool cubes soaked in medium with sucrose (S3) or in the same medium devoid of sucrose (S0). Vessels (10 L) were made from food storage containers (Fig. 1D**)** and were equipped with 0.2 µm filters to receive forced ventilation with CO_2-enriched air (2000 ppm; 1 min/h), as described for chestnut (Vidal et al. 2017).

Acclimation and effect of the type of soil

Rooted shoots obtained by CIS under photomixotrophic and photoautotrophic conditions were selected for evaluating different soils during the acclimation step. Both groups of plants were transferred to a phytotron and planted in commercial peat for a first acclimation step. One month later, the plantlets were measured and their roots were carefully washed to eliminate the rests of peat. Then, the plantlets were transferred to new pots in the greenhouse.

Three types of soil were used:
1) commercial peat,
2) soil from an oak forest, with high organic matter content,
3) crop soil with low organic matter content.

Six weeks later, the plants were removed from the soil, and parameters related to the growth response of the 6 groups of plants (2 micropropagation systems and 3 soils) were recorded. In addition, the hydrolytic enzymatic activities of the soils were determined as described by Trasar-Cepeda et al. (2008) and dehydrogenase activity by the method of Camiña et al. (1998).

Results

Proliferation by temporary immersion
The appearance of willow shoots cultured in standard conditions with 3% sucrose both in SSM and in LM by temporary immersion is presented in **Fig. 2**, and the proliferation obtained in each type of container is presented in **Fig.3.**

Figure 2*. Willow shoots cultured with 3% in standard conditions. (A) Glass jars. (B) RITA®. (C) plantform™.*

As can be observed in Fig. 3, TIS favored shoot proliferation. Similar results were observed in other woody plants such as calabash tree, apple, teak and pistachio (Murch et al. 2004, Zhu et al. 2005, Quiala et al. 2012, Akdemir et al. 2014). The highest multiplication coefficient was observed in plantform™ bioreactors, confirming the results obtained in a previous study (Regueira et al. 2018). Similar results were reported for chestnut (Vidal et al. 2015). Basal shoots produced more segments than apical explants, and consequently were used for experiments in CIS.

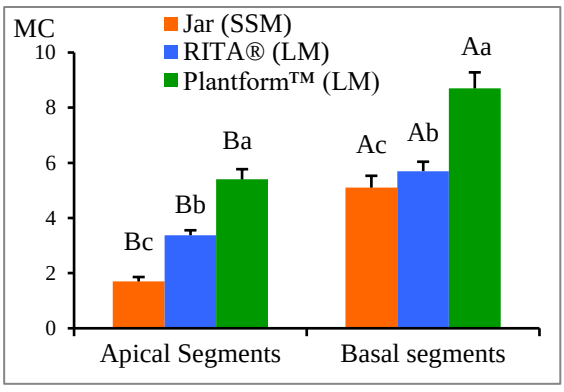


Figure 3*. Effect of type of explant and the culture system on the multiplication coefficient (MC) of willow shoots. SSM: semisolid medium. LM: liquid medium.*

Proliferation by continuous immersion
Basal shoots were successfully propagated in continuous immersion photoautotrophic conditions (high PPF and CO_2 supply), both with supplementation of 3% sucrose (photomixotrophic growth) and without

sucrose (photoautotrophic growth). Shoots cultured without sucrose presented a vigorous aspect (Fig. 4A). All shoots formed roots spontaneously (Fig. 4B).

***Figure 4**. Proliferation of willow under photoautotrophic conditions. (A) Willow shoots cultured in CIS without sucrose. (B) Shoots with roots formed spontaneously in the proliferation medium.*

The multiplication coefficient of shoots proliferated in CIS is shown in Fig. 5. The differences between shoots cultured with or without sucrose were not significant for any of the parameters tested, although the explants cultured without sugar showed a slightly higher multiplication coefficient. The beneficial effects of culturing woody plants in photoautotrophic conditions using large vessels with forced ventilation has been reported by other authors as Zobayed (2005) and Xiao et al. (2011).

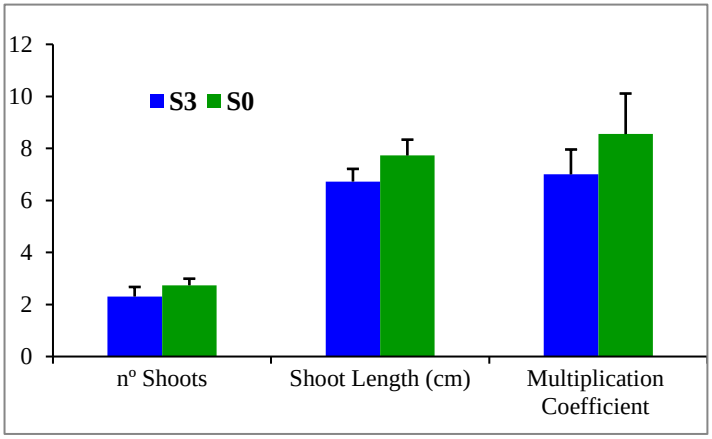


***Figure 5**. Proliferation of willow under photomixotrophic and photoautotrophic conditions.*

Acclimation and effect of the type of soil

Willow plantlets obtained in CIS in S0 and S3 were transferred to commercial peat in a phytotron for a first acclimation step. After 4 weeks, plantlets were measured (Fig. 6A) and distributed evenly between the soil treatments described in the Materials and methods section. After 6 weeks in the greenhouse, both plants and soils were characterized (Fig. 6B, Fig. 7, Fig. 8). All plants survived acclimation, although differences in the height and vigor of plants growing in the three types of soil were observed. However, the supplementation of sucrose during micropropagation did not affect the posterior growth of the plantlets (Fig. 6B, Fig. 7).

The height of the aerial and root zone of the plants after six weeks in the greenhouse is shown in Fig. 6B. Peat produced higher shoots than the other soils, whereas the highest development of the roots was obtained in crop soil (Fig. 6B). Acclimation in a soil that allows a relatively good growth of the aerial part and a good development of the root system could be advantageous for the subsequent transplanting of willows to field conditions. Usually the soils of plantations are not as rich in nutrients as the other soils used in this study (peat and forest soil), and plants cultured in the crop soil seemed to be well adapted to these restrictive conditions.

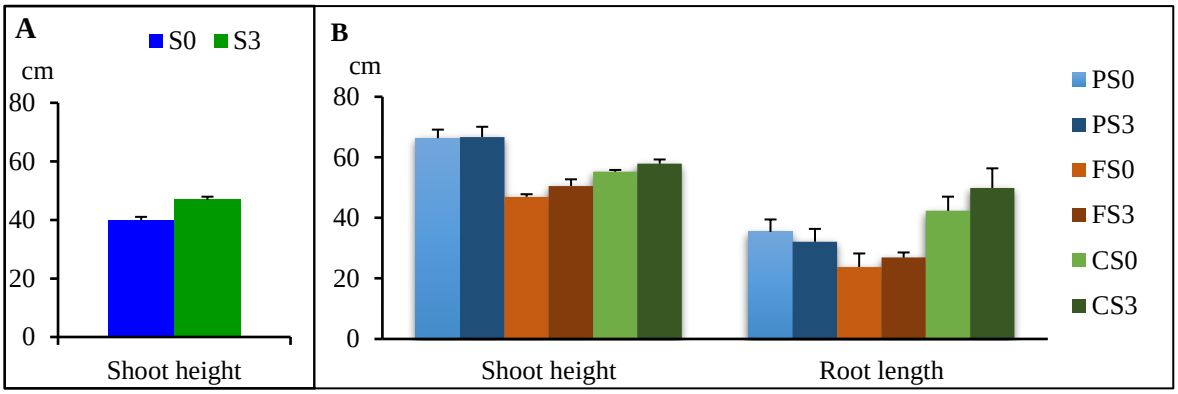


***Figure 6**. A) Shoot height of willow plantlets grown in CIS with 3% sucrose (S3) and without sucrose (S0) after one month in the phytotron. B) Shoot and root length of willow plantlets cultured for 6 weeks in the greenhouse in three types of soil. PS0, PS3: plants grown in peat and micropropagated without sucrose (PS0) or with 3% sucrose (PS3). FS0, FS3: plants grown in a forest soil and micropropagated without sucrose (FS0) or with 3% sucrose (FS3). CS0, CS3: plants grown in a crop soil and micropropagated without sucrose (CS0) or with 3% sucrose (CS3)*

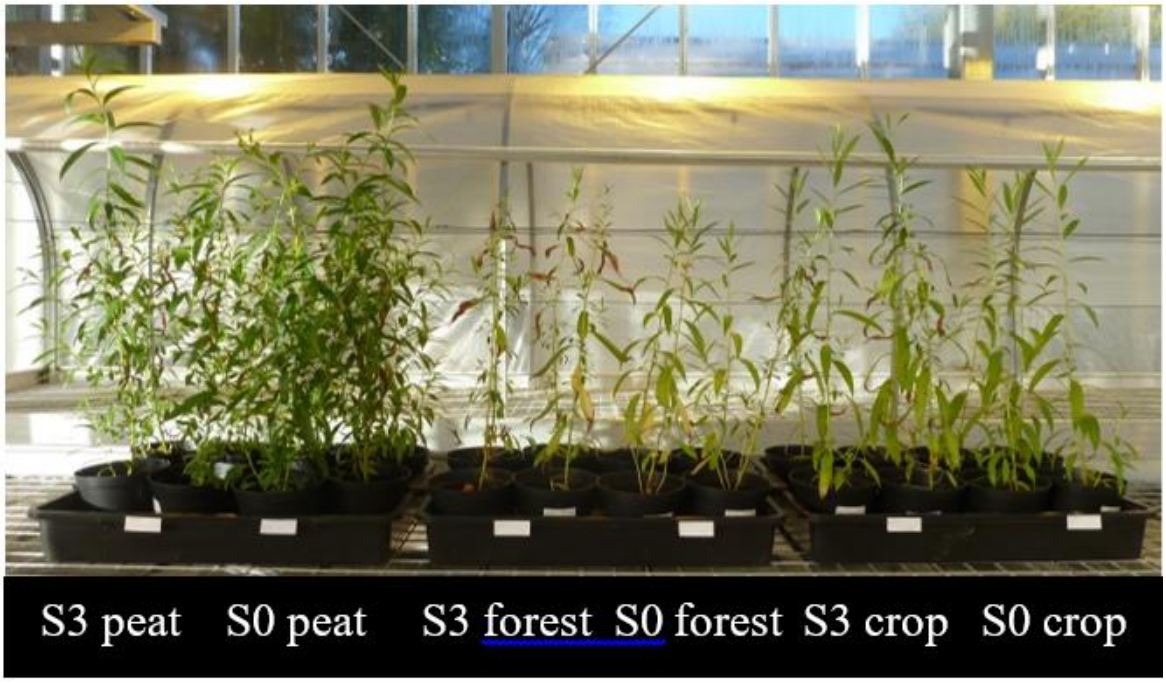


***Figure 7**. Willow shoots micropropagated with (S3) or without sucrose (S0) growing in different types of soil (peat, forest soil and crop soil).*

The preliminary enzymatic assays indicated that the values of the enzymatic activities were not affected by the micropropagation conditions of the plantlets. For this reason, and to simplify the graphic representation, the enzymatic activities corresponding to plantlets growing in the same soil were pooled together (Fig. 8), irrespective that the shoots were cultured with or without sucrose during the micropropagation stage.

The results indicate that the enzymatic activities were more influenced by the type of soil than by the presence of plants (Fig. 8). Enzyme activities were analyzed using the bulk soil. The lack of significant differences between the enzymatic activity of the soil with and without plants suggests both that the

main influence of plants and plant roots occurred on the rhizhosphere soil, and also more than six weeks are probably required to detect this influence in the bulk soil.

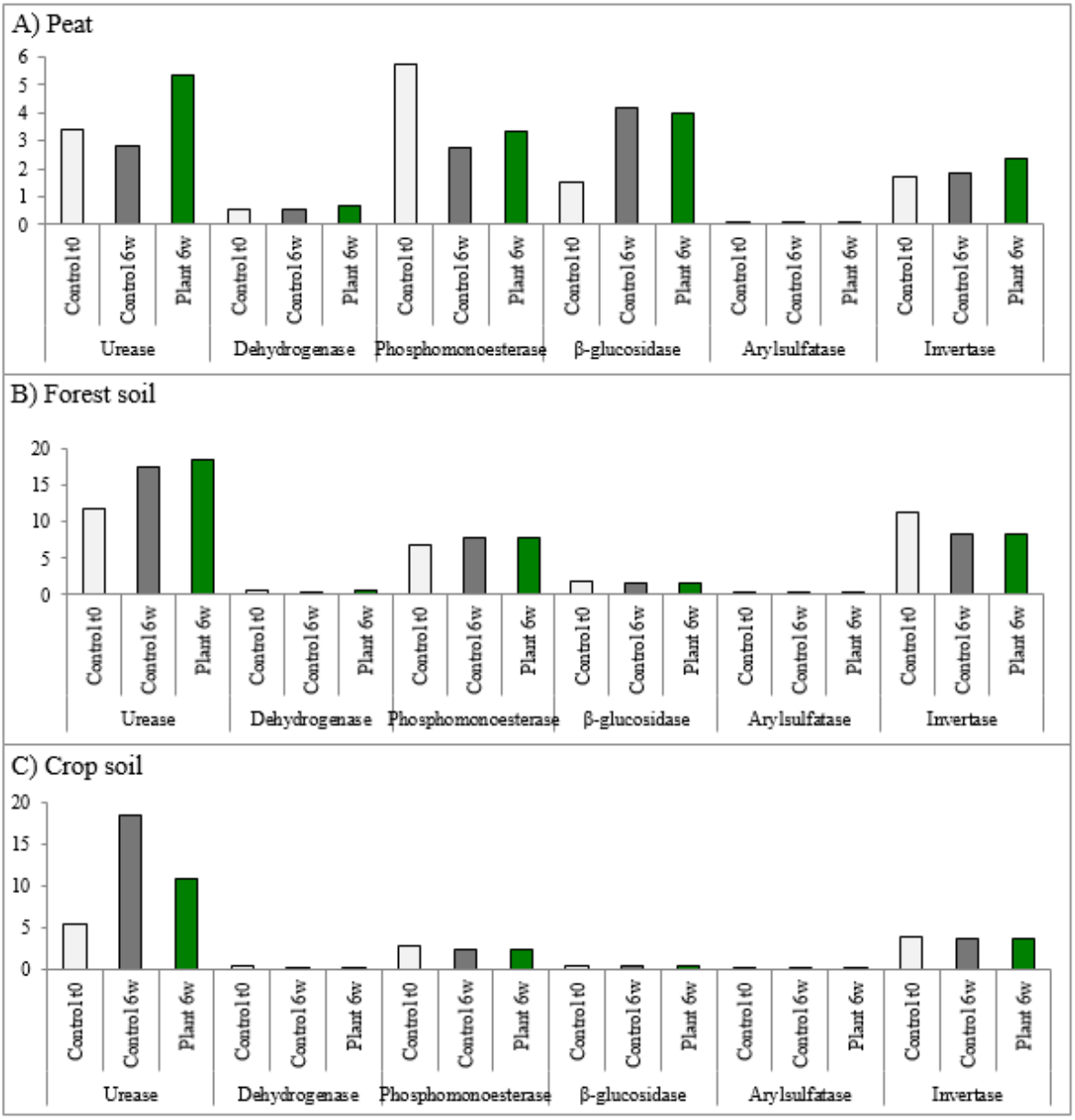


***Figure 8**. Effect of the type of soil, the time of sampling and the presence of plant on enzymatic activities. Control t0: control without plant at time 0; Control 6w: control without plant after 6 weeks; Plant 6w: samples with plants after 6 weeks. Enzymatic activities in µmoles of product g^{-1} h^{-1}.*

In conclusion, willow shoots were successfully propagated in liquid medium by temporary immersion with sucrose supplementation and by continuous immersion without sucrose. Shoots propagated under photomixotrophic and photoautotrophic conditions were satisfactorily acclimated. Commercial peat, forest soil, and crop soil were valid for willow acclimation. Plants grown in peat showed higher aerial growth, but those grown in crop soil showed more roots.

Acknowledgements: This research was partly funded by the Xunta de Galicia (Spain) through the Contrato Programa 2017-2018 and the project IN607A 2017/6. We thank Ana Isabel Iglesias Tojo and Purificación Covelo Abeleira for technical assistance.

References

Akdemir H, Süzerer V, Onay A, Tilkat E, Ersali Y, Çiftçi YO (2014) Micropropagation of the pistachio and its rootstocks by temporary immersion system. Plant Cell Tissue Organ Cult 117:65-76

Amo-Marco JB, Lledo MD (1996) In vitro propagation of *Salix tarraconensis* Pau ex Font Quer, an endemic and threatened plant. In Vitro Cell Dev Biol Plant 32:42-46

Bergman L, von Arnold S, Eriksson T (1985) Effects of N6 benzyladenine on shoots of five willow clones (Salix spp.) cultured in vitro. Plant Cell Tissue Organ Cult 4: 135-144

Bhojwani SS (1980) Micropropagation method for a hybrid willow (*Salix matsudana* × *alba* NZ-1002), New Zealand J Bot 18:209-214

Camiña F, Trasar-Cepeda C, Gil-Sotres F, Leirós C (1998) Measurement of dehydrogenase activity in acid soils rich in organic matter. Soil Biol Biochem 30:1005-1011

Mashkina OS, Tabatskaya TM, Gorobets AI, Shestibratov KA (2010) Method of clonal micropropagation of different willow species and hybrids. Appl Biochem Microbiol 46:769-775

Mleczek M, Rutkowski P, Rissmann I, Kaczmarek Z, Golinski P, Szentner K, Strażyńska K, Stachowiak A. (2010) Biomass productivity and phytoremediation potential of Salix alba and Salix viminalis. Biomass Bioenerg 34:1410-1418

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473-497

Murch SJ, Chunzhao L, Romero RM, Saxena PK (2004) In vitro culture and temporary immersion bioreactor production of *Crescentia cujete*. Plant Cell Tissue Org Cult 78:36-68

Palomo-Ríos E, Macalpine E, Shield I, Amey J, Karaoğlu C, West J, Hanley S, Krygier R, Karp A, Jones HD (2015) Efficient method for rapid multiplication of clean and healthy willow clones via *in vitro* propagation with broad genotype applicability. Can J For Res 45:1662-1667

Park SY, Kim YW, Moon HK, Murthy HN, Choi YH, Cho HM (2008) Micropropagation of Salix pseudolasiogyne from nodal explants Plant Cell Tissue Organ Cult 93: 341-346

Quiala E, Cañal MJ, Meijón M, Rodriguez R, Chavéz M, Valledon L, Feria M, Barbón R (2012) Morphological and physiological responses of proliferating shoots of teak to temporary immersion and BA treatments. Plant Cell Tissue Organ Cult 109:223-234

Read PE, Garton S, Tormala T (1989) Willows (*Salix* spp.) In Bajaj YPS (ed). Biotechnology in Agriculture and Forestry, Vol 5: Trees II. Springer Verlag Berlin Heilderberg, pp 370-386

Regueira M, Rial E, Blanco B, Bogo B, Aldrey A, Correa B, Varas E, Sánchez C, Vidal N (2018) Micropropagation of axillary shoots of *Salix viminalis* using a temporary immersion system. Trees 32:61–71

Skálová D, Navrátilová B, Richterová L, Knitl M, Sochor M, Vašut RJ (2012) Biotechnological methods of in vitro propagation in willows (Salix spp.) Cent Eur J Biol 7:931-940

Trasar-Cepeda C, Leirós MC, Gil-Sotres F (2008) Hydrolytic enzyme activities in agricultural and forest soils. Some implications for their use as indicators of soil quality. Soil Biol Bioochem 40:2146-2155

Touceda-González M, Álvarez-López V, Prieto-Fernández A, Rodríguez-Garrido B, Trasar-Cepeda C, Mench M, Puschenreiter M, Quintela-Sabarís C, Macías-García F, Kidd PS (2017) Aided phytostabilisation reduces metal toxicity, improves soil fertility and enhances microbial activity in Cu-rich mine tailings. J Environ Manag 186:301–313. doi: 10.1016/j.jenvman.2016.09.019

Vidal N, Aldrey A, Blanco B, Correa B, Sánchez C, Cuenca B (2017) Proliferation and rooting of chestnut under photoautotrophic conditions. In: Bonga J.M., Park Y.-S., and Trontin J.-F. (Eds.). Proceedings of the 4th International Conference of the IUFRO Unit 2.09.02 on "Development and application of vegetative propagation technologies in plantation forestry to cope with a changing climate and environment". September 19-23, 2016. La Plata, Argentina. pp 119-127

Vidal N, Blanco B, Cuenca B (2015) A Temporary Immersion System for Micropropagation of Axillary Shoots of Hybrid Chestnut. Plant Cell Tiss Organ Cult 123:229-243

Welander M, Persson J, Asp H, Zhu LH (2014) Evaluation of a new vessel system based on temporary immersion system for micropropagation. Sci Hortic 179:227-232

Xiao Y, Niu G, Kozai T (2011) Development and application of photoautotrophic micropropagation

plant system. Plant Cell Tiss Organ Cult 105:149-158

Zobayed SMA (2005) Ventilation in micropropagation. In: Kozai T, Afreen F, Zobayed SMA (eds) Photoautotrophic (sugar-free medium) micropropagation as a new micropropagation and transplant production system. Springer, Dordrecht, pp 147–186

Zhu LH, Li XY, Welander M (2005) Optimisation of growing conditions for the apple rootstock M26 grown in RITA® containers using temporary immersion principle. Plant Cell Tissue Organ Cult 81:313-318

In vitro propagation and callus formation of *Morus alba*.

Juan J. Silva[1], Yanexi Y. Fonseca[1], Marisel Bahi[1], Jorge L. Pérez[1], Stefaan Werbrouck[2]

[1]Centro de Estudios de Biotecnología Vegetal, Facultad de Ciencias Agrícolas, Universidad de Granma, Bayamo, Granma, Cuba.
[2] Laboratory of Applied In Vitro Plant Biotechnology, Dept. Plant & Crops, Faculty of Bioscience Engineering, University Ghent, Belgium
jsilvap@udg.co.cu

Abstract
Mulberry is a multi-purpose tree with leaves that are rich in protein. It has traditionally been used to feed silkworm and cattle, and is highly valuable for its fruit as well as for its wood production. An initiation and micropropagation protocol was established for *M. alba* 'Acorazonada' and callus was obtained for somatic embryogenesis induction. Culture media containing Murashige and Skoog salts (1962) were used for establishment, multiplication and rooting stages. In the establishment stage, 66.6% of the established nodal segments reacted on 2 mg.l^{-1} of BAP. In the rooting stage, 1.0 mg.l^{-1} of IBA was added and this yielded 70% of rooted plants. Different explants for callus formation were also evaluated, of which the petioles reacted the best on a medium with 1.0 mg.l^{-1} of 2,4-D.

Keywords: micropropagation, callus, tissue culture, *morus*

Introduction

The mulberry is a multiple-use tree that has traditionally been used as a food for the silkworm (Imran et al. 2010). Depending on the locality, it is also appreciated for its fruit that can be consumed freshly or conserved, or, as in some countries, as a vegetable where its leaves and tender stems are consumed, and for its medicinal properties.

In the 1980s, research institutions in Latin America and the Caribbean began to study its use as animal feed, mainly due to its high biomass yield, palatability and high nutritional value (Sánchez, 2002; Yongkang, 2002). The leaves can have more than 46% of essential amino acids (Machii, 1992), more than 20% of crude protein and between 70 and 80% of in vitro digestibility of dry matter (Benavides et al. 1994). The leaves also contain a level of minerals (up to 17% ash), with notably high values of calcium and phosphorus (Sánchez, 1999).

Initially, Oka and Ohyama (1974) obtained the first complete plantlets from axillary buds. Since then, more types of explants have been used for tissue culture (Kavyashree, 2007, Vijayan, 2010). The objective of this study was to evaluate in vitro plant propagation and embrogenic callus formation in Morus alba 'Acorazonada', a cultivar with outstanding agronomic performance, in order to produce large quantities of clean plantlets.

Materials and methods

Medium composition and initiation
The basal culture medium consisted of Murashige and Skoog salts (1962), thiamine 1 mg. l^{-1}, myoinositol 100 mg. l^{-1}, sucrose 30 g. l^{-1}, agar BioCen 6.0 g. l^{-1} and the pH was adjusted to 5.7

before autoclaving. Subsequently, the culture medium was distributed in 24 x 140 mm test tubes, each containing 10 ml. The sterilization was performed at 121 °C temperature and 1.2 kgf.cm^{-2} pressure for 20 minutes. The growing conditions in the growth chambers were: temperature: 25° C; relative humidity: 70 to 80%; (day) light intensity 60 μE.m^{-2} S^{-2} and a photoperiod of 12 light hours.

Stem sections of Morus alba 'Acorazonada', 20 cm long, were collected from the germplasm bank of the University of Granma. They were placed in distilled water for 15 days to obtain elongated axillary shoots (Fig. 1). They were cut into sections, the leaves were removed and they were carefully washed with water with detergent for 30 minutes in continuous agitation. Then they were submerged in a 1% sodium hypochlorite solution for 20 minutes, followed by six rinses with sterile distilled water. Subsequently, the sections were cut into single nodes (Fig. 1) and transferred to test tubes.

Figure 1. *Sprouting of buds, ready for initiation*

Initiation medium
The basal medium was supplemented with 6-BAP 0.5; 1 or 2 mg.l^{-1}. Each treatment consisted of 50 initiated explants. A completely randomized design was used. The experiment was evaluated after 28 days.

Multiplication of axillary shoots
The basal medium was supplemented with combinations of 6-BAP (0.5 and 2 mg.l-1) and GA3 (0 and 5 mg.l-1). Each treatment consisted of 25 explants (nodal segments). The evaluation of the results was carried out after 28 and 42 days, by counting the number of nodes per explant. A completely randomized design was applied.

In vitro rooting
The basal medium was administered with IBA of 0.5; 1 and 1.5 mg.l^{-1}. Each treatment consisted of 25 nodal segments. The evaluation was carried after 28 days by scoring the percentage of rooted explants. A completely randomized design was applied.

Callus formation
The basal medium was supplemented with 2,4-D 1 mg.l^{-1}. Explants consisted of stems segments of 1 cm and leaves of 0.5 x 0.5 cm in surface, obtained from sprouted shoots with two buds. They were initiated as previously described. One segments was transferred per tube containing 10 ml of culture medium. They were incubated in the dark at a temperature of 25 ° C and relative humidity between 60 and 65%. After four weeks, the following variables were evaluated: zone of callus formation, callus color, start and percentage of callus formation and the presence of roots.

Stadistical analyses
The variables expressed in percentages, were analyzed by proportional comparison according to Castillo and Miranda (2014) and the number of nodal segments was processed according to a nonparametric analysis of Kruskal-Wallis with the software InfoStat (Di Rienzo et al. 2010).

Results and discussion

Initiation medium
During initiation, he highest percentage of sprouting (66,6%) was obtained with 2 mg.l^{-1} BAP. The highest concentration inhibited apical dominance and promoted shoot formation. The contamination caused by fungi and to a lesser extent by bacteria affected the percentages of in vitro establishment and was taken into account to calculate the effect of the cytokinin concentration.

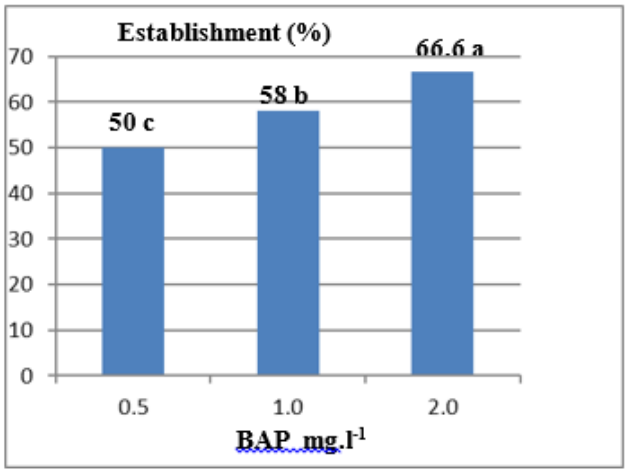


Figure 2. *Effect of BAP on the percentage of initiated shoots showing bud break*

BAP has been the main cytokinin used in the in vitro establishment of mulberry. Balakrishnan et al. (2009) assessed similar concentrations as used in our work and the results in both cases were between 70 % and 100 % of response, better than when other cytokinins such as kinetin were used or if the cytokinin was combined with auxins such as NAA and IAA. It was also observed that the already induced flower buds flowered in vitro and set fruit (figure 3) during the establishment phase that took 40 days.

Figure 3. *In vitro fruit set during in vitro establishment multiplication of axillary shoots*

Table 1 shows the number of new nodal segments per explant after 28 and 42 days. Significant differences were obtained between 0,5 and 2 mg/l BAP, whereas the addition of GA3 showed no significant effect. After 42 days, although the number of nodal segments increased slightly, no significant differences were obtained anymore between the treatments.

***Table 1**. Effect of 6-BAP and AG_3 on number of nodal segments in the multiplication phase at 28 and 42 days of culture.*

Treat.	Growth regulators ($mg.l^{-1}$)		Number of buds	
	BAP	GA_3	28 days	42 days
T_1	0.5	0	3.00 a	3.65
T_2	0.5	5	2.00 ab	2.43
T_3	2	0	1.27 b	2.36
T_4	2	5	1.89 ab	2.44
Significance 5%			*	N.S.

*N.S. Not significant difference *: Significant difference*

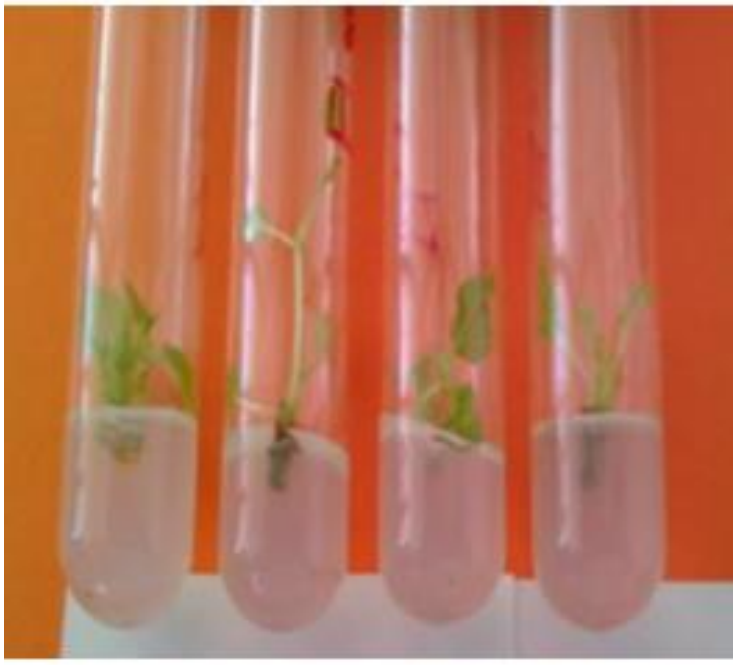


***Figure 4.** Effect of the different BAP/GA3 combinations on the axillary buds*

Figure 4 shows that GA3 induced shoot elongation. Salas et al. (2005) combined 0.5 $mg.l^{-1}$ BAP with 0.5 mg.l-1NAA using 'Criolla', achieving 5.33 buds per initial explant, superior to that presented in this work, probably caused by genotype effects, as we used 'Acorazonada'.

In vitro rooting

Figure 5 shows the results of the rooting experiment. Applying 1 mg/l IBA was slightly but significantly better (70%) then the other concentrations. Sajeevan et al. (2011) obtained 100% in vitro rooting of

mulberry by using 0.5 mg.l-1 IBA in combination with 1 g.l^{-1} activated charcoal. These results indicate that the rooting of 'Acorazonada' phase can be improved.

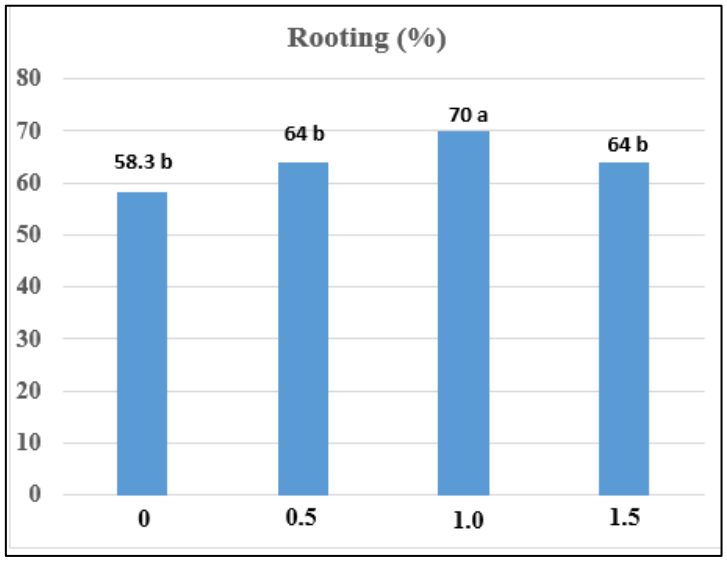


Figure 5. *Effect of IBA on the percentage of rooting.*

Embryogenic callus formation.

In the first week of cultivation, morphological changes and a bulging at the cut ends of petioles and stem segments were observed. They started to form callus, considered to be a defense mechanism to prevent the penetration of foreign agents. The leaves started to make callus at the cut through leaf veins. Figure 6 shows the formation of calluses on the different explants after 30 days of culture, showing the formation in the primary and secondary veins of the leaf. In the petioles, the calluses were observed at the base of the explants but also longitudinally on their surface. The induction and formation of roots at the base of the petioles was also observed, which may be related to the use of 2,4-D, although this is not the main physiological effect of auxins of this type. The coloration of the calluses was similar for all the explants, being yellowish brown. The calluses showed a friable consistency and proembryogenic structures were observed as well. The percentage of callus formation differed significantly between the different mulberry explants. The best result was obtained from petioles of which 65% responded. Leaf explants and stem segments showed a response of 27.5 and 1.9%, respectively.

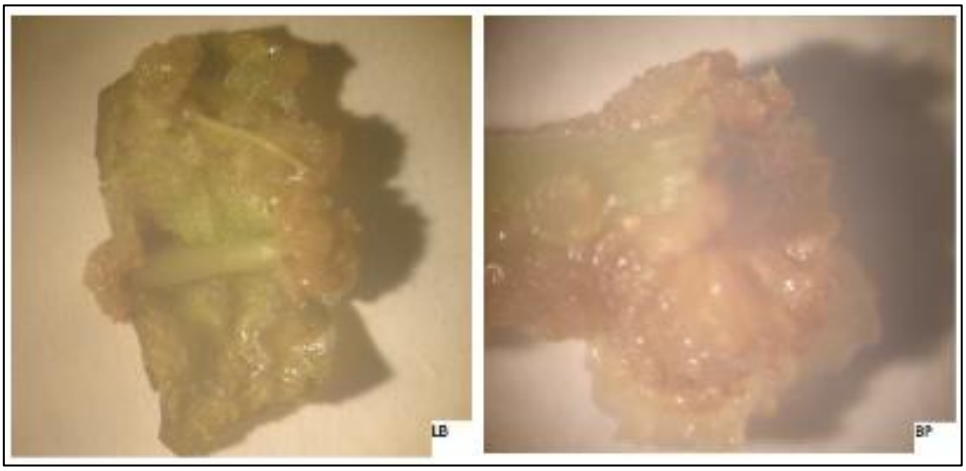


Figure 6. *Formation of calluses on leaf disks and petioles. LB. Leaf disk; BP: base of the petiole*

Conclusions

A methodology was established for the in vitro propagation of Morus alba 'Acorazonada‘ in natural light conditions, using a classical approach with BAP and NAA. The formation of embryogenic calluses was obtained from the directly initiated explants, as well as the formation of roots by direct organogenesis on petioles.

Acknowledgements: This research was supported VLIR UOS, Belgium (In vitro plan biotechnology to increase the alimentary security in the eastern region of Cuba, CU2017TEA438A103).

References

Balakrishnan V, Latha MR, Ravindran K, Robinson JP (2009) Clonal propagation of *Morus alba* L. through nodal and axillary bud explants. Bot Res Intern 2(1): 42-49

Benavides JE, Lachaux M, Fuentes M (1994) Efecto de la aplicación de estiércol de cabra en el suelo sobre la calidad y producción de biomasa de morera (Morus sp.). En: Árboles y arbustos forrajeros en América Central (Ed. J.E. Benavides). CATIE, Turrialba, Costa Rica. Serie Técnica, Informe Técnico No. 236, vol. II: 495p

Castillo Y, Miranda I (2014) COMPAPROP: Sistema para comparación de proporciones múltiples. Revista Protección Vegetal 29(3): 231-234

Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Robledo CW (2010) InfoStat versión. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina

Imran M, Khan H, Shah M, Khan R, Khan F (2010) Chemical composition and antioxidant activity of certain Morus species. Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology) 11(12): 973-980. doi: 10.1631/jzus.B1000173

Kavyashree R (2007) A repeatable protocol for in vitro micropropagation of mulberry variety S 54. Ind J Biotechnol 6(3): 385

Machii H (1992) Organogenesis from immature leaf cultures in mulberry (Morus alba L.). J Sericulture Sci Japan 61: 512

Murashigue T, Skoog F (1962) A revisied medium for rapid growth and biossays with tobacco tissue culture. Physiol Plant 15(3): 173-197

Oka S, and Ohyama K (1974). Studies on in vitro culture of excised bud growth in mulberry tree. I. Effects of growth substance on the development of shoots and organ formation from winter buds. Japan Seric. Sci 43: 230-235

Sajeevan R, Jeba S, Nataraja K, Shivanna M (2011) An efficient in vitro protocol for multiple shoot induction in mulberry, Morus alba L variety V1. Intern Res J Plant Sci 2(8): 254-261

Salas J, Agramonte D, Jiménez-Terry F, Pérez M, Collado R, Barbón R, Chávez M (2011) Propagación de plantas de Morus alba variedad Criolla con el uso de sistemas de inmersión temporal. Biotecnología Vegetal 11(2): 77 -88

Sánchez M (1999) Mulberry: an exceptional forage available almost worldwide. World Animal Review 93(2): 36-46.

Sánchez, M. (2002). Mulberry: and exceptional forage available almost worldwide. Animal Production and Health Paper N. 147, 161-172.

Vijayan K (2010) The emerging role of genomic tool in mulberry (*Morus alba*) genetic improvement. Tree Genet Genom 6(4): 613

Yongkang H (2002) Mulberry cultivation and utilization in China. Animal Production and Health Paper N. 147: 11-43.

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Somatic embryogenesis of Norway spruce in Finland – seven years from start to first commercial pilots

Mikko A. Tikkinen, Saila A. Varis, Sakari M. Välimäki, Teijo O. Nikkanen, Tuija S. Aronen

Natural Resources Institute Finland (LUKE), Production Systems, Forest Tree Breeding. Finlandiantie 18, FI-58450 Punkaharju, Finland; mikko.tikkinen@luke.fi

Abstract

A targeted effort to adapt somatic embryogenesis (SE) as a complementary propagation technology of Norway spruce (*Picea abies* L. Karst) was launched in Finland in 2011. Motivation for this attempt was raised by a shortage of high-quality, bred seed for forest regeneration, caused by irregular flowering of the species and problems in seed orchard production. Simultaneously, need for hardy ornamental conifers for the growing landscaping sector was recognized. Later on, Christmas tree producers presented a great interest towards spruce varieties with desired crown form. Building up a SE-pipeline in Finland has mostly relied on published protocols. It was observed, however, that cryopreservation of SE materials and the later phases of SE required further development to be successful enough with Finnish material. Thus research has been focused on developing a reliable cryo-protocol, and laboratory-nursery interface. Currently, over 3000 SE-lines originating from the elite trees of the tree breeding program have been initiated and cryopreserved by the Natural Resources Institute Finland (Luke). Of these over 850 are in a testing process covering embryo production in the laboratory, embling conversion and early growth in the nursery, and also the first field tests with emblings have been established. In addition, over 300 SE-lines from trees with special forms have been cryostored and are being tested for their suitability as ornamental or Christmas tree varieties. Regulatory issues involved in SE propagation have been managed in close collaboration with the Finnish Food Authority. The first lot of SE material was registered as forest regeneration material in 2017 (basic material: *Parents of families*, *Qualified*), and authorized for commercial production. First two master certificates for Norway spruce emblings in Finland were issued during 2018. In practice, this involves bulk propagation of SE-lines originating from 12 full-sib families. SE-lines with ornamental value are also available for commercial propagation. To enhance SE propagation, the price of emblings needs to be reduced. Cost analysis has shown that manual labor, especially in the later phases of SE, creates most of the production costs. The research for automation is ongoing.

Keywords: ornamental varieties, *Picea abies*, registration of forest regeneration material

Introduction

In the future forestry will face challenges caused by climate change and simultaneously the demand for bio-based raw materials is expected to increase (Pop et al. 2014; Ruotsalainen 2014; Börjesson et al. 2017; Jansson et al. 2017). As rotations are fairly long in the Nordic countries it is difficult to prepare for the changes which will occur during the coming decades (Ruotsalainen 2014). Tree breeding can contribute to solving these future challenges (Ruotsalainen 2014).

Breeding results have been implemented in practice by producing genetically improved planting stock in seed orchards (White et al. 2007). In Finland, the age distribution of Norway spruce (*Picea abies* (L.) Karst.) seed orchards is uneven; the orchards are either at the end of their operational lifespan or they are still juvenile and not yet producing seed with their full potential (Haapanen et al. 2017). The availability of improved forest regeneration material can change rapidly due to the age structure of seed orchards and fluctuating seed yields, which are caused by the irregular flowering of Norway spruce (Ruotsalainen 2014; Haapanen et al. 2017; Jansson et al. 2013). One possible way to implement breeding results more efficiently is to apply vegetative propagation to produce planting stock, which would also ease the availability problems for improved forest regeneration material (Högberg et al. 1998; Haapanen and Mikola 2008; Varis 2018).

Breeding objectives are conventionally different from the ones desired by the landscaping sector and Christmas tree growers, although the breeding tools are the same and the same species can also be utilized (Nikkanen et al. 2013). Hardy ornamental conifers adapted to Nordic climate are preferred in Finnish landscaping to replace imported species, which tend to be less resilient (Nikkanen et al. 2013). Ornamental traits are often recessive which makes vegetative propagation an ideal way to produce numerous trees that present the desired trait (Nikkanen et al. 2013).

As the interest towards vegetative propagation increased the initiative to build a somatic embryogenesis (SE)-pipeline for Norway spruce in Finland was launched in 2011. The aim was to develop a production protocol which could be applied to enhance tree breeding, to produce genetically improved planting stock from genetically diverse origins for silviculture and to propagate ornamental forms for the landscaping sector and Christmas tree growers. In this paper, the developmental work and research performed to reach these goals are reviewed briefly, as well as achievements are summarized.

SE protocols applied and developed

Introduction of SE propagation of Norway spruce in Finland has been largely based on application of protocols published for the species, and on further modification of those procedures. Following the choice of basal medium, embryo maturation treatment was refined together with storage and germination conditions of cotyledonary embryos. Furthermore, cryopreservation of embryogenic cultures was studied to find the protocol resulting in the best possible regeneration percentages. SE initiation, propagation, cryopreservation, and storage were carried out in the laboratory facilities of the Natural Resources Institute Finland (Luke) in Punkaharju, Southern Finland. All the experiments have been performed with materials from several unrelated full-sib families, including dozens or hundreds of genotypes to develop robust protocols that would suit genetically diverse materials. The main focus has been in developing a SE pipeline with high genetic throughput enabling the utilization, on a practical scale for breeding and commercial purposes.

Culture medium, initiation and proliferation of embryogenic cultures
In 2011, a comparative study was performed to follow induction of SE on media published for Norway spruce: the modified Litvay's medium (mLM) (Litvay et al. 1985; Klimaszewska et al. 2001a), and the LP-medium (von Arnold and Eriksson 1981, as modified by Högberg et al. 1998). The average SE initiation rate among 12 full-sib families, tested with 3175 explants, was much better on mLM (62%) than on LP (17%), and so was also embryo maturation frequency (Heiska et al. 2012). The mLM has thus been used as basal medium in all the further work.

The current SE initiation and proliferation protocol used for Norway spruce in Finland was presented by Varis (2018): Immature cones are collected in the summer when the heat sum is around 800 degree days and they can be stored in 4 °C. Cones are cleaned with 70% ethanol, seeds are dissected, washed, and surface sterilized in 70% ethanol for two minutes. Megagametophytes are opened and only the zygotic embryos used as explants. The same mLM medium containing 10 µM 2.4-dichlorophenoxyacetic acid (2.4-D) and 5 µM 6-benzyladenine (BA) as plant growth regulators, and 1%

(w/v) sucrose is applicable both for the induction and proliferation of embryogenic cultures of Norway spruce. The medium is solidified with gellan gum (Phytagel™, Sigma, 4 g/l) but can be used in liquid form, for example, in temporary immersion system (TIS) bioreactors. During initiation, the explants are kept in the dark (24 °C) for 2 to 8 weeks without subculturing, until embryogenic tissue starts to grow. Established embryogenic tissues are subcultured bi-weekly on the same medium and proliferated in the dark.

Development of a reliable cryopreservation protocol and storage of SE-materials
For the mass propagation program, the cryopreservation method applied should be able to handle large numbers of samples reliably and at low cost. The classic slow-cooling technique, performed using a programmable freezer and sorbitol together with dimethylsulfoxide (Me2SO) as cryoprotectants, has resulted in good recovery rates: 61% of the 72 embryogenic lines of Norway spruce tested survived cryopreservation every time tested, and 96% in part of the trials performed by Norgaard and co-workers (1993). With the Finnish material, however, the first attempts to apply Norgaard's method resulted in low recovery (on an average 25%). This could be related to genetic background, age of the material, different basal medium (BMI-S1 used by Norgaard et al. versus mLM) or longer duration of cryostorage (a few hours by Norgaard et al. versus several months for Finnish materials). Therefore a study was performed to compare different pretreatments, cryoprotectants and slow-cooling devices for cryopreservation of Norway spruce embryogenic cultures, with 12 different methods and a total of 136 spruce genotypes (Varis et al. 2017).

As a result, the current cryopreservation protocol used for Norway spruce in Finland was presented by Varis et al. (2017). Highest recovery of samples was obtained when fresh growth from young embryogenic tissues was selected for cryopreservation, and the following protocol was applied: (i) pretreatment of embryogenic tissues on a semi-solid medium with increasing sucrose concentration – 0.1 M for 24 h, 0.2 M for another 24 h; (ii) use of cryoprotectant – a mixture of polyethylene glycol 6000, glucose, and dimethylsulfoxide, 10% w/v each; and (iii) slow cooling of the samples in programmable freezer at 0.17 °C/min before subjecting them to liquid nitrogen. On average, 87% of the genotypes can be recovered, without any effect on their genetic fidelity, as shown by microsatellite markers and embryo production capacity

The current cryostorage contains samples of embryogenic masses from over 3000 genotypes derived from controlled crossings of 60 progeny tested plus trees from Southern Finland. The cell lines have been initiated in 2011, 2012, 2014 and 2015. The testing of these genotypes for their breeding characteristics and suitability for propagation is ongoing. Additionally over 300 genotypes from special formed Norway spruce genotypes have been cryostored and are being tested for their suitability for landscaping and Christmas tree production.

Maturation of somatic embryos
For further development and maturation of proembryos, the growth regulators 2.4-D and BA in proliferation medium are replaced by (±)-abscisic acid (ABA). In the original mLM medium, the amount of ABA is 60 µM. In the series of experiments, lower concentrations of ABA, i.e. 30 µM and 20 µM were tested, and 30 µM ABA found the best for somatic embryo production from a pragmatic perspective (Tikkinen et al. 2018b). Thus, the current protocol includes mixing of 180 (±20) mg of embryogenic tissue, five to seven days from the last subculture, in 3 ml liquid mLM medium without plant growth regulators. The suspension is poured onto a paper filter in a Büchner funnel, and the liquid drained by suction, and the filter is placed on mLM maturation medium with 30 µM ABA. Developing embryos are kept in the dark at 24 °C on the same medium for eight weeks (Varis 2018).

Storage of mature embryos
Mature somatic embryos of Norway spruce can be partially desiccated to lower endogenous ABA levels and thus prevent ABA's negative effect on embryo germination (Högberg et al. 2001). This can be done by placing mature embryos on filter paper in a petri dish which is placed in a larger vessel together with a petri dish containing sterile water, and by incubating this package sealed with Parafilm™ (Bemis) in

the dark for 16 to 24 days (Bozhkov and von Arnold 1998; Högberg et al. 2001). This procedure is, however, laborious, and was therefore replaced by a more simple method that is based on cold storage. As described by Varis et al. (2018), following maturation treatment, the Petri dishes with mature somatic embryos on filter paper on maturation medium, are transferred to refrigerator unit (2 to 4 °C), and can be stored there for one to six months. Tikkinen et al. (2018a) found that cold storage on filter paper prevented precocious germination and enhanced embling survival in the nursery. Cold storage works also as a 'buffer', making it possible to produce somatic embryos in the laboratory over a longer period of time, and combine them into bigger lots for germination at the desired time.

Germination and acclimatization of somatic embryos
Following the cold storage period, mature somatic embryos of Norway spruce need to be germinated *in vitro* before transfer to greenhouse conditions. Traditionally, a five-week germination period on plant growth regulator free medium has been applied (Klimaszewska et al. 2001), but our studies have shown that much shorter, e.g. a one-week germination period improves both survival rate and height growth of the emblings remarkably (Tikkinen et al. 2018b).

Thus, in the current germination protocol, mature somatic embryos are placed on petri dishes, in a way that the cotyledons face up when the petri dishes are placed in a slightly tilted position. The light intensity during germination is gradually increased from 5 to 150 µmol/m^2/s. Light intensity should be high enough to support height growth, but the temperature in the germination room adjusted so that also the temperature inside the germination vessels stays within a tolerable range. Both fluorescent lamps and light emitting diodes (LEDs) with red and blue wavelengths can be used. The photoperiod of the 16:8 day/night should be introduced at least for northern genotypes, while with southern genotypes slightly shorter day length may be enough (Varis 2018, Tikkinen et al. 2018b).

After germination *in vitro*, emblings are transferred to greenhouse conditions where they are grown until planted. The majority of the Norway spruce seedlings used for forest regeneration in Finland are grown for one to two growing seasons in the nursery (Rikala 2012). The emblings are transferred from semisolid media to peat based growth media and subjected to greenhouse environment. This phase can be rather dramatic for the emblings and cause severe mortality. The emblings are transplanted to growing containers by using the 'pricking out' method (Landis et al. 2010). The environmental control was found to be sufficient for small emblings in commercial greenhouses during germination of Norway spruce seeds by Tikkinen et al. (2018b). Additional measures differing from seedlings are unnecessary after one week of *in vitro* germination and transplanting of emblings into growing containers in the nursery when the planning of the embling production is well coordinated (Tikkinen 2018).

Cost structure of manual Norway spruce SE

Cost structure (labor, material and overall) was estimated for different phases of embling production; planning, thawing proliferation, maturation and germination (Tikkinen 2018). The estimation covered the processing of 120 genotypes, ten genotypes from each of 12 full sib families, which had been selected for production by their embryo production capacity and survival rate in the nursery (Tikkinen 2018, Tikkinen et al. 2018a). Labor costs included the work time consumed to the handling of the material and the preparation of material and equipment needed in each production phase (Tikkinen 2018). Initiation of the cultures, initial cryopreservation, laboratory testing for embryo yield, and producing emblings for field testing, were taken into account in the calculations and fractioned for the maximum amount of vegetative propagules allowed to be produced from the *parents of families* –basic material in *qualified* category (Tikkinen 2018). Most of the overall costs (60%) occurred in the germination phase, where the embryos were handled individually (Tikkinen 2018). Planning accounted for 6% of the overall costs and the remaining production phases accounted for 34% (Tikkinen 2018). The cost evaluation underlined the importance of the process development.

Registration of SE material for forest regeneration in Finland and commercial pilots

Finland is a member of European Union, and thus the marketing of forest reproductive material is regulated by Council Directive 1999/105/EC. Directive 1999/105/EC has been implemented in Finland by the Act on Trade in Forest Reproductive Material (241/2002) and the Decree on Trade in Forest Reproductive Material (1055/2002). These legislative documents set the boundaries for forest reproductive material that can be produced and marketed in Finland. Vegetative propagules can be marketed in following types of basic materials: clone, clonal mixture and parents of family (2 § 1055/2002) (Fig. 1a). Vegetative propagules can be marketed only in categories *selected*, *qualified* or *tested* (6 § 1055/2002). The Finnish Food Authority (National Designated Authority) should issue a master certificate for all material derived from approved basic material (Directive 1999/105/EC).

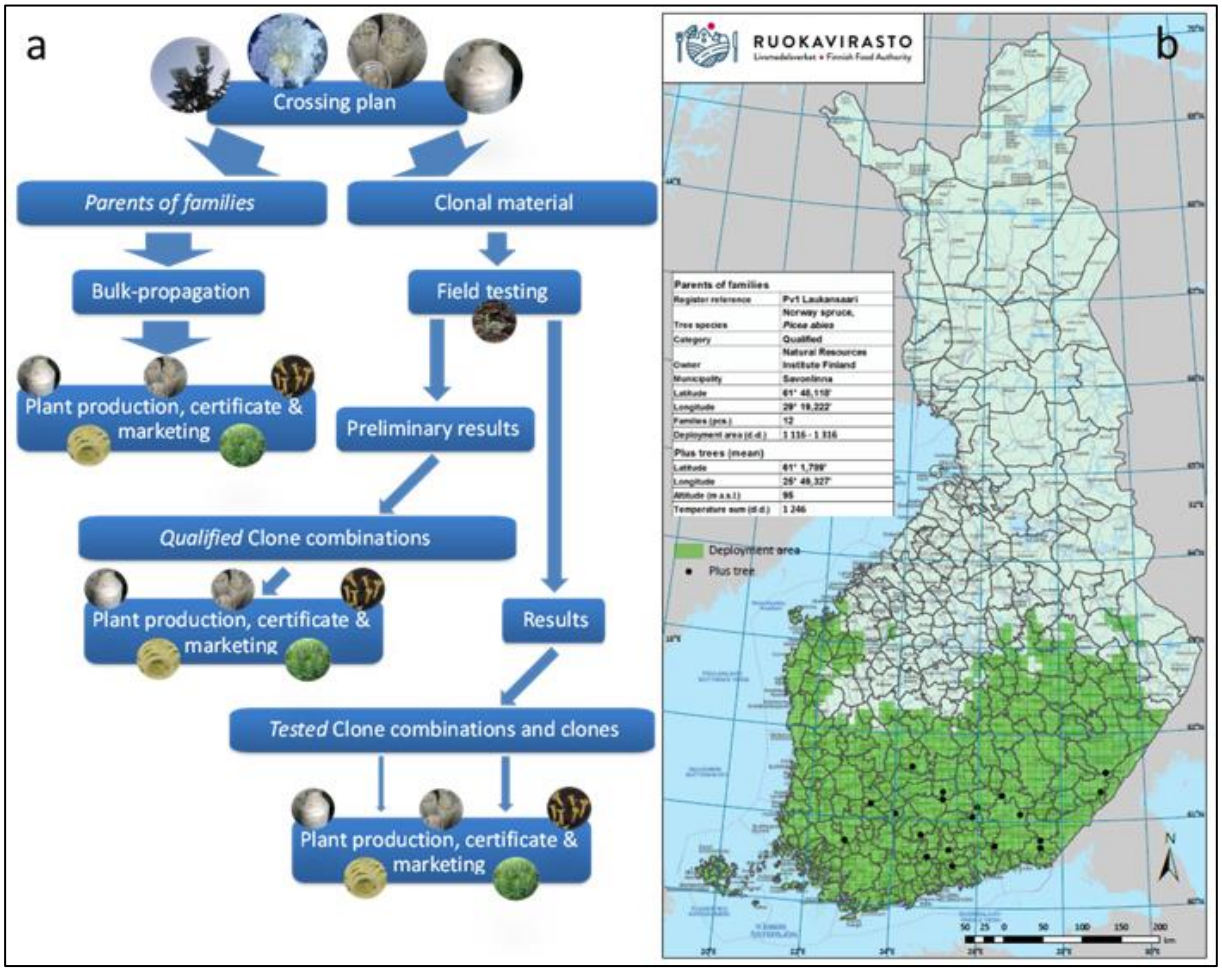


Figure 1 a *Illustration of the registration process of forest regeneration material produced with vegetative propagation in Finland.* ***b*** *Map of the area of utilization for Pv1 Laukansaari material*

The process of issuing a master certificate for vegetatively propagated material in parents of family follows the same procedure used for seed orchards (Directive 1999/105/EC Annex VIII, part B). In clones and clone mixtures, the master certificate will be according to Directive 1999/105/EC Annex VIII, part C. Finnish legislation has limited the production of vegetatively produced plants in the *qualified* category, whereas in the *tested* category the production is not limited (4 § 1055/2002). Four million plants can be marketed from each family in the *qualified* parents of family type of basic material (1055/2002). In clonal mixture, one million plants per single clone can be marketed in the *qualified* category. Additional restrictions regarding the marketing of vegetatively propagated material are found in the Act (241/2002) and the Decree (1055/2002).

In Finland, the first basic material propagated via SE was registered in 2017, in the type parents of families in the category *qualified* (Tikkinen 2018). The registered material was named Pv1 Laukansaari (Fig. 1b). The material includes cell lines of 12 full-sib families from progeny tested plus trees from southern Finland (Tikkinen et al. 2018a). The first master certificates were issued in 2018 during commercial pilot projects (Tikkinen 2018).

First commercial pilots, in which *in vitro* germinated emblings were sold to nurseries, were carried out in 2018. Samples of embryogenic tissue from 120 genotypes, ten from each of the 12 registered full-sib families, were thawed, proliferated and matured. After cold storage cotyledonary embryos from 108 genotypes were germinated *in vitro* and sold to customers.

Conclusions and future views

The development of a Norway spruce SE-pipeline in Finland has resulted in refined protocols, with high and stable recovery of embryogenic tissues from cryopreservation (Varis et al. 2017), and an acceptable propagation efficiency in a laboratory-nursery interface (Tikkinen 2018). Combining these outcomes with the high initiation rates of embryogenic lines developed earlier has resulted in high genetic throughput in manual SE. High genetic throughput, from the basal material (zygotic embryos) to the final product (emblings), can be considered as the core of the SE system, which makes it a powerful tool for plant breeders and propagators. High genetic throughput enables large-scale field testing and selection of genotypes based on their breeding values and production of a large number of emblings from the desired genotypes.

Successful development of the manual SE-pipeline, together with the immense effort put in the initiation of SE material, works as a solid foundation for future development of the SE system into a commercial scale. The first SE derived combination of full-sib families has been registered as basic material in type *parents of families* in category *qualified* and can be used commercially for forest regeneration.

Nevertheless, production numbers need to be increased and costs reduced before Norway spruce SE can be considered a mass production propagation system. A pragmatic approach to increase production numbers is to combine SE with the production of rooted shoot cuttings, by using Norway spruce emblings as donor plants for cuttings (Tikkinen et al. 2018c). Promising new technologies are emerging for scaling up SE, e.g. using TIS bioreactors to grow embryogenic tissue and to produce cotyledonary embryos (Salonen et al. 2017; Välimäki et al. *submitted*). However, the bioreactors and the protocols used need to be tailored to suit the specific needs of Norway spruce SE. Besides producing cell masses and embryos on a large scale, automation is needed in the final steps of embling production when individual emblings and embryos are manually handled. Although significant increases in production numbers may be reached by growing emblings in several locations with overlapping lots, eventually the production will be limited by the manual system.

To enhance SE-prodution pipeline in Finland, new laboratory facilities (KASVU1) will be completed by the end of 2019 in Savonlinna. These facilities will allow a more streamlined SE process and larger annual embling production capacity. In KASVU1 further development of the SE-pipeline together with preservation of genetic forest resources and extensive research regarding forest biotechnology will be conducted by the Natural Resources Institute Finland (Luke). New cell lines will be initiated from full sib families of progeny tested plus trees as soon as flowering of Norway spruce will allow it. Field testing of genotypes will be carried out by the Finnish tree breeding programme and more elite forest regeneration materials will be combined and registered for further use.

Acknowledgements: The authors would like to thank Kari Leinonen from the Finnish Food Authority for providing valuable comments to improve the manuscript. **Funding:** The writers would like to thank the European Regional Development Fund, South Savo Regional Council, Savonlinna Business Services Ltd. and Savonlinna municipality for funding this research.

References

Bozhkov PV, von Arnold S (1998) Polyethylene glycol promotes maturation but inhibits further development of Picea abies somatic embryos. Physiol Plant 104:211–224

Börjesson P, Hansson J, Berndes G (2017) Future demand for forest-based biomass for energy urposes in Sweden. Forest Ecology and Management 383:1 17–26. https://doi.org/10.1016/j. foreco.2016.09.018

COUNCIL DIRECTIVE 1999/105/EC (2000) Official Journal of the European Communities

Haapanen M, Mikola J (2008) Metsänjalostus 2050 ─ pitkän aikavälin metsänjalostusohjelma [Forest tree breeding 2050 – long term breeding plan]. Metlan työraportteja / Working Papers of the Finnish Forest Research Institute 71. 50 p

Haapanen M, Leinonen H, Leinonen K (2017) Männyn ja kuusen siemenviljelyssiemenen taimitarhakäytön kehitys 2006–2016: Alueellinen tarkastelu [Usage of improved Norway spruce and Scotch pine seed in nurseries 2006–2016: Regional analysis]. Metsätieteen aikakauskirja [1241] 4p. http://dx.doi.org/10.14214/ma.7716

Heiska S, Jaakola L, Varis S, Aronen T (2012) Towards mass-propagation of Norway spruce in Finland. In: Park YS & Bonga JM (eds) Proceedings of the IUFRO Working Party 2.09.02 conference on "Integrating vegetative propagation, biotechnologies and genetic improvement for tree production and sustainable forest management". June 25-28, 2012, Brno Czech Rebublic. http.//www.iufro20902.org/

Högberg K-A, Ekberg I, Norell L, von Arnold S (1998) Integration of somatic embryogenesis in a tree breeding programme: a case study with *Picea abies*. Can J For Res 28:10 1536–1545. https://doi.org/10.1139/x98-137

Högberg K-A, Bozhkov PV, Grönroos R, von Arnold S (2001) Critical factors affecting *ex vitro* performance of somatic embryo plants of *Picea abies*. Scand J For Res 16:295–304 http://dx.doi.org/10.1080/02827580116772

Jansson G, Danusevičius D, Grotehusman H, Kowalczyck J, Krajmerova D, Skrøppa T, Wolf F (2013) Norway Spruce (*Picea abies* (L.) H.Karst.). In: Paques LE (ed), Forest Tree Breeding in Europe: Current State-of-the-Art and Perspectives. Managing Forest Ecosystems, Volume 25. Springer. Dordrecht Heidelberg New York London. p. 123–176 https://doi.org/10.1007/978-94-007-6146-9_3

Klimaszewska K, Lachance D, Pelletier G, Lelu M-A, Séguin A (2001) Regeneration of transgenic *Picea glauca, P. mariana* and *P. abies* after cultivation of embryogenic tissue with *Agrobacterium tumefaciens*. In vitro Cell Dev Biol Plant 37:748–755

Laki metsänviljelyaineiston kaupasta 241/2002 (2002) [Act on Trade in Forest Reproductive Material] http://www.finlex.fi/fi/laki/ajantasa/2002/20020241

Landis TD, Dumroese RK, Haase D (2010) The Container Tree nursery manual. Seedling Propagation. Agricultural handbook, vol 6. U.S. Department of Agriculture, Forest Service, Washington, DC, p 674

Litvay JD, Verma DC, Johnson MA (1985) Influence of a loblolly pine (Pinus taeda L.) culture medium and its components on growth and somatic embryogenesis of the wild carrot (Daucus carota L.). Plant Cell Rep 4:325–328

Maa- ja metsätalousministeriön asetus metsänviljelyaineiston kaupasta 1055/2002 (2002) [Decree on Trade in Forest Reproductive Material] http://www.finlex.fi/fi/laki/alkup/2002/20021055

Nikkanen T, Heiska S, Aronen T (2013) New ornamental conifers for harsh northern conditions through cutting propagation of special forms of Norway spruce. In: Park YS & Bonga JM (eds) Proceedings of the IUFRO Working Party 2.09.02 conference on "Integrating vegetative propagation, biotechnologies and genetic improvement for tree production and sustainable forest management", June 25-28, 2012, Brno, Czech Republic. p. 98–109

Norgaard JV, Duran V, Johnsen Ø, Krogstrup P, Baldursson S & von Arnold S (1993) Variations in cryotolerance of embryogenic *Picea abies* cell lines and the association to genetic, morphological, and physiological factors. Can J For Res 23(12): 2560–2567. https://doi.org/10.1139/x93-317

Pop J, Lakner Z, Harangi-Rákosa M, Fáric M (2014) The effect of bioenergy expansion: Food,

energy, and environment. Renewable and Sustainable Energy Reviews 32: 559–579. https://doi.org/10.1016/j.rser.2014.01.056

Rikala R (2012) Metsäpuiden paakkutaimien kasvatusopas [Container Seedling Growing Manual for Forest Trees]. The Finnish Forest Research Institute, Suonenjoki, 247 p

Ruotsalainen S (2014) Increased forest production through forest tree breeding, Scan J For Res 29:4 333–344. https://doi.org/10.1080/02827581.2014.926100

Salonen F, Varis S, Aronen T (2017) From Petri dishes to bioreactors–First experiences on optimization of Norway spruce SE-process for bioreactors. In: Park YS & Bonga JM (eds) Proceedings of the 4th international conference of the IUFRO unit 2.09.02. on Development and application of vegetative propagation technologies in plantation forestry to cope with a changing climate and environment, La Plata, Argentina, 19–23 Sept 2016, p. 293–297

Tikkinen M (2018) Improved propagation efficiency in a laboratory–nursery interface for somatic embryogenesis in Norway spruce. Dissertationes Forestales 265. 35 p https://doi.org/10.14214/df.265

Tikkinen M, Varis S & Aronen T (2018a) Development of Somatic Embryo Maturation and Growing Techniques of Norway Spruce Emblings towards Large-Scale Field Testing. Forests. 9(6) 325. https://doi.org/10.3390/f9060325

Tikkinen M, Varis S, Peltola H & Aronen T (2018b) Improved germination conditions for Norway spruce somatic cotyledonary embryos increased survival and height growth of emblings. Trees 32(6) 1489–1504. https://doi.org/10.1007/s00468-018-1728-6

Tikkinen M, Varis S, Peltola H & Aronen T (2018c). Norway spruce emblings as cutting donors for tree breeding and production. Scan J For Res 33(3): 207–214. https://doi.org/10.1080/02827581.2017.1349925

Välimäki S, Paavilainen L, Tikkinen M, Salonen F, Varis S & Aronen T (2019) Production of Norway spruce embryos in a temporary immersion system (TIS) *submitted*

Varis S (2018) Norway Spruce *Picea abies* (L.) Karst. In: Jain SM, Gupta P (eds) Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants, Volume 1, second edition, Forestry Sciences 84. Springer International Publishing AG. Cham, Switzerland. p. 255–267. https://doi.org/10.1007/978-3-319-89483-6_19

Varis S, Ahola S, Jaakola L, Aronen T (2017) Reliable and practical methods for cryopreservation of embryogenic cultures and cold storage of somatic embryos of Norway spruce. Cryobiology 76:8–17. https://doi.org/10.1016/j.cryobiol.2017.05.004

von Arnold S, Eriksson T (1981) *In vitro* studies of adventitious shoots of *Pinus contorta*. Can J Bot 59:870–874

White TL, Adams WT, Neale DB (2007) Forest genetics. Wallingford: CABI Publishing 29

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Flowering traits as a component of reproductive success in maritime pine clonal seed orchards

Jean-François Trontin[1], Pierre Alazard[1], Sandrine Debille[1], Laurent Bouffier[2,3]

[1] FCBA, Biotech & Advanced Forestry Dpt, 71, Route d'Arcachon – Pierroton, 33610 Cestas, France
[2] INRA, BIOGECO, UMR 1202, 33610 Cestas, France
[3] University of Bordeaux, BIOGECO, UMR 1202, 33400 Talence, France
jean-francois.trontin@fcba.fr

Abstract
Open-pollinated clonal seed orchards are a cost-effective way to deliver expected genetic gain in new varieties provided that female and male contributions of selected (grafted) genotypes are well balanced and that contamination by foreign pollen is low. Molecular analyses of maritime pine clonal orchards revealed high pollen contamination rates, therefore questioning the mating structure in relation with flowering precocity and intensity of progenitors. A strong genetic control of flowering traits is suggested from the preliminary survey of 3 clonal orchards during two consecutive seasons. Female flowering precocity could not be related with pollen contamination rate but male flowering intensity could partly explain reproductive success.

Keywords: *Pinus pinaster*, progenitors, grafting, flowering precocity, flowering intensity, mating structure

Introduction

Maritime pine (*Pinus pinaster* Ait.) is the main conifer species for plantation forestry in France with over 45 million seedlings produced per year (latest annual statistics 2016-2017, Ministry of Agriculture, https://agriculture.gouv.fr), almost exclusively from improved genetic resources. Most French standing resources (ca. 1 million ha) are located in the region "Nouvelle Aquitaine" (Landes de Gascogne forest) where significant reforestation efforts have been undertaken since 10 years after extensive damages caused by the "Klaus" 2009 storm (more than 200,000 ha devalued).

Third-generation clonal seed orchards currently producing the "VF3" commercial seedlots (qualified category) were established in Nouvelle Aquitaine between 2002 and 2006 by the maritime pine breeding cooperative, a group of scientific interest established in 1995 (GIS "Pin maritime du Futur") bringing together INRA, FCBA and other stakeholders involved in development of improved varieties and adequate silvicultural practices. Selected material (52 genotypes overall) was propagated via the same source through conventional grafting and used as clonal progenitors in these orchards. They have been producing improved seeds since 2011 in an open pollination setting, a method that drastically reduces production costs compared to controlled crossings. The full achievement of the expected genetic gain, however, assumes that pollen contribution from outside the orchard (pollen contamination) is low, while that of the orchard's clonal progenitors is well-balanced. In practice, a recent genetic study of VF3 seedlots from 3 clonal orchards (Bouffier et al. 2017) based on SNP markers (Single Nucleotide Polymorphism), developed by Vidal et al. (2015), revealed that the pollen contamination rate could be high and variable according to the year, the orchard (site) and the maternal genotype. Moreover, the vast majority of paternal genotypes do contribute to offspring but with great heterogeneity.

In this work our objective was to carry out a pilot study of female flowering precocity as well as intensity of female and male flowering of all progenitors in the same 3 clonal seed orchards investigated by Bouffier et al. (2017). Relative genotype ranking for flowering traits was then tentatively used to investigate how flowering behavior of clonal orchard progenitors can affect pollen contamination rate and parental contribution in maritime pine VF3 seedlots.

Material and methods

Clonal seed orchards

The 3 clonal seed orchards investigated in this work (Picard, Vaquey and Saint-Sardos, Table 1) are producing third-generation improved seedlots for the "Vigour-Form" traits (VF3). Picard is located in the northern part of the Landes de Gascogne forest while Vaquey and Saint-Sardos are located about 20 km east to this large forest massif (Figure 1). They have been established in contrasting pedo-climatic conditions and, accordingly (P. Alazard, multi-year observations), Vaquey (clay loam soil) and Saint-Sardos (compact clay) show an earlier flowering time of 7-10 days compared to Picard (sandy wet soil). Clay soils have a greater thermal inertia compared to sandy soils and warm up more permanently in spring.

Table 1. *VF3 clonal seed orchards investigated in this study (Picard, Vaquey, Saint-Sardos). The basic genetic material available in these orchards (52 selected genotypes overall) is almost the same (Raffin 2014).*

Orchard (area)	Location	Reference	Setting up	Commercial production	Genotypes (N)	Operator
Saint-Laurent2-VF3 (15 ha)	Picard (Saint-Laurent-Médoc, 33)	PPA-VG-014	2006	2014-2024	46	Vilmorin
Beychac-VF3 (15 ha)	Vaquey (Beychac-et-Caillau, 33)	PPA-VG-011	2002 2003	2011-2021	47	Forelite
Saint-Sardos-VF3 (6 ha)	Saint-Sardos (47)	PPA-VG-015	2003	2013-2023	48	Forelite

Evaluation of flowering traits

Three flowering traits were scored for each investigated tree per clone (ramet):

i) the predominant development stage of female strobili (stage 1-10, from unopened floral buds until the end of strobili' receptivity to pollen); this scoring system, which was adapted from reference conifer protocols (especially *Pinus sylvestris*, Ducci et al. 2012) was used to estimate female flowering precocity.
ii) the intensity of female flowering (number of strobili),
iii) the intensity of male flowering (score 0-4, density of male strobili from low to high).

Data were collected for all genotypes (mean of 7 ramets/clone) in the 3 clonal seed orchards during both springs 2015 and 2016 (overall 7570 data). All observations were made in predefined orchard zones (1 central zone for Picard and Saint-Sardos, 2 east and north zones for Vaquey) by a single, experienced investigator. Such a procedure was designed to minimize "operator" effects that could be significant for partially subjective observations (i.e. development stage, density score). Regular visits to orchards were organized in order to roughly estimate when the receptivity of female strobili (development stages 4-8) was effective for about 50% of the trees. The phenotypic variability may be too small to be discriminatory in case of too early or late observations. Data were then tentatively collected during a single visit to each orchard at the appropriate time in 2015 (April 15-22) and 2016 (April 5-14). Most data could be obtained except the density score of male strobili at Saint-Sardos in 2015.

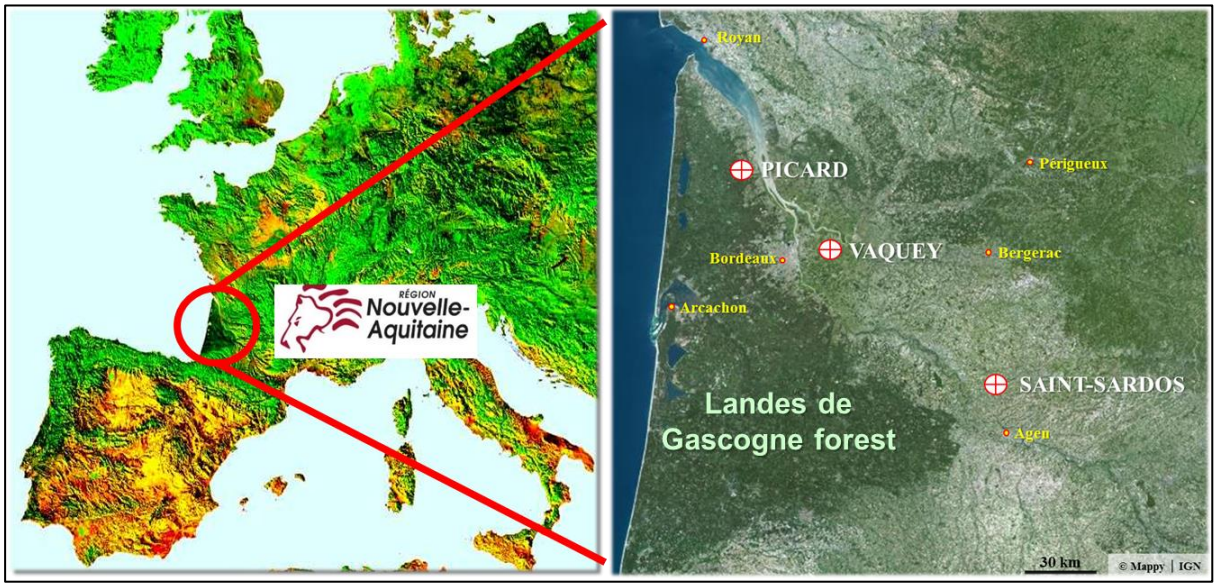


***Figure 1**. The 3 clonal seed orchards investigated (Picard, Vaquey, Saint-Sardos) are located close to the Landes de Gascogne forest (Region Nouvelle Aquitaine, South-West France).*

Genotype ranking procedure

For each variable (development stage and number of female strobili, density score of male strobili) and descriptive information (orchard, year, zones), all genotypes were first ordered (ranking out of max 52 genotypes) according to mean values calculated from all observed ramets. Relative ranking score (1-52) were assigned from earliest to latest (female flowering precocity) or from highest to lowest (female or male flowering intensity). For visual presentation of results, a color code has been assigned to the 17 earliest or most flowering genotypes (green), or, on the opposite, the 17 latest or least flowering genotypes (pink). The remaining 18 genotypes ranking with an intermediate behavior have been assigned an orange color code. For each genotype, a global ranking score could be calculated for each variable based on mean ranking obtained for each orchard and each observation year.

Results and discussion

Maritime pine flowering in clonal seed orchards

Although some annual differences could be observed among sites, strikingly, average ranking of each genotype for female flowering precocity or intensity and male flowering intensity is quite similar in the 3 orchards for the 2 investigated years (**Figure 2**). A more detailed analysis of the Vaquey orchard in 2 zones (east and north) also does not reveal any significant flowering differences within orchard (data not shown). This is a remarkable and quite robust result considering that flowering assessment is a difficult task on large trees (9-13 years old in this study) and that these orchards are located in contrasting pedo-climatic conditions. A strong genetic control of flowering traits in maritime pine is therefore suggested from these data. Moreover a positive relationship was observed between female flowering precocity and female or male flowering intensity (Figure 3) suggesting some genetic correlation as reported for other forest tree species (e.g. eucalypts, Cané-Retamales et al. 2011, Contreras-Soto et al. 2016). However observation bias cannot currently be excluded from this preliminary study, i.e. underestimated flowering intensity for late female flowering genotypes. Additional annual observations are required in these orchards at regular intervals along the flowering season to better characterize flowering phenology of progenitors.

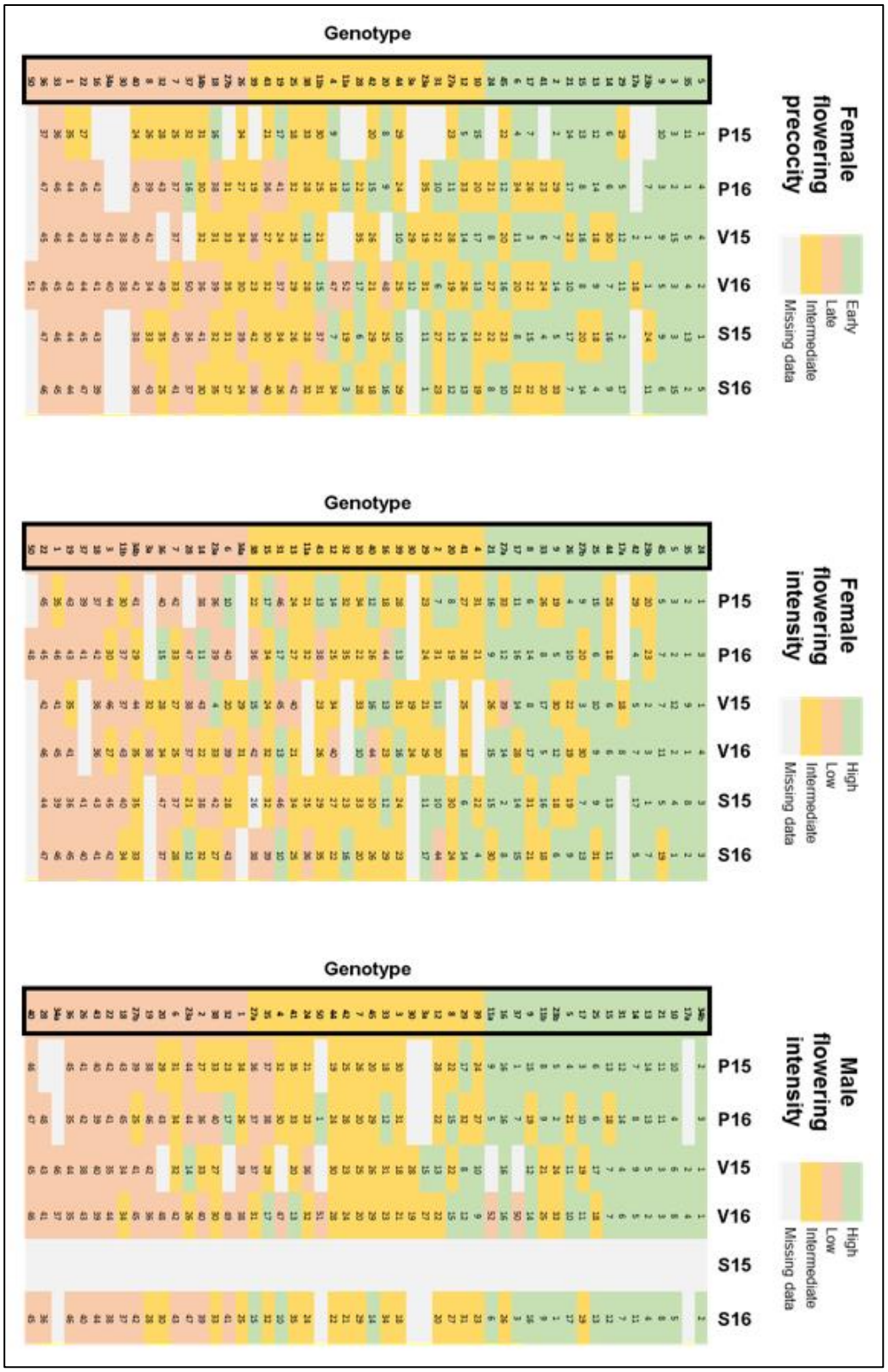


Figure 2. *Genotype ranking for female flowering precocity or intensity and male flowering intensity in maritime pine clonal seed orchards during 2 consecutive springs. For each trait, the global ranking of genotypes is shown on the left (black boxes).* ***P15, P16****: Picard 2015, 2016;* ***V15, V16****: Vaquey 2015, 2016;* ***S15, S16****: Saint-Sardos 2015, 2016.*

Figure 3. *Relationship between female flowering precocity (●) and male (●) or female (●) flowering intensity (all 2015 and 2016 data from the 3 clonal seed orchards). Linear regressions are shown as dotted lines in grey (male flowering intensity, $r^2 = 0.1858$) or orange (female flowering intensity, $r^2 = 0.1688$).*

Possible relationship of flowering precocity and intensity with pollen contamination rates and paternal contributions in clonal seed orchards

Genotype flowering precocity and intensity estimated from these multi-site (3 orchards) and multi-year (2 seasons) datasets were tentatively used as variables that could explain observed pollen contamination rates and paternal contributions in VF3 seedlots from clonal orchards (SNP analysis, Bouffier et al. 2017).

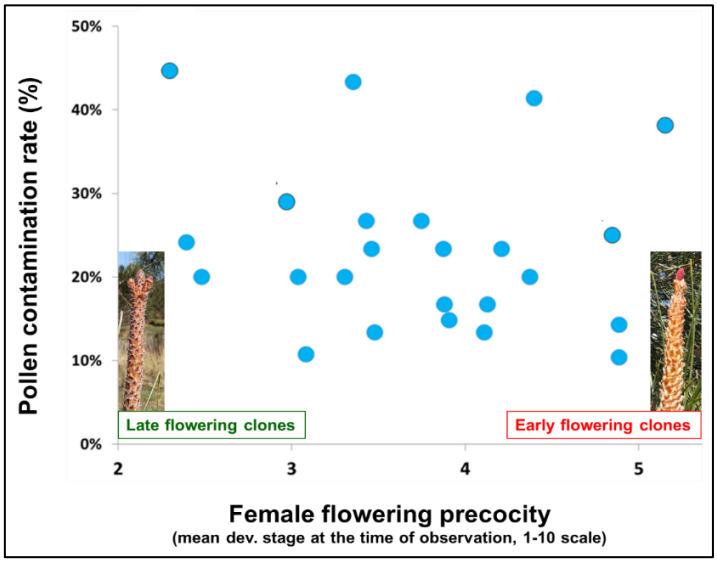


Figure 4*. Relationship between female flowering precocity and pollen contamination rate of 24 maternal genotypes (●) from the Vaquey clonal seed orchard. SNP data for calculating the pollen contamination rate (Bouffier et al. 2017) were obtained from seedlots harvested in fall 2014 (pollination in spring 2013). Female flowering precocity of each maternal genotype is estimated from the mean developmental stage of strobili at the time of observation (this work, all 2015 and 2016 data from the 3 clonal seed orchards investigated).*

Surprisingly, we did not find any significant difference in pollen contamination rate of the precocious and late genotypes for female flowering (Figure 4). This could theoretically result in phenological shifts with pollen emission period by foreign (outside orchard) source trees. A slight trend towards lower pollen contamination of early genotypes is however detected and should be further investigated.

As expected, male flowering intensity could partly explain the heterogeneity of paternal contributions to VF3 seedlots as revealed by the SNP analysis (Figure 5). Most paternal genotypes with high or intermediate male flowering intensity showed greater propensity to contribute to seedlots. As no significant relationship could be detected between paternal contributions and the number of ramets per clone in clonal seed orchards (Bouffier et al. 2017) it is suggested that reproductive success is more related to flowering capacity of progenitors than to orchard structure.

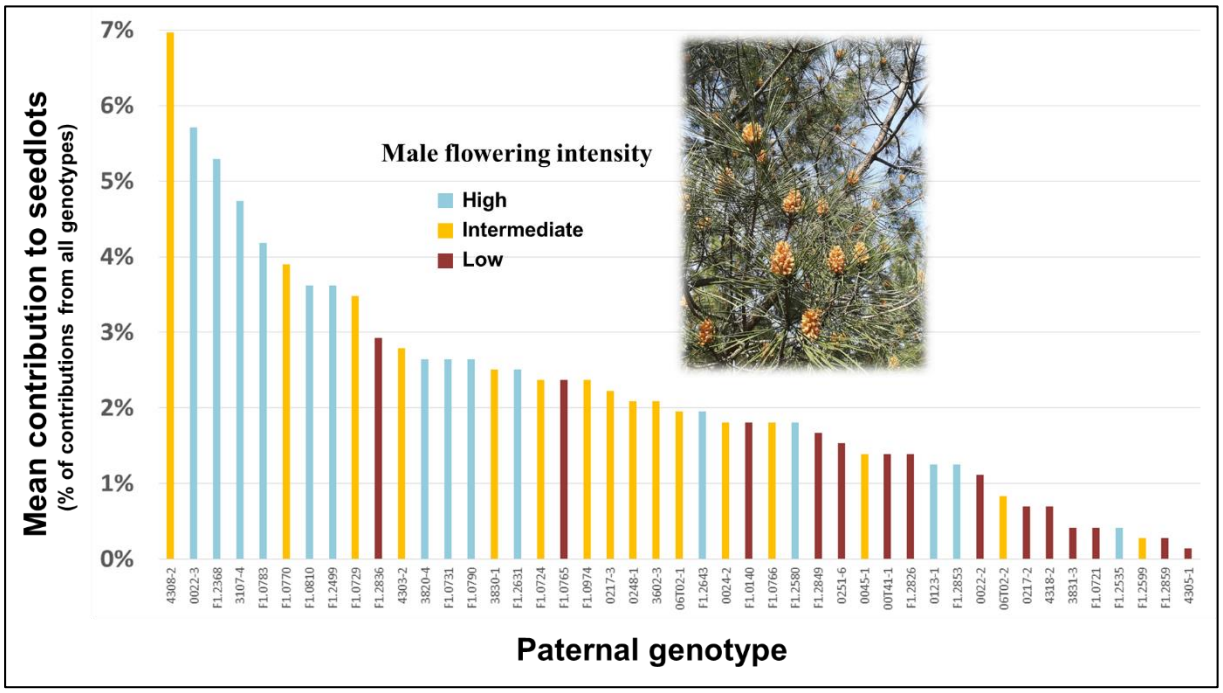


Figure 5*. Relationship between paternal contributions to seedlots and male flowering intensity of 45 genotypes from the Vaquey clonal seed orchard. SNP data for calculating the paternal contributions (Bouffier et al. 2017) were obtained from seedlots harvested in fall 2012 and 2014 (pollination in spring 2011 and 2013, respectively). Male flowering intensity of each paternal genotype is estimated from the density score of male strobili at the time of observation (this work, all 2015 and 2016 data from the 3 clonal seed orchards investigated).*

Conclusion and prospects

Flowering phenology and intensity of progenitor trees in clonal seed orchards are still largely unknown but could be critical to understand the mating structure (especially heterogeneity of paternal contribution) and how it affects real genetic gain in commercial seedlots. Our preliminary study shows that flowering traits of progenitors could be estimated in a fairly robust way by cross-observation of 3 clonal seed orchards established in contrasting pedo-climatic conditions over 2 seasons. Overall rankings of genotypes are in good accordance with observations in the three orchards and highlight the genetic control of these flowering traits. Further analyses are needed to better estimate the phenology and intensity of flowering and avoid putative observation bias of selected progenitors. Beyond the flowering process, it would be useful to collect data on pollen viability, compatibility and/or competitiveness among progenitors as another main component of reproductive success in clonal seed orchards.

Acknowledgements: This project was funded by the French Ministry of Agriculture (MAA/DGAL, QUASEGRAINE project/2014-352/coord. ONF). We thank the GIS "Pin maritime du Futur" as well as Vilmorin and Forelite for providing continuous access to the orchards for flowering monitoring. SNP genotyping data to which we refer in this study (Bouffier et al. 2017) were performed at the Bordeaux Genomic Facility granted by Region Nouvelle Aquitaine (20030304002FA, 20040305003FA), FEDER funds from European Union (2003227) and the French National Research Agency (XYLOFOREST platform/ANR-10-EQPX-16).

References:

Bouffier L, Debille S, Alazard P, Pastuszka P, Raffin A, Harvengt L, Lelu-Walter MA, Musch B, Trontin JF (2017) Pollen contamination and mating structure in maritime pine clonal seed orchards. IUFRO 2.09.01 Seed Orchard Conference, 04-06 sept. 2017, Bålsta (Sweden). Poster, pp. 65 (scientific paper in prep.).

Cané-Retamales C, Mora F, Vargas-Reeve F, Perret S, Contreras-Soto R (2011) Bayesian threshold analysis of breeding values, genetic correlation and heritability of flowering intensity in *Eucalyptus cladocalyx* under arid conditions. Euphytica 178: 177-183.

Contreras-Soto R, Ballesta P, Ruiz E, Mora F (2016) Identification of ISSR markers linked to flowering traits in a representative sample of *Eucalyptus cladocalyx*. Journal of Forestry Research 27: 239-245.

Ducci F, De Cuyper B, Pâques LE, Proietti R, Wolf H, *Compilers* (2012) Reference protocols for assessment of trait and reference genotypes to be used as standards in international research projects. Ed. CRA SEL - Arezzo, Italy: p. 82, ISBN: 978-88-901923-6-4.

Raffin A (2014) Les variétés actuelles et futures de pin maritime (Current and future maritime pine varieties). GIS Pin maritime du Futur, Les Cahiers de la Reconstitution 4 (June 2014): 2-4 (in French).

Vidal M, Plomion C, Harvengt L, Raffin A, Boury C, Bouffier L (2015) Paternity recovery in two maritime pine polycross mating designs and consequences for breeding. Tree Genetics & Genomes 11: 1-13. doi:10.1007/s11295-015-0932-4.

Extended Abstracts

Biotechnology and bio-prospection of native species from Monte desert Patagonia, as strategies for the development of regional bio-economy

Patricia Boeri[1,2,3] ,Lucrecia Piñuel[1,2,3], Irina Lopez Dumrauf[1], Daniela Dalzotto[1], Inti Sabanes[1], Sandra Sharry [2,3,4,5]

[1]Lab. de Tecnología de Alimentos y Biotecnología, Universidad Nacional de Río Negro, Sede Atlántica. Ruta Provincial N° 1 y Rotonda Cooperación. [2]Viedma, Río Negro, Argentina. CIT -Rio Negro - CONICET. Viedma, Río Negro, Argentina. [3]UIISA. Unidad Integrada para la Innovación del Sistema Agroalimentario de la Patagonia Norte. Universidad Nacional de Río Negro-Viedma, Río Negro, Argentina. [4]Laboratorio de Investigaciones de la Madera. LIMAD-FCAyF-UNLP. CC 31.La Plata (1900) Buenos Aires, Argentina. [5]Comisión de Investigaciones Científicas-Buenos Aires (CICPBA), Argentina. Email: pboeri@unrn.edu.ar; lpinuel@unrn.edu.ar; danielacdalzotto@gmail.com; irina_071@hotmail.com; intisabanes@gmail.com; ssharry@gmail.com

Keywords: biodiversity, shrubs plants, vegetative propagation, tissue culture, arid and semiarid region.

Introduction

The dry-land surface (i.e. semi-arid and arid regions) covers approximately one-third of the world's land area and are inhabited by almost 400 million people. During the last 50 years, there has been growing interest in the cause of desertification due to its impact on the global environment, economy and society. These ecosystems provide a series of ecological services essential for the sustainability of human life and the development of productive activities. In Argentina, the arid and semi-arid environments represent approximately one-third of the country's surface. Particularly, the Patagonia region in Argentina has 80% of surface containing signs of deterioration. In Latin America and the Caribbean (LAC) interest for the bio economy has increased significantly. Argentina is at the forefront and the Province of Buenos Aires and Patagonia region has already approved a Bio economy Strategy. Argentina offers a wide range of options for the local development of bio economy**.** Our country has a vast territory, a remarkable climate variety and biodiversity, a significant natural and planted forested area, plus highly competitive agricultural and cattle breeding sectors**.** Besides, biotechnological advances were implemented at an early stage, and advanced scientific and technological skills are available**.** The conservation of biodiversity depends largely on knowledge generation about our own resources and enhances the native plants that are part of the cultural heritage of the region. The current boom of native species and the lack of studies forces improvement of conservation and propagation methods in addition to the quality of planting material and production in nurseries. At the Universidad of Rio Negro we carried out a Project named *Regional bioeconomy. Propagation and domestication of medicinal, ornamental, aromatic or forest species for the province of Rio Negro (Bioeconomía Regional. Propagación y domesticación de especies medicinales, ornamentales, aromáticas y/o forestales para la Provincia de Río Negro*" (PI-UNRN 40C352). The main purpose was to re-evaluate the properties of native species from argentine Monte (PATAGONIA) and their bio products as unconventional functional foods, nutraceuticals, and dietary supplements, cosmetics and phytotherapy and find propagation strategies through biotechnology to promote multiplication and reintroduction of species to the ecosystem and sustainably manage and conserve them.

The project objectives are:

General objective:

- Propagation and domestication of native species of potential economic value of the Flora Rionegrina, Patagonia.

Specific objectives:

- To apply and adjust techniques of sexual and vegetative propagation of native species.
- Develop and adjust commercial scale harvesting, processing and storage of seeds and in vitro plant production techniques.
- Promote in situ and ex situ conservation of native germplasm in order to maintain biological diversity.
- To form human resources in propagation and nursery of native plants.
- To disseminate the technical and/or theoretical knowledge obtained with the development of the research.
- Establish and strengthen relations in the field of extension and research with provincial government agencies on the issue of the propagation of native species, encouraging the exchange of knowledge and information achieved in the research.
- Integrate different disciplines and institutions with a common goal by training human resources and strengthening the pre-existing institutional infrastructure.

The aim of this communication is to contribute to the body of knowledge of native species from desert and semi-desert regions, in order to provide alternatives for conservation and sustainable use of these resources and to enable them to be included in ecological reforestation and restoration programs in degraded environments. The species studied were: *Prosopis alpataco*, *Prosopis caldenia*, *Geoffroea decorticans*, *Acantholippia seriphioides*, *Condalia microphylla*, *Senecio* sp, *Larrea divaricata* and *Boungavillea spinosa (Figure 1)*.

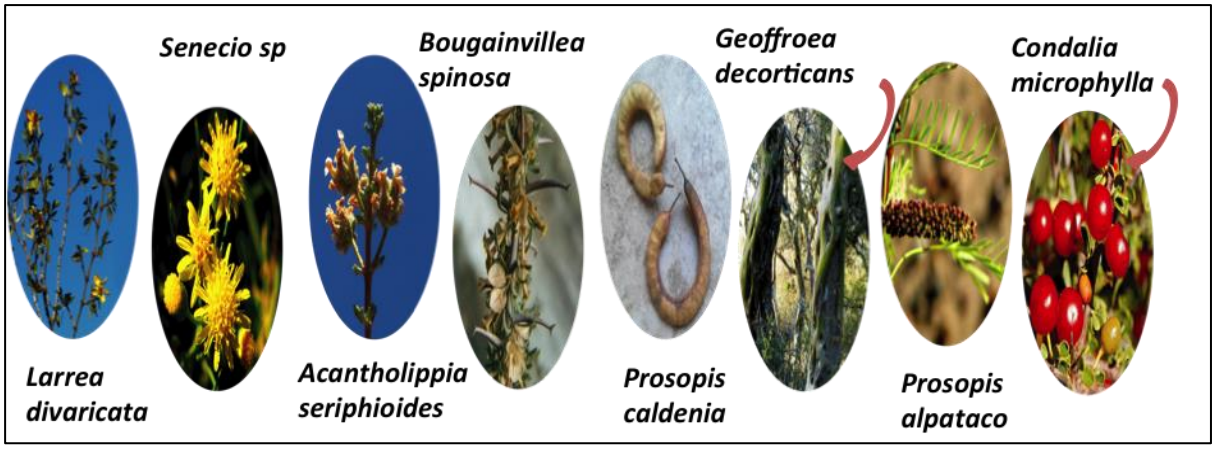
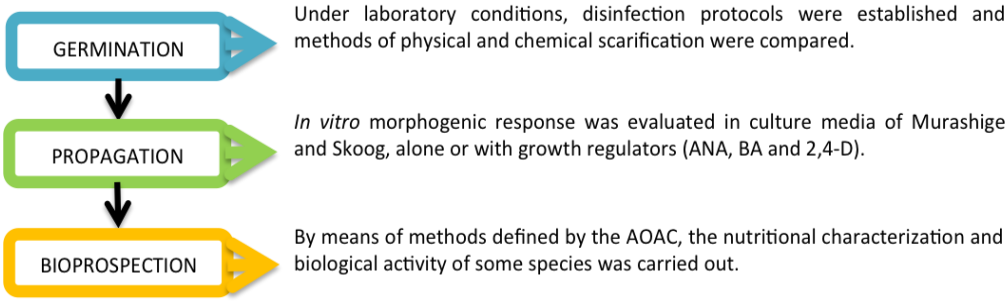


Figure 1. *Species studied.*

Material and Methods

The mother plants were collected from the field in the Monte Plant Formation, in Rio Negro Province, Argentina. We assayed germination, propagation and bio prospection in different species:



For in vitro studies we used as explants the seeds, cotyledons, embryos, and shoot and nodal section from vitroplants. The selected species from arid areas of the Monte desert, generally share the presence of hard and impermeable integuments. Therefore, for each case, we used different types of scarification treatments to optimize the germination process (see Boeri et al., 2018). The embryogenic callus induction of *P. alpataco* from cotyledonary explants was developed as described in Boeri and Sharry (2018). All the culture steps were incubated in a plant growth chamber under controlled conditions of temperature (25 ± 2 ºC), under a 16 hr light: *8* hr *dark photoperiod* (provided by fluorescent tubes) and average photosynthetic photon flux of 50 µmol $m^{-2}s^{-1}$. Basic medium composition was Murashige-Skoog (1962). The pH was adjusted to 5.8 with 1N KOH or 0.5 N HCl prior to autoclaving at 121ºC for 18 min. The nutritional value was determined according to the Association of Official Analytical Chemists (AOAC) methodology, and phenolic compounds were quantified by diode-array detection (HPLC-DAD). Antioxidant activity *in vitro* and *in vivo* was analysed through the use of the radical species 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and zebra fish model, respectively (Boeri et al, 2017a).

Main Results

Micropropagation was achieved through direct and indirect organogenesis of *P. alpataco* (Fig. 2 a), *G. decorticans* (Fig. 2b), *B. spinosa* and *A. seriphioides (*Fig. 2c) and Somatic embryogenesis of *P. alpataco* (Fig. 2d) (Boeri and Sharry, 2018).

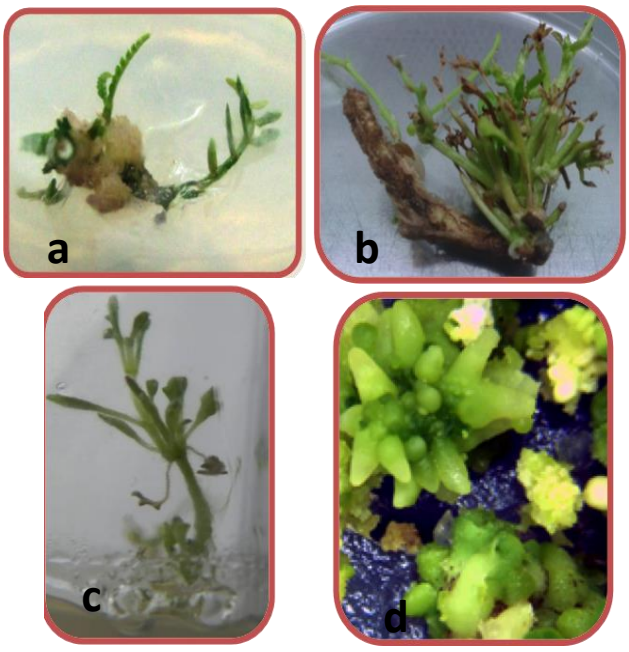


Figure 2. *Indirect organogenesis of P. alpataco (a), Direct Organogenesis of G. decorticans (b) Organogenesis A. seriphioides (c) and Somatic embryogenesis of P. alpataco (d)*

The genetic diversity of regional resources provides the ability to obtain a wide selection of products by bioprospecting, in order to find new foods with high nutritional value for human consumption. The pods of *Prosopis alpataco* constitute a potential resource for both forage and human food. Whole flour was obtained from the full pod. To assess the nutritional value of integral flour (317.15 Kcal / 100 g) the percentage composition was determined. Whole pod flour has a high content of protein and carbohydrates (10 and 62% of dry weight, respectively) and thus pods of this species are a potential food resource. The fat content determination showed that the flour of *P. alpataco* has a low lipid content (3.23%) and the ratio of unsaturated / saturated fat was 4: 1. The presence of antinutritional factors such as polyphenols and phytoaglutinins were evaluated. The flour contained 33.8 mg GAE / 100 g and 0.35 weight HA / mg ml total protein, respectively. In conclusion, the results suggest that integral flour could be used as a dietary supplement for the food industry, either in the preparation of food for human or animal consumption (Boeri et al, 2017b). The integral flour of *P. alpataco* and the pulp of *C. microphylla indicate* could be an important source of carbohydrates. *P. alpataco*, in addition, had a high content of fibers and proteins. *C. microphylla* possesses antioxidant and hemagglutinating activity (Boeri et al, 2017a) (Figure 3).

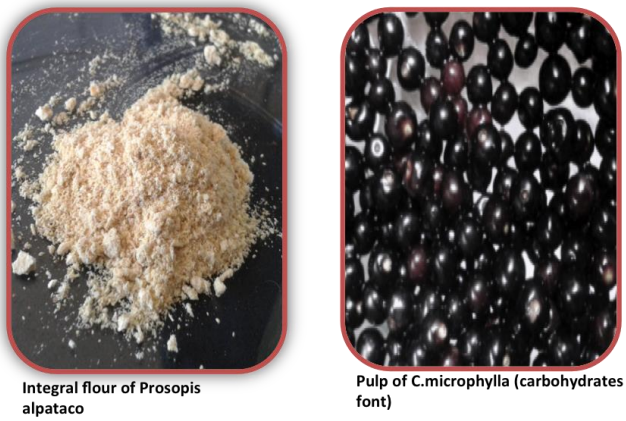


Figure 3. *Prosopis alpataco* flour and *Condalia microphylla* fruits

Condalia microphylla has hemagglutination activity (Boeri et al, 2017a). The results of the biological characterization indicated that the drupes of *C. microphylla* possess a significant antioxidant activity *in vitro* and *in vivo*. Therefore, piquillin fruit is a promising source of antioxidant molecules that could exert a preventive effect against oxidative stress-associated disorders and might be used as a natural additive for food. In addition, the information generated in this work could lengthen the list of bioactive compounds for current scientific evaluation. Further studies are needed to investigate the bioavailability and mechanism of action of the compounds included in piquillin fruits.

The disinfection and germination of the seeds of "thyme", "Senecio", "Chañar" and "Caldén" were adjusted for their subsequent introduction in in vitro conditions (Figure 4).

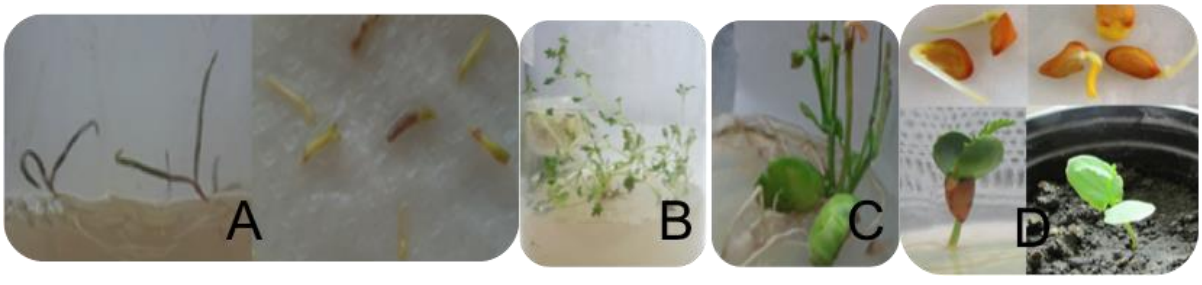


Figure 4. *In vitro germination of seeds. A. A.serifoides , B. Senecio sp. C. Geoffrea decorticans D. Prosopis caldenia.*

Transfer-Social Impact

The results of the project, mainly the plants obtained were transferred through agreement and support to the Ministry of agriculture, livestock and fisheries of the province - project PFIP-09 (Cofecyt): "Multiplication of native species of Rio Negro xerophyte monte, from a basic laboratory of plant biotechnology" (Figure 5) (Boeri et al, 2018b).

Figure 5. *Plantlets of "alpataco" after rustication and the field of Ministry of agriculture, Rio Negro, Argentina.*

Conclusions

The species under study have been systematically used as sources of energy, food or even medicinal use. This has exposed them to extractive type conditions, compromising their future availability, such as *A. seriphioides*, a species that is currently in danger of extinction. This project has allowed to generate knowledge about the propagation of the native flora of the arid and semiarid region, indispensable for the implementation of restoration strategies of degraded ecosystems and the ex situ conservation of these species. The conservation of biodiversity depends to a large extent on generating knowledge of our own resources and re-evaluing native plants, which are part of the cultural heritage of the region. The lack of studies of this native species obliges to optimize strategies of propagation for its use-based bioenergy and to generate methodologies of bio prospection that allow knowing its nutritional and medicinal value. The development of the bio-resources area has become a strategic axis for the country, with a strong impact on the regional socio-economic and environmental areas. The evaluations of the medicinal and nutritional properties of the selected species, allowed the identification of products with current or potential uses, and are fundamental for the use and rational protection of biodiversity. On the other hand, this project has allowed to generate knowledge about the propagation of the native flora of the arid and semiarid region, indispensable for the implementation of restoration strategies of degraded ecosystems and the *ex situ* conservation of these species. The great physiological and morphological variability of these species allows their inclusion in programs of reforestation of arid lands in Argentina. The optimization of different propagation strategies and the increased knowledge of chemical prospecting of these resources, adapted to the environmental stress factors that characterize the region, allow us to initiate plans for the restoration of degraded ecosystems in the Monte desert of Patagonia Argentina. This work allowed us to expand the information base of Patagonian biodiversity, recover threatened species and collaborate with the maintenance of regional biodiversity. Biodiversity is the basis of the bio-economy and the sustainable management of regional resources is a fundamental principle of the new circular economy paradigm. In this context, the development of the bio-resources area has become a strategic axis for the country, with a strong regional socio-economic impact.

References

Boeri P, Piñuel L, Sharry S, Tombari A. and Barrio D (2017a) Chemical and Biological Characterization from *Condalia microphylla* Fruits, a Native Species of Patagonia Argentina. J Agric Sci Technol B 7: 395-405 D doi: 10.17265/2161-6264/2017.06.004

Boeri P, Piñuel L, Sharry S and Barrio D (2017b) Nutritional characterization of the whole carob flour (*Prosopis alpataco*) of the northern Patagonia Argentina". Revista de la Facultad de Agronomía, La Plata, 2017. Vol 116 (1): 129-140

Boeri P and Sharry S (2018) Somatic embryogenesis of alpataco *(Prosopis alpataco L.).* In: Forestry Sciences, Jain SM and Gupta P (Eds). Vol 85: Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants, Vol II Chapter 16: 189:198. ISSN 0924-5480. DOI 10.1007/978-3-319-79087-9 Springer International Publishing. 451pp

Boeri P, Piñuel L, Dalzotto D, Barrio D, Romero Alves M, Cedrés Gazo M and Sharry S (2018) Sustainable management of biodiversity in woody ecosystems: biotechnology and bioprospecting of native species from monte desert, Patagonia. In: "Forest Conservation: Methods, Management and Challenges", Nova Science Publishers, Long Island, USA. (www.novapublishers.com/catalog/index.php)

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Comparative analysis of primary metabolome and secondary metabolites in the somatic embryogenesis of tamarillo (*Solanum betaceum* Cav.)

André Caeiro[1], Ivana Jarak[2], Sandra Correia[1,4], Rui Carvalho[1], Jorge Canhoto[1,4]

[1] Department of Life Sciences, University of Coimbra, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal. afcaeiro91@gmail.com;
[2] Laboratory of Cell Biology and Unit for Multidisciplinary Research in Biomedicine (UMIB), Department of Microscopy, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Porto, Portugal
[3] Health Sciences Research Centre (CICS–UBI), University of Beira Interior, Covilhã, Portugal
[4]Centre for Functional Ecology, University of Coimbra, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal

Keywords: Embryogenic cells, metabolomic profile, NMR, non-embryogenic cells, solanaceae.

Introduction

Tamarillo (*Solanum betaceum* Cav.) is a small solanaceous tree endogenous to South America. It is an economically important species because of its edible fruits, which can be consumed fresh or processed. Different cloning techniques for mass propagation have been developed for this species, namely somatic embryogenesis (SE) (Canhoto *et al.*, 2005). SE can be defined as a multi-step process by which a somatic cell or tissue undergoes ontogenetic changes that culminate in a structure morphologically similar to a zygotic embryo that can ultimately evolve into a full plant (Joshi and Pramod, 2013). This process has been described for a great variety of species and has several advantages when compared to other cloning methods. However, many of its molecular mechanisms are still poorly understood and several species remain recalcitrant or scarcely responsive to SE.

SE was first described in tamarillo by Guimarães *et al.* (1988) and since then the process has been extensively studied and optimized (Correia and Canhoto, 2018). Furthermore, several approaches including proteomic studies have been carried out to understand the molecular events involved in the formation of somatic embryos and its regulation (Correia *et al.*, 2012). In short, the induction protocol from tamarillo leaf explants or zygotic embryos (Lopes *et al.*, 2000; Canhoto *et al.*, 2005) produces embryogenic (EC) and non-embryogenic (NEC) calluses from the same explant, allowing a direct comparison of the molecular features responsible for embryogenic competence since both calluses have the same genetic background and are originated in the same experimental conditions. Both EC and NEC can be maintained *in vitro* and sub-cultured independently. In this work, previously induced embryogenic and non-embryogenic calluses from leaf origin of *in vitro* growing plantlets were used. The induction protocol (Lopes *et al.* 2000) uses a basal Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with high sucrose levels (9 %) and picloram.

Metabolomics

Metabolomics can be broadly defined as a systematic study all the metabolites in a given organism, tissue or cell (the metabolome). The results acquired by metabolomic research are typically

complementary to other "omics" approaches such as genomics, transcriptomics and proteomics (Hong *et al.,* 2016). Several metabolomic approaches have been undertaken to study SE in plants (Kim *et al.,* 2010), particularly aspects related to the analysis of differently expressed small metabolites such as amino acids, which have been consistently found to be preferably linked to either embryogenic or non-embryogenic *calli* (Mahmud *et al.,* 2014). This analysis is typically based on fold changes found in the metabolic profile (Krishnan *et al*., 2004).
The main detection techniques currently used in metabolomic research are Mass Spectrometry and Nuclear Magnetic Resonance (NMR) spectroscopy. Each technique has its own advantages and limitations that have been extensively reviewed (Saito and Matsuda, 2010 and references therein). NMR detection is less sensitive than mass spectrometry, however it benefits from a higher reproducibility and, in general, better structural information and quantitative aspects extracted from the data. The sample preparation also tends to be simpler and the analysis time is shorter (Krishnan *et al*., 2005). Therefore, NMR techniques are suitable for broad spectrum analysis as well as a large number of samples. The data acquired is also easily interpreted via mathematical models that extrapolate the importance of the metabolites either comparatively or in a time-scale projection (Dowlatabadi *et al.,* 2009; Mahmud *et al.,* 2014).

^{1}H-NMR analysis was used in this work and the resulting spectra were used to construct latent projection models that identify the differently expressed metabolites in each cell line (obtained from EC and NEC). Additionally, secondary compounds, namely phenolic acids were also quantified as these compounds have been shown to intervene in the SE process (Moriguchi *et al.,* 1999; Reis *et al.,* 2008). These compounds form large complex families, which are also a possible target for metabolic fingerprinting with several separation techniques readily available for separation and analysis (Seal, 2016).

Metabolic Profile

The results showed an increase in sugar content (glucose, fructose, sucrose), and some amino and organic acids (alanine, malic acid and 4-aminobutyrate) in NEC, while chlorogenic acid, phenylalanine, choline and proline content were detected mainly in EC. The embryogenic lines seem to have more stress-responding mechanisms (predominance of proline and glutamine) whereas the NEC lines appear to have a more energy-driven metabolism. The presence of chlorogenic acid and phenylalanine in EC lines also seem to indicate a higher synthesis of secondary compounds, as both molecules are precursors of important biosynthesis pathways. In fact, for both secondary compound classes assayed (phenolic and flavonoid compounds) the concentration appeared about two-fold higher in EC, suggesting the importance of these metabolites in the maintenance of embryogenic competence, although the specific mechanism of action cannot be inferred from the data presented.

Conclusions and future perspectives

The results presented show clear metabolic differences between the two cell lines. These differences may reflect the differences in the organization of the two types of calluses as well their specific metabolic pathways and developmental programs: whereas EC develops to somatic embryos upon transfer to an auxin free medium whereas NEC cells became necrotic and died.

Future perspectives include a more detailed analysis of the differential primary metabolism in the cell lines and its integration with other available results, namely proteomic studies. The secondary metabolite pool can also be further characterized by separating phenolic and flavonoid pools and identifying the main compounds present in each phase of induction and describe its influence in the regulation of SE.

References

Canhoto J, Lopes M, Cruz G. (2005) Protocol of somatic embryogenesis in tamarillo (*Cyphomandra*

betacea (Cav.) Sendtn.). In: Jain SM, Gupta PK (eds) Protocol for somatic embryogenesis in woody plants, Springer, Dordrecht pp 379-389

Correia S, Canhoto JM (2018) Somatic Embryogenesis of Tamarillo (*Solanum betaceum* Cav.). In: Jain S, Gupta P (eds) Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants. Forestry Sciences, pp 171-179

Correia SI, Vinhas R, Manadas B, Lourenço A, Veríssimo P, Canhoto JM (2012) Comparative proteomic analysis of auxin induced embryogenic and non-embryogenic tissues of the solanaceous tree *Cyphomandra betacea* (tamarillo). J Proteome Res, 11: 1666−1675

Dowlatabadi R, Weljie AM, Thorpe TA, Yeung EC, Vogel HJ (2009) Metabolic footprinting study of white spruce somatic embryogenesis using NMR spectroscopy. Plant Physiol Biochem, 47: 343-50

Guimarães ML, Cruz, GS, Montezuma-de-Carvalho JM (1988) Somatic embryogenesis and plant regeneration in *Cyphomandra betacea* (Cav.) Sendt. Plant Cell Tissue Organ Cult 15: 161–167

Hong J, Yang L, Zhang D, Shi J (2016) Plant metabolomics: an indispensable system biology tool for plant science. Int J Mol Sci 17: 767-783

Joshi R, Pramod K (2013) Regulation of somatic embryogenesis in crops: A review. Agric Rev 3: 1-20

Kim HK, Verpoorte R (2010) Sample preparation for plant metabolomics. Phytochem Anal, 21: 4-13.

Krishnan P, Kruger NJ, Ratcliffe RG (2004) Metabolite fingerprinting and profiling in plants using NMR. J Exp Botany 56: 255-265

Lopes ML, Ferreira MR, Carloto JM, Cruz GS, Canhoto JM. (2000) Somatic embryogenesis induction in tamarillo (*Cyphomandra betacea*). In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol. 6. Kluwer Academic Publishers, Dordrecht pp 433-455

Mahmud I, Thapaliya M, Boroujerdi A, Chowdhury K (2014) NMR-base metabolomics study of the biochemical relationship between sugarcane callus tissues and their respective nutrient culture media. Ann Bioanal Chem 406: 5997-6005

Moriguchi T, Kita M, Tomono Y, Endo-Inagaki T, Omura M (1999) One type of chalcone synthase gene expressed during embryogenesis regulates the flavonoid accumulation in Citrus cell cultures. Plant Cell Physiol 40: 651-655

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15: 473-497

Reis E, Batista T, Canhoto JM (2008) Effect and analysis of phenolic compounds during somatic embryogenesis induction in *Feijoa sellowiana* Berg. Protoplasma, 232: 193-202

Saito K, Matsuda F (2010) Metabolomics for functional genomics, systems biology, and biotechnology. Annu Rev Plant Biol 61: 463-489

Seal T (2016) Quantitative HPLC analysis of phenolic acids, flavonoids and ascorbic acid in four different solvent extracts of two wild edible leaves, *Sonchus arvensis* and *Oenanthe linearis* of North-Eastern region in India. J Appl Pharm Sci 6: 157-166

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Cork oak somatic embryogenesis as a system model to study QsMYB1, a transcription factor highly expressed in phellem

Tiago Capote[1], Leonor Morais-Cecílio[2], Sónia Gonçalves[1,†], Liliana Marum[1,3]

[1] Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo (CEBAL) / Instituto Politécnico de Beja (IPBeja), 7801-908 Beja, Portugal.
[2] Linking Landscape, Environment, Agriculture and Food, Instituto Superior de Agronomia, University of Lisbon, Lisboa, Portugal.
[3] Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, Pólo da Mitra, 7002-554 Évora, Portugal
† Currently at the Wellcome Trust Sanger Institute, Hinxton, Cambridge CB10 1HH, UK
tiago.capote@cebal.pt; lmorais@isa.ulisboa.pt; sg19@sanger.ac.uk; liliana.marum@cebal.pt

Keywords: somatic embryos, QsMYB1, ChIP-Seq, RNA-Seq, Cork oak.

Introduction

Cork oak (*Quercus suber*) is a woody species with high socio-economic value due to the production and industrial exploitation of cork tissue. Cork derives from phellogen, a secondary meristem, which differentiates in phellem and is characterized by suberized cells. Cork is mainly composed by two macropolymers, suberin and lignin, among polysaccharides, extractives and other compounds.

The study of the molecular regulatory mechanisms of cork formation and differentiation are still scarce. QsMYB1 is a R2R3 transcription factor highly expressed in suberized tissues (Almeida et al. 2013). In previously work, we identified the putative DNA-binding of QsMYB1 in a genome wide-scale investigation of cork oak by ChIP-Seq, unravelling the putative genes regulated by this transcription factor (Capote 2018). In this context, a cork oak somatic embryogenesis system was established and somatic embryos overexpressing *QsMYB1* were generated. Therefore, it is of interest to understand how overexpression of QsMYB1 affects the expression of genes implicated in cork development. In addition it is of great importance to unravel the genetic basis of cork associated biosynthetic pathways, namely related with suberin and lignin biosynthesis.

Material and Methods

Experiments and bioinformatic analysis are described in detail in Capote (2018).

Results and Discussion

The transcriptomic analysis of somatic embryos overexpressing *QsMYB1* was performed using a NGS platform, in order to study how QsMYB1 is modulating the expression of the target genes.

We took advantageous of the cork oak somatic embryogenesis system overexpressing QsMYB1 in order to ChIP-Seq and therefore identify the targets of QsMYB1. In parallel, we sequenced the transcriptome of those embryos overexpressing QsMYB1. The results showed 66,693 expressed genes from which 10,575 ($p<0.01$) are differentially expressed when compared with non-transformed embryos. The differential expressed genes were then subject to a Gene Ontologies (GO) enrichment analysis. GO

biosynthetic processes terms are largely overrepresented among the differentially expressed genes. This result indicates an association between QsMYB1 and the modulation of several cellular biosynthetic processes (Figure 1). In order to clarify these QsMYB1-associated molecular processes, we generated GO graphs (Level 4). Results show that 2,670 differentially expressed genes are related with macromolecules metabolic processes. These macromolecular metabolic associated genes were then crossed with the *loci* of QsMYB1 genome binding-sites in order to accurately identify the genes regulated by QsMYB1, *in vivo*. Furthermore, these candidate genes were integrated with the Kyoto Encyclopedia of genes and genomes (KEGG) database, in order to understand the high-level function of the gene-coding enzymes related with suberin and lignin biosynthetic pathways.

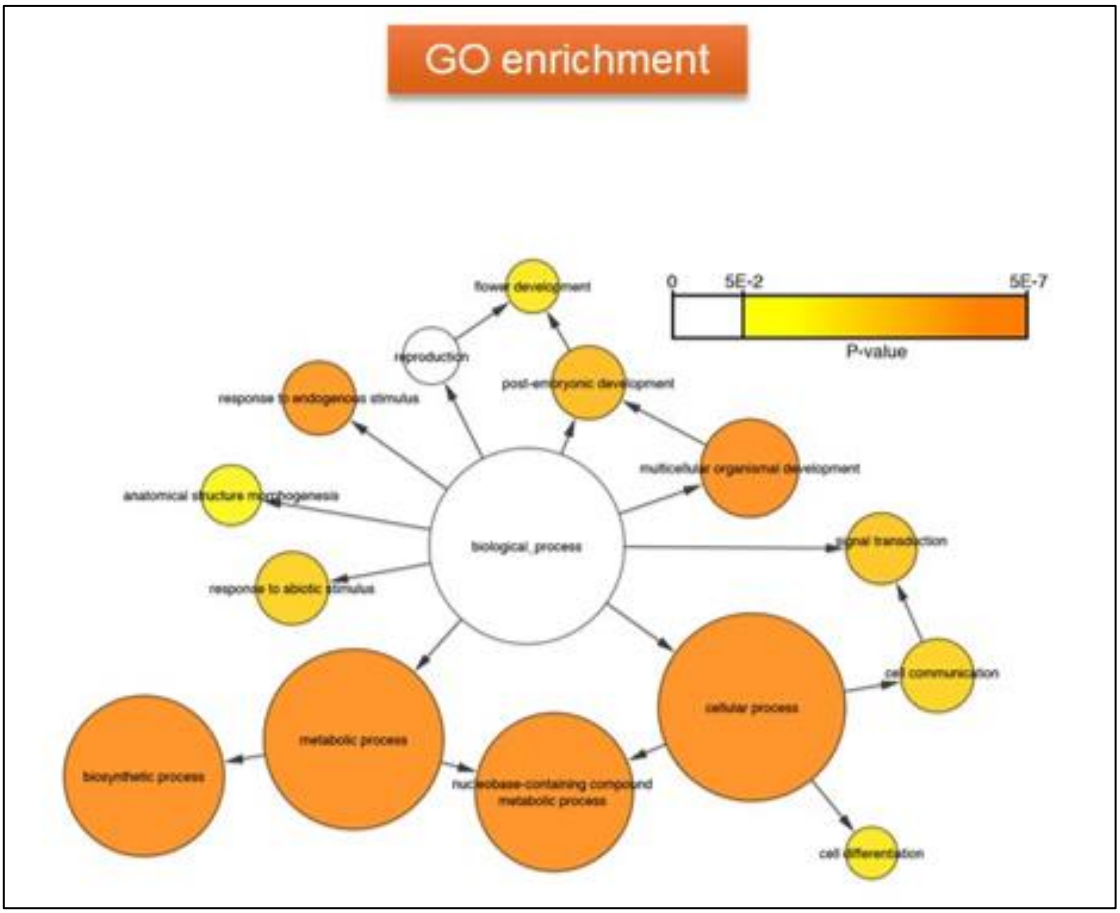


Figure 1. *Gene Ontologies (GO) enrichment analysis*

Our study shows that a group of genes identified in the ChIP-Seq data are overexpressed in the genetically modified embryos overexpressing QsMYB1. Importantly we identified gene encoding for enzymes which play key roles in the suberin and lignin pathways as wells as in the phenylpropanoid and fatty acid biosynthethic pathways. We identified the following; 4-Coumarate:CoA ligase (4CL), Cinnamyl alcohol dehydrogenase (CAD), class III plant peroxidase (PPO), Long-chain acyl-CoA synthetase (LACS), β-ketoacyl-CoA synthase (KCS), Cytochrome P450 enzymes subfamily 86A1 (CYP86A1), Cytochrome P450 enzymes subfamily 86A8 (CYP86A8), Glycerol 3-phosphate acyltransferase (GPAT), Acyl-CoA oxidase (ACX), Fatty acid synthase (FAS) and Acyl-carrier protein (ACP).

Conclusions

This work supports the idea that somatic embryogenesis is an attractive working-stage to perform functional studies in a non-model plant species such as cork oak. Using the somatic embryogenesis

system we performed for the first time a ChIP-Seq assay in cork oak, which allowed the identification of QsMYB1 binding-sites. The results provide evidence that QsMYB1 is a master regulator of suberin and lignin biosynthesis. Finally, by using cork oak somatic embryogenesis, we identified several genes regulated by QsMYB1 which will be the basis of future functional studies that will unravel the molecular mechanisms behind cork formation and differentiation.

Acknowledgements: Work was supported by Alentejo2020, through FEDER under Lentidev-ALT20-03-0145-FEDER-000020 project and by Fundação para a Ciência e a Tecnologia (FCT) through the PhD grants SFRH/BD/69785/2010.

References

Almeida T, Menéndez E, Capote T, et al (2013) Molecular characterization of Quercus suber MYB1, a transcription factor up-regulated in cork tissues. J Plant Physiol 170:172–178

Capote T (2018) Quercus suber MYB1 transcription factor - an in vivo approach for the elucidation of its regulatory networks. PhD thesis. Instituto Superior de Agronomia, University of Lisbon. Lisbon.

In vitro germination of *Brachychiton populneus* (Schott & Endl.) R.Br.

Tatiana Cinquetti [1], Maite Romero Alves[1], Sandra Sharry[1.2]

Laboratorio de Investigaciones de la Madera (LIMAD)
Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Argentina
Universidad Nacional de Río Negro sede Viedma, Argentina
tatianacinquetti@gmail.com; mromeroalves@gmail.com; ssharry@gmail.com

Introduction

The Brachychiton (*Figure 1*) is an evergreen, small to medium tree (8 to 20 m) from Australia where it is known as Kurrajong. It is usually used as an ornamental, appreciated for its high forage value supplementing the diet of animals in winter when the grass is scarce. It is found in diverse habitats from wet coastal districts to semi-arid in land areas. It grows mainly on red-brown earths and red earth and it is very drought-resistant. In Argentina, it is used as an ornamental tree that will grow even on relatively poor sites. No study of tissue culture of this species is known. A critical stage in the introduction of plants to tissue culture is to obtain cultures free of microbial contamination.

The aim of this study was to establish the conditions of in vitro germination of Brachychiton seeds that will provide aseptic seedlings as a source of explants for in vitro propagation.

Figure 1. *Braquiquito tree in the Faculty of Agricultural and Forestry Sciences in La Plata, Argentina.*

Materials

1. Fungicide: Funguicap® (Captan); 70% ethanol; 30% commercial bleach; tween 80; sterile water.
2. Water treatment equipment; balances; autoclave; laminar-flow hood; scissors, forceps and scalpels; glass bottles; baby food jars (55 mm diameter, 72 mm height), plastic film and Parafilm®; growth chambers
3. Isolation medium (agar 7,5 g/L with 30 g/L of ordinary sugar)
4. Seeds. (*Figure 2*)

Figure 2. *Size of the cleaned seed*

Material and Methods

The mother plant was chosen because of its desirable characteristics like good health, no presence of diseases, good form and large quantity of fruits from a tree in the garden of the Faculty of Agronomy and Forestry Sciences, La Plata city. Seed were collected between February and March from dried fruits. The seeds were washed with regular water. The seed coat was removed with forceps and water and then was treated with fungicide for 1-hour by shaking the solution from time to time. After that the coat-free seeds were washed with regular water. Then they were submerged in 70% ethanol for 1 minute and then in 30% commercial bleach plus a drop of Tween 80® for 30 minutes. They were then rinsed three times with sterile distilled water in the laminar-flow hood. Five de-coated seeds were placed in glass bottles filled with a medium where they will germinate and sealed with Parafilm® (*Figure 3*). The glass bottles were placed in a growth chamber at 25°C and a 16-h photoperiod for 15 days to initiate germination.

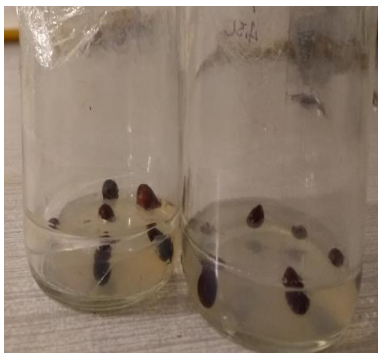


Figure 3. *Seeds in the initial stage in germination medium*

Results

Maintenance of an aseptic condition is a prerequisite for successful *in vitro* seed germination and proliferation. An efficient protocol has been optimized for seed surface sterilization and germination in this study. The germination rate was 80% after 15 days (*Figure 4*) with only a 5% contamination rate.

Some plantlets were acclimatized under greenhouse conditions, in order to check their viability. All of them survived and were transferred to field conditions.

Figure 4: Germinated seeds after two weeks.

Different explants were excised from the vitroplants (cotyledons, hypocotyl and radicle). The explants were cultured on initiation medium with 1,5 mgL^{-1} IBA (indolebutyric acid) and 1 mgL^{-1} BAP (6-benzylaminopurine) in order to induce morphogenic responses. Callus was obtained on this medium after 30 days of culture, first 15 days in darkness and then in light, where it continued growing (*Figure 5*).

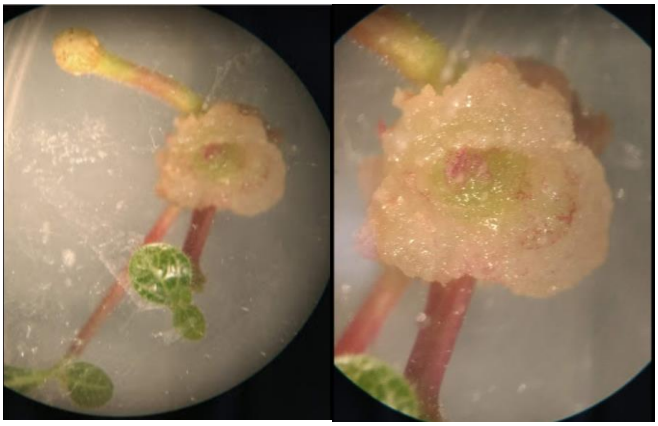


Figure 5*. Callus induction on initiation medium with IBA and BAP.*

Conclusions

In vitro manipulation of tree seeds requires high-quality sterile seedlings as a source of hypocotyl and cotyledon explants for initiation of embryogenic cultures of embryo apexes for shoot production. The present study has shown successful in vitro germination and callus induction from different explants derived from in vitro germinated plants. As the germination time is very long, we are planning to try to shorten it as a first step. Our protocol can successfully be used to sterilize seeds of Brachychiton.

References

Lake M. (2019). Australian Forest Woods: Characteristics, Uses and Identification. CSIRO PUBLISHING.

Ayuntamiento de Murcia (2019). Concejalía del Medio Ambiente y Calidad Urbana. http://www.ayto-murcia.es/medioambiente/parquesyjardines/material/ Brachychiton %20populneus.pdf

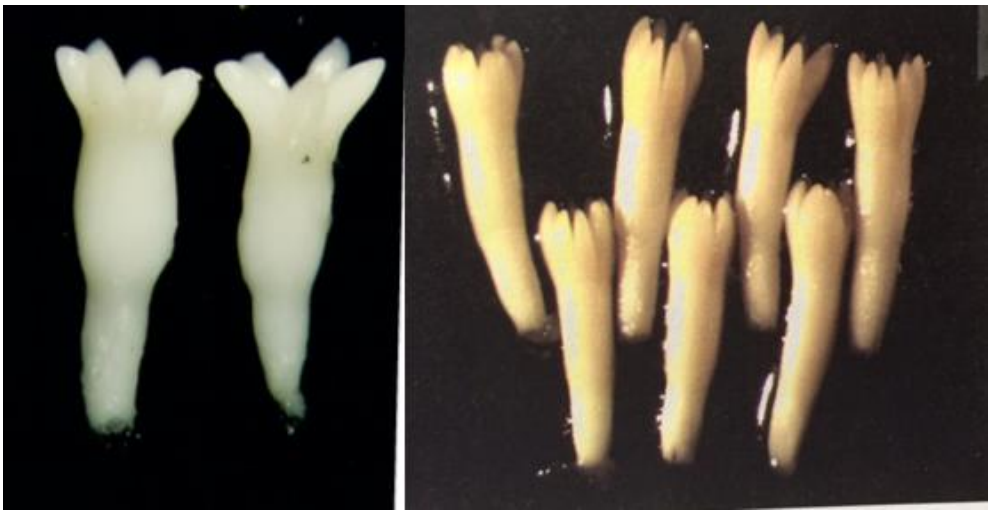
A method of initiation and multiplication by - culture - cycling of somatic polyembryogenesis of Loblolly pine.

Pramod K Gupta

Trees for the future LLC. 29329 3rd Ave SW Federal Way WA 98023 USA,
Pramodgupta.trees@gmail.com, www.treesforthefuture.tech

Good quality zygotic-like somatic embryo (SE) development of pine is a major problem. Industries were not able to commercialize this technology because of this. In pine SE we initiate the cultures from cleaving embryos (standard method) which, inside the seed, are going to degenerate at a later stage. Unfortunately, we cannot initiate cultures from the dominant zygotic embryo when it is fully mature. Most SE cultures of other conifer species are initiated from dominant embryos (*Abie*s sp., Douglas-fir, Spruce sp.). Abrahamsson et al. 2017 suggested that SE of Scots pine, and probably of all Pinus species, if established from cleavage zygotic polyembryos, are at a high risk of becoming problematic. Therefore, we must initiate cultures from dominant zygotic embryos. Culture cycling during multiplication is another problem due to cleavage polyembryony. Cultures do not produce embryos all the time after plating, they decline in embryo yield and finally no embryos are formed, or they are of poor quality. This presentation showed a new method of initiating and possibility of establishing cultures from dominant zygotic embryos, instead of from cleaving polyembryos, by culture cycling during multiplication.

Pine embryo quality is a major problem in somatic embryogenesis. Morphologically and biochemically pine somatic embryos are different from zygotic embryos. This is not a major problem with Spruce, *Abies* species and Douglas-fir. For these there is more focus on scale-up, automation and artificial seed formation but not on somatic embryo quality improvement.



Somatic embryos *Zygotic embryos*

***Figure 1**. Somatic embryos have a swollen hypocotyl and open cotyledons as compared to zygotic embryos*.

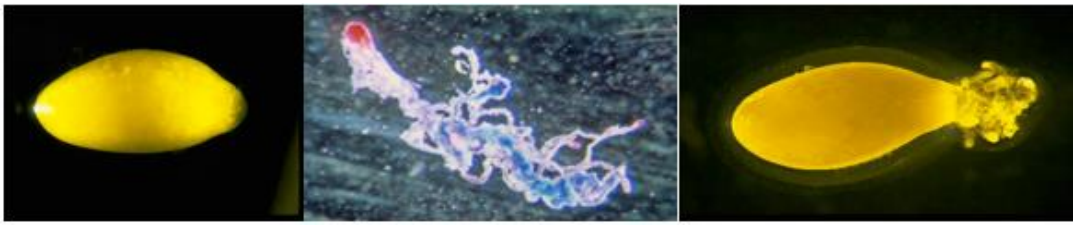


Figure 2. *Standard method of initiation*

Figure 3*. Somatic embryo quality obtained by standard initiation methods*

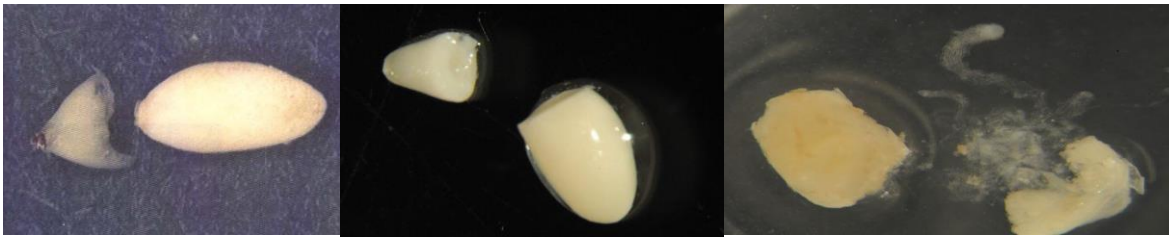


Figure 4. *New method of initiation*

Figure 5*. Somatic embryos obtained by the new initiation method are of better quality (not much swelling of the hypocotyl and with not fully open cotyledons, i.e., still not similar to zygotic embryos but better than embryos produced by cultures initiated by the old method). We also found a 20% increase in germination percentage.*

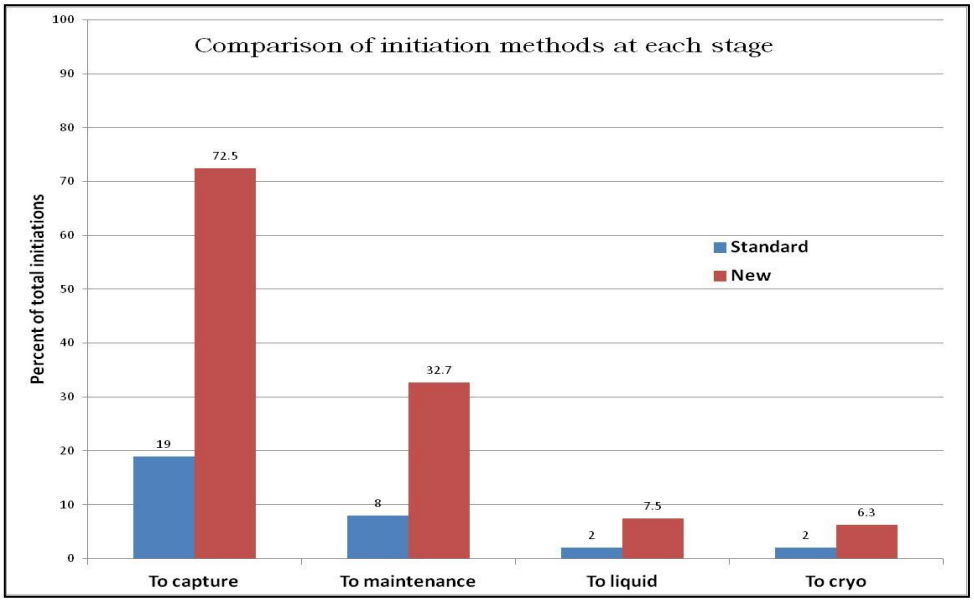


Figure 6. *We found a 3.5X increase in initiation, maintenance, liquid culture establishment and cryostorage with the new initiation method as compared to old standard (whole gametophyte) method.*

Multiplication culture cycling:

Embryo yield and quality declined after longer time maintenance (3-6 months) and also that it fluctuated every 2-3 weeks after plating as shown in Fig. 7

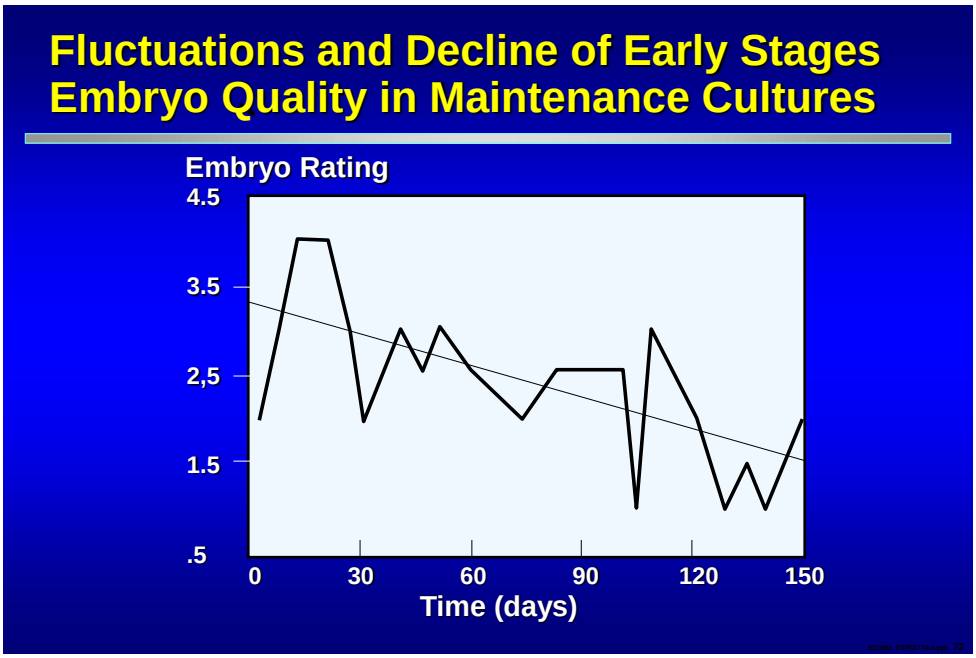


Figure 7A. *Decline in embryo yield, quality and fluctuation due to multiplication via cleavage polyembryony as shown in Fig. 7 B.*

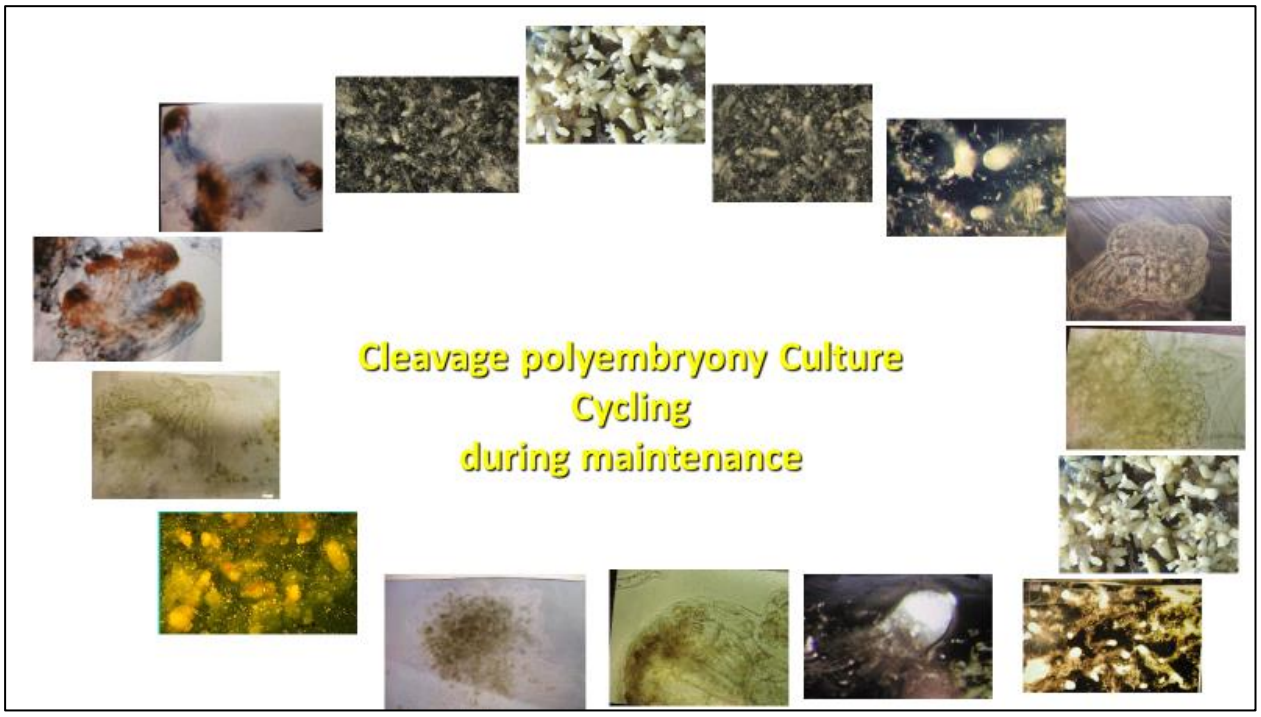


***Figure 7B**. Stressed or cleaving embryonal heads do not produce good quality embryos. Culture observation is very important before plating.*

Embryonal heads get stressed and disorganized after 3 to 4 weeks of subculture. During this time cultures do not produce embryos or produce poor quality embryos. However, with the new method embryonal heads become again organized, without showing stress symptoms, and cultures produce good quality embryos. Embryonal head quality is very important before plating.

Conclusion

1. A new initiation method improved embryo quality.
2. It is possible to initiate pine cultures from dominant zygotic embryos with the new initiation method.
3. By this new method it is possible to avoid the stress that occurs during plating of cultures during the cleavage polyembryony multiplication cycle.

References

Abrahamsson M, Valladares S, Merino I, Larsson E and von Arnold S (2017) Degeneration pattern in somatic embryos of *Pinus* sylvestris L. In Vitro Cell Dev Biol - Plant 53: 86-96

Gupta PK (2016) Method for initiation of somatic embryos of pine.US Patent 9,374,95

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Effects of exogenous phytosulphokine and polyamines in somatic embryogenesis of stone pine

Inmaculada Hernández, Mar Ruiz-Galea, Dolores López-Vela, Mariano Toribio, Cristina Celestino

Departamento de Investigación Agroambiental. Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (IMIDRA). Finca El Encín, Apdo. 127, 28800 Alcalá de Henares-Madrid, Spain. inmaher.sanchez@gmail.com

Keywords: ageing, conifer, embryo development, micropropagation, *Pinus pinea* L.

The stone pine (*Pinus pinea* L.) is a Mediterranean forest species considered as a fruit tree for edible pine nut production (Mutke et al. 2013). Plant regeneration by somatic embryogenesis (SE) from immature megagametophytes with enclosed zygotic embryos was achieved in this species (Carneros et al. 2009, 2017). A general phenomenon observed in conifers is the loss of the maturation ability with increasing age of embryogenic tissue, in relation to a degradation of the normal morphology of immature embryos (Breton et al. 2006). The importance of the suspensor for the normal development of somatic embryos of *Larix leptolepis* was discussed (Umehara et al. 2004). Early embryos carrying suspensor cells in excess of the normal number was observed in embryogenic lines that yielded abnormal cotyledonary embryos (Abrahamsson et al. 2012). Furthermore, a continuous loop of embryo degeneration and differentiation of new embryos was produced (Abrahamsson et al. 2017).

Manipulations of culture conditions early in the process can improve normal somatic embryo production (Klimaszewska et al. 2016). Culture condition could induce modifications of gene expression related with the variation in embryo maturation ability (Lelu-Walter et al. 2016).

The effects of phytosulphokine (PSK) on cell division and embryogenesis were examined in various species (Matsubayashi et al. 1999, Kobayashi et al. 1999). PSK belongs to the group of plant peptide growth factors that are bioactive at nanomolar concentrations (Sauter 2015). The stimulatory effect of PSK in SE was confirmed in *Cryptomeria japonica* (Igasaki et al. 2003) and *Larix leptolepis* (Umehara et al. 2005).

Changes in polyamine (PA) metabolism during SE were reported (Baron and Stasolla 2008). These authors suggested that differences in cellular PA content or ratios could serve as indicators of regeneration-embryogenic potential. An inverse relationship between total content of free PAs and embryogenic potential was reported in *Pinus nigra* (Noceda et al. 2009). An increase in the spermidine (Spd) content relative to that of putrescine (Put) was associated with the development of *Pinus radiata* somatic embryos (Minocha et al. 1999). The effects of exogenous PAs on the formation of somatic embryos have been investigated. Spd added to the maturation media promoted elongation of suspensors and Put increased the production of somatic embryos in *Picea glehnii* (Nakagawa et al. 2011). By contrast, after Put treatment, polyembryogenic complexes did not successfully release single embryos and the frequency of development of malformed embryos was high in *Picea abies* (Vondráková et al. 2015).

In an attempt to recover the ability to produce somatic embryos of three stone pine embryogenic lines that had been subcultured for a long time, cultures were exposed to either PSK during proliferation or different PAs during maturation. Culture morphology and maturation ability of the embryogenic lines were examined.

Embryogenic lines were established following the protocol described by Carneros et al. (2009). Lines were subcultured in darkness at 23ºC for a long-term as clumps on M-mLV medium (M) that consisted of modified LV nutrient medium (Litvay et al. 1985) containing 9.5 µM 2,4-dicholophenoxyacetic acid (2,4-D), 4.5 µM benzylaminopurine (BAP), 20 gl^{-1} sucrose, 4 gl^{-1}gellan gum (Gelrite®, ref. G1910, Sigma-Aldrich, USA). The aged lines showed different maturation ability: line 2F47 showed the best performance, line 5F62 showed low maturation ability and line 1F62 declined over time. In the PSK experiment, individual clumps (0.5 g) were subcultured biweekly on M medium supplemented with 32 nM and 50 nM PSK during 4 weeks in darkness at 23ºC. Solutions of PSK-alpha (SC1465, PolyPeptide Group, France) were filter-sterilized and added to the cooling autoclaved medium. Maturation ability of the embryogenic lines was tested according to the method described in Carneros et al. (2017). Briefly, peripheral parts of embryogenic masses (EMs) were transferred to a preconditioning medium (half-strength mLV nutrient medium, 10 gl^{-1}maltose, 10 gl^{-1}Gelrite and 10 gl^{-1} activated charcoal) for 2 weeks prior to maturation. Then, 50 mg of preconditioned tissue were suspended in liquid M medium lacking PGRs and glutamine and poured onto a filter paper disk placed on a stack of blotting paper. The filter paper disks were maintained under a laminar flow hood to drain the liquid medium and then transferred to maturation medium (ABA-M) that consisted of mLV medium, 121 µM ABA, 60 gl^{-1} maltose and 10 gl^{-1} Gelrite, for 16 weeks in darkness at 28ºC. In the PAs experiment, the effects of the addition of Put, Spd or spermine (Spm) at 100 µM to maturation medium were evaluated. In each medium tested, clumps of line 2F47 maintained onto M medium were transferred onto maturation condition as described above. Solutions of Put (P0927, Duchefa-Biochemie), Spd (S1369, Duchefa-Biochemie) and Spm (S3256, Sigma-Aldrich) were filter-sterilized and added to the cooling autoclaved ABA-M medium. The plates were sealed with paper (3M Micropore™, 1530-0, Germany). Culture morphology was examined under a Nikon SMZ745T stereomicroscope connected to Nikon's Digital Sight DS-L2 Camera Control System. Formation of morphologically normal cotyledonary somatic embryos after maturation conditions were examined as well.

Our results showed that the addition of PSK to the proliferation medium had a slight stimulatory effect on the formation of early somatic embryos in stone pine embryogenic lines (Fig. 1A-F), but complete normal maturation was not obtained. In most of the individual EMs, PSK stimulated cell proliferation, especially in lines 2F47 and 1F62, which showed the best growth, but not its subsequent somatic embryo development. The addition of 32 nM PSK was slightly less effective (Fig. 1B, E) than 50 nM PSK after 4 weeks of treatment (Fig. 1C, F). In an embryogenic line of *Cryptomeria japonica* that was subcultured for more than 2 years, the addition of 32 nM PSK maintained both the ability to proliferate and regenerate (Igasaki et al. 2003). These authors suggested that PSK might take part in the maintenance of cell division and the juvenility of embryogenic cells. When embryo proper lacking a suspensor were treated with 50 nM PSK, cell division of embryo proper and regeneration of the suspensor were stimulated in *Larix leptolepis*, therefore PSK might be a supporting factor for plant embryogenesis (Umehara et al. 2005). We have observed in aged embryogenic lines of *P. pinea* a degeneration pattern that was similar to the one reported in *P. sylvestris*, so that many of the early embryos produced new abnormal early embryos and abnormal late embryos with a cone-shaped embryonal mass and supernumerary suspensor cells (Abrahamsson et al. 2017). Thus according to data reported by these authors, we suggest that aged embryogenic lines of stone pine remain in a persistent polyembryony throughout the maturation process, producing abnormal late embryos with supernumerary suspensor cells and cotyledonary embryos with aborted hypocotyls.

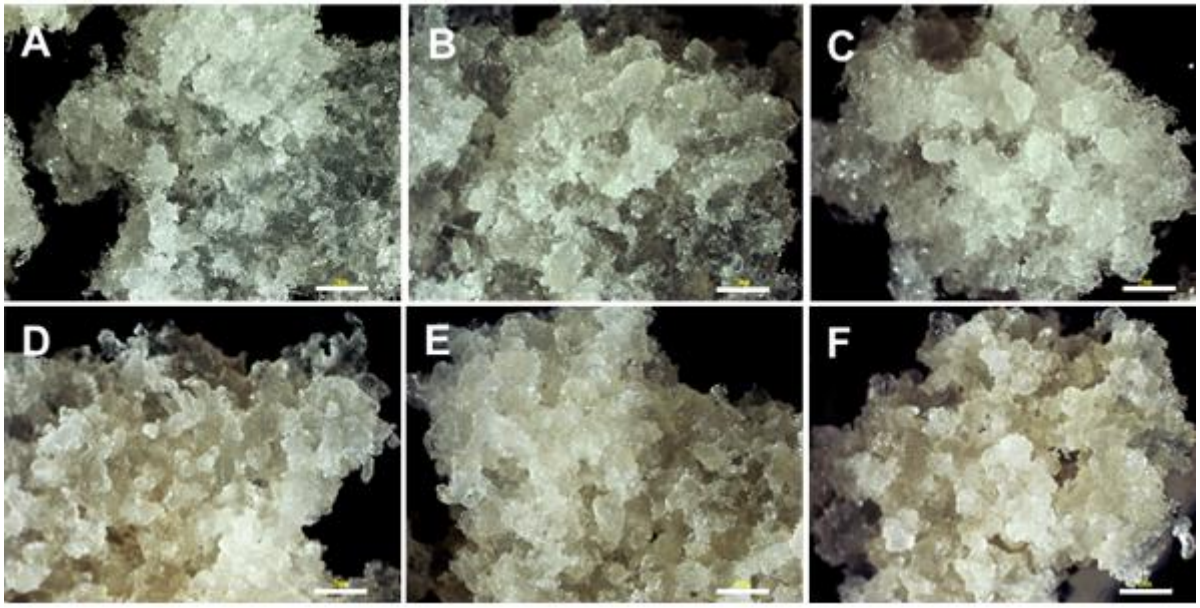


Figure 1. *Effects of PSK added to proliferation medium on the somatic embryo development in aged embryogenic cultures of stone pine, after 4 weeks of culture. (A) Line 2F47 showing early somatic embryos on medium lacking PSK or (B) containing 32 nM PSK or (C) containing 50 nM PSK. (D) Line 1F62 showing early somatic embryos on medium lacking PSK or (E) containing 32 nM PSK or (F) containing 50 nM PSK. Bars=2 mm.*

In the PAs experiments, we found that the addition of Put, Spd or Spm at 100 µM to maturation medium promoted early embryo formation in aged embryogenic cultures, but with abnormal morphology. Line 2F47 showed differentiation of early somatic embryos on maturation medium lacking PAs (Fig. 2A) although this line also developed abnormal mature somatic embryos (Fig. 2B). Exogenous Put slightly reduced culture growth and stimulated the formation of elongated somatic embryos (Fig. 2C), but embryos with embryogenic heads structurally disorganized by the formation of polyembryogenesis were also developed (Fig. 2D). A similar situation was reported in *Picea abies*, showing that meristem cell division and enlargement were stimulated, but single embryos were not rescued from polyembryonic centers and the frequency of development of malformed embryos was high (Vondráková et al. 2015). Spd slightly increased culture growth and stimulated the formation of clusters of early somatic embryos through polyembryogenesis, but normal mature embryos were not developed (Fig. 2E, F). Conversely, the addition of Spd at the same concentration enhanced the formation of somatic embryos in *Picea glehnii* (Nakagawa et al. 2011). In embryogenic cultures of *Pinus sylvestris*, the exogenous Spd retarded cell proliferation and growth but increased embryo-forming capacity (Niemi et al. 2002). The presence of Spm in the maturation medium reduced culture growth and abnormal development of somatic embryos was observed (Fig. 2G, H). Nakagawa et al. (2011) reported that exogenous Spm inhibited the maintenance of bipolar structures in *Picea glehnii*. The thickened embryos obtained in these experiments could resemble those somatic embryos that showed supernumerary suspensor cells in *Pinus sylvestris* (Abrahamsson et al. 2012).

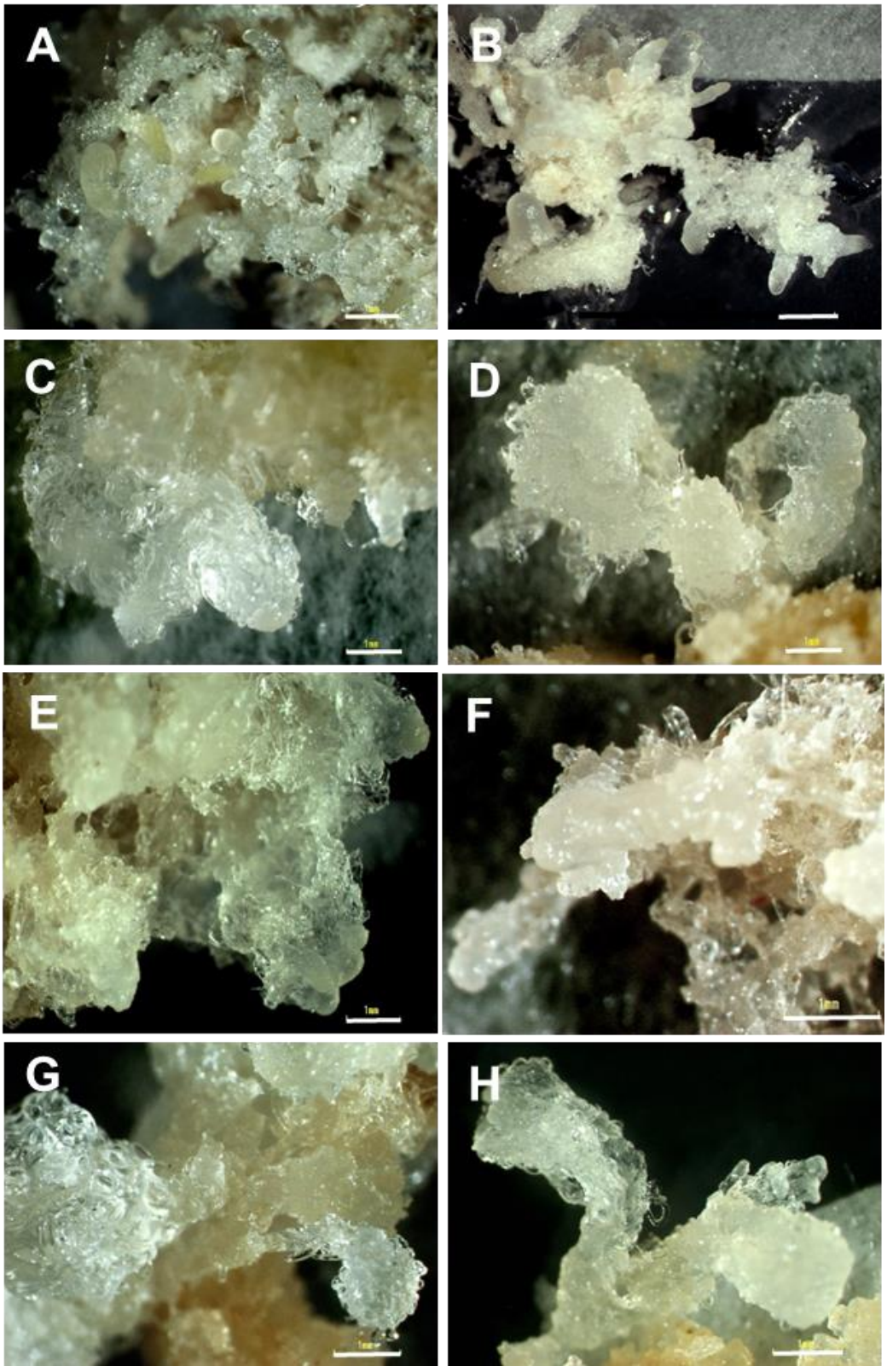


Figure 2. *Effects of PAs added to maturation medium on the somatic embryo development in aged line 2F47. (A) Precotyledonary somatic embryos on medium lacking PAs after 12 and (B) 16 weeks of culture; (C) on medium containing Put after 12 and (D) 16 weeks of culture; (E) on medium containing Spd after 12 and (F) 16 weeks of culture (Bars=2 mm); (G) on medium containing Spm after 12 and (H) 16 weeks of culture. Bars=1 mm.*

In conclusion, the loss of the ability to produce normal mature somatic embryos in the aged cultures of *P. pinea* could not be reversed with the exogenous supply of either PSK during proliferation and PAs during maturation.

Acknowledgments: This work was funded by the Spanish National Project AGL2013-47400-C4-1R.

References

Abrahamsson M, Valladares S, Larsson E, Clapham D, von Arnold S (2012) Patterning during somatic embryogenesis in Scots pine in relation to polar auxin transport and programmed cell death. Plant Cell Tissue Organ Cult 109:391-400. doi:10.1007/s11240-011-0103-8

Abrahamsson M, Valladares S, Merino I, Larsson E, von Arnold S (2017) Degeneration pattern in somatic embryos of *Pinus sylvestris* L. In Vitro Cell Dev Biol-Plant 53:86-96. doi:10.1007/s11627-016-9797-y

Baron K, Stasolla C (2008) The role of polyamines during *in vivo* and *in vitro* development. In Vitro Cell. Dev. Biol.-Plant 44:384-395. doi:10.1007/s11627-008-9176-4

Breton D, Harvengt L, Trontin JF, Bouvet A, Favre JM (2006) Long-term subculture randomly affects morphology and subsequent maturation of early somatic embryos in maritime pine. Plant Cell Tissue Organ Cult 87:95-108. doi:10.1007/s11240-006-9144-9

Carneros E, Celestino C, Klimaszewska K, Park Y-S, Toribio M, Bonga JM (2009) Plant regeneration in Stone pine (*Pinus pinea* L.) by somatic embryogenesis. Plant Cell Tissue Organ Cult 98: 165-178. doi:10.1007/s11240-009-9549-3

Carneros E, Toribio M, Celestino C (2017) Effect of different cryoprotectant procedures on the recovery and maturation ability of cryopreserved *Pinus pinea* embryogenic lines of different ages. In Vitro Cell Dev Biol-Plant 53:469-477. doi:10.1007/s11627-017-9833-6

Igasaki T, Akashi N, Ujino-Ihara T, Matsubayashi Y, SakagamiY, Shinohara K (2003) Phytosulfokine stimulates somatic embryogenesis in *Cryptomeria japonica*. Plant Cell Physiol 44:1412-1416. doi:10.1093/pcp/pcg161

Klimaszewska K, Hargreaves C, Lelu-Walter MA, Trontin JF (2016) Advances in conifer somatic embryogenesis since year 2000. In: Germaná MA, Lambardi M (eds.) In Vitro Embryogenesis in Higher Plants. Methods in Molecular Biology, 1359:131-166. Humana Press, New York. doi:10.1007/978-1-4939-3061-6-7

Kobayashi T, Eun CH, Hanai H, Matsubayashi Y, Sakagami Y, Kamada H (1999) Phytosulphokine-α, a peptidyl plant growth factor, stimulates somatic embryogenesis in carrot. J Exp Bot 50:1123-1128. doi:10.1093/jxb/50.336.1123.

Lelu-Walter MA, Klimaszewska K, Miguel C, Aronen T, Hargreaves C, Teyssier C, Trontin JF (2016) Somatic embryogenesis for more effective breeding and deployment of improved varieties in *Pinus* spp.: bottlenecks and recent advances. In: Loyola V, Ochoa-Alejo N (eds) Somatic embryogenesis: fundamental aspects and applications. Springer International Publisher, Switzerland, pp 319-365. doi:10.1007/978-3-319-33705-0-19

Litvay JD, Verma DC, Johnson MA (1985) Influence of a loblolly pine (*Pinus taeda* L.) culture medium and its components on growth and somatic embryogenesis of the wild carrot (*Daucus carota* L.). Plant Cell Rep 4:325–328. doi:10.1007/BF00269890

Matsubayashi Y, Morita A, Matsunaga E, Furuya A, Hanai N, Sakagami Y (1999) Physiological relationships between auxin, cytokinin, and a peptide growth factor, phytosulfokine-α, in stimulation of asparagus cell proliferation. Planta 207: 559-565. doi:10.1007/s004250050518

Minocha R, Smith DR, Reeves C, Steele KD, Minocha SC (1999) Polyamine levels during the development of zygotic and somatic embryos of *Pinus radiata*. Physiol Plant 105:155-64. doi:10.1034/j.1399-3054.1999.105123.x

Mutke S, Pastor A, Picardo A (2013) Toward a traceability of European pine nuts “from forest to fork”. In: Mutke S, Piqué M, Calama R (eds.). Mediterranean stone pine for agroforestry. Options Méditerranéennes A, 105:105-109. CIHEAM, Zaragoza, Spain. ISBN: 2-85352-508-2

Nakagawa R, Kurushima M, Matsui M, Nakamura R, Kubo T, Funada R (2011) Polyamines promote the development of embryonal-suspensor masses and the formation of somatic embryos in *Picea glehnii.* In Vitro Cell Dev Biol-Plant 47:480-487. doi:10.1007/s11627-011-9366-3

Niemi K, Sarjala T, Chen X, Häggman H. (2002) Spermidine and methylglyoxal bis (guanylhydrazone) affect maturation and endogenous polyamine content of Scots pine embryogenic cultures. J Plant Physiol 159: 1155-1158. doi:10.1078/0176-1617-00634

Noceda C, Salaj T, Pérez M, Viejo M, Cañal MJ, Salaj J, Rodriguez R (2009) DNA demethylation and decrease on free polyamines is associated with the embryogenic capacity of *Pinus nigra* Arn. cell culture. Trees 23:1285-1293. doi:10.1007/s00468-009-0370-8

Sauter M (2015) Phytosulfokine peptide signalling. J Exp Bot 66:5161-5169. doi:10.1093/jxb/erv071

Umehara MS, Ogita H, Sasamoto H, Kamada (2004) An *in vitro* culture system used to investigate possible interactions between the embryo proper and the suspensor in embryogenesis in Japanese larch (*Larix leptolepis* Gordon). Plant Biotechnol 21:169-171. doi:10.5511/plantbiotechnology.21.169

Umehara M, Ogita S, Sasamoto H, Eun CH, Matsubayashi Y, Sakagami Y, Kamada H (2005) Two stimulatory effects of the peptidyl growth factor phytosulfokine during somatic embryogenesis in Japanese larch (*Larix leptolepis* Gordon). Plant Sci 169:901-907. doi: 10.1016/j.plantsci.2005.06.008

Vondráková Z, Eliásová K, Vágner M, Martincová O, Cvikrová M (2015) Exogenous putrescine affects endogenous polyamine levels and the development of *Picea abies* somatic embryos. Plant Growth Regul 75:405-414. doi:10.1007/s10725-014-0001-2

Detection and expression of somatic embryogenesis regulatory genes in *Tilia amurensis, Tilia insularis* and *Tilia mandshurica*

Hae-In Kang, Kyu-Suk Kang

The Department of Forest Sciences, Seoul National University, 08826 Seoul, Korea
emmahi@snu.ac.kr , kangks84@snu.ac.kr

Keywords: gene detecting, gene expression

Introduction

The genus *Tilia* is in high demand as honey producing trees or landscape plants. Somatic embryogenesis could be used for mass propagation of *Tilia* spp. as an alternative propagation system. While previous research regarding somatic embryogenesis has been conducted to develop inducible conditions, there has been no research of the genetics involved in *Tilia* somatic embryogenesis. Hence, it is necessary to define the unknown mechanism of somatic embryogenesis of *Tilia* spp. In this research, we studied whether the induction condition of *T. insularis* and *T. mandshurica* was identical to that of *T. amurensis.* Also, seven genes which were known to be associated with SE were tested for their existence in the *T. amurensis* genome. Subsequently expression of genes detected throughout SE development was measured.

Materials and Methods

Somatic embryogenesis

For the study, immature seeds of *T. amurensis* Rupr., *T. insularis* Nakai and *T. mandshurica* Rupr. *et* Maxim. were collected from a seed orchard. After that, immature embryos dissected from seeds were cultured on MS medium with 1.0 mg/L 2,4-D. Four developmental stages during somatic embryogenesis were defined as described in Kim et al. (2006); globular stage (SEG), heart stage (SEH), torpedo stage (SET), cotyledonary stage (SEC).

Detection of genes

Total RNA was isolated from SEs at different developmental stages and cDNA was synthesized. Seven gene (*PtSERK*, *PtWOX2*, *BBM*, *LEC1*, *WOX4*, *PICKLE*, *VAL1*) primers from references were used in polymerase chain reaction (PCR) and sequencing of amplified DNA fragment was performed (Rupps et al. 2016, Nic-Can et al. 2013, Perez et al. 2015). EST homologies were analyzed with BLAST, querying the NCBI database. To confirm that isolated ESTs were homologous, translated amino acid sequences were aligned with arabidopsis (*Arabidopsis thaliana*), cork oak (*Quercus suber*), poplar (*Populus trichocarpa*) and soybean (*Glycine max*).

Expression analysis

Then gene-specific primers were designed using Primer3 web 4.1.0 and real time qPCR was performed. Relative expression values were calculated and expressed as fold-change using the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001). Changes in gene expression among SE developmental stages were analyzed by analysis of variance (ANOVA).

Results and Discussion

SEs developed to the cotyledonary stage, the final stage of somatic embryogenesis in *T. amurensis* and *T. Insularis*. SEs at the globular stage were induced in *T. mandshurica* (Figure 1).

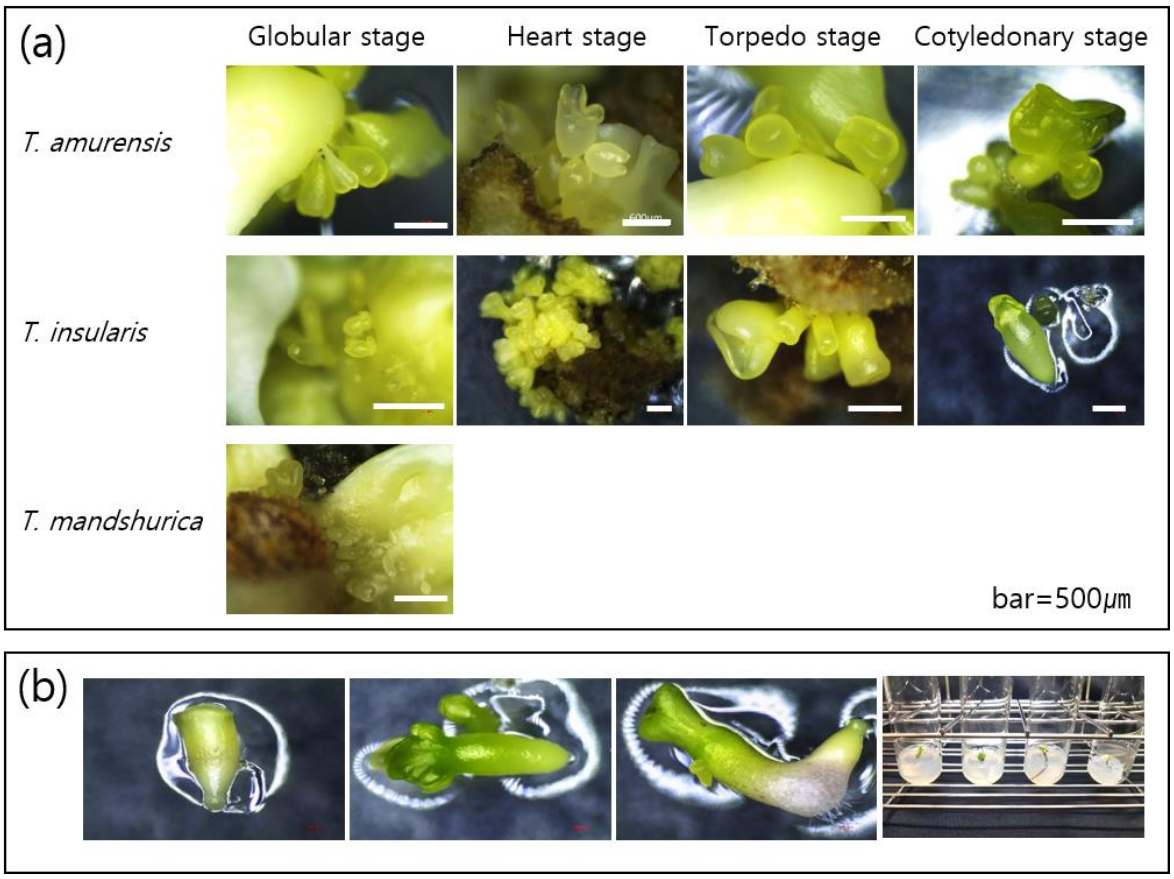


Figure 1. *(a) Somatic embryogenesis stages obtained. (b) Somatic embryos that germinated on plant regeneration medium.*

Partial sequences of putative homologs of *SERK*, *PICKLE* and *VAL1* of *T. amurensis* were identified. The genes were referred to as *pTaSERK*, *pTaPICKLE* and *pTaVAL1*. When translated sequences were compared to those of arabidopsis, poplar, soybean and cork oak, *SERK*, *PICKLE* and *VAL1* showed 100%, 89.4,% and 58.4% similarity, respectively.

The expression of *pTaSERK* was the highest at the heart stage in *T. amurensi*s and *T. insularis*. It increased in early somatic embryogenesis and decreased expression followed. *pTaPICKLE* showed similar expression pattern as SERK in *T. amurensis*, while it showed opposite pattern to *pTaSERK* in *T. insularis*. *pTaVAL1* expressed in *T. amuerensis* showed high expression at hearth stage and cotyledonary stage, while the expressions of all stages in *T. insularis* were lower than the control (Figure 2).

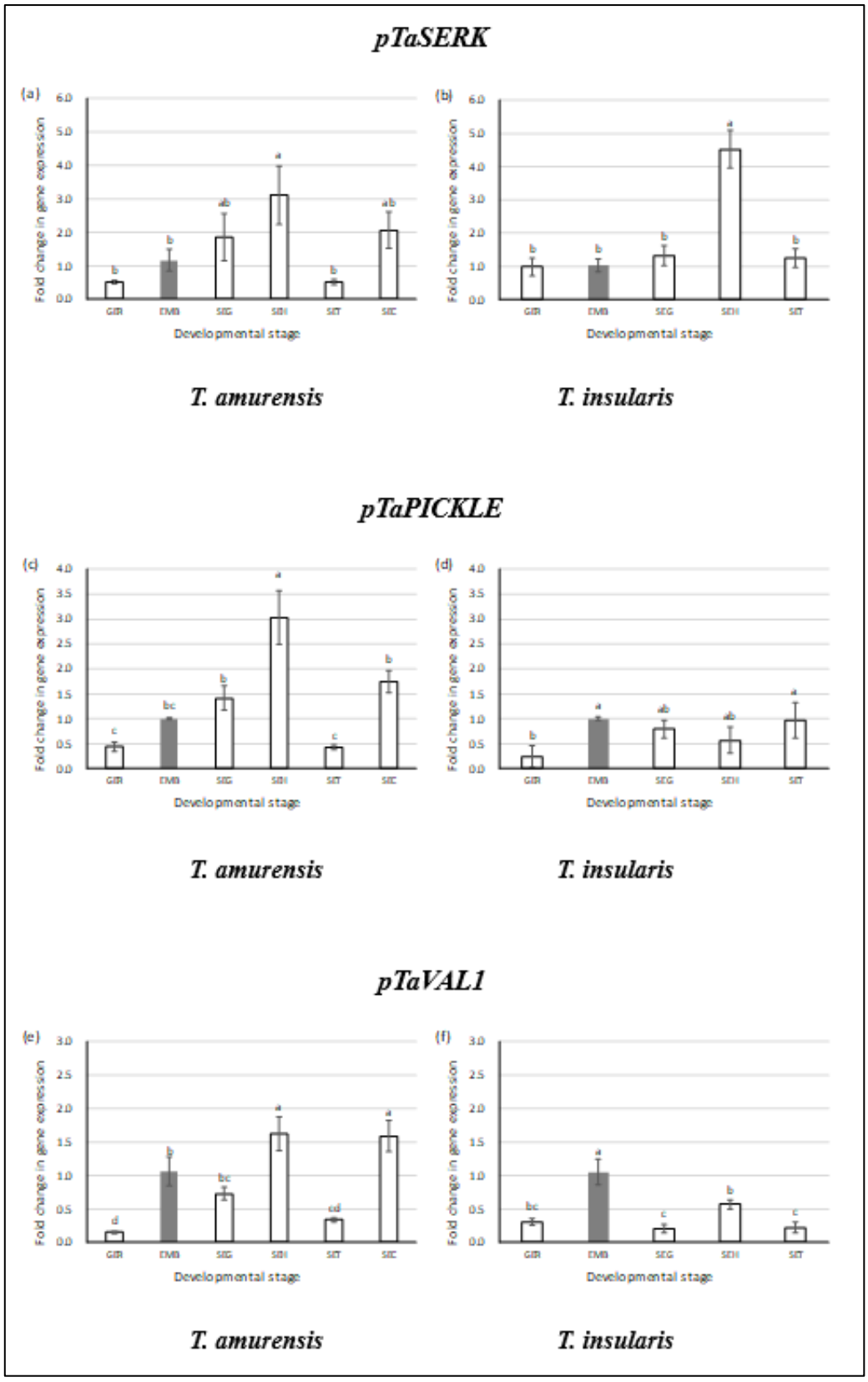


Figure 2. *Relative gene expression of pTaSERK, pTaPICKLE and pTaVAL1 during the somatic embryogenesis of Tilia amurensis and Tilia insulgaris, assessed by real time qPCR. Expression is relative to ACTIN gene and normalized for EMB (in gray). GER, germinated seed; EMB, immature embryo; SEG, globular stage; SEH, heart stage; SET, torpedo stage; SEC, cotyledonary stage.*

References

Kim TD, Choi YE, Lee BS, Kim YJ, Kim TS, Kim IS (2006) Micropropagation of *Tilia amurensis* via repetitive secondary somatic embryogenesis. J Plant Biotech 33: 243-248

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408

Nic-Can GI, Lopez-Torres A, Barredo-Pool F, Wrobel K, Loyola-Vargas VM, Rojas-Herrera R, De-la-Pena C (2013) New insights into somatic embryogenesis: leafy cotyledon1, baby boom1 and WUSCHEL-related homeobox4 are epigenetically regulated in *Coffea canephora*. PLoS One 8, e72160

Perez M, Canal MJ, Toorop PE (2015) Expression analysis of epigenetic and abscisic acid-related genes during maturation of *Quercus suber* somatic embryos. Plant Cell Tissue Organ Cult 121: 353-366

Rupps A, Raschke J, Rummler M, Linke B, Zoglauer K (2016) Identification of putative homologs of *Larix decidua* to BABYBOOM (BBM), LEAFY COTYLEDON1 (LEC1), WUSCHEL-related HOMEOBOX2 (WOX2) and SOMATIC EMBRYOGENESIS RECEPTOR-like KINASE (SERK) during somatic embryogenesis. Planta 243: 473-88

Biochemical characterization of cryopreserved *Pinus radiata* embryogenic tissue

Yenny Lineros [1], Darcy Ríos [2], Ximena Muñoz [1], Claudio Balocchi [1]

[1] Bioforest S.A, Unidad de Biotecnología, Camino a Coronel km 15 s/n, Coronel, Chile.
[2] Laboratorio de Cultivo de Tejidos Vegetales, Centro de Biotecnología, Facultad de Ciencias Forestales, Universidad de Concepción, Barrio Universitario s/n, Concepción, Chile
yenny.lineros@arauco.cl

Keywords: somatic embryogenesis, cryopreservation, proline, dimethyl sulfoxide.

Introduction

Cryopreservation of plants involves the storage of plant tissue in liquid nitrogen at -196 °C or in its liquid nitrogen vapor phase at -136 °C. The main advantage of cryopreservation is that the tissues can be preserved, in principle, indefinitely, since at ultra-low temperatures all metabolic processes are stopped (Kaczmarczyk et al., 2012).

Preserving the viability of plant tissue depends on the ability to minimize the stress caused by cryopreservation (Zeliang and Pattanayak 2012). Before being cryopreserved, embryogenic conifer tissue is treated mainly with a mixture of sorbitol and dimethyl sulfoxide (DMSO) at different concentrations (Hargreaves et al., 2002, Mathur et al., 2003, Carneros et al., 2017). The method most frequently used for this type of tissue is the slow cooling method developed by Whiters and King (1980). This method allows progressive dehydration to the point where the cellular content will not form ice crystals when reaching cryogenic temperatures.

Most cell damage results from the severe dehydration that occurs during freezing. When ice forms in the intercellular spaces, there is a decrease in the water potential outside the cell, which causes a movement of unfrozen water due to the gradient of water and chemical potentials from the inside the cells towards the intercellular spaces (Tomashow 1998).

Dehydration induced by freezing could have a series of effects that produce cell damage, such as the denaturation of proteins and the precipitation of several molecules. Numerous studies have shown that low temperatures induce the accumulation of metabolites, including carbohydrates such as glucose, fructose, maltose, raffinose and amino acids such as proline and glutamine. These metabolites play an important protective role in tolerance to freezing in plant cells (Burrit 2012). The total protein content has been used as a marker of the integrity and functionality of cell membranes after exposure to certain stress events (Martínez-Montero et al., 2002). The objective of our study was to determine if cryopreservation affects stress related metabolites in conifer embryogenic tissue.

Material and methods

The material used in this study came from immature cones of *Pinus radiata* that resulted from controlled crosses carried out in seed orchards owned by Arauco S.A. Forest Company (Chile). These cones were

selected for the induction of embryogenic cell lines. The crossings were performed in 2015 in seed orchards located in the Biobío Region of Chile.

Cryopreservation of the material was performed with a modified slow freezing technique (Hargreaves et al., 2002) using 5 cryoprotective pretreatments with 0.4 M sorbitol as base. The pretreatments were: 5% (v/v) dimethyl sulfoxide (DMSO5); 10% (v/v) dimethyl sulfoxide (DMSO10); 5% (v/v) dimethyl sulfoxide and 0.09 M L-proline (DMSO5P); 10% (v/v) dimethyl sulfoxide and 0.09 M L-proline (DMSO10P); and 0.09 M L-proline (DMSO0P).

A sample of 100 mg of fresh embryogenic tissue of 4 genotypes was taken in the multiplication phase on the same day of cryopreservation (control) and during 4 weeks after growth reactivation. The samples were stored in a freezer at -80 °C until they were processed. The following biochemical parameters were evaluated: total content of soluble carbohydrates (TSCs), total content of proteins and proline.

The content of total soluble sugars was determined using the method of Yemm and Willis (1954) with 15 mg / ml anthrone and 72% sulfuric acid (v / v). For the preparation of the standard curve, 1 mg/ml of glucose was used, dissolved in distilled water. For the extraction of the samples 2 ml of 85% ethanol (v/v) for 12 hours was used. For quantification, 200 µl per sample in 96 well Trueline microplates was used. The quantification was carried out by measuring of the absorbance at 625 nm in a Biotek brand microplate spectrophotometer, model Epoch. The obtained values were contrasted against the glucose standard curve.

For protein extraction, an extraction buffer of 100 mM Tris-HCl at pH 7, 6.5 mM EDTA, 1% (w/v) of PVP, 50 mM NaCl and 0.2% (v/v) of β-Mercapto etanol was used. For the preparation of the standard curve, 1.45 mg / ml of Bovine Serum Albumin (BSA) was used. The samples were shaken on ice during 60 minutes and then centrifuged at 8.000 rpm during 20 min at 4 °C. The supernatant was used for the determination of total soluble proteins with the method of Bradford (1976). For protein quantification, 200 µl per sample in 96 well Trueline microplates was used. The quantification was carried out by measuring of the absorbance at 595 nm in a Biotek brand microplate spectrophotometer, model Epoch. The obtained values were contrasted against the BSA standard curve.

For the proline extraction, 3% sulfosalicylic acid (w/v) was used, then each sample was stirred during 60 minutes and centrifuged at 6.000 rpm during 30 minutes at 10 °C. The supernatant was used for the determination of proline. The determination of proline was carried out by the method of Bates et al. (1973) using 25 mg/ml of acid ninhydrin dissolved in glacial acetic acid and 6 M of phosphoric acid. For the preparation of the standard curve a 40 µM standard of L-proline (Merck) was used. The proline quantification was carried out by measuring the absorbance at 520 nm in a Biotek brand microplate spectrophotometer, model Epoch. The obtained values were contrasted against the proline standard curve.

The experimental design was randomized complete blocks. Each treatment was repeated 3 times with 4 samples by each week of evaluation. The obtained data in quantitative observations were analyzed statistically with an analysis of variance (ANOVA) and a Tukey's multiple comparison test at a confidence level of 95% ($P \leq 0.05$) was performed using the statistical software "Statistica", Data Analysis Software System, version 913, 2015.

Results and Discussion

Total soluble carbohydrates (TSCs)

For TSCs content, significant differences were observed only during the first week of subculture, recovering their initial values from week 2 of subculture (Fig. 1). In general, the same genotype behavior was observed for all cryoprotective pretreatments for which the embryogenic tissue was recovered once thawed. Lipavska and Kondradova (2004), point out that TSCs play an indispensable protective role

under stress conditions. It has been reported for *Pinus* species that glucose is the preferred carbohydrate in the metabolic demands of the species (Treat et al., 1989). In a study in *Picea mariana* and *Picea abies* it was observed that the endogenous levels of sucrose remained constant during the proliferation of embryogenic tissue and a fluctuation in the raffinose contents towards the end of the subculture cycle was observed (Iraqui and Tremblay 2001).

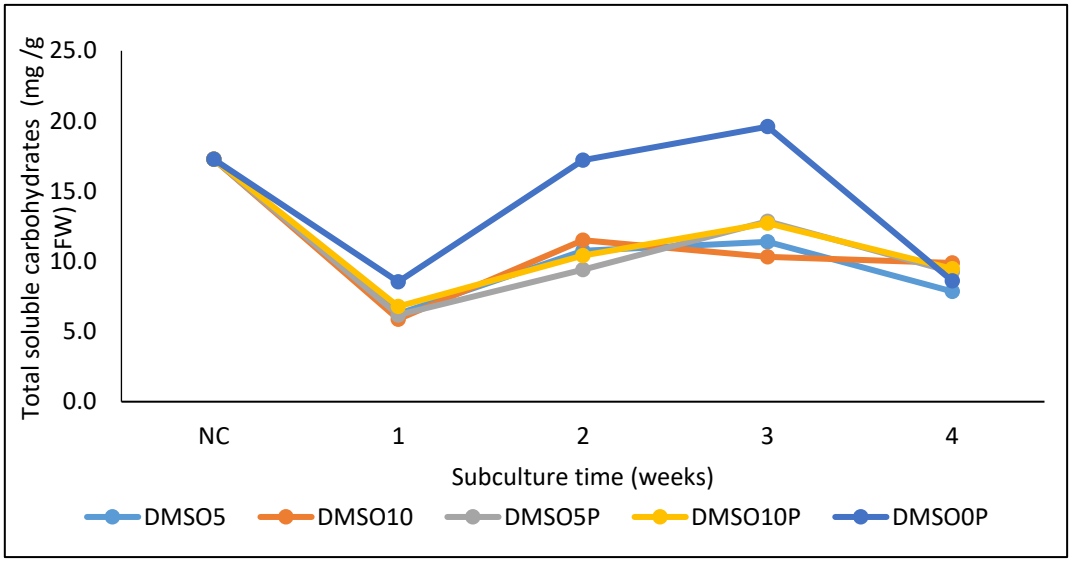


Figure 1. *Total soluble carbohydrates (TSCs) content in embryogenic tissue of 4 P. radiata genotypes after cryopreservation with 5 cryoprotective pretreatments.*

Total protein

The protein quantification results show that no significant differences between the weeks after thawing and the initial values were observed before cryopreservation of the tissue. In Fig.2, the same behavior was observed with all the cryoprotective pretreatments during the entire evaluation period, except for the pretreatment with absence of DMSO (DMSO0P) that showed an increase in week 2. In general terms, all the genotypes reached their values of total basal proteins at week 4 after its growth was reactivated.

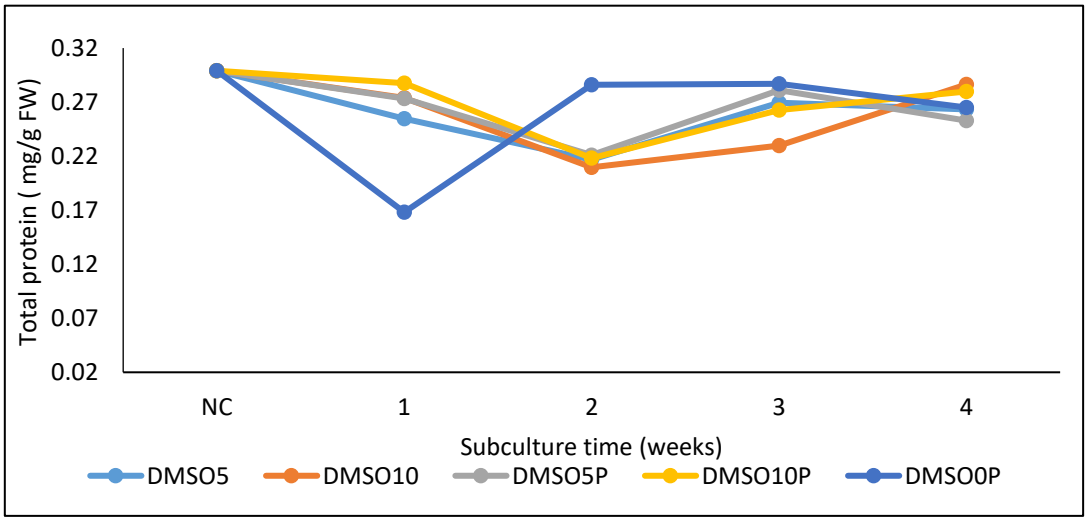


Figure 2. *Total protein content in embryogenic tissue of 4 P. radiata genotypes after cryopreservation with 5 cryoprotective pretreatments.*

Although an increase in total protein content after cryopreservation was expected in this study, as observed by Tomashow (1998), this was not observed in the time periods under evaluation. It is presumed that with the time elapsed between the defrosting of the embryogenic tissue and the first post-cryopreservation evaluation, the tissue was able to recover its initial values. In the study in embryogenic tissue of *Saccharum sp*, Martínez-Montero et al. (2002) identified an increase in the total protein content on the second day after being thawed, observing a gradual decrease until recovering their initial values on the fourth day of subculture, which agrees with the results of this study.

Proline

No significant differences were observed in the proline content related to the initial values. In Fig. 3 it is observed that the content of this amino acid did not have great variations once the tissue was reactivated after being cryopreserved. It was observed for all the genotypes that the content of proline did not have greater variations on either in the evaluated period or among the cryoprotective pretreatments used. The greatest variations were observed in the pretreatment in the absence of DMSO.

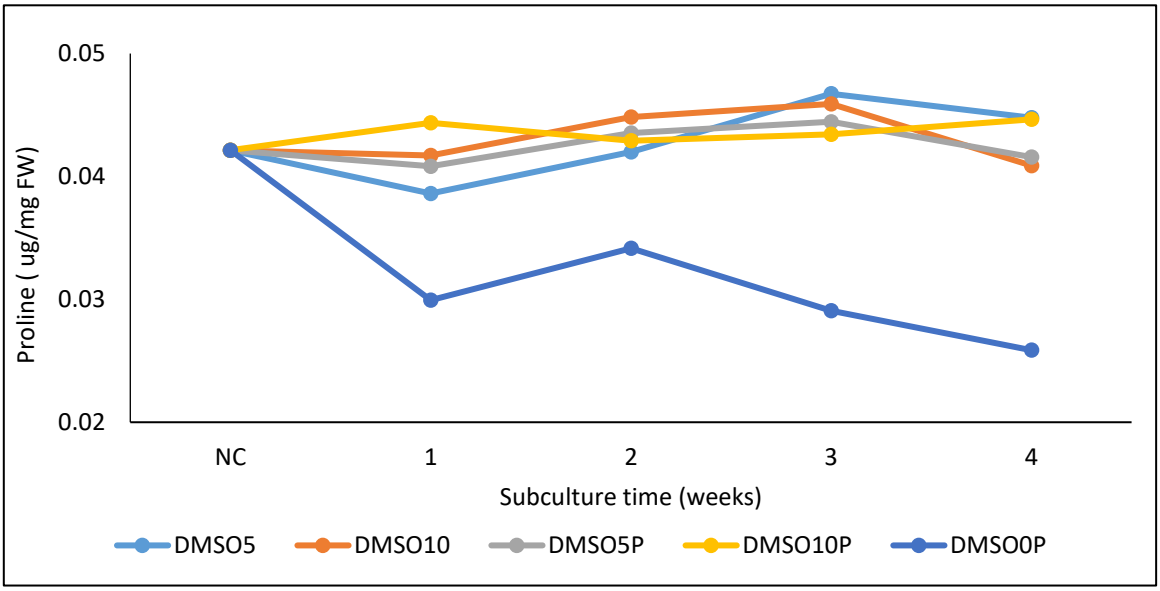


Figure 3. *Proline content in embryogenic tissue of 4 P. radiata genotypes after cryopreservation with 5 cryoprotective pretreatments.*

The exogenous application of L-proline as a supplement in the cryoprotective pretreatments together with sorbitol and DMSO, had no effect on the proline contents of the embryogenic tissue once its growth was reactivated.

The behavior pattern of the biochemical parameters studied in embryogenic tissue of cryopreserved *P. radiata* depends on the genotype, but not on the cryotolerance or the pretreatment used. The genotypes with similar cryotolerance did not present the same biochemical pattern in the different evaluated pretreatments.

We can conclude that despite the effects caused by stress during the process of cryopreservation, it does not significantly affect the primary metabolism, since after the second subculture, once the growth was reactivated, the parameters studied returned to their initial values.

References

Bates L (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39: 205-207

Bradford M (1976) A rapid and sensitive method for the quantitation of micro-gram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72: 248-254

Burrit D (2012) Proline and the cryopreservation of plant tissue: Functions and practical aplications. In: Katkov I (ed), Current frontiers in Cryopreservation. Intech, Croatia. pp 415-430

Carneros E, Hernández I, Toribio M, Díaz-Sala C, Celestino C (2017) Effect of differen cryoprotectant procedures on the recovery and maturation ability of cryopreserved *Pinus pinea* embryogenic lines of different ages. In Vitro Cell Dev Biol - Plant 53: 469-477

Hargreaves C, Grace L, Holden D (2002) Nurse culture for efficient recovery of cryopreserved *Pinus radiata* D. Don embryogenic cell lines. Plant Cell Rep 21: 40-45

Iraqui D, Tremblay F (2001) Analysis of carbohydrate metabolism enzymes and cellular contents of sugar and proteins during spruce somatic embryogenesis suggests a regulatory role of exogenous sucrose in embryo development. J Exp Bot 52: 2301- 2311

Kaczmarczyk A, Funnekotter B, Menon A, Phang P, Al-Hanbali A, Bunn E, Mancera R (2012) Current Issues in Cryopreservation. In: Katkov I (ed) Current Frontiers in Cryobiology. Intech, China, pp 417-438

Lipavska H, Kondradova H (2004) Somatic embryogenesis in conifers: The role of carbohydrate metabolism. In Vitro Cell. Dev Biol - Plant 40: 23-30

Mathur G, Alkutkar V, Nadgauda R (2003) Cryopreservation of embryogenic culture of *Pinus roxburghii*. Biol Plant 46: 205-210

Martínez-Montero M, Mora N, Quiñones J, González-Arnao M, Engelmann F, Lorenzo J (2002) Effect of cryopreservation on the structural and functional integrity of cell membranes of sugarcane (*Saccharum sp*) embryogenic calluses. CryoLetters 23: 237-244

Tomashow M (1998) Role of Cold-Responsive Genes in Plant Freezing Tolerance. Plant Physiol 118: 1–7

Treat W, Engler C, Soltes E (1989) Culture of photomixotropic soybean and pine in a modified fermentor using a novel impeller. Biotechnol Bioengng 34: 1191-1202

Withers L, King P (1980) A simple freezing unit and routine cryopreservation method for plant cell cultures. CryoLetters 1: 213-220

Yemm E, Willis A (1954) The estimation of carbohydrates in plants extract by anthrone. Biochem J 57(3): 508-514

Zeliang P, Pattanayak A (2012) Fundamental Cryobiology and Basic Physical, Thermodynamical and Chemical Aspects of Plant Tissue Cryopreservation. In: Abdurakmonov I (ed) Plant Breeding, IntechOpen, Croatia, pp 41-56

Improving somatic embryogenesis in grapevine

Óscar Martínez[1], Verónica Arjones[1], Yosvanis Acanda[1,3], Cristina Domínguez[1], María Victoria González[4], Manuel Rey[1,2]

[1] Departamento de Biología Vegetal y Ciencia del Suelo, Facultad de Biología, Universidad de Vigo. 36310 Vigo, Spain
[2] CITACA, Agri-Food Research and Transfer Cluster, Campus da Auga, Universidad de Vigo, 32004 Ourense, Spain
[3] Citrus Research Education Center, UF-IFAS, Department of Plant Pathology, 700 Experiment Station Rd., Lake Alfred, FL 33850, USA
[4] Departamento de Biología Funcional, Facultad de Farmacia, Universidad de Santiago de Compostela, Campus Sur, 15872 Santiago de Compostela, Spain
mrey@uvigo.es (Manuel Rey)

Keywords: abscisic acid, histone deacetylation, induction, polyamines, somatic embryo maturation, *Vitis vinifera* L.

Somatic embryogenesis is an excellent system for woody plant propagation. Because somatic embryos have a unicellular origin in many species, including grapevine, it has been used for several purposes, including basic developmental research, mutagenesis, genetic transformation and functional genomics. Although protocols are available for several grapevine cultivars, the application of somatic embryogenesis is still essentially empiric, with several limitations remaining. During the last years, in our lab we have developed specific somatic embryogenesis protocols for some autochthonous grapevine cultivars (Prado et al. 2010; Acanda et al. 2013). However, as a result of this research we have observed that the induction of somatic embryos and their maturation are limiting stages leading to a reduction in the efficiency of regeneration of normal plants.

The induction of embryogenic cultures depends on multiple factors such as genotype, the origin of explants and their developmental stage. Most protocols in grapevine use floral explants for induction. This represents an important temporal limitation because these structures are available only a few days every year. Our goal was to evaluate the possibility of obtaining grapevine somatic embryos in a flowering-independent way. With this goal we tested the effect of two histone deacetylase (HDAC) enzymes inhibitors on the embryogenic competence of different explants.

In addition, asynchrony and precocious germination were often observed during the differentiation and maturation of somatic embryos. These phenomena negatively affect to subsequent plant conversion of germinated embryos (Prado et al. 2010). To solve this problem, we used a semipermeable membrane placed between the culture medium and the embryogenic aggregates during their differentiation.

To test the effect of the application of HDAC enzymes inhibitors, four types of grapevine (cv. Mencía) explants (somatic embryos at the cotyledonary stage, germinated somatic embryos, and shoot tips and leaves from micropropagated plants) were cultured in induction SEIM4 medium (Acanda et al. 2013) supplemented with different concentrations of HDAC enzymes inhibitors: sodium butyrate (NaB; 0.5 mM, 2 mM or 5 mM) and Trichostatin A (TSA; 0.5 µM, 2 µM or 5 µM). Embryogenic responses were recorded after eight weeks of culture.

Treatment with 0.5 mM NaB significantly increased the embryogenic response when cotyledonary somatic embryos were used as explants (Table 1). This treatment also produced the overexpression (Martínez 2018) of genes encoding HDAC enzymes, as well as others related to the embryogenic competence (VvSERK2 and VvWOX2). The NaB also increased slightly the embryogenic response when germinated somatic embryos were used (no significant difference over the control, Table 1). In contrast, all the TSA treatments failed in increasing the embryogenic response (Table 1). Although further research is needed, these results open the possibility to obtain somatic embryos in grapevine in a flowering-independent way.

Table 1. *Effect of the application of inhibitors of histone deacetylase enzymes (NaB and TSA) on the embryogenic response of cotyledonary and germinated grapevine somatic embryos. Percentage of explants with embryogenic response (mean ± standard error) after 8 weeks of culture in SEIM4 medium containing each of the indicated inhibitor treatments was represented. Different letters indicate significant differences ($p < 0.05$) in columns.*

	Embryogenic response (%) from somatic embryos	
Treatment	Cotyledonary	Germinated
SEIM 4 (control)	7 ± 3 bc	9 ± 7
0.5 mM NaB	30 ± 7 a	16 ± 6
2 mM NaB	10 ± 3 b	18 ± 8
5 mM NaB	0 c	6 ± 6
0.5 µM TSA	3 ± 1 bc	5 ± 3
2 µM TSA	2 ± 1 c	0
5 µM TSA	1 ± 1 c	3 ± 3

The effect of a semipermeable membrane on the maturation of grapevine somatic embryos was tested using grapevine (cv. Mencía) embryogenic aggregates induced and maintained in SEIM4 medium (Acanda et al. 2013). To test the membranes, embryogenic aggregates were transferred to differentiation DM1 medium (Prado et al. 2010) supplemented with 6 % of sucrose and cultured on a cellulose-acetate semipermeable membrane placed over the culture medium for five weeks. The relative water content and the abscisic acid endogenous content of the somatic embryos were recorded over the time of culture in those conditions (Acanda 2015). At the end of the experiment a histological analysis, using calcofluor white, was performed and single somatic embryos were collected to test their germination ability using our standard protocols (Prado et al. 2010; Acanda 2015; Martínez 2018). Somatic embryos cultured in the same DM1 medium but without the membrane were used as controls.

The use of the semipermeable membrane during the differentiation process allowed to significantly improve the maturation of somatic embryos as shown by the histological analyses (Figure 1), avoiding precocious germination and increasing the subsequent germination rates (22 %) in respect to the control somatic embryos (1 %) cultured without the membrane. The somatic embryos cultured on the membrane showed a reduction in their water content (Figure 2) but a significant increase in their endogenous ABA content, mainly after two weeks of culture (Figure 3). The results suggested an overall effect of the membrane on the water state of the somatic embryos and a relationship with their ABA content, which could help to explain the observed improvement of the maturation of the somatic embryos.

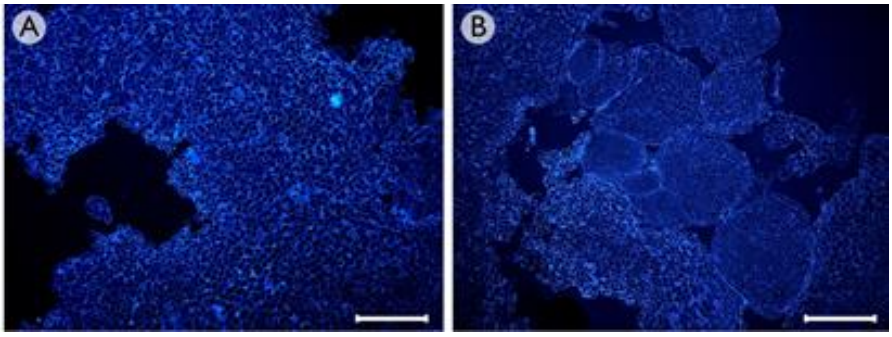


Figure 1. *Histological analysis of grapevine embryogenic aggregates obtained after 5 weeks of culture in DM1 medium without (A) or with a semipermeable membrane (B). Proliferation of globular somatic embryos are shown in (B) but not in (A). Sections were stained with calcofluor white. Bars = 200 µm.*

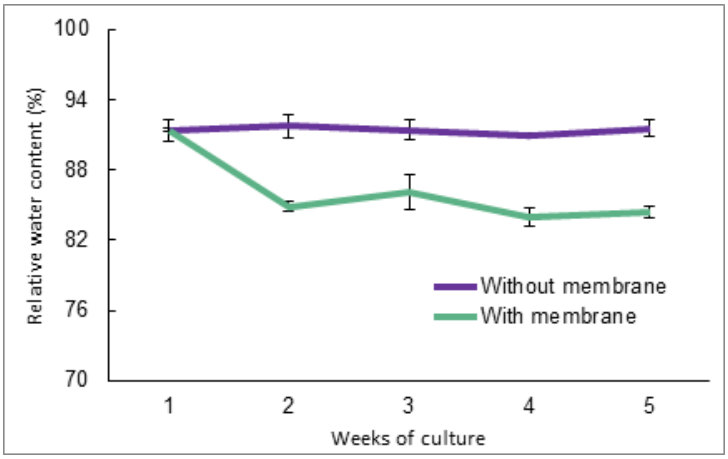


Figure 2. *Relative water content (mean ± standard error) of grapevine embryogenic aggregates cultured during 5 weeks on DM1 differentiation medium supplemented with 6 % sucrose and on a semipermeable membrane.*

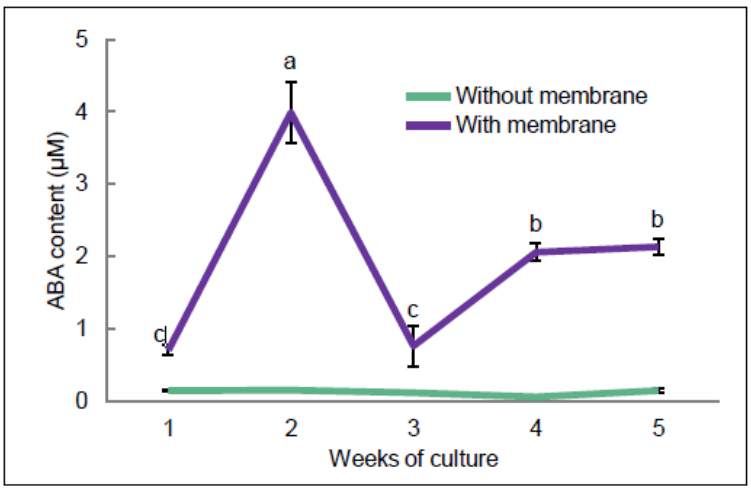


Figure 3. *Endogenous ABA concentrations (mean ± SE) of grapevine embryogenic aggregates cultured during 5 weeks on DM1 differentiation medium supplemented with 6 % sucrose on a semipermeable membrane. Different letters indicate significant differences among times of culture ($p < 0.05$).*

Furthermore, expression analysis carried out during the differentiation of the somatic embryos showed a complex regulation of ABA and polyamine metabolism and a potential interaction between their pathways (Martínez 2018). Differential expression of biosynthetic (*VvNCED1*; *VvBG2*) and catabolic (*VvHyd2*; *VvUGT*) ABA-related genes appeared related to maintain a steady-state ABA level. In addition, biosynthetic polyamine genes *VvADC* and, mostly in cultures over membrane *VvSAMDC*, were overexpressed during the differentiation of the embryogenic aggregates.

Acknowledgements: This research was partially funded by the Spanish Ministry of Economy and Competitivity (grant AGL2009-07488). Óscar Martínez thanks the Spanish Ministry of Education, Knowledge and Sport for the support with a FPU scholarship. Yosvanis Acanda thanks the Spanish Ministry of Foreign Affairs and Cooperation for support with a MAEC-AECID scholarship. The authors thanks to María José Graña and Julián Benéitez for their help during plant material collection at the "Centro de Formación y Experimentación de Viticultura y Enología de Ribadumia" (Pontevedra, Spain). This research is a contribution of the Interuniversity Research Group in Biotechnology and Reproductive Biology of Woody Plants (BioVitAc Research group, code 09IDI1705).

References

Acanda Y, Prado MJ, González MV, Rey M (2013) Somatic embryogenesis from stamen filaments in grapevine (*Vitis vinifera* L. cv. Mencía): changes in ploidy level and nuclear DNA content. In Vitro Cell Dev Biol Plant 49:276-284

Acanda Y (2015) Somatic Embryogenesis in Grapevine: Control of Somatic Embryo Maturation and Generation of New Variability *in vitro*. Ph.D. Thesis. Universidad de Vigo, Spain

Martínez O (2018) Molecular Analysis of the Regulation of Somatic Embryo Maturation in Grapevine (*Vitis vinifera* L.). Ph.D. Thesis. Universidad de Vigo, Spain

Prado MJ, Grueiro MP, González MV, Testillano PS, Domínguez C, López M, Rey M (2010) Efficient plant regeneration through somatic embryogenesis from anthers and ovaries of six autochthonous grapevine cultivars from Galicia (Spain). Sci Hortic 125:342-352

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Strawberry tree (*Arbutus unedo* L.) breeding: hybridization and polyploidization

João F. Martins[1], Glória Pinto[2], Jorge M. Canhoto[1]

[1] *Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, Portugal.*
[2] *CESAM, Department of Life Sciences, University of Aveiro, Portugal*
joao.martins@uc.pt

Keywords: breeding, hybridization, micropropagation, pollen, polyploids

Strawberry tree (*Arbutus unedo*, Ericaceae) is a small tree growing around the Mediterranean basin and Atlantic coast, including in Portugal. It is well adapted to stress conditions and shows a great regenerative capacity following forest fires making it an interesting species for reforestation programs. Due to its ability to prosper in poor marginal soils and its fairly tolerance to common pathogens, strawberry tree has been seen as a good alternative for farmers due to its potential for fruit production as well as being an ornamental. During the last years a considerable effort has been carried out in order to convert strawberry tree into a species more interesting for fruit production through the selection and propagation of selected trees by *in vitro* culture methods and hybridization assays in order to obtain different genetic combinations. Polyploidization experiments can also be extremely helpful as the polyploid plants can have enhanced characteristics, such as bigger organs and fruits and improved tolerance to biotic and abiotic stresses. The objective of this work was the induction of polyploid plants, as well as achieving a better understanding of the pollination mechanisms of the strawberry tree and obtaining hybrid plants.

Several phenotypes were selected from wild strawberry tree populations, based on fruit production. These plants were established *in vitro* and propagated through different *in vitro* culture techniques, such as axillary shot proliferation and organogenesis on Andersson medium supplemented with 2mgL^{-1} benzylaminopurine (Martins et al. 2019). For tetraploid induction 300 *in vitro* shoots (2n) from 3 different genotypes were submitted to a treatment with two c-mitotic agents (colchicine and oryzalin), for different periods of time and at different concentrations. The survival rate was close to 20% and plant ploidy was evaluated by flow cytometry. Samples were prepared with WPB extraction buffer and nuclei were stained with propidium iodide. *Solanum lycopersicum* was used as a control. 15 of the induced shoots (Fig. 1A) were found to be mixoploid but only 3 tetraploid plants were produced, a conversion rate of 1%. Moreover, all the mixoploid plants reverted to its initial ploidy level.

The strawberry tree phenological cycle is very long. The flower buds arise during summer, and are pollinated in autumn and early winter. The fruits take almost a year to develop and ripen the next autumn. For this reason, flowers and fruits can be found on the tree at the same time, making it an attractive ornamental. The flowers are grouped on hanging panicles, and the bell-shaped flowers contain 10 anthers, with two whiskers each, and a penta-loculated ovary with numerous ovules. A morphological study of the pollen was carried out, as well as germination tests. Strawberry tree pollen grains are grouped on tetrahedral tetrads, with a medium size and a psilated surface. They have a long and narrow *colpus* with a granulated membrane on contiguous pollen grains, and a circular pore. Pollen can be easily germinated on a medium with H_3BO_3, $CaCl_2$, KNO_3 and a carbon source. Different carbon sources were tested and best results are achieved with 15% sucrose. Fructose was found to inhibit pollen germination. The effect of different plant growth regulators was also tested on strawberry tree pollen germination, such as: Indol-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA) and gibberellic acid (GA_3). Both

IBA and NAA completely inhibit pollen germination at higher concentrations. On the other hand, GA_3 greatly promotes pollen germination and pollen tube elongation, making it a potential pollen extender for artificial pollination on orchards, increasing fruit production.

Controlled *in vitro* and field pollinations were used to understand the pollination process and to obtain hybrids between selected trees. No auto-incompatibility barriers were found on the *in vitro* assays and some plantlets from the controlled crosses have been obtained following seed germination. The new genetic combinations (Fig. 1B) arising from these crosses will be screened for stress tolerance and propagated by *in vitro* culture techniques. Finally, the selected genotypes will be transferred to the field for phenotype evaluation and productivity performance.

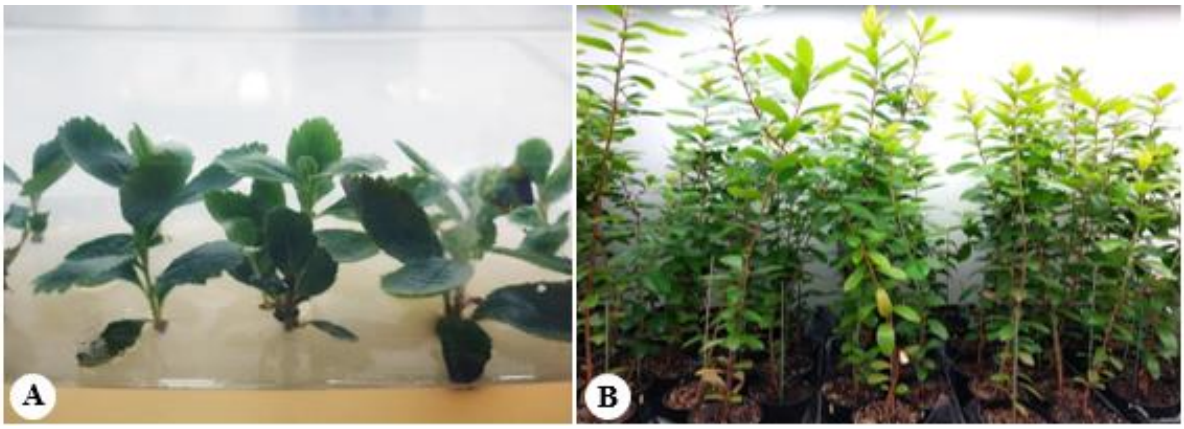


Figure 1. *Micropropagated strawberry tree plants after treatment with c-mitotic agents. Hybrid plants obtained in the field from controlled crosses between selected plants.*

Acknowledgements: Foundation for Science and Technology (Portugal) supported J. Martins PhD Fellowship (SFRH/BD/122478/2016), G. Pinto (SFRH/BPD/101669/2014) and J. Canhoto (PTDC/AGR-FOR/3746/2012). The authors also acknowledge CESAM within PT2020 Partnership Agreement and Compete 2020 (UID/AMB/50017/2013-POCI-01-0145-FEDER-007638) and ReNATURE project (CENTRO-01-0145-FEDER-000007).

References

Martins J, Correia B, Correia S, Pinto G, Canhoto J (2019) Shoot proliferation and organogenesis on *Arbutus unedo*: physiological analysis under water stress. Biol Plant 63: 278-286

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

The ups and downs of developing hybrid sweetgum varieties for the U.S. bioenergy and pulp and paper industries: a 20-year case study

Scott Merkle

Warnell School of Forestry and Natural Resources, University of Georgia, Athens, GA, USA
smerkle@uga.edu

Keywords: *Liquidambar styraciflua*, *Liquidambar formosana*, hybrid breeding, somatic embryogenesis, cryostorage

The southeastern U.S. is well-known as a prime location for plantation-based forestry, possessing millions of hectares of plantations for production of timber and paper products. The state of Georgia alone has almost 9 million hectares of commercial forest land, with forest industries bringing $35 billion into the state annually. The vast majority of forest plantations in the U.S. southeast are southern pines (*Pinus taeda* and *Pinus elliottii*). In fact, since prices for pine in the region have traditionally been significantly higher than prices for hardwood, and the supply of hardwoods from natural stands has been plentiful, little investment in hardwood plantations has occurred. This situation has been changing over the past ten years, as demand for hardwood fiber has risen, so that currently, the price for hardwood fiber in the region equals or slightly exceeds that of pine fiber. Thus, the case for purpose-grown hardwoods in the southeastern U.S. is becoming stronger, in particular in areas where a reliable supply of hardwood fiber is needed year-round, since purpose-grown trees have the ability to stabilize wood costs and provide predictable price caps for mills. The region is home to several fast-growing hardwood species, e.g. yellow-poplar (*Liriodendron tulipifera*), American sycamore (*Platanus occidentalis*), sweetgum (*Liquidambar styraciflua*) and eastern cottonwood (*Populus deltoides*). Sweetgum plantations, first established in the southern U.S. in the 1960s, experienced some limitations that have largely been overcome with the development of improved planting stock (Wright and Cunningham 2008). However, overall, the lack of investment in developing hardwood resources means that genetic improvement programs to develop elite families or clones of these hardwood species have lagged.

Two tools that could be applied to accelerate genetic improvement of hardwoods in the southeastern U.S. are hybrid breeding and clonal forestry, including such propagation techniques as somatic embryogenesis. The application of hybrid breeding to forest trees has resulted in very useful and productive genotypes for some forest crops. Notable successes include hybrid poplars (e.g. *Populus trichocarpa* x *Populus deltoides*) and hybrid Eucalyptus (e.g. *E. urograndis*). However, these hybrids have not been shown to be suitable for plantation forestry in the southeastern U.S. In fact, neither hybrid breeding nor clonal forestry have been applied commercially in the region. Somatic embryogenesis, which at one time appeared to have great potential for clonal propagation of elite southern pine genotypes or "varietals", has yet to attain a significant percentage of southern pine annual regeneration. However, embryogenic culture systems have been developed for multiple southern hardwoods, and this approach may actually be much more amenable to commercial scale-up for hardwoods than for pines.

The combination of SE technology with hybrid breeding generates a very powerful approach that could change the landscape of hardwood forestry in the southeastern U.S. Two examples of southern hardwoods with great potential for improvement via hybrid breeding and that can be mass propagated by SE are sweetgum and yellow-poplar. Both trees grow rapidly up to 65 m tall, with sweetgum making

its best development on bottomlands and yellow-poplar performing best on upland sites. Of the two species, sweetgum has become more important to the region's pulp and paper industry, with increasing quantities of fiber from the tree incorporated into pulp mixes over the past 20 years. Each of these species has an Asian counterpart, from which it has been separated for at least 10 million years, and with which it remains interfertile. *Liquidambar formosana* is native to Taiwan and China. Santamour (1972a) reported the first interspecific *Liquidambar* hybrids. Similarly, *Liriodendron chinense* is native to China and other parts of southeastern Asia. *Liriodendron* interspecific hybrids were described by Santamour (1972b) and Parks et al. (1983) reported heterotic growth rates for some of the hybrid trees. Furthermore, potentially scalable SE-based propagation systems have been reported for both *L. tulipifera* (Merkle et al. 1990) and *L. styraciflua* (Merkle et al. 1998). Thus, all the components were in place by 1998 to test the potential of combining hybrid breeding and SE to produce elite clones of these two genera. We began a project in cooperation with International Paper Co., Weyerhaeuser Corp. and the Consortium for Plant Biotechnology Research in 1999 to generate hybrid *Liriodendron* and hybrid *Liquidambar* clones using SE. Since, this paper focuses on the hybrid *Liquidambar* variety story, I will not discuss the hybrid *Liriodendron* research further here. Details on that research can be found in Merkle et al. (1993) and Dai et al. (2004).

To incorporate the use of embryogenic cultures for clonal propagation of elite hybrid genotypes, our approach to producing the hybrid clones is reversed from the usual sequence of steps. Usually, horticultural breeders perform the hybrid crosses, plant out the hybrid seedlings for evaluation, and choose the best performers to clonally multiply to create a hybrid cultivar. In our approach, which emulates that used by companies such as ArborGen Inc. and CellFor Inc. for pine varietal production, we initiated embryogenic cultures from the hybrid seeds and cryostored copies of every hybrid culture while somatic seedlings from those cultures were evaluated in the field. Then, based on the performance of the somatic seedlings in field tests, we recovered from cryostorage those cultures from which the very best performing somatic seedlings were derived and scale up production of those clones using SE to create elite varieties. Specifically, to generate hybrid sweetgum varieties, pollen was collected from three mature *L. formosana* trees growing in a U.S. Forest Service demonstration planting near Saucier, MS in March 1999 and used by International Paper (IP) Co. breeders to conduct controlled pollinations with three *L. styraciflua* selections from the N.C. State Hardwood Program at IP's Southlands Experiment Forest near Bainbridge, GA, for a total of nine crosses. Immature fruit were collected from the *L. styraciflua* mother trees in June and July and shipped to the Merkle lab at UGA, where the immature seeds were used for culture initiation. Immature fruits were surface-disinfested using a sequence that included Clorox; then the fruits were dissected and immature seeds removed, nicked with a scalpel blade and cultured on a semi-solid induction-maintenance medium (IMM), which was a modified Blaydes (Witham et al. 1971) medium that included 2 mg/L 2,4-D (Vendrame et al. 2001). A second round of hybrid breeding and culturing was conducted in 2005. Seed explants were transferred to fresh medium after one month and cultures were evaluated for evidence of SE after another month.

Approximately 2% of the cultured seeds produced proembryogenic masses (PEMs; Fig. 1A) within 2 months following culture initiation. PEMs could be maintained by monthly transfer to fresh medium and grew more rapidly once inoculated into liquid IMM to produce embryogenic suspension cultures. Suspension cultures were size-fractionated on stainless steel sieves, and a selected size fraction between 38 µm and 140 µm, collected on filter paper using a Büchner funnel, produced up to 6000 synchronously developing somatic embryos per 0.5 g of PEMs (Fig. 1B) within 2 months following plating on semi-solid basal medium, which was the same as IMM but lacking 2,4-D (Dai et al. 2004). Eighty to 90 percent conversion of the somatic embryos to somatic seedlings could be obtained following an 8-week pre-germination cold treatment at 10º C (Merkle et al. 2010). Recently, further improvements in PEM production were obtained using air-lift bioreactors, which produced up to twice the volume of PEMs as shaken flasks within 6 weeks (Lu and Merkle, in preparation). Early growth of somatic seedlings was accelerated using RITA® temporary-immersion bioreactors (Lu and Merkle, in preparation). Several hundred hybrid sweetgum somatic seedlings, representing multiple varieties, were produced, transferred to potting mix and planted in field tests in different locations in the southeastern U.S. by IP and later by ArborGen collaborators over the past 10 years. The hybrid trees can be distinguished from the parent

species by their leaf shape, which is intermediate between the five-lobed *L. styraciflua* leaf and the three-lobed *L. formosana* leaf. In addition, embryogenic cultures and trees derived from them were verified to be hybrids using RAPDs (Vendrame et al. 2001).

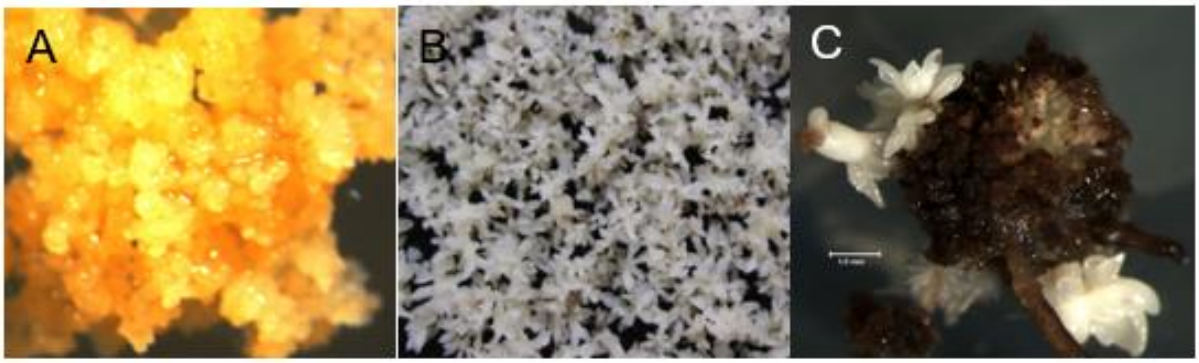


Figure 1. *Hybrid sweetgum somatic embryogenesis.* ***A.*** *Proembryogenic masses derived from hybrid seed explants.* ***B.*** *Embryogenic cultures produced thousands of hybrid sweetgum somatic embryos.* ***C.*** *Somatic embryos emerging from somatic tissue of a staminate inflorescence explant.*

The hybrid somatic seedlings produced displayed a range of growth rates and habits in field tests on multiple sites. In the oldest planting, established in 2002 on IP property near Aiken, SC, a small number of the varieties showed faster growth rates than elite native sweetgum genotypes, as well as significantly higher wood specific gravity. For example, one variety in the test had a DBH of over 26 cm and a height of 21 m at age 12 (Fig. 2A), with a wood specific gravity of almost 0.55, compared to an average DBH of 18.5 cm, height of 18 m and specific gravity of 0.45 for native American sweetgum seedlings. This variety and two others displaying similar performance were selected by ArborGen for commercial production. Other hybrid varieties showed potential for use as landscape trees and ornamentals, with dwarf phenotypes and striking purple fall leaf color. The second round of breeding and culturing in 2005 resulted in the production of another eight hybrid varieties, which were planted in field tests at four locations in 2008. One of the tested varieties showed superior growth rates at all four sites and so was selected as a fourth commercial variety.

Copies of the hybrid cultures were cryostored following the protocol of Vendrame et al. (2001) with the intention of thawing and scaling up production from those cultures that produced the best trees, based on the results of the field tests. However, the desired varieties could not be successfully re-grown following recovery from cryostorage. Fortunately, we had previously optimized a protocol for initiation of embryogenic cultures from mature sweetgum trees using inflorescence tissues as explants. Briefly, we found that both staminate and pistillate inflorescences, collected from fully dormant buds of mature sweetgum trees and cultured on medium with NAA or thidiazuron, or even on basal medium, could produce repetitively embryogenic cultures or PEMs (Fig. 1C), while leaves from the same buds could not be induced to do so (Merkle et al. 1998, Merkle and Battle 2000). These cultures, which arose from flower parts as well as inflorescence axes, were of somatic tissue origin and thus were diploid rather than haploid. Thus we were able to use this protocol to start new embryogenic cultures from the top hybrid varieties growing in the Aiken, SC field test by collecting dormant buds from the 8-year old trees and culturing the staminate inflorescences in those buds.

Plantlets regenerated from the inflorescence-derived cultures were used by ArborGen personnel to establish hedges for scaled-up production of rooted cuttings. During the period 2014-2017, approximately 200,000 trees per year (Fig. 2B), representing four elite hybrid varieties, were produced by ArborGen and planted by landowners in four states, with the goal of providing a ready source of hardwood fiber for pulp mills in their areas. Unfortunately, ArborGen suspended production of the hybrid trees in 2018. However, landowners who have planted the hybrid varieties report they have performed well so far (Fig. 2C) and demand from landowners for more planting stock continues, so it is possible ArborGen will resume production in the future.

Figure 2. *Hybrid sweetgum varietal trees.* ***A.*** *Elite hybrid clone after 12 years of growth.* ***B.*** *Rooted cuttings produced by ArborGen. Photo was taken in autumn. Photo courtesy of Dr. Mike Cunningham, Arborgen, Inc.* ***C.*** *Hybrid sweetgum varietals in Morris County, TX, following one season of growth in the field. Photo courtesy of Mr. Ed Hurliman.*

Overall, the 20-year effort to generate commercially useful hybrid sweetgum varietals by combining hybrid breeding with somatic embryogenesis should be considered a success. While the hybrid sweetgum varietal planting stock that was ultimately marketed by ArborGen was not somatic seedlings, SE technology did play a vital role in generating the hybrid varieties. I believe that with additional research and investment in automation, production of hybrid sweetgum somatic seedlings could be scaled up to become price-competitive with trees derived from rooted cuttings. Thus, the story of these hybrid sweetgum SE clones may yet continue.

Acknowledgements: This research was supported by funding from the Consortium for Plant Biotechnology Research, International Paper Co. and ArborGen Inc. I would like to thank Mike Cunningham for supplying information on the hybrid variety performance and photos of ArborGen's rooted cutting operation and field tests, Ed Hurliman for the photo of his plantation, Ron Schmidtling for helping us get access to Chinese sweetgum material and Wagner Vendrame, Paul Montello, Jianliang Dai, Siran Lu and Taryn Kormanik for technical assistance.

References

Dai J, Vendrame W, Merkle SA (2004) Enhancing the productivity of hybrid yellow-poplar and hybrid sweetgum embryogenic cultures. In Vitro Cell Dev Biol - Plant 40:376-383

Merkle SA, Sotak RJ, Wiecko AT, Sommer HE (1990) Maturation and conversion of *Liriodendron tulipifera* somatic embryos. In Vitro Cell Dev Biol 26:1086-1093

Merkle SA, Hoey MT, Watson-Pauley BA, Schlarbaum SE (1993) Propagation of *Liriodendron* hybrids via somatic embryogenesis. Plant Cell Tissue Organ Cult 34:191-198

Merkle SA, Neu KA, Battle PJ, Bailey RL (1998) Somatic embryogenesis and plantlet regeneration from immature and mature tissues of sweetgum (*Liquidambar styraciflua*) Plant Sci 132:169-178

Merkle SA, Battle PJ (2000) Enhancement of embryogenic culture initiation from tissues of mature sweetgum trees. Plant Cell Rep 19:268-273

Merkle S, Montello, P, Kormanik T, Le H (2010) Propagation of novel hybrid sweetgum phenotypes for ornamental use via somatic embryogenesis. Propag Ornamental Plants 10:220-226

Parks CR, Miller NG, Wendell JF, McDougal KM (1983) Genetic divergence within the genus *Liriodendron* (Magnoliaceae). Annals of the Missouri Botanical Garden 70:658-666

Santamour FS (1972a) Interspecific hybridization in *Liquidambar* Forest Sci 18:23-26

Santamour FS (1972b) Interspecific hybrids in *Liriodendron* and their chemical verification. Forest Sci 18:233-236

Vendrame WA, Holliday CP, Merkle SA (2001) Clonal propagation of hybrid sweetgum (*L. styraciflu*a

X *L. formosana*) via somatic embryogenesis. Plant Cell Rep 20:691-695
Witham FH, Blaydes DF, Devlin RM (1971) Experiments in Plant Physiology. Van Nostrand-Reinhold Co., New York. 245 p
Wright J, Cunningham M (2008) Sweetgum plantations for sawtimber, energy, pulp, and other uses. Forest Landowner 67(3):26-28

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Implementing genomic selection for multi-varietal forestry of white spruce (*Picea glauca*) in New Brunswick, Canada

Yill-Sung Park[1*], Chen Ding[2,6], Patrick Lenz[2,3], Simon Nadeau[3], Greg Adams[4], Shona Millican[5], Jean Beaulieu[2,3], Jean Bousquet[2]

*[1]Natural Resources Canada, Canadian Wood Fibre Centre, Fredericton, Canada; [2]Canada Research Chair in Forest Genomics, Institute for Systems and Integrative Biology, Université Laval, Québec, Canada; [3] Natural Resources Canada, Canadian Wood Fibre Centre, Québec, Canada; [4] JD Irving, Limited, Sussex, NB, Canada; [5]New Brunswick Department of Natural Resources, Fredericton, Canada; [6]Present address: Western Gulf Forest Tree Improvement Program, TAMU, College Station, TX, USA; *Corresponding author: yillsung.park@canada.ca*

Keywords: BLUP, Rooted cuttings, SNP, Somatic embryogenesis

Introduction

Genomic selection (GS) yields large genetic gains through shortening the duration of tree breeding by avoiding the need for lengthy testing periods. For example, in multi-varietal forestry (MVF), with the aid of vegetative propagation techniques such as somatic embryogenesis (SE) and rooted cuttings, the time savings of GS can be from 15 to 25 years depending on the trait considered. Mature commercial traits such as volume growth and wood density are difficult to assess at an early age, and the early selection using seed orchard-based breeding in conifers has been largely inefficient. We evaluated the feasibility of implementing GS in a white spruce (*Picea glauca* [Moench.] Voss) breeding program in eastern Canada. For GS modeling, we assessed the growth traits of a clonally replicated genetic test at age 24 that contains 15,000 trees propagated by rooting of cuttings. We genotyped 351 clones with ~4,100 single-nucleotide polymorphism (SNP) markers using a *P. glauca* Infinium chip designed as part of the *FastTRAC* project. This forest genomics endeavour, funded by Genome Canada and Genome Quebec, aims at developing and applying GS in current spruce breeding programs in eastern Canada. We found that GS applied at a very early age, combined with vegetative propagation through somatic embryogenesis or rooted cuttings, could result in about 75% greater volume productivity than the use of the conventional pedigree-based selection method in conjunction with seed orchard production, as well as that it eliminated the need for genetic field testing of the selected juvenile material.

Materials and methods

Plant material and phenotyping

GS modeling was carried out using a clonally-replicated genetic test of white spruce established in 1990, which contained 377 clones from 75 full-sib families created from a disconnected diallel mating design representing 20 unrelated parents selected for the breeding program in New Brunswick (NB). The test was established in three locations (Acadia, Sussex, and St. Quentin) in NB using a randomized complete block design with 16 blocks of single tree plots at each test site. Measurements were taken at regular intervals and the latest measurements (phenotyping) were taken at age 24 (2014) for height and DBH. Tree volume was estimated using the Honer's tree volume table (Honer et al. 1983). For genotyping of the clones in the test, foliage samples were taken at the Acadia site and DNA was extracted following standard procedures (Pavy et al. 2013a).

White spruce genomic resources and genotyping
Prior to the *FastTRAC* project, a white spruce SNP marker atlas was constructed from EST (expressed sequence tags) sequencing and alignment of sequences against the white spruce gene catalog GCAT largely derived from full-length cDNAs (Rigault et al. 2011). About 213K high-confidence SNP markers (with true-positive rate of 92%) distributed in 15K expressed genes were discovered and released with open access for research (Pavy et al. 2013b). Based on these markers and following published protocols (Pavy et al. 2013a), a SNP genotyping chip was built to obtain a multi-locus genomic profile for each of the clones. To do so, the iSelect Infinium SNP array technology (Illumina, San Diego, CA) was used and a chip containing ~5,300 successfully manufactured SNPs was assembled, representing as many distinct gene loci well distributed over the 12 linkage groups of white spruce (Pavy et al. 2017). This chip was distinct from previous ones used in earlier GS proof of concept work in white spruce (Beaulieu et al. 2014a,b). Genotyping was conducted by Neogene Canada Inc. After removing non-segregating markers and applying in-house quality control filters, such as low rate of missing data (average of 1% per locus) and minor allele frequency $\geq$ 0.01, high quality genotyping data from 4,075 SNPs were retained for GS analyses, which is more than sufficient to obtain high accuracy of GS models in spruce advanced-breeding populations (Beaulieu et al. 2014b, Lenz et al. 2017, 2019). A total of 351 clones were genotyped.

Statistical modeling and BLUP analyses
Three statistical methods for predicting the genetic values of clones and families were evaluated: (1) traditional pedigree-based best linear unbiased prediction (ABLUP) to calculate the estimated genetic values (EGV); (2) marker-based GBLUP; and (3) the blended HBLUP to calculate the genomic estimated genetic values (GEGV). We applied linear mixed models to partition the fixed block and diallel set effects, and random genetic effects as following for single sites for height and DBH traits:

$$Y = X\beta + Z_1 a + Z_2 u + E$$

where Y is the phenotypic vector; X is the design matrix of fixed effects; β is the parameter of fixed values to be estimated; Z_1 is the design matrix of additive genetic random effects; Z_2 is the design matrix of non-additive genetic random effects; *a* is the parameter of random additive genetic effect of individual clones where $a \sim N(0, A\,\sigma_A^2)$, and A is the kinship matrix in ABLUP. For estimation of GBLUP, the A-matrix was replaced by the genomic relationship matrix (G-matrix), or a blended relationship matrix (H-matrix) in HBLUP; and E is the random error $E \sim N(0, I\,\sigma_E^2)$; u is the random non-additive effect based on pedigree information $u \sim N(0, I\,\sigma_{NA}^2)$, where I is the identity matrix. The G- and H-matrices were constructed following the methods of Legarra *et al*. (2009).

Results

Genetic gains form GS using vegetative propagation:
Based on ABLUP, the selection of the top 10 clones achieved 25, 32, and 75%, respectively, of height, DBH and volume gain over the test average, while selecting the top 13% (i.e., 50 clones) yielded 15, 24 and 54 % gain for height, DBH and volume, respectively (Table 1), although calculated volume tended to be over-estimated. The genetic gain was comparable between the GBLUP and ABLUP methods for these traits. Since all the offspring from selected elite parents were included in the test, the test average represents the gains from conventional seed orchard breeding.

Correlation among ABLUP, GBLUP, and HBLUP:
The clone rankings of the pedigree-based (ABLUP) and marker-based (GBLUP) estimates were positively correlated, with correlation coefficients ranging from 0.94 to 0.96 for height and DBH. The top elite (10%) clones overlapped between the ABLUP and GBLUP predictions. Overall ranking of GBLUP and HBLUP approximately matched that of the pedigree-based method.

Table 1. *Realized genetic gains, expressed in percentage above the trait means, for varying degrees of selection intensity from the clonally replicated test of white spruce containing 351 clones at age 24. The volume was calculated based on the selection scenario based on DBH. Note that trait means represent the seed orchard output if the parental clones were used in the seed orchard.*

No of top Ranking	Percent selected	Height (Mean = 12.04 m)		DBH (Mean = 14.2 cm)		Volume (Mean = 0.08 m^3)	
		ABLUP	GBLUP	ABLUP	GBLUP	ABLUP	GBLUP
10	3	24	21	32	33	75	82
15	4	23	22	32	35	71	84
20	5	20	22	32	35	72	81
30	8	20	17	28	27	65	66
50	13	15	15	24	22	54	51

Application of GS in current tree breeding programs

Progeny testing of plantations established during the 1970s and 1980s now provide important data on the growth and quality at the rotation age that enable the development of GS model for the mature traits. As well, these tests are the source of material in applying GS for the next generation. For example, from existing progeny test plantations with the mature phenotype, one can develop a GS model, and select the best ABLUP/GBLUP parents (about 30 trees). These parents are used to produce about 250 candidate lines through controlled crosses. These lines are vegetatively propagated either by SE or by rooted cuttings from SE-derived donor plants. These lines are then genotyped and applied to the GS model developed at the parental generation to obtain the GBLUP values. Based on the GBLUP values, an appropriate number of varieties are deployed in the commercial plantations. This will eliminate lengthy field testing for the mature commercial traits that may take 15 to 25 years for white spruce while providing more flexibility in the context of climate change (Fig. 1). However, it will be highly desirable

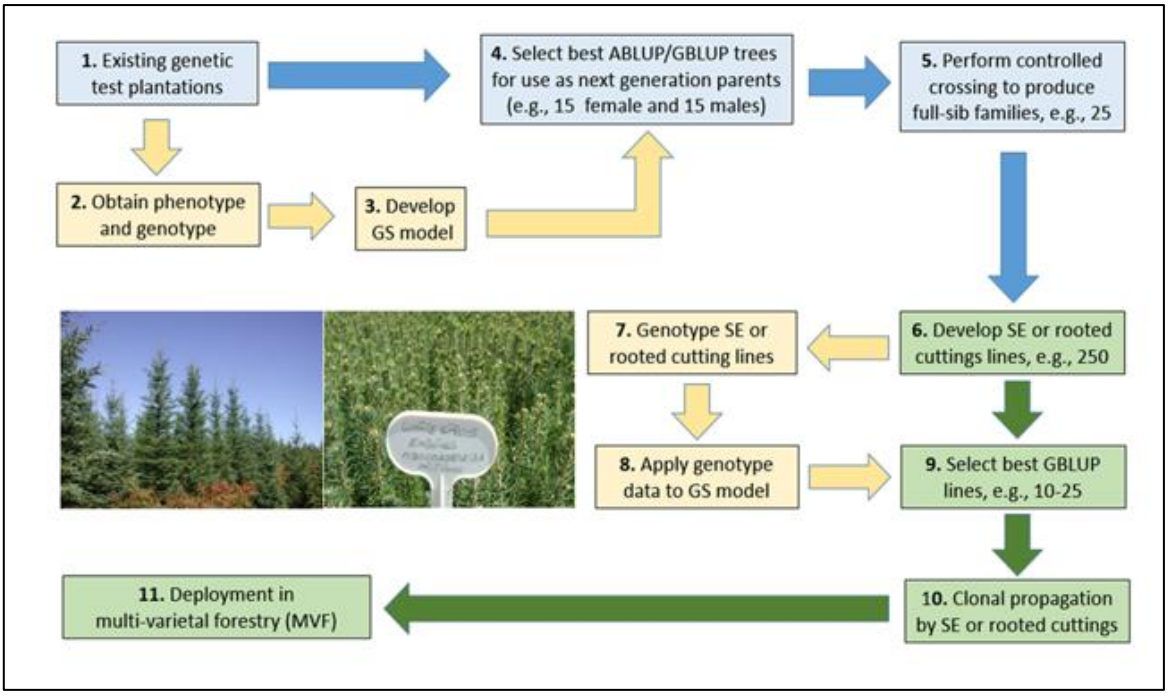


Figure 1. *Genomic selection implementation plan for white spruce breeding in New Brunswick, Canada, using somatic embryogenesis and rooted cuttings for seedling production.*

Acknowledgements: This research was funded by Genome Canada and Genome Quebec for the *FastTRAC* Project (Fast Tests for Rating and Amelioration of Conifers) that aims to implement GS at the operational level for spruce breeding in eastern Canada with partners Université Laval, Natural Resources Canada, Canadian Wood Fibre Centre, Province of New Brunswick, Province of Québec, J.D. Irving Limited and FPInnovations. Drs. Fred Raley and Tom Byram, and Mr. Benjamin Bartlett (Western Gulf Forest Tree Improvement Program) provided constructive comments and in-kind support for the editing.

References

Bartholomé J., Van Heerwaarden J., Isik F., Bour, C., Vidal M., Plomion C. *et al.* 2016 Performance of genomic prediction within and across generations in maritime pine. *BMC Genomics*, **17**, 604.

Beaulieu J., Doerksen T.K., Clément S., Mackay J. and Bousquet J. 2014a. Accuracy of genomic selection models in a large population of open-pollinated families in white spruce. *Heredity,* **113**, 343-352.

Beaulieu J., Doerksen T.K., Mackay J, Rainville A. and Bousquet J. 2014b. Genomic selection accuracies within and between environments and small breeding groups in white spruce. *BMC Genomics*, **15,** 1048 (16p.).

Honer T.G., Ker M.F, Alemdag I.S. 1983 Metric timber tables for the commercial tree species of central and eastern Canada. Maritimes Forestry Research Centre. Information report M-X-140. 139 p.

Legarra A., Aguilar I. and Misztal I. 2009 A relationship matrix including full pedigree and genomic information. *Journal of Dairy Science*, **92**, 4656-4663.

Lenz P., Beaulieu J., Clément S., Mansfield S., Desponts,M. and Bousquet J. 2017. Factors affecting the accuracy of genomic selection for growth and wood quality traits in an advanced-breeding population of black spruce (*Picea mariana*). *BMC Genomics* **18**, 335 (17p.).

Lenz P., Nadea, S., Mottet M.-J., Perron M., J. Beaulieu J. and Bousquet J. 2019. Multi-trait genomic selection for weevil resistance, growth and wood quality traits in Norway spruce. *Evolutionary Applications* (in review).

Pavy N., Gagnon F., Rigault P., Blais S., Deschênes A., Boyle B, Pelgas B., Deslauriers M., Clémen, S., Lavigne P., Lamoth M., Cooke J.E.K., Jaramillo-Correa J.P., Beaulieu J., Isabel N., Mackay J. and Bousquet J. 2013a. Development of high-density SNP genotyping arrays for whitespruce (*Picea glauca*) and transferability to subtropical and nordic congeners. *Molecular Ecology Resources*, **13**, 324-336.

Pavy N., Deschênes A., Blais S., Lavigne P., Beaulieu J., Isabel N., MacKay J. and Bousquet J. 2013b. The landscape of nucleotide polymorphism among 13,500 genes of the conifer *Picea glauca*, relationships with functions, and comparison with the angiosperm *Medicago truncatula*. *Genome Biology and Evolution.* **5**, 1910-1925.

Pavy N., Lamothe M., Pelgas B., Gagnon F., Birol I., Bohlmann J., Mackay J., Isabel N., Bousquet, J. 2017. A high resolution reference genetic map positioning 8.8 K genes for the conifer white spruce: structural genomics implications and correspondence with physical distance. *The Plant Journal*, **90,** 189-203.

Rigault P., Boyle B. Lepage P., Cooke J.E.K., Bousquet J. and MacKay J. 2011. A white spruce gene catalogue for conifer genome analyses. *Plant Physiology,* **157,** 14-28.

Induction of somatic embryogenesis in *Chorisia speciosa (A.St.-Hil.)* Ravenna

Maite Romero Alves[1], Tatiana Cinquetti[1], Sandra E. Sharry[1,2]

1.Laboratorio de Investigaciones de la Madrea (LIMAD). Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Argentina.
2. Universidad Nacional de Rio Negro, Sede Viedma, Argentina
mromeroalves@gmail.com, tatianacinquetti@gmail.com, ssharry@gmail.com

Introduction

The "Palo Borracho" (*drunken stick*) is a native tree from Argentina that has a bulge in its trunk that makes it take the form of a barrel (*Figure 1*). It grows disseminated in the eastern Chaco Park and in the Tucuman-Bolivian Forest, Argentina, (Tortorelli, 1956). It is cultivated for its ornamental characteristics. The bulging trunk, full of stings, is one of its main attractions, in addition to its beautiful flowers that, depending on the species, may be pink if it's *Chorisia speciosa* or white-yellowish if it's *Chorisia insignis*. It has finger-like leaves with 5 serrated edge leaflets. The fruit is a large dehiscent capsule of up to 15 cm of length that has numerous seeds trapped inside a species of cotton called "paina", of a slightly yellowish white colour that can have textile applications. The species we used in this work was *Chorisia speciosa*, where the parts of the flowers can be easily recognized in the explant. There are no records of *in vitro* tissue culture of this species, that is why it is important to develop *in vitro* propagation protocols.

Figure 1. *Palo borracho's tree (Taken by Google Photos).*

Materials and Methods

The mother plants were randomly selected from the public woodland of La Plata city following the recommendations of Sharry *et al*. (2015). As explant were used closed button flowers, harvested totally closed and with no marks of any pathogen attack in March of 2018. (*Figure 2*)
For the disinfection and culture establishment were used the following items

1) 96% ethanol
2) Burner
3) Sterilized water
4) Water treatment equipment; balances; autoclave; laminar-flow hood; scissors, forceps and scalpels; glass bottles; baby food jars (55 mm diameter, 72 mm height), plastic film and Parafilm®; growth chambers
5) Insulation medium (agar 7,5 g/L with 30 g/L of ordinary sugar)
6) Closed button flowers (free of pathogens)

All the items used except the Parafilm® were sterilized in an autoclave for 20 minutes at 1 atmosphere of overpressure and 121 ºC.

Fig.2. *Floral button*

For the *in-vitro* establishment we used the following steps:

1) The button flowers were immersed in 96% alcohol and flamed for 3 seconds above the burner turning the explant around once per second, then the flame was extinguished in a glass with sterilized water, all in the laminar-flow hood.
2) The button flowers were sectioned in androecium and gynoecium. Then they were placed in Petri dishes filled with 10 ml of insulation culture medium, and sealed with Parafilm®.
3) The Petri dishes were placed in a growth chamber at 25 ºC and a 16-h photoperiod.
4) After 15 days, the explants were introduced in Petri dishes filled with 10 ml of Woody Plant Medium (McCown, 1981) at half concentration with 1,5 ppm of 2,4-Dichlorophenoxy acetic acid and 1 g/L of activated charcoal.
5) The Petri dishes were placed in a growth chamber at 20-24 ºC in darkness to induce callus formation and possibly somatic embryogenesis.

Results and Discussion

The disinfection was efficient and after 15 days of culture in the dark, friable calluses were formed (*Figure 3*). Both androecium and gynoecium gave calluses, that means that all the explants had divisions ending in calluses. These calluses were sub-cultivated in an induction of embryogenesis medium,

composed of WPM salts at half strength where the formation of pro-embryos was observed. Actually, these tissues, still in darkness, induced proliferation and maturation of somatic embryos.

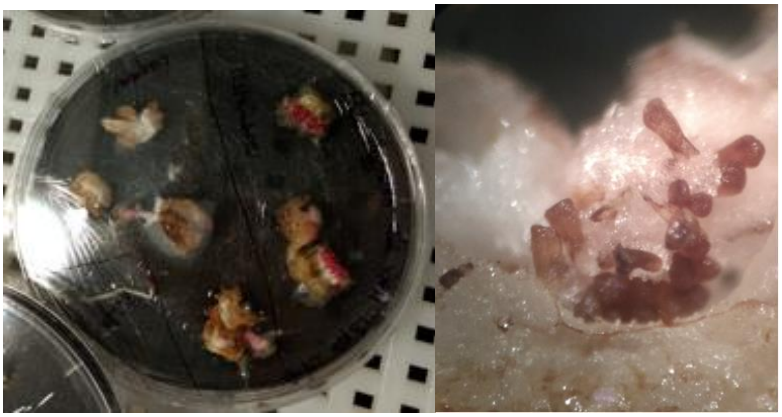


Figure 3: Calluses formation in explants of Ceiba

The use of flower buttons to induce somatic embryogenesis is not common but it is used in the propagation of cacao. So, for example, Urrea Trujillo et al. (2011) induced somatic embryogenesis from flower explants in two clones of *Theobroma cacao*.

The somatic embryogenesis of Palo borracho has not been reported before, that is why this exploratory study could lead to and adjusted protocol from a non-commonly used explant in micropropagation.

References

McCown BH (1981) Woody Plant Medium (WPM) a mineral nutrient formulation for microculture for woody plant species. Hort. Sci., 16, 453.

Sharry S, Adema M & Abedini W (2015) Plantas de probeta: manual para la propagación de plantas por cultivo de tejidos in vitro. Coordinación general: Sandra Sharry; Marina Adema; Walter Abedini. - 1a ed. adaptada. - La Plata: Universidad Nacional de La Plata. ISBN 978-950-34-1254-1. 240pp.

Tortorelli LA (1956) Maderas y bosques argentinos. pp. 910.

Urrea Trujillo AI., Atehortúa Garcés L & Gallego Rúa AM (2011) Regeneración vía embriogénesis somática de una variedad colombiana élite de Theobroma cacao L. Revista Colombiana de Biotecnología, [S.l.], v. 13, n. 2, p. 39-50. ISSN 1909-8758. Available in: https://revistas.unal.edu.co/index.php/biotecnologia/article/view/27916/38317

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Ex situ conservation of the Brazilian conifer *Podocarpus lambertii* (Podocarpaceae) by means of cryopreservation

Leila N. Vieira[1,2], Patrick D. Marques[1], Angelo S. Heringer[1], Hugo P.F. Fraga[2], Daniela Goeten[1], Miguel P. Guerra[1,3,*]

[1]Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal, Programa de Pós-graduação em Recursos Genéticos Vegetais, Centro de Ciências Agrárias, Universidade Federal de Santa Catarina, Florianópolis, SC, 88034-001, Brazil.
[2]Laboratório de Micropropagação Vegetal, Programa de Pós-graduação em Botânica, Setor de Ciências Biológicas, Universidade Federal do Paraná, Curitiba, PR, 81531-970, Brazil.
[3]Tree and Timber Institute, National Research Council of Italy, Via Madonna del Piano 10, 50019 Sesto Fiorentino, Florence, Italy
**Corresponding author: miguel.guerra@ufsc.br Tel: +55 48 3721-5331.*

Keywords: Seeds, zygotic embryo, droplet-vitrification

Introduction

Podocarpus L'Hér. ex Pers. (Podocarpaceae) is the second largest genus among conifers, comprising 97 species mainly distributed in the Southern Hemisphere (Farjon and Filer 2013). Species of the genus *Podocarpus* are important timber, wax, tannin, and ornamental trees in their native areas (Abdillahi et al. 2010). However, relatively little is known about the Brazilian species *Podocarpus lambertii* Klotzsch ex Endl. This species is indigenous to one of the 25 biodiversity hotspots of the world, the subtropical moist forest ecoregion of the Atlantic Forest biome - Brazil (Myers et al. 2000; Farjon and Filer 2013). *Podocarpus lambertii* conservation status is classified as near threatened in the IUCN red list (Farjon 2013). Thus, the development of *in situ* and *ex situ* conservation strategies is necessary.

Cryopreservation is a widely used process for long-term *ex situ* conservation (Engelmann 2004). It is based on the preservation of biological material (i.e. organelle, cells, tissue) at ultra-low temperatures, usually liquid nitrogen (LN, -196 °C) (Engelmann 2004). Vitrification is a cryopreservation technique that uses a highly concentrated cryo-protective solution, allowing the super-cooling of plant material to very low temperatures without undergoing crystallization (Sakai and Engelmann 2007). In the droplet-vitrification technique, the explants are treated with the vitrification solution and frozen in individual micro droplets of the vitrification solution placed on aluminum foils, which are immediately immersed in liquid nitrogen (Sakai and Engelmann 2007). The droplet-vitrification method has been successfully applied to the cryopreservation of embryos from many plant species (Martinez-Montero et al. 2008; Engelmann 2011; Gantait et al. 2015; Bradaï et al. 2017).

The cryopreservation of *P. lambertii* excised embryos might enable the long-term *ex situ* conservation of genotypes from endangered populations. Thus, in the present study we propose a cryopreservation protocol for *P. lambertii* embryos using droplet-vitrification technique.

Materials and methods

Plant material
Mature seeds of *P. lambertii* were collected in a natural population at Curitiba - Brazil (latitude 25° 25′ S, longitude 49° 16′ W, altitude 925 m). Seeds were surface sterilized with 70% ethanol for 1 min and 2% sodium hypochlorite for 20 min, followed by triple rinsing with sterile deionized water. Embryos were excised in a laminar flow chamber with the aid of a stereomicroscope.

In vitro *germination of embryos*
Excised embryos (n = 40) were inoculated in test tubes (25 mm x 200 mm) with 15 mL of culture medium consisting of Woody Plant Medium (WPM; Phytechnology Laboratories, Shawnee Mission, KS; Lloyd and McCown 1981), supplemented with 88 mM sucrose, 2 ml L^{-1} Plant Preservative Mixture™ (PPM), 3 g L^{-1} gellan gum (Phytagel™). The pH was adjusted to 5,8 before autoclaving at 121 °C for 15 min. The embryos were incubated in the dark for 72 h, and then transferred to 16 h photoperiod (60 µmol m^2 s^{-1}) and 25 ± 2 °C for 30 days. After 30 days in culture, the green germinants were counted.

Effect of incubation time in PVS2 (-LN)
Embryos were subjected to different incubation times in PVS2 (composition described below) for 0, 20, 40, and 60 min and germinated in the same culture medium described above. In this step, the focus was to evaluate the possible toxicity of cryoprotectants agents and its effect on embryos germination. Thus, excised embryos were subjected to the loading solution composed by 2 M glycerol and 0.4 M sucrose (Matsumoto et al. 1994) plus WPM basal salts and vitamins. After 20 min in darkness at room temperature, the loading solution was replaced by PVS2 solution consisting of 3.26 M glycerol, 2.42 M ethylene glycol, 1.9 M dimethyl sulfoxide and 0.4 M sucrose (Sakai et al. 1990), plus WPM basal salts and vitamins. Different incubation times in PVS2 (0, 20, 40 and 60 min.) at 0 °C were evaluated. Subsequently, all embryos were subjected to *in vitro* germination.

Cryopreservation by droplet-vitrification (+LN)
Cryopreservation procedures were carried out using the droplet-vitrification method on aluminum foil strips (Panis et al. 2005). Excised embryos were incubated in PVS2 at 0 °C for 0, 20, 40 and 60 min. Then, the embryos were surrounded by a droplet of PVS2, rapidly transferred to sterilized aluminum foil strips and immersed in LN for at least 24 h. Re-warming was performed in a 40 ºC deionized water bath (40 s) with basal WPM medium containing 1.2 M sucrose. Explants were kept in this unloading solution (Sakai et al. 1990) for 20 min and then transferred for the unloading solution 2 (Nishizawa et al. 1993), consisting of basal WPM medium containing 0.3 M sucrose, and were incubated for 24 h. After that, all explants were inoculated into germination culture medium.

Statistical analysis
The experimental design was completely randomized with 15 embryos per treatment (n = 15). The experimental unit consisted of one test tube with one embryo. Data of green embryo formation and morphological features were recorded at 60 days of culture. Data were compared by chi-square ($\chi 2$) test ($p<0.05$) with Yates correction. Pairwise comparisons were carried out in 2x2 contingency tables in order to detect differences between treatments.

In vitro rooting of shoots
Sixty days after the re-warming from LN, 20 rootless plantlets obtained from cryopreserved embryos were subjected to *in vitro* rooting in the germination culture medium supplemented with 12 µM indole -3-butyric acid (IBA) (Castro et al. 2011). The plantlets were incubated in the dark for 72 h, and then transferred to 16 h photoperiod and 25 ± 2 °C. Data of *in vitro* rooting was recorded after 60 days in culture.

Results and discussion

Embryos subjected to *in vitro* germination showed 77.7% of green embryos and subsequent plantlet formation (Table 1). Similar rates of green embryos formation were observed for all incubation times in PVS2 (-LN). As our results did not indicate negative effects on green embryo formation after the different incubation times in PVS2 (-LN), we tested all PVS2 incubation times (0, 20, 40, and 60 min) in the cryopreservation by the droplet-vitrification protocol.

Embryos subjected to cryopreservation by droplet-vitrification (+LN) showed contrasting results. The lowest rate of green embryos formation was observed in treatment with PVS2 incubation for 0 min (13.33%). Treatments with PVS2 incubation for 20 and 40 min indicated high green embryos formation, reaching the same rates observed in treatments not subjected to liquid nitrogen (Table 1). Longer exposure time to PVS2 (60 min) showed deleterious effect and decreased rate (20%).

The best results found for *P. lambertii* embryos cryopreservation was with 20 and 40 min incubation in PVS2 (+LN), showing 53.33% and 46.66% of green embryos, respectively. Prudente et al. (2016) reported a cryopreservation protocol for *Araucaria angustifolia* embryo shoot tips, a species belonging to family Araucariaceae, a family sister to Podocarpaceae. In that study, the authors obtained 53.2% survival of shoot tips after treatment with PVS2 for 15 min, with a decreased explant survival after 30 min incubation. In our study, decreased survival was only observed after 60 min incubation.

Table 1. *P. lambertii embryos recovery and injury rates after different cryo-treatment incubation times (0, 20, 40 and 60 min) after 60 days in culture.*

PVS2 incubation time		Green embryos (%)	Damaged embryos (%)**
Control	0'	77.70 a	-
(-)LN	0'	73.33 a	-
	20'	73.33 a	-
	40'	73.33 a	-
	60'	66.66 a	-
(+)LN	0'	13.33 c	66.67*
	20'	53.33 a	60.0*
	40'	46.66 a	60.0*
	60'	20.0 b	80.0*

Different letters in column indicate statistical difference between different PVS2 incubation times, compared by chi-square ($\chi 2$) contingency test (95%). (*) indicate no statistical difference. (**) Embryos with embryonic axis broken in two pieces. (-)LN: non-subjected to liquid nitrogen; (+)LN: subjected to liquid nitrogen. Control treatment: n = 40; (-)LN and (+)LN treatments: n=15.

However, an intriguing event occurred to all embryos subjected to liquid nitrogen. A rupture of the root apical zone was observed in these embryos (Fig. 1A and B). This injury occurred in 66.67% of embryos, in average, without significant difference (Table 1). Importantly, before the cryopreservation procedures, the embryos indicated intact shoot and root apical zones (Fig. 1C).

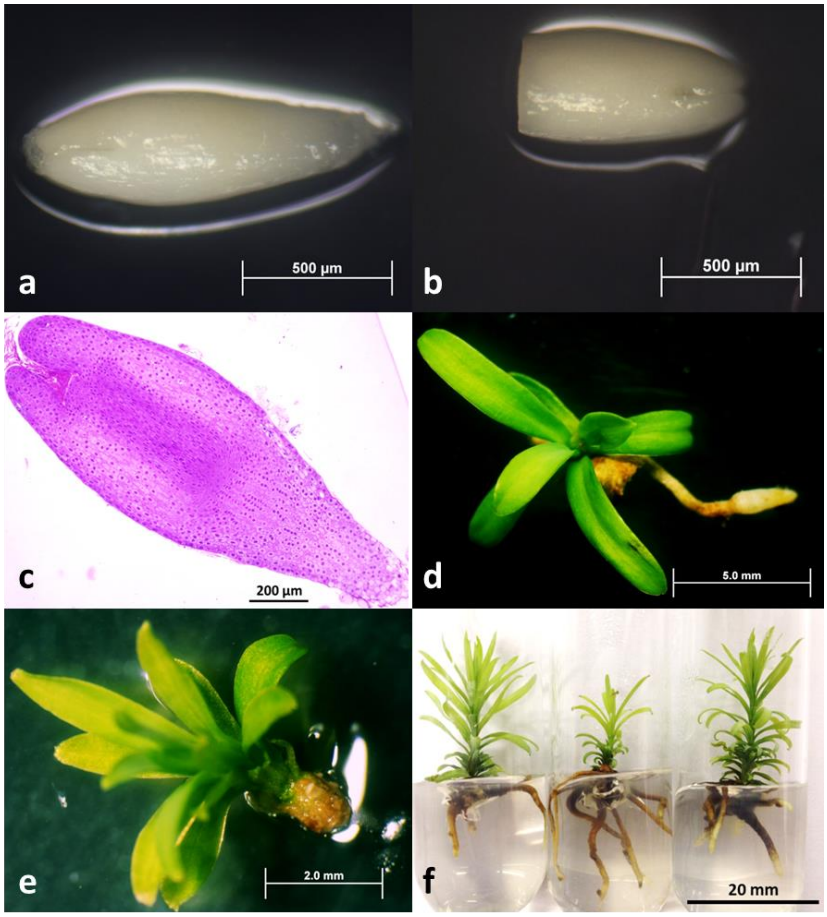


Figure 1. *Morphological features of P. lambertii embryos cryopreservation. Excised embryos before (A) and after (B) cryopreservation procedures, indicating the loss of root apical zone. (C) Histological section of the embryo at the cotyledonary stage showing the intact shoot and root apical zones. Germinated embryos non-subjected (D) or subjected (E) to liquid nitrogen indicating the presence and absence of root, respectively. (F) Rooted plantlets after in vitro rooting stage*

Embryos not subjected to liquid nitrogen indicated normal plantlet development during the germination process, with root formation (Fig. 1D). Embryos cultured after the complete cryopreservation procedure showed rootless plantlet development (Fig. 1E). In order to avoid the consequences of the root structures absence, which could compromise the subsequent plantlet development, rootless plantlets were subjected to *in vitro* rooting. High rooting rates could be achieved in this step (40%), allowing the obtainment of complete plantlets and, consequently, improving the feasibility of the cryopreservation protocol (Fig. 1F).

Conclusion

In the present study, a suitable protocol for cryopreservation of *P. lambertii* embryos is described. This protocol represents the first *ex situ* conservation approach reported for this species, an endangered Brazilian conifer. In addition, this is the first cryopreservation protocol described for the family Podocarpaceae. Thus, this cryopreservation protocol may be useful for future studies with other Podocarpaceae species.

Funding: This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) under Grant numbers 438692/2016-0, 457726/2013-0, and 306126/2013-3.

References

Abdillahi HS, Stafford GI, Finnie JF, Van Staden J (2010) Ethnobotany, phytochemistry and pharmacology of *Podocarpus* sensu latissimo (sl). S Afr J Bot 76:1-24

Bradaï F, Almagro-Bastante J, Sánchez-Romero C (2017) Cryopreservation of olive somatic embryos using the droplet-vitrification method: The importance of explant culture conditions. Sci Hortic 218:14-22

Castro MR, Belo AF, Afonso A, Zavattieri MA (2011) Micropropagation of *Juniperus navicularis*, an endemic and rare species from Portugal SW coast. Plant Growth Regul 65:223-230

Engelmann F (2004) Plant cryopreservation: progress and prospects. In Vitro Cell Dev Biol-Plant 40:427–433

Engelmann F (2011) Use of biotechnologies for the conservation of plant biodiversity. In Vitro Cell Dev Biol-Plant 47:5-16

Farjon A (2013) *Podocarpus lambertii*. The IUCN Red List of Threatened Species. Version 2017-3. <www.iucnredlist.org>. Downloaded on 31 December 2017

Farjon A, Filer D (2013) An atlas of the world's conifers: an analysis of their distribution, biogeography, diversity and conservation status. Leiden: Koninklijke Brill.

Gantait S, Sinniah UR, Suranthran P, Palanyandy SR, Subramaniam S (2015) Improved cryopreservation of oil palm (*Elaeis guineensis* Jacq.) polyembryoids using droplet vitrification approach and assessment of genetic fidelity. Protoplasma 252:89-101

Martinez-Montero ME, Martinez J, Engelmann F. 2008. Cryopreservation of sugarcane somatic embryos. CryoLetters 29:229-242

Matsumoto T, Sakai A, Yamada K (1994) Cryopreservation of in vitro-grown apical meristems of wasabi (*Wasabia japonica*) by vitrification and subsequent high plant regeneration. Plant Cell Rep 13:442-446

Lloyd G, McCown BH (1981) Woody plant medium (WPM) - a mineral nutrient formulation for microculture of woody plant-species. HortScience 16:453-453

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. Nature 403:853–858

Nishizawa S, Sakai A, Amano Y, Matsuzawa T (1993) Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant regeneration by vitrification. Plant Sci 91:67-73

Panis B, Piette B, Swennen R (2005) Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all Musaceae. Plant Sci 168:45-55

Prudente DO, Paiva R, Paiva PDO, Silva LC (2016) Cryopreservation of shoot tips excised from zygotic embryos of *Araucaria angustifolia* (Bertol.) Kuntze. Acta Hortic 1113:257-264

Sakai A, Kobayashi S, Oiyama I (1990) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. Plant Cell Rep 9:30-33

Sakai A, Engelmann F (2007) Vitrification, encapsulation-vitrification and droplet-vitrification: a review. CryoLetters 28:151-172

Short Abstracts

Transcriptional identification and characterization of differentially expressed miRNAs involved in conifer embryogenesis

Ana Alves[1,2,3], Andreia Rodrigues[1,2], Susana Lopes[1,2], Pedro Perdiguero[1,2], Bruno Costa[1,2], Inês Chaves[1,2], Ana Confraria[4], Célia Miguel[1,2,3]

1 Instituto de Biologia Experimental e Tecnológica (iBET), Oeiras, Portugal. acalves@itqb.unl.pt
2 Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa (ITQB).
3 Biosystems & Integrative Sciences Institute, Faculdade de Ciências, Universidade de Lisboa (FCUL), Lisboa, Portugal.
4 Instituto Gulbenkian de Ciência (IGC), Oeiras, Portugal.

Keywords: auxin responses, embryo development, gymnosperms, sRNA transcriptome

The molecular regulation of plant embryogenesis has been mostly studied using angiosperm model species such as *Arabidopsis*. However, gymnosperm and angiosperm lineages are estimated to have driven apart over 300 million years ago and their differences, in particular at the embryogenic phase, are well described (Axtell, 2013). As major regulators of gene expression during developmental processes in plants, it is expected that sRNAs play a relevant role in the regulation of embryogenesis and, most probably, in the emergence of differential characteristics of the conifer embryo (Bologna & Voinnet, 2014). During this transition, conifer embryos develop multiple cotyledons while maintaining a radial symmetry which contrasts with the *Arabidopsis* embryo where a bilateral symmetry is acquired (Williams, 2009). Based on previous studies in our lab that generated an overview of the *Pinus pinaster* sRNA transcriptome in several tissues including embryos at several developmental stages, a set of candidate miRNAs and corresponding target transcripts have emerged as potential embryogenesis regulators. Centered on the differential expression analysis of identified miRNAs throughout embryo development, we hypothesize that specific miRNA-target regulatory nodes, potentially involved in processes such as auxin responses and miRNA biogenesis, are crucial developmental regulators. Among these, *miR160* and *miR162*, putatively targeting an *ARF10* and *DCL1*, respectively, are interesting candidates to further characterize based on their expression patterns, either specific of zygotic embryo tissues/developmental stages or markedly differentially expressed during embryo development (Rodrigues et al., 2017). By using a conifer *in vitro* embryogenesis model system(Filonova et al., 2000; Vestman et al., 2011), and a combination of cell, molecular biology and genetic tools, we are working on the functional characterization of a short list of selected candidate miRNA-target regulatory pairs. As a first step of this characterization, the validation of *in vivo* interaction of selected miRNAs and their putative target genes, predicted mostly by in silico analysis, is being performed using a co-transient expression with the luciferase reporter system in *Arabidopsis* protoplasts (Martinho et al., 2015). Preliminary results already obtained up to now validate the existence of *in vivo* interaction between miR160 and its target ARF transcript.

References

Axtell MJ (2013) Classification and Comparison of Small RNAs from Plants. Annual Review of Plant Biology, 64(1), 137–159

Bologna NG, Voinnet O (2014) The Diversity, Biogenesis, and Activities of Endogenous Silencing Small RNAs in Arabidopsis. Annual Review of Plant Biology, 65(1), 473–503

Filonova LH, Bozhkov PV, Brukhin VB, Daniel G, Zhivotovsky B & von Arnold S (2000) Two waves

of programmed cell death occur during formation and development of somatic embryos in the gymnosperm, Norway spruce. Journal of Cell Science, 113(24), 4399–4411

Martinho C, Confraria A, Elias CA, Crozet P, Rubio-Somoza I, Weigel D, Baena-González E (2015) Dissection of miRNA pathways using Arabidopsis mesophyll protoplasts. Molecular Plant, *8*(2), 261–275

Rodrigues A, Chaves I, Costa B, Lin Y, Lopes S, Correia, Miguel CM (2017) Identification of regulatory miRNA-target nodes across embryo development in *Pinus pinaster*. *In* Bonga JM, Park YS, Trontin JF (Eds.), Proc of the 4th IUFRO 2.02.09 Conference. Sept 19-23, 2016. La Plata, Argentina

Vestman D, Larsson E, Uddenberg D, Cairney J, Clapham D, Sundberg E, von Arnold S (2011) Important processes during differentiation and early development of somatic embryos of Norway spruce as revealed by changes in global gene expression. Tree Genetics & Genomes, *7*(2), 347–362

Williams CG (2009) Conifer reproductive biology. Dordrecht, Springer

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

An automated active compounds screening system allows high-throughput optimization of somatic embryogenesis in *Coffea Arabica*

Rayan Awada[1,2,3*], Dorothée Verdier[1], Solène Froger[1], Eric Brulard[1], Hervé Etienne[2,3], David Breton[1]

[1] *Nestlé R&D Center Tours, 101 avenue Gustave Eiffel, 37097 Tours Cedex 2*
[2] *CIRAD, UMR IPME, F-34398 Montpellier, France*
[3] *UMR IPME, Univ Montpellier, CIRAD, IRD, F-34394 Montpellier, France*
** Corresponding author: rayan.awada@cirad.fr*

Keywords: *Coffea arabica*, somatic embryogenesis, miniaturization, high-throughput screening, histone deacetylase inhibitors

Somatic embryogenesis (SE) is a very promising biotechnological tool for the rapid and large-scale vegetative propagation of elite varieties. For *Coffea arabica* F1 hybrids, research has led to successful industrialization and commercial application of SE in Latin America. However, SE research on *C. arabica* remains essentially empirical and at a low-throughput level, resulting in many drawbacks, especially an overall slow technical progress over the last few years. While production today can reach 1-2 million SE-derived plants per year, a scale-up is needed to meet market demands (50-100 million per year). A high-throughput approach can be a solution to optimize SE protocols by testing a wider range of active compounds (nutrients, growth regulators, etc.), multiplying experimental conditions, and reducing volumes as well as required spaces and manpower. An automated and miniaturized system is a pre-requisite to establish this approach. We here present an automated high-throughput screening system able to optimize SE in *C. arabica*. Our focus was on the production of embryos from established cell suspensions. This SE stage is classically done in 250 ml flasks so miniaturization in 24-well plates was necessary. Arabica calli were also miniaturized in order to fit the pipetting platform. The automated platform was then programmed in a way to validate homogeneity of calli distribution as well as callus-to-embryo conversion. After a successful establishment of the system, a screening proof of concept was carried out. Four compounds (Oxamflatin, SAHA, Scriptaid and Trichostatin A) belonging to the histone deacetylase inhibitors family were tested at three different concentrations: 0.1, 1 and 10 µM, as potential active compounds on *C. arabica* embryo differentiation. Only calli treated with 1 µM Trichostatin A showed a significant increase in the number of torpedo-shaped embryos (3-fold increase). As a validation, this treatment was re-tested in standard conditions (250 ml flasks) and there also showed a significant increase in the number of embryos obtained (3.5-fold increase), their size (30%) and the overall fresh weight (60%). Our results show that our system can be well-suited to screen thousands of compounds in a restrained time period. Further analysis should be carried out to understand the Trichostatin A molecular effect on embryo production stimulation and plantlet conversion efficiency.

Effect of priming during SE maturation on the phenotype of maritime pine plants after a short-term heat treatment

Eva Cañizares[1], Alex Alborch[1], María Cano[1], Alberto Guillén[1], Juan Segura[1], Ester Sales[2], Isabel Arrillaga[1]

[1] ISIC/ERI BIOTECMED, Department of Plant Biology, University of Valencia, Av. Vicent Andrés Estellés, 46100 Burjassot, Valencia, Spain,
[2] Departamento de Ciencias agrarias y del Medio Natural, Escuela Politécnia Superior, University of Zaragoza, Ctra. Cuarte s/n 22071 Huesca, Spain
isabel.arrillaga@uv.es

Keywords: heat stress, resilience, photosynthesis, maritime pine.

Maritime pine (*Pinus pinaster* Aiton) is the most abundant conifer in the Mediterranean basin with a surprisingly ample ecological niche. Biotechnological tools such as somatic embryogenesis (SE) and gene transfer have been developed in this species for breeding, with the aim of generating elite genotypes with improved growth characteristics under stress conditions. The main goal of our research is to obtain maritime pine clones with a better adaptation to high temperatures, by "priming" somatic embryos (SE) during the maturation process.

Maritime pine embryogenic lines, induced and proliferated at 28ºC, were maturated at 18º, 23º or 28ºC as described in Arrillaga et al. (2019). Mature SEs were germinated and transferred to the greenhouse. After one year, plants were exposed during 10 days to high temperatures, which ranged from 22ºC to 50ºC for 4 hours, and then was maintained over 3 hours. Data from photosyntetic parameters, i.e. proline and relative water content, as well as anatomical characteristics were determined in sampled needles at the beginning (T0) and at the end (T10) of the 10 days-heat treatment and after another 10 days to measure plant recovery (TR).

Our preliminary results suggest that a drastic temperature decrease (10ºC) during SE maturation produced plants with a higher adaptation to heat stress in terms of higher water content, which also correlated with higher proline levels in plant needles. In addition, reduction in net photosynthesis during heat stress was lower in the plants derived from SE maturated at 18ºC than in those derived from SE maturated at 23ºC or 28ºC (at T10) and increased after 10 days recovery (TR). Finally, we also found that after heat stress, the needle chlorophyllic parenchyma in these plants was wider than in those from SE maturated at warmer temperatures. A possible cross-tolerance between priming with low temperatures during maturation and heat stress resilience is under study.

References

Arrillaga I, Morcillo M, Zanón I, Lario F, Segura J and Sales E (2019) New Approaches to Optimize Somatic Embryogenesis in Maritime Pine. Front Plant Sci 10:138. Doi: 10.3389/fpls. 2019.00138

Acknowledgements: This work was supported by the MINECO and EU (AGL2016-76143-C4-1-R) and the Generalitat Valenciana (PROMETEOII/2014/052).

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Dynamics of DNA methylation and effects of de-methylating agents on somatic embryogenesis of *Quercus suber* L

Elena Carneros[1], Yolanda Pérez-Pérez[1], Ivett Bárány I.[1], Beatriz Pintos[2], Aránzazu Gómez-Garay[2], María C. Risueño[1], Pilar S. Testillano[1]*

[1] Pollen Biotechnology of Crop Plants, Biological Research Center, CIB-CSIC, Madrid, Spain
[2]Dep. Genetics, Physiology and Microbiology, UCM, Madrid, Spain
**Corresponding author, testillano@cib.csic.es*

Epigenetic marks are involved in the regulation of global gene expression programs, among these marks, DNA methylation, accomplished by DNA methyltransferases, is a key epigenetic modification of the chromatin, associated with gene silencing that can change during cell proliferation and differentiation processes. Somatic embryogenesis is a widely used biotechnological tool for *in vitro* plant regeneration but, unfortunately, often still with limited efficiency in woody species. Cell reprogramming, totipotency acquisition and somatic embryogenesis initiation involve changes in the developmental program of the cell, which affect global genome organization. Recent studies in rapeseed and barley have reported that microspore embryogenesis initiation is associated with DNA hypomethylation (Solís et al. 2012, El-Tantawy et al. 2014), and its efficiency can be improved by the DNA demethylating agent 5'-azacytidine (AzaC) (Solís et al. 2015). However, in trees, little is known about DNA methylation dynamics during somatic embryogenesis, except for a few reports that have revealed the association of somatic embryogenesis initiation with reduced DNA methylation (Rodríguez-Sanz et al. 2014, Corredoira et al. 2017).

In this work we analyzed the changes in global DNA methylation levels and nuclear distribution of methylated DNA by immunofluorescence with anti-5-methyl-deoxycitidine (5mdC) antibodies (Testillano et al. 2013), as well as gene expression profiles of several DNA methyl transferases during somatic embryogenesis progression in *Quercus suber* L. (cork oak), by biochemical, molecular and immunocytochemical approaches. Furthermore, effects of AzaC treatments on somatic embryogenesis were analyzed.

Results showed low levels of global DNA methylation at early stages of somatic embryogenesis, in proembryogenic masses, followed by a progressive increase in DNA methylation at later stages, during somatic embryo differentiation. This pattern correlated with the expression profile of the DNA methyl transferase *QsMET1* which was up-regulated during somatic embryo development, suggesting the involvement of MET1 in the process. AzaC treatment reduced global DNA methylation of proembryogenic masses and promoted the proliferation of somatic embryogenesis cultures, favoring the initial stages of somatic embryogenesis. However, at advanced stages AzaC prevented embryo differentiation, an effect that could be reverted by eliminating the drug from culture medium.

These findings provide new insights into the epigenetic regulation underlying somatic embryogenesis in cork oak, a forest species of high economic and ecologic value, opening up new intervention pathways, by using epigenetic modulators, to improve somatic embryogenesis yields for forestry breeding and propagation programs.

Acknowledgments: Work supported by project AGL2017-82447-R funded by Spanish Ministry of Science, Innovation and Universities (MCIU) and European Regional Development Fund (ERDF/ FEDER).

References

Corredoira E, Cano V, Bárány I, Solís MT, Rodríguez H, Vieitez AM, Risueño MC, Testillano PS. (2017) Initiation of leaf somatic embryogenesis involves high pectin esterification, auxin accumulation and DNA demethylation in *Quercus alba*. J Plant Physiol 213: 42–54

El-Tantawy AA, Solís MT, Risueño MC, Testillano PS. (2014) Changes in DNA methylation levels and nuclear distribution patterns after microspore reprogramming to embryogenesis in barley. Cytogenet Genome Res 143: 200-208

Rodríguez-Sanz H, Manzanera JA, Solís MT, Gómez-Garay A, Pintos B, Risueño MC, Testillano PS. (2014) Early markers are present in both embryogenesis pathways from microspores and immature zygotic embryos in cork oak, *Quercus suber* L. BMC Plant Biol 14: 224

Solís MT, Rodríguez-Serrano M, Meijón M, Cañal MJ, Cifuentes A, Risueño MC, Testillano PS. (2012) DNA methylation dynamics and MET1a-like gene expression changes during stress-induced pollen reprogramming to embryogenesis. J Exp Bot 63: 6431-6444.

Solís MT, El-Tantawy AA, Cano V, Risueño MC, Testillano PS (2015). 5-azacytidine improves microspore embryogenesis initiation by decreasing global DNA methylation but prevents subsequent embryo development in rapeseed and barley. Front Plant Sci 6: 472

Testillano PS, Solís MT, Risueño MC (2013). The 5-methyl-deoxy-cytidine (5mdC) localization to reveal in situ the dynamics of DNA methylation chromatin pattern in a variety of plant organ and tissue cells during development. Physiol Plantarum 149: 104-114

Characterization of the influence of nitric oxide donors on metabolism of polyamines and amino acids in embryogenic cell cultures of Brazilian pine (*Araucaria angustifolia* (Bertol.) Kuntze)

André L.W. dos Santos[1*], Giovanni V. Cerruti[1], Alana S. Silva[1], Ana S.R.L.A. Duque[2], Manuel P.S. Fevereiro[2], Eny I.S. Floh[1]

[1]Universidade de São Paulo, Instituto de Biociências, Departamento de Botânica, Laboratorio de Biologia Celular de Plantas (BIOCEL), São Paulo, Brazil.
[2]Universidade Nova de Lisboa, Instituto de Tecnologia Química e Biológica, Plant Cell Biotechnology Laboratory (BCV), Portugal.
*Corresponding author: alwsantos6@gmail.com

Keywords: NO donors, GSNO, SNP, stress tolerance, cell lines

High levels of stress and/or the application of exogenous plant growth regulators have been used for the induction of embryogenesis in cell cultures. Stress response accelerates somatic embryo formation and increases the transcription of genes related to stress treatment. Thus, the levels of stress tolerance exhibited by individual cell lines may influence both embryogenic cell viability and metabolism, as well as their adaptation to environmental conditions during *in vitro* culturing. Polyamines (PAs) and nitric oxide (NO) are molecules associated to several plant growth and development processes, including adaptive responses to biotic and abiotic stress. PAs (putrescine, spermidine, and spermine) are biochemically related to NO through arginine, a common precursor in their biosynthetic routes, suggesting that alteration in NO homeostasis can affect PAs bioavailability and vice-versa. Moreover, NO has been shown to be produced from PAs through a still uncharacterized mechanism (Tun et al. 2006; Silveira et al. 2006). The overlapping roles between PAs and NO raise the question of how both molecules may act in coordination during plant development. Somatic embryogenesis (SE) associated to cryopreservation can represent a useful strategy for ex situ conservation of Brazilian pine, a native endangered conifer of South America. In Brazilian pine, PAs and NO seem to be involved in the regulatory mechanisms responsible for proliferation of proembryogenic masses and differentiation in somatic embryos. In order to elaborate an appropriate condition to improve SE in Brazilian pine, we analyzed the influence of NO donors (GSNO and SNP) during somatic embryo formation in three embryogenic cell lines. In addition, endogenous NO production, PAs and amino acids levels were recorded after cultivation in the presence of NO donors for 30 min, 24 and 72h. These findings will be important for evaluation of embryogenic culture responses to changes in media culture formulation and increase of somatic embryo formation in different Brazilian pine genotypes.

Acknowledgments: This work is supported by *FAPESP* (Fundação de Amparo à Pesquisa do Estado de São Paulo), Young Investigators Grants 15/21075-4 and 17/01284-3.

References

Silveira V, Santa-Catarina C, Tun NN, Scherer GFE, Handro W, Guerra MP, Floh EIS. 2006. Polyamine ffects on the endogenous polyamine contents, nitric oxide release, growth and differentiation of embryogenic suspension cultures of *Araucaria angustifolia* (Bert) O. Ktze. Plant Sci. **171**:91-98

Tun NN, Santa-Catarina C, Begum T, Silveira V, Handro W, Floh EIS, Scherer GFE. 2006. Polyamines induce rapid biosynthesis of nitric oxide (NO) in *Arabidopsis thaliana* seedlings. Plant Cell Physiol. **47**(3):346-354

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Field performances of hybrid poplar leaf and wood traits in both micropropagated plants and plants propagated from root cuttings: ecophysiological, vascular, nanomechanical and cell wall compositional assessments

Jaroslav Ďurkovič[1], Hana Husárová[1], František Kačík[2], Rastislav Lagaňa[3], Ingrid Čaňová[1]

[1] *Department of Phytology, Technical University, 96053 Zvolen, Slovakia*
[2] *Department of Chemistry and Chemical Technologies, Technical University, 96053 Zvolen, Slovakia*
[3] *Department of Wood Science, Technical University, 96053 Zvolen, Slovakia*
jaroslav.durkovic@tuzvo.sk

Understanding the physiological, vascular and biomechanical processes that allow micropropagated plants to modify their phenotype in response to environmental conditions can help to improve both field performance and plant survival. To identify differences between the hybrid poplar *Populus tremula* × (*Populus* × *canescens*) plants propagated from in vitro multiple shoot cultures and those from root cuttings, we assessed any changes in both the leaf traits and the woody cell wall composition during the sixth and seventh growing seasons following planting in the field. Measurements and comparisons were made for leaf growth, photosynthetic and vascular traits, nanomechanical properties, content of cell wall components, lignin monomer composition, as well as for the macromolecular properties of both lignin and cellulose. With regard to the leaf traits, the micropropagated plants showed significantly higher values for leaf area, leaf length, leaf width and leaf dry mass. The increased leaf area and leaf size dimensions resulted from a higher transpiration rate. The micropropagated plants also achieved higher values for the nanomechanical dissipation energy of tracheary element cell walls which may indicate a higher damping capacity within the primary xylem tissue under abiotic stress conditions. The performance of the plants propagated from root cuttings was superior for instantaneous water-use efficiency signifying a higher acclimation capacity during a severe drought. Similarities between the two stock types were found for the majority of the examined leaf traits, including leaf mass per area, stomatal conductance, net photosynthetic rate, hydraulic axial conductivity, indicators of primary xylem vascular architecture, modulus of elasticity, adhesive force and deformation. This research revealed that there was no decrease in the leaf physiological performance which could be attributed to the micropropagated plants.

From a viewpoint of the woody cell wall composition, the performance of the micropropagated plants was superior for the content of cellulose, relative proportions of both D-glucose and D-mannose, and weight-average molecular weight of dioxane lignin. The plants propagated from root cuttings achieved significantly higher values for the content of hemicelluloses, total yield of neutral saccharides, and both absolute yields and relative proportions of D-xylose and L-arabinose. Non-significant differences between the stock types were found again for the majority of the examined wood traits, including the macromolecular properties of cellulose and the vascular traits. Although no direct preference to either stock type can be clearly underlined, there were also no substantial drawbacks found in either the chemical profiles of the wood components or in the anatomical profiles of the vascular traits which could be attributed to the micropropagated plants. In addition, no significant differences were found in lignin monomer composition or in the relative proportion of syringyl units between the stock types. Thus, seen from a phytopathological point of view, no advantage could be attributed to either stock type for an enhanced resistance against cellulose biodegradation.

The effect of different air humidity during desiccation on the development of Norway spruce somatic embryos

Lucie Fischerová, Lenka Gemperlová, Jan Kuderna, Alena Trávníčková, Jiří Malbeck, Kateřina Eliášová, Zuzana Vondráková, Milena Cvikrová, Martin Vágner

Institute of Experimental Botany of the Czech Academy of Sciences, Prague, Czech Republic.
fischerova@ueb.cas.cz

Keywords: autophagy – related genes, desiccation, glucanases, chitinases, polyamines

The protocols of coniferous somatic embryogenesis (SE) are constantly evolving with the aim to find application of these protocols for a wide spectrum of species. Although individual genera, sometimes species, need protocol modifications, the goal remains the same – fully developed somatic embryos mimicking zygotic embryos. Desiccation, as the final phase of embryonic development, leads to biochemical changes in somatic embryos approaching those in its zygotic counterparts (e.g. Gemperlova et al. 2009) and appears to be important in the transition from the embryogenic phase to germination. The objective of the presented study was to follow morphological and selected biochemical characteristics induced by various air humidity during desiccation of Norway spruce (*Picea abies* Karst.) somatic embryos. The fully developed embryos were desiccated in three different levels of air humidity (90%, 95%, and 100%) for 10 days; for another 10 days the embryos were kept in 100% air humidity. We determined changes of polyamine content as an indicator of the level of drought stress in embryos. Concurrently we observed the expression of two beta glucanase and two chitinase genes and selected ATG (autophagy related) genes, since they are assumed to play a role both in development and reaction of plants to various stresses (e.g. Veluthakkal et al. 2012).

The basic morphology of desiccated embryos was comparable in all three variants, however germination of somatic embryos cultivated in 90% of air humidity was negatively affected. Desiccation in 100% relative air humidity (control variant) affected polyamine levels; free polyamines were lowered and higher forms of polyamines (spermidine and spermine) were favored. Low humidity at the start of desiccation led to an increase of putrescine level, which dropped after subsequent rehydration at the end of desiccation. Expression of endo-1,4-beta glucanase increased during desiccation in 100% air humidity while in lower air humidity it remained unchanged. The expression of beta-1,3-glucanase was highly up-regulated after low humidity desiccation, the same pattern of expression was observed with class IV chitinase Chia4-Pa and putative ATG12. Expression levels of putative class I chitinase and ATG8 genes were slightly up-regulated in all desiccation treatments. Somatic embryos were highly active during desiccation both on the level of polyamine metabolism and expression of selected genes. From this we conclude that desiccation of somatic embryos is a metabolically active process affected by relative air humidity.

Acknowledgement: This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (grants no. LTC17036 and LD13050)

References

Gemperlova L et al. (2009). Polyamine profiles and biosynthesis in somatic embryo development and comparison of germinating somatic and zygotic embryos of Norway spruce. Tree Physiol 29:1287–1298.

Veluthakkal R et al. (2012). Tree chitinases - stress- and developmental-driven gene regulation. For Pathol 42:271–278.

Effect of megagametophyte priming on short-term response to high temperatures in somatic embryogenesis-derived plants of maritime pine

Juan G. Haro[1], Juan Segura[1], Alex Alborch[1], María Cano[1], Ester Sales[2], Isabel Arrillaga[1]

[1] *ISIC/ERI BIOTECMED, Department of Plant Biology, University of Valencia, Av. Vicent Andrés Estellés, 46100 Burjassot, Valencia, Spain,*
[2] *Departamento de Ciencias agrarias y del Medio Natural, Escuela Politécnia Superior, University of Zaragoza, Ctra. Cuarte s/n 22071 Huesca, Spain*
isabel.arrillaga@uv.es

Keywords: *Pinus pinaster*, heat stress, *HSP70*, *WRKY*, *PEROX1*, somatic embryogenesis, resilience.

In the context of climate change, efforts in forest research should be addressed to provide improved genotypes with increased resilience to high temperature events. One of the goals of our group is to obtain improved maritime pine (*Pinus pinaster*) clones that include such a characteristic by "priming" our somatic embryos (SE) at different periods of the SE production. To this end, we have applied high temperature pulses (37ºC and 50ºC) to isolated megagametophytes just at the beginning of the SE induction phase. Afterwards, embryogenic masses from these primed megagametophytes went through proliferation and maturation stages as described in Cano et al. (2018). Finally *in vitro* germinated emblings from one of these clones were used to study whether this initial "priming" would improve resilience after further heat events. In order to do this, *in vitro* growing plantlets were subjected to different heat treatments, at 23ºC, 37ºC or 50ºC for 3 hours, and needle samples were collected at 0, 24 and 72 hours after the heat treatment. The relative expression of *HSP70*, *WRKY* and *PEROX1* genes, all related to defense responses, was determined through quantitative-PCR (qPCR).

Expression of the *WRKY* gene was higher for *in vitro* plants derived from primed megagametophytes than in those from control explants, and its expression remained unaltered after heat treatments, indicating some epigenetic changes on this gene. In contrast, expression of the *HSP70* gene increased after the heat treatment in plants derived from megagametophytes primed at 37º, indicating an earlier response to heat stress. Neither the priming nor further heat treatments affected *PEROX1* gene expression.

Our results suggest that priming initial explants might accelerate the heat response of plants under *in vitro* conditions and thereby increase resilience. Further assays will be performed after transferring these plants to *ex-vitro* conditions.

Acknowledements: This work was supported by the MINECO and EU (AGL2016-76143-C4-1-R) and the Generalitat Valenciana (PROMETEOII/2014/052).

References

Cano M, Morcillo A, Humánez A, Mendoza-Poudereux I, Alborch A, Segura J, Arrillaga I (2018) Maritime Pine (*Pinus pinaster* Aiton). In: Jain SM and Gupta PK (eds), Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants: Volume I pp 167-179 Forestry Sciences 84. Springer International Publishing. https://doi.org/10.1007/978-3-319-89483-6_13

Recent progress in somatic embryogenesis of Japanese conifers

Tsuyoshi E. Maruyama[1], Hosoi Yoshihisa[2]

[1]Forestry and Forest Products Research Institute (FFPRI), Research Planning and Coordination Department, Matsunosato 1, Tsukuba, 305-8687 Japan
[2]Forestry and Forest Products Research Institute (FFPRI), Department of Forest Molecular Genetics and Biotechnology, Matsunosato 1, Tsukuba, 305-8687 Japan
tsumaruy@ffpri.affrc.go.jp , yh2884@ffpri.affrc.go.jp

Keywords: clonal propagation, gellan gum, polyethylene glycol, somatic embryo desiccation, somatic plants, tissue culture

Sugi (*Cryptomeria japonica*), Kuromatsu (*Pinus thunbergii*), Akamatsu (*P. densiflora*), Yakutanegoyou (*P. armandii* var. *amamiana*), Ryukyumatsu (*P. luchuensis*), Hinoki (*Chamaecyparis obtusa*), Sawara (*C. pisifera*), Yatsugataketouhi (*Picea koyamae*), and Himebaramomi (*P. maximowiczii*) are important in Japan for reforestation and landscaping. However, these species are affected by various biological problems and need urgent measures for their propagation. Somatic embryogenesis (SE) is the most promising technique for mass propagation of clones, and for plant regeneration in genetic transformation protocols used in basic studies and in tree improvement programs. In addition to the potential of SE as a powerful tool for efficiently and economically mass producing of clonal planting stock, the most attractive advantage is that the embryogenic cultures can be cryopreserved without changing their genetic make-up and without loss of juvenility.

In this presentation we report recent progress over the last decade in protocol development for SE in Japanese conifers. After the first report of plant regeneration via SE in *Cryptomeria japonica* (Maruyama et al. 2000, Plant Biotechnology), several results on enhancement of somatic embryo production and plant conversion efficiency were published regarding Japanese conifers. The status in protocol development for SE in Japanese conifers, including induction of embryogenic tissues, embryogenic culture maintenance/proliferation, somatic embryo production, germination, and plant regeneration from somatic embryos are described.

SE was initiated from megagametophytes containing zygotic embryos. Embryogenic cultures were maintained and proliferated in a medium supplemented with 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine, sucrose, and glutamine. Then, somatic embryo maturation experiments were performed in darkness at 25°C, culturing the embryogenic tissues on maturation media containing maltose, activated charcoal, abscisic acid, and polyethylene glycol (PEG). The addition of PEG to the medium dramatically stimulated embryo maturation and resulted in an enhanced yield of mature embryos as the PEG concentration is increased. Although the cotyledonary embryo production varied according to the species, supplementation of medium with 100-150 g l–1 PEG was found to be suitable for high-quality embryo production of Japanese conifers. Mature somatic embryos germinated and then converted into plantlets after their transfer to plant growth regulator-free medium. However, for the Japanese pine species, desiccation of somatic embryos after PEG-maturation was found to be essential for achieving both high germination and high conversion rates. Desiccation of somatic embryos at high relative humidity resulted not only in a marked increment in germination frequency but also, subsequently, in an improved plant conversion rate. In addition, this treatment resulted in a considerable improvement of the synchronization of the germination period, compared to that of the untreated control. In contrast, when somatic embryos of Japanese pines were matured on PEG-free medium but containing

a high concentration of gellan gum, somatic embryos readily germinated without any post-maturation treatments.

Although the improved protocol represents a promising perspective for efficient mass propagation of Japanese conifers, it is necessary to refine the protocol to enhance embryogenic tissue induction frequencies, as well as to improve the productivity of high quality embryos in liquid medium. In addition, even more efforts are necessary to develop an effective and practical, reproducible technique to produce somatic plants from adult vegetative material for rapid acceleration of many tree improvement programs.

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Development of an *in vitro* screen for *Phytophthora cinnamomi* resistance in hybrid and transgenic chestnut trees

Scott A. Merkle[1], Marjorie G. MacKnight[1], Heather J. Gladfelter[1], Steven N. Jeffers[2]

[1]*Warnell School of Forestry and Natural Resources, University of Georgia, Athens, GA USA*
[2]*Department of Plant & Environmental Sciences, Clemson University, Clemson, SC, USA*
smerkle@uga.edu, mgm71556@uga.edu, hjgladf@uga.edu, sjffrs@clemson.edu

Keywords: *Castanea dentata*, *Castanea mollissima*, *Phytophthora* root rot, shoot cultures

The lack of resistance to Phytophthora cinnamomi, an oomycete pathogen that causes *Phytophthora* root rot disease in American chestnut (*Castanea dentata*) and other woody species, may pose a major barrier to introducing the products of The American Chestnut Foundation's (TACF) breeding program to the southern portion of the American chestnut's original range in the U.S. The integration of genes for P. cinnamomi resistance from Chinese chestnut (*Castanea mollissima*) into TACF's hybrid backcross chestnuts is now proceeding, but will take additional years of breeding and selection. Combining somatic embryogenesis-based propagation of chestnuts with a reliable in vitro assay for resistance to P. cinnamomi would help to more rapidly evaluate hybrid backcross chestnut clones thought to carry resistance genes—which in turn will accelerate the production of elite chestnut varieties with resistance to both chestnut blight (*Cryphonectria parasitica*) and *P. cinnamomi* for planting throughout the eastern U.S. Our goal was to define a quantitative in vitro screening approach that could be applied to identify *P. cinnamomi*-resistant hybrid backcross chestnuts (B3 and BC3F3 generations), and to test transgenic American chestnut carrying the candidate *P. cinnamomi* resistance genes RPH and NPR3/4, using pure American chestnuts and Chinese chestnuts as susceptible and resistant controls, respectively. Clones were screened using micropropagated shoots "planted" into agar gel in test tubes and inoculated with a 3 mm plug of a locally-collected isolate of *P. cinnamomi*. In three experiments, the growth rate of a dark lesion from the base of the shoot to the tip was used to compare resistance among the different chestnut genotypes. The results indicated that within 30 days of inoculation, Chinese chestnuts showed significantly shorter stem lesions compared to pure American chestnuts and hybrid backcross chestnuts. In addition, it appeared that some of the hybrid backcross chestnuts had intermediate resistance between the susceptible American chestnut and Chinese chestnut genotypes tested, as might be expected, although others were no more resistant than pure American chestnuts. None of the tested transgenic chestnut shoots showed evidence of enhanced resistance compared to pure American chestnut.

CYTED NET: BIOALI, Biotechnology to improve breeding programs of species of socioeconomic interest

Paloma Moncaleán*, BIOALI Consortium (www.bioali.es)

*NEIKER-TECNALIA. Centro de Arkaute. 01080 Vitoria-Gasteiz. Spain. *pmoncalean@neiker.eus*

Keywords: Breeding, food, productivity, social benefit, trees.

The Ibero-American Program of Science and Technology for Development (CYTED) has approved the financing of the BIOALI network "Biotechnology to strengthen improve programs of species with socioeconomic interest". The network that groups 18 research groups, 3 companies of the agri-food and 1 of the environmental sector and 85 researchers from research centers and universities, including professionals interested in the development and application of biotechnological tools (tissue culture, genetic, physiological and molecular techniques) for the breeding of species of social and economic interest. The general objective of the BIOALI network (www.bioali.es) will be to contribute to the sustainable improvement of forest tree productivity and nutritional value of food species, of social and economic interest, via the generation and exchange of knowledge among members. This improvement will be carried out through the exploration of their genomes, the fortification of their products, and the development of early diagnosis methods of its main pathogens through phenotyping, physiological and metabolomic techniques and obtaining indicators of abiotic and biotic stresses derived from climate change. This big goal is being carried out by establishment of a research consortium, training, exchange of information between multidisciplinary groups in different fields, education and social awareness, actions that will be carried out to satisfy the needs of present generations, without endangering the future ones.

Acknowledgements: This work is founded by CYTED (Programa Iberoamericano de Ciencia y Tecnología para el Desarrollo) through BIOALI net and by DECO from the Basque Government, Spain.

Current results in somatic embryogenesis for *Pinus koraiensis*, an ecologically and economically very important pine species in East Asia

Chunxue Peng, Fang Gao, Hailong Shen, Ling Yang, Peng Zhang, Chunping Liu

State Key Laboratory of Tree Genetics and Breeding, School of Forestry, Northeast Forestry University, Harbin 150040, China

*First author: 1325835674@qq.com , *Corresponding author: shenhl-cf@nefu.edu.cn*

Keywords: explant effects, embryo development, culture conditions, micropropagation

Korean pine (*Pinus koraiensis* Sieb. et Zucc.) (KP) is an ecologically and economically very important pine species distributed in Northeast China, the Russian Fast East and the northern part of the Korean peninsula, and has scattered distribution at high mountain areas in Japan. It is the dominant and community-constructive species (edificato) in the cool-temperate zonal climax forest of mixed Korean pine and broad leaved trees in its distribution areas and produces high quality pine timber, nutritious edible nuts and products like turpentine. Especially, Korean pine nut production has become the supporting industry in Northeast China's forest regions and, therefore, timber harvest has had to be halted in the natural Korean pine forest and no timber can currently be harvested in Korean pine plantations in China. There are 3 reports related to somatic embryogenesis (SE) in Korean pine (Bozhkov et al. 1997; Shen et al. 2005; Wang et al. 2009). Our studies on SE of KP was begun in 2007 and SE was first achieved in 2016 (Gao 2017). At present, a relatively effective KP SE system has been established by using immature zygotic embryos (ZE) as explants, but not yet by using mature ZE. The main results were as follows: (1) explant collection time for KP SE was at the end of June or at the begging of July, and strongly depended on the developmental status of ZE. This was influenced by the stock trees, location of cones on the stock tree crown and location of seeds in the cone; (2) good embryogenic callus was obtained from immature ZE by using DCR medium solidified by 6.5 g/L agar and supplemented with 35.0 g/L sucrose and supplemented with 2 mg/L NAA, 1.5 mg/L 6-BA and 500 mg/L acid hydrolyzed casein. The embryogenic callus could be maintained and proliferated by DCR medium with 30.0 g/L sucrose, 0.5 mg/L 2,4-D, 0.1 mg/L 6-BA, 500 mg/L acid hydrolyzed casein and 500 mg/L glutamine; the subcullture period should be 15 days; somatic embryos were obtained but not in ideal quantity and good status; (3) improved embryogenic callus, a large quantity of somatic pre-embryos and good matured somatic embryos were obtained recently on DCR and modified LV media solidified by 12 g/L gellan gum and supplemented with 68.5 g/L sucrose and 21.1 mg/L ABA, but mLV was much better than DCR for somatic embryo development of KP. These results provide scientific support for the establishment of a large-scale, high-efficiency micropropagation system of genetic improved material of KP via somatic embryogenesis.

References

Bozhkov PV, Ahn IS, Park YG (1997) Two alternative pathways of somatic embryo origin from polyembryonic mature stored seeds of *Pinus koraiensis* Sieb et Zucc [J]. Can J Bot 75(3): 509-512

Gao F (2017) Somatic embryogenesis and plantlet regeneration of *Pinus koraiensis* [D]. Master thesis, Northeast Forestry University (China), 2017. (in Chinese with English abstract)

Shen XH, Jiang XN, Park YS, Mahedrappa MK (2005) Somatic embryogenesis (SE) in Korean pine (*Pinus koreiensis* Sieb. et Zucc) [J]. J Chengdu Univ (Natural Sci) 24(1): 11-14 (in Chinese with English abstract)

Wang G (2009) Somatic embryogenesis and cryopreservation of *Pinus koraiensis* Sieb. et Zucc.[D]. Master thesis, Shanghai Jiao Tong University (China) (in Chinese with English abstract)

Clonal test of *Sequoia sempervirens* (D. Don) Endl. in southern Brazil

Pereira MO[1], Navroski MC [1], Angêlo AC[2]

[1] *Department of Forest Engineer of the Santa Catarina State University - Udesc, Lages, Santa Catarina, Brazil.* marcio.navroski@udesc.br
[2] *Department of Forest Engineer of the Federal University of Paraná - UFPR, Curitiba, Paraná, Brazil.* alessandrocangelo@gmail.com

Keywords: clonal silviculture, conifer, field establishment, frost damage

Sequoia sempervirens (D. Don) Endl., popularly known as sequoia or redwood, is a species with valuable wood properties, its wood has a reddish color, easy workability and high natural durability. It has the potential for planting in places outside its natural habitat, as shown by deployments in Chile and New Zealand. The successful implementation of a species such as sequoia may represent a forest alternative for Brazil, especially for the southern half, a place that presents climatic analogies with the place of origin of the species. Cloning of the species suggests a greater ease of genetic gain and the possibility of selecting specific clones for different sites. The objective of the study was to implement sequential clonal tests in two regions of Santa Catarina (Brazil), seeking information on adaptation and growth. The implantation occurred in two locations in the State of Santa Catarina - Brazil: Campo Belo do Sul (Cfb climate, average annual temperature 16ºC, and occurrence of strong frosts in winter) and Arabutã (Cfa climate, average temperature 19.2ºC, and rare occurrence of frost). The material of origin (cuttings) were obtained by means of the rescue of trees approximately 40 years old (São Joaquim (SC) and São Francisco de Paula (RS)), propagation by cutting, assembly of a clonal mini-garden, and finally production of cuttings by minicutting. Planted cuttings were around 30 cm in height and lap diameter of more than 4 mm. In Campo Belo do Sul, implantation was carried out in December 2015 with three clones (SJ, A140 and A228), with evaluation of growth and frost damage at 6, 12 and 24 months. Four clones were used in Arabutã (A127, A138, A140 and A228). Planting was carried out in December 2016, with growth assessment at 6 and 12 months. The sequoia cuttings implanted in the colder region (Campo Belo do Sul) suffered severe frost damage, with survival of only 2% observed after 24 months of implantation. In contrast, in the warmer area (Arabutã) (there was no record of frost during that period) survival was greater than 80% at 12 months, with an average height of more than 50 cm and 7.5 mm in the lap diameter. Other strategies should be adopted to seek the establishment of sequoia in colder areas, seeking to minimize the effect of frosts on seedlings. More evaluations are needed, however, the species initially shows potential for areas that are without the occurrence of frost.

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Cell wall remodeling by pectin esterification and AGP expression underlies somatic embryogenesis of cork oak

Yolanda Pérez-Pérez[1], Elena Carneros[1], María-Teresa Solís[1,2], Ivett Bárány[1], Beatriz Pintos[2], Aránzazu Gómez-Garay[2], María C. Risueño[1], Pilar S. Testillano[1]

[1] Pollen Biotechnology of Crop Plants, Biological Research Center, CIB-CSIC, Madrid, Spain
[2]Dep. Genetics, Physiology and Microbiology, UCM, Madrid, Spain
**Corresponding author, testillano@cib.csic.es*

Somatic embryogenesis (SE) is a system for *in vitro* regeneration, with many biotechnological applications in woody species, but the regulating mechanisms of the process are largely unknown. Changes in cell wall mechanics controlled by the methylesterification/de-methylesterification status of pectins, mediated by pectin methylesterases (PME) and pectin methylesterase inhibitors (PMEI) underlie organogenesis initiation and embryogenesis progression in various species (Solís et al. 2016). Nevertheless, the functional meaning of pectin-related cell wall remodeling in different cell types and processes still remains unclear. Arabinogalactan proteins (AGPs) are a large family of highly glycosylated proteins that are present in cell walls, on the surface of plasma membranes and extracellular secretions, playing a key role in several plant developmental processes, including different aspects of sexual reproduction and embryogenesis (El-Tantawy et al. 2013). Addition of exogenous AGPs to culture medium has been reported to promote somatic embryogenesis, however, the precise role of endogenous AGPs in the regulation of somatic embryogenesis remains poorly understood.

In this study, we have investigated changes in pectin esterification and AGPs during SE in *Quercus suber*, cork oak. Expression analysis of several PME, PMEI and AGP genes, by qRT-PCR, immunofluorescence and confocal analysis were performed by using monoclonal antibodies to AGPs, high- and low-methylesterified pectins (LM6, LM2, LM19, LM20, JIM7, JIM5). Furthermore, functional analyses were approached by *in vitro* pharmacological treatments with catechin, an inhibitor of PME activity, and Yariv reagents, that block AGPs.

Results allowed the characterization of the distribution patterns of AGPs and pectin esterification/de-esterification during SE progression, as well as their correlation with the expression patterns of *QsPME*, *QsPMEI*, *QsLys-rich-AGP18*, *QsLys-rich-AGP17*, and *QsAGP16L1* genes. At early SE stages, cells of proembryogenic masses showed high levels of esterified pectins and expression of *QsPMEI* gene. Advanced SE stages, i.e., differentiating cells of heart, torpedo and cotyledonary embryos, exhibited walls rich in de-esterified pectins, and progressively increased expression of the *QsPME* gene. AGPs were detected in cell walls of proembryogenic masses and somatic embryos. This correlated with the expression profiles of *QsLysrich-AGP18*, *QsLys-rich-AGP17*, and *QsAGP16L1* that increased with embryogenesis progression. SE cultures treated with both catechin and Yariv reagent impaired the progression of embryogenesis, indicating that pectin de-esterification and increase in AGP levels were necessary for embryo development (Pérez-Pérez et al. 2019). This indicates a role for pectins and AGPs during somatic embryogenesis of cork oak, promoting the cell wall remodeling during the process. These findings give new insights into the regulating mechanisms of the process in woody species, for which information is still scarce, opening up new possibilities to improve *in vitro* embryo production in tree breeding programs.

Acknowledgments: Supported by project AGL2017-82447-R funded by Spanish Ministry of Science, Innovation and Universities (MCIU) and European Regional Development Fund (ERDF/ FEDER).

References

El-Tantawy AA, Solís MT, Costa ML, Coimbra S, Risueño MC, Testillano PS (2013) Arabinogalactan protein profiles and distribution patterns during microspore embryogenesis and pollen development in *Brassica napus*. Plant Reproduc 26: 231-43

Pérez-Pérez Y, Carneros E, Berenguer E, Solís MT, Bárány I, Pintos B, Gómez-Garay A, Risueño MC, Testillano PS (2019) Pectin de-methylesterification and AGP increase promote cell wall remodeling and are required during somatic embryogenesis of *Quercus suber*. Front Plant Sci 9: 1915

Solís, MT, Berenguer E, Risueño MC, Testillano PS (2016) *BnPME* is progressively induced after microspore reprogramming to embryogenesis, correlating with pectin de-esterification and cell differentiation in *Brassica napus*. BMC Plant Biol 16: 176

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

The transcriptome of maritime pine across embryo development

Andreia S. Rodrigues[1,2], José J. De Vega[3], Célia M. Miguel[1,2,4*]

1 Instituto de Biologia Experimental e Tecnológica (iBET), Apartado 12, 2780-901, Oeiras, Portugal
2 Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa (ITQB NOVA), Av. da República, 2780-157, Oeiras Portugal
3Earlham Institute, Norwich Research Park, NR4 7UZ, Norwich, UK
4 Universida de de Lisboa, Faculdade de Ciências, BioISI - Biosystems & Integrative Sciences Institute, Campo Grande, 1749-016, Lisboa, Portugal. cmmiguel@fc.ul.pt

Keywords: Zygotic embryo, *Pinus pinaster*, embryogenesis, RNA-seq, developmental stages

Embryo development presents clear differences between angiosperm and gymnosperm species. Most of the knowledge currently available on transcriptomics and transcriptional regulation during plant embryogenesis has been obtained from studies conducted with angiosperms species, and most often with the model plant *Arabidopsis thaliana* (reviewed by De Smet et al., 2010). In gymnosperms, conifers have also been the subject of a few reports on transcript profiling of embryogenesis (reviewed by Miguel et al., 2016 and by Lelu-Walter et al., 2016). Such studies have shown the presence of many putative embryo-specific transcripts without an assigned function. To gain further knowledge on the transcriptomic expression during conifer embryogenesis and to extend our previous transcriptomic analysis using a microarray approach (De Vega-Bartol et al., 2013), we sequenced the transcriptome of zygotic embryos in several developmental stages covering most of *Pinus pinaster* (maritime pine) embryogenesis (Gonçalves et al., 2005).

Total RNA samples collected from five zygotic embryo developmental stages as previously described (De Vega-Bartol et al., 2013) were sequenced with Illumina technology. Since the genome of *Pinus pinaster* is not yet publicly available, *de novo* assembly of the transcriptome was performed using a comprehensive approach (Visser et al., 2015). The transcriptome of reference for the period of zygotic embryogenesis in maritime pine contains 67,429 transcripts, which likely encode 58,527 proteins. The annotation shows that 30.8% of predicted proteins are exclusively present in pine embryogenesis.

A differential expression analysis (FDR < 0.05) between each pair of consecutive stages of development retrieved 1738 transcripts (2.6%) differentially expressed in at least one developmental transition. Functional and enrichment analyses of the differentially expressed transcripts pointed to the over-representation of carbohydrate transport and metabolism in early embryo stages. This was evidenced by the identification of many putative glycoside hydrolases, which may be associated with cell wall modification, and carbohydrate transport transcripts. Additionally, functions related to chromatin remodeling events were highlighted in early to middle embryogenesis, associated with an active synthesis of histones and their post-translational modifiers related to increased transcription, and silencing of transposons.

Our results significantly expand the catalogue of transcripts expressed during embryogenesis and further elucidate transcriptional regulation during zygotic embryogenesis in conifer species. These results are a valuable resource to support further improvements in somatic embryogenesis for vegetative propagation of conifer species. Specific transcripts associated with carbohydrate metabolism, monosaccharide

transport and epigenetic regulation seem to play an important role in pine early embryogenesis and may be a source of reliable molecular markers for early embryogenesis.

References

De Smet I, Lau S, Mayer U, Jürgens G (2010) Embryogenesis - the humble beginnings of plant life. The Plant Journal, 61, 959–70.

Miguel CM, Rupps A, Raschke J, Rodrigues AS, Trontin JF (2016) Impact of molecular studies on somatic embryogenesis development for implementation in conifer multivarietal forestry. In: Park YS, Bonga JM, Moon HK, editors. Vegetative propagation of Forest trees. Seoul: National Institute of Forest Science; p. 373–421.

Lelu-Walter MA, Klimaszewska K, Miguel C, Aronen T, Hargreaves C, Teyssier C, et al. (2016) Somatic embryogenesis for more effective breeding and deployment of improved varieties in Pinus spp.: bottlenecks and recent advances. In: Somatic Embryogenesis - Fundamental Aspects and Applications. Loyola-Vargas VM, Ochoa-Alejo N, editors. Springer International Publishing; p. 319–365.

De Vega-Bartol JJ, Simões M, Lorenz WW, Rodrigues AS, Alba R, Dean JFD, Miguel CM (2013) Transcriptomic analysis highlights epigenetic and transcriptional regulation during zygotic embryo development of *Pinus pinaster*. BMC Plant Biology, 13:123.

Gonçalves S, Cairney J, Maroco J, Oliveira MM, Miguel C (2005) Evaluation of control transcripts in real-time RT-PCR expression analysis during maritime pine embryogenesis. Planta, 222, 556–63.

Visser EA, Wegrzyn JL, Steenkmap ET, Myburg AA, Naidoo S (2015) Combined de novo and genome guided assembly and annotation of the Pinus patula juvenile shoot transcriptome. BMC Genomics, 16, 1057.

Proceedings of the Fifth International Conference of the IUFRO Unit 2.09.02 on
"Clonal Trees in the Bioeconomy Age: Opportunities and Challenges"
September 10-15, 2018 | Coimbra, Portugal

Ex situ conservation of *Prunus lusitanica* by micropropagation techniques

Alberto Rodríguez[1], Nieves Vidal[1], Purificación Covelo[1], Saleta Rico[1], Conchi Sánchez[1]

[1]*Dpto. Fisiología Vegetal. Instituto de Investigaciones Agrobiológicas de Galicia. IIAG (CSIC). Avda de Vigo s/n. 15705 Santiago de Compostela, Spain.*
alberto.rodriguez.acevedo@gmail.com, nieves@iiag.csic.es, pcovelo@iiag.csic.es, saleta@iiag.csic.es, conchi@iiag.csic.es

Keywords: biodiversity, genetic resources, Portuguese cherry laurel, vulnerable species

Prunus lusitanica is a woody plant of high ecological and botanical interest. It is considered a characteristic species of the relict subtropical flora that persists in Southern Europe, currently in regression due to both human action and climate change. *P. lusitanica* is listed on Appendix I of the Bern Convention and in Annex II of the EU Habitats Directive. In the Azores and Madeira this species is being threatened by the alteration of the water regime, the cutting of trees, invasive alien species and lack of pollinators and dispersal agents (Duarte et al. 2011). Both subspecies that occur in Spain (ssp. *lusitanica* and spp. *hixa*) are listed as Vulnerable in the 2008 Spanish Red List.

With the goal of preserving and increasing its genetic diversity, both *in situ* and *ex situ* approaches must be developed and combined. Advances in plant biotechnology may provide new alternatives to establish germplasm collections, and to facilitate the long- and short-term multiplication and storage of the species biodiversity. At present, only a protocol for *in vitro* germination of immature seeds of this species has been reported (Schulze et al. 2017).

The aim of this work was to develop specific protocols for *in vitro* establishment and propagation of juvenile and mature material of *P. lusitanica* populations.

Actively growing shoots were harvested from three mature plants of different ages (3- and 30 year-old plants) and were used for *in vitro* establishment. For each genotype, different culture lines were established from a single-node or apex that was subcultured and propagated separately. For propagation, different media, such as MS (Murashige and Skoog, 1962), DKW (Driver and Kuniyuki, 1984) and WPM (Lloyd and McCown, 1980), supplemented with N^6-benzyladenine (0.5 and 1.0 mg L^{-1}) or Metatopoline (0.4 mg L^{-1}) in combination with indole-3-butyric acid (0.05 mg L^{-1}) were used. Proliferation was evaluated in terms of number of shoots, length of longest shoot and rooting percentage. The effect of genotype, culture line and the different media and plant growth regulators combinations was studied.

In vitro establishment was successfully achieved in the three genotypes evaluated. The *in vitro* response of the initial explants was dependent on the age of the starting material. Proliferation rates indicated a culture line-dependent response to culture medium. Rooting was achieved in proliferation medium and rooted plants were *ex vitro* adapted and successfully established in field conditions.

Acknowledgements: This research was partly funded by the Xunta de Galicia (Spain) through the Contrato Programa 2017-2018 and the project IN607A 2017/6. We thank Alejandro Díaz, Rafael Sánchez and Patricia Val for technical assistance.

References

Driver JA, Kuniyuki AH (1984) In vitro propagation of Paradox walnut *Juglans hindsii* x *Juglans regia* rootstock. HortScience 19: 507-9.

Duarte MC, Draper Munt, D, Branca F, Donnini D, Tavares M (2011) *Prunus lusitanica*. The IUCN Red List of Threatened Species 2011: e.T62857A12596811. (http://www.iucnredlist.org/details/full/62857/1#conservation_in_place)

Lloyd G, McCown BH. (1980) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Com Proc Int Plant Propagators Soc 30: 421-427

Murashige T, Skoog FM (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473-497

Schulze JA, Lattier JD, Contreras RN (2017) In vitro germination of immature *Prunus lusitanica* seed. HortScience 52:1122-1124

Effect of genotype, sucrose concentration and glutamine supply on the protein and lipid content in embryogenic suspension cultures of cork and holm oak

Mar Ruiz-Galea, Inmaculada Hernández, Celia Cano, Eva Friero, Cristina Celestino, Mariano Toribio

Departamento de Investigación Agroambiental. Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (IMIDRA). Finca El Encín, Apdo. 127, 28800 Alcalá de Henares, Madrid, Spain. mdelmar.ruiz@madrid.org

Keywords: *Quercus suber*, *Quercus ilex*, somatic embryogenesis, large scale production.

Cork oak (*Quercus suber* L.) and holm oak (*Quercus ilex* L.) are representative species of Mediterranean forest and of the agro-silvo-pastoral system called "Dehesa" in Spain or "Montado" in Portugal. Their acorns are mainly used for hog feeding because of their high carbohydrate, protein and lipid content. Oak acorns are morphologically characterized by the absence of endosperm and the presence of an embryo with big cotyledons in which nutrients are stored. Somatic embryos produced in repetitive embryogenic cultures can therefore be used not only for the production of clonal seedlings, but also for the large scale production of nutritive compounds and other metabolites. Somatic embryogenesis was initiated from leaf tissues of adult cork oak trees and from female flowers of holm oaks. Embryogenic cultures were maintained by subculturing in SH liquid medium without PGRs. Total protein and lipid content of embryogenic tissues at the proliferation stage was determined. Five genotypes of each species were analysed following standard methods. Variability between and within species was observed. The mean protein content was 31.7%DW in cork oak, ranging from 21.4 to 38.5%DW depending on genotype. In holm oak it was 36.0%DW ranging from 29.3 to 48.4%DW. Regarding lipid content, it was 6.6%DW ranging from 3.2 to 10.5%DW in cork oak and 7.2 %DW ranging from 4.6 to 8.9%DW in holm oak. The effects of sucrose concentration and the presence of glutamine in the culture medium were also evaluated. Increasing sucrose concentration decreased the crude protein content in cork oak but did not influence that of holm oak. Sucrose concentration hardly affected the lipid content in both species. The supply of glutamine increased the crude protein levels in both species while the lipid content remained unchanged.

Public perception in university and educational institutions about clonal forestry and forest biotechnology in Argentina and Venezuela

Sandra Sharry [1,3,4], Iselen Trujillo [2], Maite Romero Alves[1], Tatiana Cinquetti[1], Sebastian Galarco[1], Patricia Boeri [3]

[1] Department of Agricultural and Forestry Technology- Laboratory Wood Research (LIMAD) of the Faculty of Agricultural and Forestry Sciences, La Plata University, La Plata, Argentina
2. Simón Rodríguez National Experimental University. Institute of Scientific and Technological Studies (IDECYT). Mara Av, Altos del Cují, Colinas de la Mariposa. San Antonio de los Altos, Miranda, Venezuela, Postal Code 1204.
3. Integrated Unit for the innovation of the agri-food system of the Northern Patagonia (UIISA). National University of Rio Negro-Viedma, Río Negro, Argentina
4. Committee on Scientific Research of the Buenos Aires province (CICPBA)
Mail. ssharry@gmail.com; iselen03@yahoo.com; mromeroalves@gmail.com; tatianacinquetti@gmail.com; sebastiangalarco@gmail.com; pboeri@unrn.edu.ar

Keywords: clonal trees; forest biotechnology, KAP survey; public perception.

The issue of clonal planting and biotechnology has captured unprecedented public interest and concern throughout the world, and South America has not kept out of this debate. The debate includes scientific aspects, ethical issues and possible environmental, economic and social impacts, as well as health, technological dependence and sovereignty. Biotechnology in South America has grown rapidly in recent years, highlighting Micro propagation as one of the most used bio-technics. However, forest biotechnology (mainly GM trees) and clonal forestry face constraints that limit their social acceptance, due to aggressive campaigns by environmental NGOs and environmentalists, directed at people who are unaware of the issue, and based on information from unreliable sources. The objective of this work was to conduct a KAP (knowledge, attitudes and practices) survey that targeted Forest engineering students and those in Environmental Sciences (Argentina), Biological Sciences and Environmental Sciences and Agro ecology (Venezuela) to determine the acceptance level in relation to the use of forestry and forest biotechnology. There were differences in responses according to country, career and gender. The results show that environmental awareness is the most important concern, focused on the loss of biodiversity. Of students 76% know what a plant clone is, but they do not know how they are produced. However, they identify different vegetative propagation techniques, the best known being the propagation through cuttings and grafts, and the least known being somatic embryogenesis in the in vitro area. All the students answered that they know what biotechnology is, but they could not select the correct definition. Points of view on these issues differ between and within countries, because South America is a cultural mosaic, where questions about the acceptance of new technologies have a different basis depending on what each nation considers most important. In conclusion, this preliminary study showed that these subjects should be included in the curriculum of the surveyed careers, incorporating in each country, characteristic local elements of importance to the environment.

What the Latin American people think about clonal forestry and forest biotech?

Sandra Sharry[1,3,4], Iselen Trujillo [2], Maite Romero Alves[1], Tatiana Cinquetti[1], Sebastian Galarco [1], Patricia Boeri[3]

[1] The Department of Agricultural and Forestry Technology- Laboratory research of wood (LIMAD) - Faculty of Agricultural and Forestry Sciences, La Plata University, Buenos Aires, Argentina.
2. Simón Rodríguez National Experimental University. Institute of Scientific and Technological Studies (IDECYT). Mara Avenue, Altos del Cují, Colinas de la Mariposa. San Antonio de los Altos, Miranda, Venezuela Postal Code 1204.
3. Integrated Unit for the innovation of the agri-food system of the Northern Patagonia (UIISA). National University of Rio Negro-Viedma, Río Negro, Argentina
4. Committee on Scientific Research of the Buenos Aires province (CICPBA)
Mail. ssharry@gmail.com; iselen03@yahoo.com; mromeroalves@gmail.com; tatianacinquetti@gmail.com; sebastiangalarco@gmail.com; pboeri@unrn.edu.ar

Keywords: forest biotechnology, clonal forestry, communication, people, and opinion poll

Significant progress has been made in LAC regions in the development of appropriate technologies to improve agricultural productivity in sustainable systems. However, these results rarely reach the field, mainly due to misunderstanding of the relationships between the components of the agricultural and forestry systems by those who run the sector, including professionals and those entities in charge of interaction with other sectors. The innovation process needs key actors of society to be summoned and supported technically, so that they can reach a consensus understanding on key issues related to the management of natural resources and the sustainability of agriculture and forestry. Framed in this context, biotechnology is emerging as a useful alternative in these development processes, even within the often complex perception that exists of it in society. Public acceptance of technologies is based not only on technological and scientific strength, but also on their social, political and economic perception. This aspect has a great influence on investment in technology, and on its influence on the quality of life in society. The use of rapid assessment methods allows us to generate fast data already "which is better information faster than none at all". The objective of this work was to conduct a rapid assessment procedure (RAP), in order to determine what perception the public has about clonal forestry and forest biotechnology. For this, an exploratory opinion web-survey was made, evaluating different aspects of the subject. The results indicate that, 84% know that biotechnology include cloning of trees; 60% know forests of clonal plantations exist, and 62% think that plantations are not forests. However, 82% think that plantations reduce the pressure on native forests. Increasingly, planted forests will have to be recognized within the community in general by the range of values provided, not just by wood. Communication and community participation and dialogue between forestry companies and stakeholders is increasingly important. We know that public attitudes towards these issues are influenced by different factors, including information, social context, cultural norms, beliefs, values and perceptions.

Somatic embryogenesis and other vegetative propagation techniques for native forest tree species in north China: current status and issues that should be resolved in the near future

Hai-Long Shen

State Key Laboratory of Tree Genetics and Breeding, School of Forestry, Northeast Forestry University, Harbin 150040, China. shenhl-cf@nefu.edu.cn

Keywords: Native tree species, north China, somatic embryogenesis, rooted cuttings, grafting, timber production, non-timber forest products

The world's round wood production in 2016 was 3737 million m³ which was increased by 1%, 8% and 19% over that of 2015, 2000 and 1980 respectively (FAO, 2016). Plantations would have to provide 50%-70% of the timber in the future (Lelu-Walter et al., 2013; Bonga, 2016; Hazubska-Przybyt et al., 2016) and also for most of non-timber forest products such as edible seeds and industrial raw materials. Hence, the development of improved materials and fast and large-scale vegetative propagation systems, i.e., somatic embryogenesis (SE), rooted cuttings (RC) and grafting are the current focus (Lelu-Walter et al., 2013, 2016ab; Hazubska-Przybyt et al., 2016; Park, 2014; Trontin et al., 2016; Miguel et al., 2016). Native tree species like *Pinus koraiensis*, *Picea koraiensis*, *P. crassifolia*, *Abies holophylla*, *Catalpa bungei*, *Fraxinus mandshurica*, *Juglans mandshurica*, *Tilia amurensis*, *Quercus mongolica*, *Q. acutissima*, *Q. variabilis*, *Acer mono*, *Syringa reticulata* var. *mandshurica* are ecologically and economically very important in north and/or northeast China. They are high-value timber production species and in which *Pinus koraiensis* and *Juglans mandshurica* are very important edible nut species while oaks are starch and potential ethanol production species. These tree species grow slowly and have a short cultivation and breeding history and almost all of them have year on and year off phenomenon. Thus, their genetically improved materials (seeds, seedlings, cuttings and scions) are insufficient. Breeding of these species should be promoted and the limited improved germplasm material that is available should be propagated as fast as possible by large-scale somatic embryogenesis and other vegetative propagation methods. But there are many issues which hinder the development and application of these vegetative propagation systems. These issues are the result of problems of biological and technological nature (such as no well-developed and practically applicable SE and RC system for most species; grafting is well developed and applicable for species like *Pinus koraiensis* but is only suitable for nut production but not for high valued timber production for which a more than 100 years long cultivation period is needed. Even so, effective grafting systems are still lacking for most species and some of them are only propagated by seed and seedlings etc.). Social and economic aspects have to be considered (researchers focus on publishing papers but often lack cognition and a sense of practical, applicable propagation techniques , the high cost of using genetically improved materials and developing and applying SE and other vegetative propagation methods, etc.). These issues should be solved urgently for those native tree species in north China.

References

Bonga J M (2016) Conifer clonal propagation in tree improvement programs. *In:* Park YS, Bonga JM and Moon HK (eds) Vegetative propagation of forest trees. National Institute of Forest Science (NiFos), Seoul, Korea, pp 3-31

FAO (2016) Global production and trade of forest products. http://www.fao.org/forestry/statistics/80938/en/2016

Hazubska-Przybyt T., Bojarczuk K. (2016) Tree somatic embryogenesis in Science and Forestry.

Dendrobiology 76: 105-116

Lelu-Walter M-A, Thompson D, Harvengt L, Sanchez L, Toribio M and Pâques LE (2013) Somatic embryogenesis in forestry with a focus on Europe: state-of-the-art, benefits, challenges and future direction. Tree Genet Genomes 9:883-899

Lelu-Walter M-A, Klimaszewska K, Miguel C, Aronen T, Hargreaves C, Teyssier C, Trontin J-F (2016a). Somatic embryogenesis for more effective breeding and deployment of improved varieties in *Pinus* spp.: bottlenecks and recent advances. In: Somatic Embryogenesis - Fundamental Aspects and Applications. Springer International Publishing pp 319-365 http://dx.doi.org/10.1007/978-3-319-33705-0_19

Lelu-Walter M-A, Teyssier C, Guérin V, Pâques L E. (2016b) Vegetative propagation of larch species: somatic embryogenesis improvement towards its integration in breeding programs. In: Park Y S, Bonga J M and Moon H-K (eds.) Vegetative Propagation of Forest Trees. National Institute of Forest Science (NIFoS). Seoul, Korea. pp 551-571

Miguel C M, Rupps A, Raschke J, Rodrigues A S, Trontin J-F (2016) Impact of molecular studies on somatic embryogenesis development for implementation in conifer multi-varietal forestry. In: Park Y S, Bonga J M and Moon H-K (eds.) Vegetative Propagation of Forest Trees. National Institute of Forest Science (NIFoS). Seoul, Korea. pp 373-421

Park YS (2014) Conifer Somatic Embryogenesis and Multi-Varietal Forestry. *In*: Trevor F (ed). Challenges and Opportunities for the World's Forests in the 21st Century. Forestry Sciences Volume 81, pp 425-439

Trontin J-F, Teyssier C, Morel A, Harvengt L, Lelu-Walter M-A (2016) Prospects for new variety deployment through somatic embryogenesis in maritime pine. *In*: Park Y S, Bonga J M and Moon H-K (eds) Vegetative Propagation of Forest Trees. National Institute of Forest Science (NIFoS). Seoul, Korea. pp 572-606

Repetitive somatic embryogenesis induced cytological and proteomic changes in embryogenic lines of *Pseudotsuga menziesii*

Caroline Teyssier[1], Florian Gautier[1,2], Kateřina Eliášová[3], Jean-Charles Leplé[1,4], Zuzana Vondráková[3], Anne-Marie Lomenech[5], Claire Le Metté[1], Philippe Label[6], Guy Costa[2], Jean-François Trontin[7], Marie-Anne Lelu-Walter[1]

1 UMR BioForA INRA –ONF, 2163 avenue de la Pomme de Pin CS 40001 – Ardon, F-45075 ORLEANS CEDEX 2, France. caroline.teyssier@inra.fr
2 Université de Limoges, Laboratoire PEIRENE, Equipe SylvaLIM, 123 avenue Albert Thomas, 87000 Limoges, France
3 Institute of Experimental Botany of the Czech Academy of Sciences, Rozvojová 263, Praha 6-Lysolaje, 165 02, Czech Republic
4 BIOGECO, INRA, Université de Bordeaux, 33610 Cestas, France
5 Université de Bordeaux, Centre de Génomique Fonctionnelle, Plateforme Protéome, F-33000 Bordeaux, France
6 Université Clermont Auvergne, INRA, PIAF, F-63000 Clermont–Ferrand, France
7 FCBA, Pôle Biotechnologie et Sylviculture Avancée, Campus Forêt-Bois de Pierroton, 71 route d'Arcachon, 33610 Cestas, France

Keywords: histology, embryogenic potential, proteomic, *Pseudotsuga menziesii*, plant growth regulators, secondary somatic embryogenesis

In Europe, Douglas-fir is a major species for reforestation with increasing demand for its wood. Therefore, adaptation of new varieties to climate change is one challenging question for ongoing breeding programs. Efficient selection and vegetative propagation of improved varieties appeared to be key issues to maintain productivity in plantation forestry (Lelu-Walter et al. 2013). Somatic embryogenesis, coupled with cryopreservation, is a promising clonal propagation system of selected trees that has been successfully improved in Douglas-fir (Reeves et al. 2018; Lelu-Walter et al. 2018). Repetitive somatic embryogenesis from cotyledonary somatic embryos (SEs) has been obtained for two unrelated Douglas-fir genotypes, producing secondary and tertiary embryogenic lines. Each one exhibited significantly higher embryogenic potential for both genotypes compared to primary or secondary lines, respectively (increase of 351 to 72%). The origin of such differences in embryogenic potential is unknown. Our objective was to study changes induced in embryonal masses (EMs) after repetitive somatic embryogenesis at the histo-cytological and molecular levels (LC-MS-based proteomics). Repetitive somatic embryogenesis improved the EM structure by increasing frequency of small, separated immature SEs together with reducing the size of polyembryogenic centers. Each cycle of embryogenesis induced a modification of the expression of proteins related with biological processes that are known to be involvedin somatic embryogenesis but that are new for embryogenic masses, i.e. plant development, defense response, metabolism, proteolysis, and stress. The innovative implementation of protein networks in our proteomic analyses has been conclusive. The networks revealed a global down or up pattern of protein expression after the first or second cycle of somatic embryogenesis, respectively. For both patterns, interactions with different plant growth signaling molecules (flavonoids and associated compounds, jasmonic acid, ABA, auxin, salicylic acid, lignin) were predominant. It is shown that cells have the ability to use different protein regulatory pathways that lead to an increased embryogenic potential (cotyledonary SE production). In Douglas-fir and in conifers, this is the first report describing cellular and molecular changes in EMs obtained after two successive cycles of repetitive somatic embryogenesis (Gautier et al. 2018). The results contribute to a

better understanding of the cytological and proteomic changes associated with the enhanced embryogenic potential of secondary and tertiary embryogenic lines.

References

Gautier F, Eliášová K, Leplé JC, Vondráková Z, Lomenech A-M, Le Metté C, Label P, Costa G, Trontin J-F, Teyssier C, Lelu-Walter MA (2018) Repetitive somatic embryogenesis induced cytological and proteomic changes in embryogenic lines of *Pseudotsuga menziesii* [Mirb.]. BMC Plant Biology 18, 164-188

Lelu-Walter M-A, Thompson D, Harvengt L, Sanchez L, Toribio M , Pâques L-E (2013) Somatic embryogenesis in forestry with a focus on Europe: state-of-the-art, benefits, challenges and future direction. Tree Genet Genomes 9: 883-899

Lelu-Walter MA, Gautier F, Eliášová K, Sanchez L, Teyssier C, Lomenech A-M, Le Metté C, Hargreaves C, Trontin J-F, Reeves C (2018) High gellan gum concentration and secondary somatic embryogenesis: two key factors to improve somatic embryo development in *Pseudotsuga menziesii* [Mirb.]", Plant Cell, Tissue Organ Cult 132, 137-155.

Reeves C, Hargreaves C, Trontin JF, Lelu-Walter MA (2018) Simple and efficient protocols for the initiation and proliferation of embryogenic tissue of Douglas-fir. Trees 32, 175-190

Temperature affects somatic embryo development in maritime pine

Jean-François Trontin[1], Isabelle Reymond[1], Francis Canlet[1], Mamadou D. Sow[2], Alain Delaunay[2], Stéphane Maury[2], Claire Le Metté[3], Caroline Teyssier[3], Marie-Anne Lelu-Walter[3]

[1] *FCBA, Biotech & Advanced Forestry, 71, Route d'Arcachon – Pierroton, 33610 Cestas, France.*
[2] *Univ. Orléans, LBLGC, EA 1207, INRA USC 1328, Rue de Chartres, 45067 Orléans, France*
[3] *UMR BioForA INRA-ONF, 2163 av. Pomme de Pin, CS40001, Ardon, 45075 Orléans, France*
jean-francois.trontin@fcba.fr

Keywords: *Pinus pinaster*, maturation, cotyledonary embryos, DNA methylation

The adaptability of forest tree varieties to the environmental stresses induced by global warming is still largely unknown. This is a major concern for foresters because climate change could affect a crucial reproductive function of trees: seed production. In some conifers such as Norway spruce, temperature during embryogenesis has been shown to affect the development of both somatic and zygotic embryos as well as, *a posteriori*, plant phenology over several years of juvenile vegetative phase (Carneros et al. 2017 and references therein). Temperature effects on embryo development are particularly difficult to investigate *in situ*, e.g. in seed orchards. As a good *in vitro* model system of embryo development, we used the best somatic embryogenesis protocols currently available for maritime pine at FCBA and INRA (Trontin et al. 2016) to study the temperature effect (18, 23 and 28°C) when applied during the maturation phase of cotyledonary embryos. We showed, for two unrelated maritime pine embryogenic lines (PN519 and AAY06006), that the temperature during embryogenesis has major impacts on the development of cotyledonary embryos (duration, yield, mass) and that these direct effects are complemented with delayed effects (estimated in up to 15-month-old emblings) on germination capacity, survival, initial growth in height and the phenology of plant development. Genotypic effects possibly related to the line's pedigree were also highlighted. Indirectly our results suggest that temperature affects the intrinsic quality of cotyledonary embryos, which could be reduced by a low temperature of 18°C or a high temperature of 28°C as compared with the reference temperature (23°C). No significant differences in global DNA methylation could be detected among the tested maturation conditions in both immature (1 week maturation) and cotyledonary embryos (10-14 weeks maturation) from line PN519. Further biochemical, proteomic and methylome analyses are ongoing to clarify the physiological and molecular mechanisms involved in embryos' perception of temperature in maritime pine.

Acknowledgements: authors thank the Conseil Régional Centre-Val de Loire (IMTEMPERIES project / 2014-00094511), the European Union (PROCOGEN project / FP7 / 289841), and the French National Research Agency (XYLOFOREST, ANR-10-EQPX-16).

References

Carneros E, Yakovlev I, Viejo M, Olsen JE, Fossdal CG (2017) The epigenetic memory of temperature during embryogenesis modifies the expression of bud burst-related genes in Norway spruce epitypes. Planta 46:553-566

Trontin JF, Teyssier C, Morel A, Harvengt L, Lelu-Walter MA (2016) Prospects for new variety deployment through somatic embryogenesis in maritime pine. In: Park YS, Bonga JM, Moon HK (Eds), Vegetative propagation of forest trees. Korea Forest Research Institute, Seoul, Korea, pp. 572-606. ISBN 978-89-8176-064-9

Primordial shoots of Norway spruce (*Picea abies*) as explants for somatic embryogenesis

Saila A. Varis[1], Krystyna K. Klimaszewska[2], Tuija S. Aronen[1]

[1] *Natural Resources Institute Finland, Savonlinna Research Unit, Savonlinna, Finland.*
[2] *Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre, Quebec, Canada.*
saila.varis@luke.fi

Keywords: clonal trees, conifers, recalcitrance, shoot buds

The recalcitrance of adult conifer explants has prevented induction of somatic embryogenesis (SE) in trees with known and desired characteristics. SE induction protocol, recently developed for white spruce (*Picea glauca*), was applied in order to examine the feasibility, frequency and timing of SE induction from primordial shoots of Norway spruce (*Picea abies*). In 2015 to 2017, 39 genotypes of four to six years old trees of SE origin as explant donors were screened. Two genotypes responded and produced 28 proliferating embryonal mass sublines from 19 primordial shoots. Sublines from both genotypes had numerous early somatic embryos that were identified in acetocarmine stained samples. However, the number of mature somatic embryos produced per g fresh mass varied from 1.6 to 142.5 between the two genotypes. Successful SE inductions occurred at the beginning of April, when the temperature sum (d.d.) started to accumulate, and at the end of October or beginning of November when the chilling unit sum was over 500.

References

Varis S, Klimaszewska K, Aronen T (2018) Somatic Embryogenesis and Plant Regeneration From Primordial Shoot Explants of *Picea abies* (L.) H. Karst. Somatic Trees. Frontiers in Plant Science 9:1551

Low cost media for improving *in vitro* propagation of woody plant species

Nieves Vidal [1], Saleta Rico[1], Salvatore Casano[2], Verónica Codesido [2], Conchi Sánchez[1]

[1] *Dpto. Fisiología Vegetal. Instituto de Investigaciones Agrobiológicas de Galicia. IIAG (CSIC). Avda de Vigo s/n. 15705 Santiago de Compostela, Spain.*
[2]*Dpto. Hibridación y Cultivo. Phytoplant Research S.L. Rabanales 21-Parque Científico Tecnológico de Córdoba. Calle Astrónoma Cecilia Payne. Edificio Centauro modulo B-1. 14014 Córdoba, Spain*
nieves@iiag.csic.es, saleta@iiag.csic.es, s.casano@phytoplant.es, v.codesido@phytoplant.es, conchi@iiag.csic.es

Keywords: Formula *β*-A, Formula *β*-H, *in vitro* propagation media, plant tissue culture, vitamins

Plant tissue culture is used for large scale plant multiplication, disease elimination, plant breeding and production of secondary metabolites. The high cost of the reagents needed for *in vitro* propagation is one of the limiting factors of these techniques. In a previous report, Phytoplant Research SL -a company specialized in developing the industrial chain of medicinal plants- reported *in vitro* propagation of six *Cannabis sativa* L. varieties using two new culture media (Formula *β*-A and Formula *β*-H). *Cannabis* micropropagation was performed in one step (growth of the aerial part and rooting simultaneously) without sucrose, agar and/or vitamins, and obtained better success rates than by using conventional medium plus vitamins (Codesido et al. 2017).

In this study, we investigated the possibility of using these new formulations for the *in vitro* propagation of several woody plant species, including forest and fruit trees. The conventional MS (Murashige and Skoog 1962) and GD (Gresshoff and Doy 1972) formulations were used as controls. Both sucrose (2-3% (w/v)) and plant growth regulators regularly used in these plant species were provided to all tested media. Conventional media were supplied with vitamins described in their formulation while no vitamins were added to the low cost media. To avoid interferences due to previous culturing in conventional medium, the explants were subcultured twice in the new low cost medium before recording the multiplication parameters. Then, survival rate, number of shoots, multiplication rate, shoot length, and rooting capacity were recorded during three subcultures.

The media prepared by using Formula *β*-H produced negative results in two species (*Eucalyptus globulus* and *Genista tridentata*), while in two others (*Betula pendula* and *Prunus avium)* a certain growth was achieved, but without reaching the levels obtained with conventional medium. In contrast, with the media prepared by using Formula *β*-A good results were obtained in five of the six species studied (*Betula pendula*, *Eucalyptus globulus*, *Pyrus cordata*, *Prunus domestica* and *Salix viminalis*). In four of them, the multiplication ability and plant quality were significantly improved with respect to their performance in conventional media.

These results demonstrate that Formula *β* based media, even without addition of vitamins, may represent a good choice for the micropropagation of some woody plant species.

Acknowledgements: This work was partly funded through the contract Phytoplant Research SL-CSIC Ref. 20174612. We thank Anxela Aldrey, Alejandro Díaz, Rafael Sánchez and Patricia Val for technical assistance.

References

Codesido V, Casano S, Meyer S (2017) Cultivo *in vitro* low cost: maximizando la productividad. SECIVTV 2017 Plants *in vitro* for the future. Madrid, September 13-15, 2017.

Gresshoff PM, Doy CH (1972) Development and Differentiation of Haploid *Lycopersicum esculentum* (Tomato)". Planta 107:161-170.

Murashige T, Skoog FM (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473-497.

Relationship between H_2O_2 accumulation and NO signal synthesis in osmotic stress-induced somatic embryogenesis of *Fraxinus mandshurica*

Ling Yang, Hailong Shen

State Key Laboratory of Tree Genetics and Breeding, School of Forestry, Northeast Forestry University, Harbin 150040, China yangl-cf@nefu.edu.cn; shenhl-cf@nefu.edu.cn

Keywords: *Fraxinus mandshurica*, somatic embryogenesis, hydrogen oxide, nitric oxide, osmotic stress

Osmotic stress promotes somatic embryogenesis of *Fraxinus mandshurica*, which is related to reactive oxygen species (ROS) accumulation, but the underlying mechanism is still unclear. The purpose of this research is to reveal the relationship between ROS accumulation and reactive nitrogen species (RNS) in osmotic stress- promoted somatic embryogenesis. Single pieces of cotyledons of *F. mandshurica* were used as explants and somatic embryogenesis was induced in these in osmotic stress medium supplemented with 75 $g \cdot L^{-1}$ sucrose. Meanwhile, the endogenous hydrogen oxide (H_2O_2) content of explant cells was modified by adding exogenous H_2O_2 or catalase (CAT) solution, so as to analyse the effects of exogenous H_2O_2 stimulation on somatic embryogenesis and intracellular H_2O_2 accumulation, as well as the relationship between ROS signals and RNS signals. The results showed that (1) the addition of exogenous H_2O_2 at an appropriate concentration increased the number of somatic embryos; after 60 days the number of somatic embryos was highest on explants treated with 200 $\mu mol \cdot L^{-1}$ H_2O_2, reaching 17.41 embryos per explants and 136.54% higher than that of control; (2) exogenous H_2O_2 treatment significantly increased the intracellular H_2O_2 content and enhanced the antioxidant enzymes activity (superoxidase dismutase and peroxidase); (3) H_2O_2 treatment activated the intracellular non-enzymatic reaction pathway to facilitate NO synthesis, but the correlation between intracellular H_2O_2 and NO was not significant. These results demonstrated that the somatic embryogenesis of broad-leaved trees was closely related to the ROS accumulation and antioxidant defense reactions; both H_2O_2 and NO as signaling molecules were involved in the process of somatic embryogenesis in broad-leaved trees. In the process of exogenous hydrogen peroxide promoting somatic embryogenesis, NO signal synthesis depended on non-enzymatic reactions. The research results provide a scientific basis for resolving the regulation mechanism of ROS in somatic embryogenesis of broad-leafed trees and establishing a reasonable and efficient technology system for regulating somatic embryogenesis of trees.

Photo gallery

The Fifth International Conference of the IUFRO Unit 2.09.02

"Somatic Embryogenesis and Other Vegetative Propagation Technologies"

Clonal Trees in the Bioeconomy Age: Opportunities and Challenges

University of Coimbra

Coimbra ◆ Portugal

September 10-15, 2018

Our Invited Speakers

C. Marques

H. Shen

H.K. Moon

I. Wendling

T. Maruyama

P. Moncaleán

P. Gupta

S. Correia

S. Merkle

S.M. Jain

V. Cano

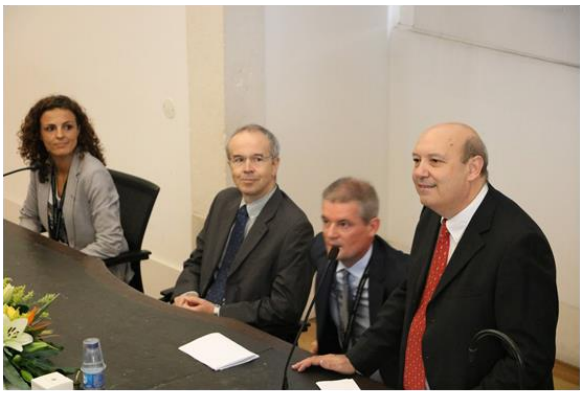
T. Almeida

Y.S. Park

Opening Ceremony (September 10, 2018)

Coimbra, Auditorium of the Museum of Science








The conference was introduced in the beautiful auditorium of the Museum of Science in Coimbra by:

Prof. **Jorge Canhoto** & Dr. **Sandra Correia**, Conference Organizers (Centre for Functional Ecology, University of Coimbra)

Dr. **Jean-François Trontin**, IUFRO 2.09.02 Coordinator (FCBA, France)

Prof. **João Gabriel Silva**, Rector of the University of Coimbra

During the sessions (September 10-14, 2018)

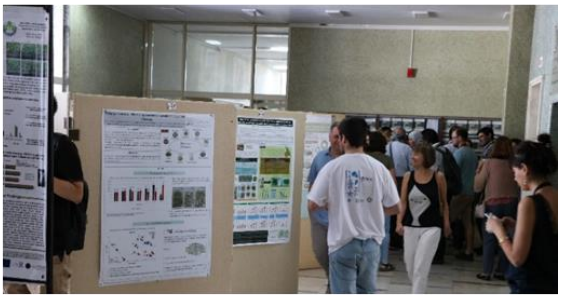
Coimbra, Museum of Science

University of Coimbra, Department of Mathematics

Coimbra, Convento de São Francisco

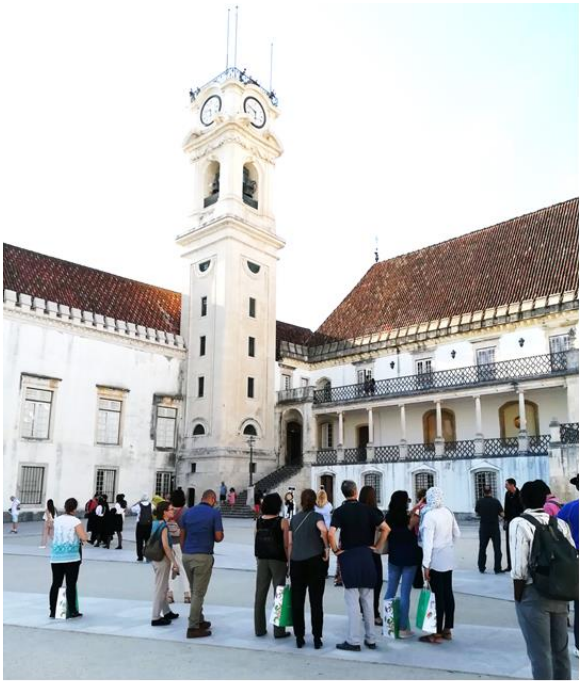






Our Speakers during the 6 sessions!

Visit of the University of Coimbra (September 10, 2018)

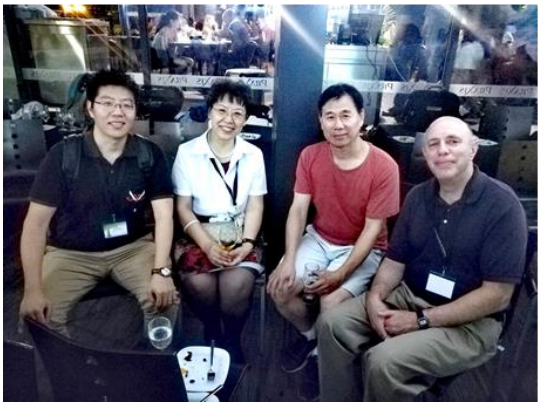


Coimbra by night and Traditional Music, student's Tund (September 10, 2018)

Beer Party with Fado de Coimbra (September 11, 2018)






Visit to Mata do Bussaco, national forest (September 12, 2018)

Luso, Portugal





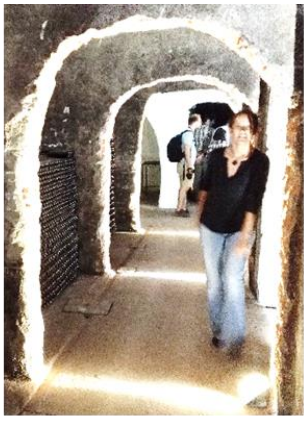







Visit and dinner at Caves Messias (Sept.ember 12, 2018)

Mealhada, Portugal



Gala Dinner, Icebreaker reception with Fado de Coimbra (September 13, 2018)

Coimbra, Convento de São Francisco















Gala Dinner, at the tables (Sept. 13, 2018)

Coimbra, Convento de São Francisco

Gala Dinner, during the Awards Ceremony (Sept. 13, 2018) of the Third Biennial IUFRO 2.09.02 Student's Scientific Competition

Coimbra, Convento de São Francisco

"Advances in tree vegetative propagation technologies & Application of somatic embryogenesis in tree breeding and biotechnology" (competition chaired by Jana Krajňáková)

The winner of the competition, **Bruno Viana NAVARRO** (University of São Paulo, Brazil), has been invited with full support to make an oral presentation during the conference.

All 8 runners-up of this well-contested competition were invited to contribute to the conference:

Chang-Ho AHN (Kangwon National Univ., Republic of Korea) – 2 posters

Ana ALVES (Univ. of Lisbon, Portugal) – Co-Chairwoman, 1 oral communication

Rayan AWADA (CIRAD and Nestlé R&D Center, France) – Co-Chairman, 1 oral communication

Jayeni Hiti BANDARALAGE (Univ. of Queensland, Australia) - 1 poster abstract

Biljana ĐORĐEVIĆ (Mendel Univ. of Brno, Czech Republic) – Chairwoman, 1 oral communication

Cátia PEREIRA (Neiker, Spain, Univ. of Coimbra, Portugal) – Co-Chairwoman, 1 oral communication

Wang QIUSHUI (Northeast Forestry Univ., China) – 1 poster abstract submitted

Cheng WEI (Northeast Forestry Univ., China) – 1 poster presentation

Gala Dinner, during the Recognition Ceremony of founding members of the IUFRO 2.09.02 Unit, 10 years ago, in 2008 (Sept. 13, 2018)

Coimbra, Convento de São Francisco

Yill-Sung PARK (Canada)
IUFRO 2.09.02 Coordinator 2008-2014

Heung-Kyu MOON (Republic of Korea)
Deputy Coordinator 2008-2014

Mariano TORIBIO (Spain)
Deputy Coordinator 2008-2014

The Organizing Committee was proud to have again the opportunity to express to Dr. PARK, Dr. MOON and Dr.TORIBIO its gratitude and above all friendship for their outstanding contribution to this IUFRO 2.09.02 Unit!

Of course, you remain our permanent honorary members!

Closing Ceremony (Sept. 14, 2018)

Coimbra, Convento de São Francisco

The time to conclude the conference after more than 50 oral presentations in a friendly atmosphere. Sandra and Jorge are tired-looking after such a 7-day marathon! Thank you so much again for making this new IUFRO 2.09.02 event a great success!

Visit to Óbidos Village (Sept. 15, 2018)




















Visit to Alcobaça Monastery (Sept. 15, 2018)

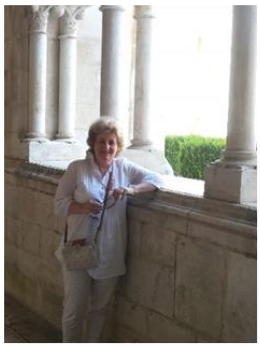
















Visit to Fátima Sanctuary (Sept. 15, 2018)











And Portugal, of course, is also gastronomy!
What a pleasure throughout Coimbra2018!









And the very last glass of Portuguese wine! Cheers to all participants and looking forward to meeting you again in next IUFRO 2.09.02 conferences!

More downloadable photos of Coimbra2018 are available from:

https://clicktime.symantec.com/a/1/4HwnfP2SKRCJ9Z1R5D8G7OUlddaPdIMiSk2wGtUs1rA=?d=ktIOweE5xs5E8_r2Qr9-aNPYdbqDcQvhLIQUTjSJfxVXAY8lwz6j4kNllM2Re_k34rLn53TVmokCzdKqgGA4OrnXwNaSfNg3-9SKVckLxooR4_zh193S70oZJYUi50AZ0VEc3Hdp5D-B85jrkLHfyHZyDPw5Y6eK_hN-bCXYc_4suqRN-vsSzHFq8AkvKMZbQxPhFJM8D1G_ItKUuVyv2rjkXLPY9W60NwWNDrcKuqmJptTMZExNp0BfT3F36CkHnA0N6tHdtLBLW3VEsNaWP4nLjopq3LTycfKb9MID7CCcaTZdqhv4PmHLHLSJ7zGImFx8khVv3e1JArGr78AkpDLs1yH-xMrdILm5JYHMzSEZjuD2tpq1BKJy_WIGH9SuWSyX7CUfEbP70admfx8vLg%3D%3D&u=https%3A%2F%2Fphotos.app.goo.gl%2F27pbd39E9XAqdsyB8

Our Very Special Thanks to the Fantastic Hosting Team in Coimbra!

Thank you Sandra!

Many thanks Jorge!

And thank you also the whole team of the local committee:

João, Ricardo, Jorge, André, Cátia, Mariana, Arlindo, Daniela, Inês & José

Your strong support to the conference was much appreciated by all participants to Coimbra2018!

Thank you, Jan Max Bonga

Our editor, colleague, and friend, Jan M. Bonga is retiring after the publication of these proceedings. Jan was involved in the editing of all our proceedings and the book, "Vegetative Propagation of Forest Trees," (2016) also available on our website.

Jan was officially retired from the Canadian Forest Service 24 years ago, but he enthusiastically continued his volunteer work as a Scientist Emeritus until today. Throughout his extended career, Jan conducted research on tissue culture, rejuvenation, recalcitrance, physiology and genetics. He was one of the first who successfully obtained SE in conifers from haploid culture of *Larix decidua* in 1985. He published widely on these subjects, hosted visiting scientists from around the world, and mentored younger scientists. We were all so fortunate to meet him and honor him for his achievements and contributions during the Brno2012 Conference. Recently, he celebrated his 89th birthday!

We wish him all the very best for his second retirement.

Thank you, Jan

From all of us in our working unit on
Somatic Embryogenesis and Other Vegetative Propagation Technologies

Participants & contact information

ATTENDEES: **in blue**

OTHER DIRECT CONTRIBUTORS to the conference: **in green**

ARGENTINA (7)

BASIGLIO CORDAL, María de los Ángeles
mbasigliocordal@ceamse.gov.ar
Coordinación Ecológica Area Metropolitana Sociedad del Estado (CEAMSE)
Subgerencia de Áreas verde, forestación y parquización, Gerencia de Saneamiento y Mantenimiento de CDF terminados, Laboratorio de Cultivo *in vitro*, Ortega 4850, Villa Dominico (1874), Buenos Aires

CABRAL, José
ingftalcabral@gmail.com;
gerencia@phytolab.com.ar
Biofábrica Misiones S.A. / Sarasola Ingeniería
Ruta Nacional 12, Km 7 ½, Posadas, Misiones

GALARCO, Sebastián Pablo
sebastiangalarco@gmail.com
National University of La Plata (UNLP)
Faculty of Agricultural and Forestry Sciences (FCAyF), Laboratorio de Investigaciones de la Madera (LIMAD). CC 31, La Plata (1900), Buenos Aires

ROMERO ALVES, Maite
mromeroalves@gmail.com
Universidad Nacional de La Plata (UNLP)
Facultad de Ciencias Agrarias y Forestales. Calle 60 s/n., La Plata, Buenos Aires

SALVATIERRA, Guillermo R.
guillermosalvatierra7@gmail.com ;
investigacion@biofabrica.com.ar
Biofábrica Misiones S.A.
Ruta Nacional 12, Km 7 ½, Posadas, Misiones

SHARRY, Sandra
ssharry@gmail.com, *ssharry@agro.unlp.edu.ar*
National University of La Plata (UNLP)
Faculty of Agricultural and Forestry Sciences (FCAyF), Department of Agricultural and Forestry Technology, Laboratory Wood Research (LIMAD), Vegetative propagation of plants Unit (UPROVE), 60 st and 119 st. PC 1900, La Plata, Buenos Aires

TACCARI, Leonardo E.
semillas.inbies@unp.edu.ar
Universidad Nacional de la Patagonia SJB
Instituto de Biotecnologia Esquel, Ruta 259 Km 16, 40, Esquel, Chubut

AUSTRALIA (1)

BANDARALAGE, Jayeni Hiti
jayeni.hitibandaralage@uq.net.au
The University of Queensland
Queensland Alliance for Agriculture and Food Innovation, Brisbane

BELGIUM (2)

MUVARNIDZE, Nino
nino.murvanidze@gmail.com
Ghent University
Fac. Bioscience Engineering, Dept. Applied Bioscience, Valentin Vaerwyckweg 1, 9000 Sint-Pietersnieuwstraat 25, OostVlaanderen, Ghent

WERBROUCK, Stefaan
stefaan.werbrouck@ugent.be
Ghent University
Fac. Bioscience Engineering, Dept. Plants and Crops, Laboratory for Applied In Vitro Plant Biotechnology, Valentin Vaerwyckweg 1, 9000 Sint-Pietersnieuwstraat 25, OostVlaanderen, Ghent

BRAZIL (14)

DOS SANTOS, André L. Wendt
alwsantos6@gmail.com
University of São Paulo
Instituto de Biociências, Departamento de Botânica, Laboratorio de Biologia Celular de Plantas (BIOCEL), São Paulo.

ELBL, Paula
paulaelbl@usp.br
University of São Paulo
Department of Botany, Institute of Bioscience, Rua do Matão, 277, São Paulo, SP, 05508-090

FRAGA, Hugo P. F.
hugofraga@ufpr.br
Universidade Federal do Paraná
Laboratório de Micropropagação Vegetal, Setor de Ciências Biológicas, Curitiba, PR
Universidade Federal de Santa Catarina
Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal, Centro de Ciências Agrárias, Florianópolis, SC

GONZÁLEZ, Esteban Roberto
estebang@suzano.com.br
Futuragene
Itapetininga, São Paulo

GUERRA, Miguel Pedro
miguel.guerra@ufsc.br
Universidade Federal de Santa Catarina (UFSC)
Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal, Centro de Ciências Agrárias, Admar Gonzaga Road, 1346, ZC 88.034-001, Florianópolis, SC

MINGOSSI, Fabiana
fabiana.mingossi@fibria.com.br
FIBRIA Celulose
Centro de Tecnología, 12340-010, Jacarei, San Pablo

NAVARRO, Bruno (Viana)
bruno_vnavarro@usp.br
University of São Paulo
Dpt. of Botany, Institute of Bioscience, Rua do Matão 277, 05508-090, São Paulo

NAVROSKI, Marcio Carlos
marcio.navroski@udesc.br
Santa Catarina State University
Department of Forest Engineer, Udesc, Lages, Santa Catarina

PAIVA, Luciano Vilela
luciano@dqi.ufla.br
Federal University of Lavras
Central Laboratory of Molecular Biology, Department of Chemistry, Lavras, Minas Gerais

QUISEN, Regina Caetano
regina.quisen@embrapa.br
Embrapa - Brazilian Agricultural Research Corporation
Embrapa Forestry, Colombo, Paraná

QUOIRIN, Marguerite
mquoirin@ufpr.br, quoirinm@hotmail.com
Federal University of Paraná (UFPR)
Department of Botany, Sector of Biological Science, C.P. 19051, CEP 81531-980, Curitiba – PR

SALGADO, Caio
caio.salgado@klabin.com.br
Klabin S.A.
Department of Forest Research, São Paulo

VIEIRA, Leila do Nascimento
leilavieira@ufpr.br
Federal University of Paraná (UFPR)
Department of Botany, Sector of Biological Science, Laboratório de Micropropagação Vegetal, C.P. 19051, CEP 81531-980, Curitiba – PR
Universidade Federal de Santa Catarina
Centro de Ciências Agrárias, Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal, Florianópolis, SC

WENDLING, Ivar
ivar.wendling@embrapa.br
Embrapa - Brazilian Agricultural Research Corporation
Embrapa Florestas, Colombo, Paraná

CANADA (4)

BONGA, Jan
jan.bonga@canada.ca
Natural Resources Canada – Canadian Wood Fibre Centre
Emeritus Scientist, Fredericton, New Brunswick

DING, Chen
holybaboon@gmail.com
Université Laval
Canada Research Chair in Forest Genomics, Institute for Systems and Integrative Biology, Québec, QC
Western Gulf Forest Tree Improvement Program
College Station, TX, USA

MOSSELER, Alex
alex.mosseler@canada.ca
Natural Resources Canada – Canadian Forest Service
Atlantic Forestry Centre, Box 4000, Fredericton, New Brunswick

PARK, Yill-Sung
yillsung.park@canada.ca
Natural Resources Canada – Canadian Wood Fibre Centre
Emeritus Scientist, Fredericton, New Brunswick

CHILE (2)

AVILÉS MALDONADO, Fabiola
Fabiola.Aviles@arauco.cl
ARAUCO BIOFOREST S.A.
Ruta 160, Km 15, Coronel, Concepción

LINEROS FUENTEALBA, Yenny
yenny.lineros@arauco.cl
ARAUCO BIOFOREST S.A..
Unidad de Biotecnología, Camino a Coronel km 15 S/N, Coronel

CHINA (9)

LI, Wan-Feng
liwf@caf.ac.cn
Chinese Academy of Forestry,
Research Institute of Forestry, State Key Laboratory of Tree Genetics and Breeding, Beijing 100091

PENG, Chun-Xue
1325835674@qq.com
Northeast Forestry University
Department of Silviculture, School of Forestry, No.26, Hexing-Lu, Harbin 150040, Heilongjiang Province

QIUSHUI, Wang
wangqiushui@126.com
Northeast Forestry University
Department of Silviculture, School of Forestry, No.26, Hexing-Lu, Harbin 150040, Heilongjiang Province

SHEN, Hailong
shenhl-cf@nefu.edu.cn
Northeast Forestry University
State Key Laboratory of Tree Genetics and Breeding, School of Forestry, No.26, Hexing-Lu, Harbin 150040, Heilongjiang Province

WEI, Cheng
Vcheng026@163.com
Northeast Forestry University
State Key Laboratory of Tree Genetics and Breeding, School of Forestry, No.26, Hexing-Lu, Harbin 150040, Heilongjiang Province

YANG, Ling
yangling082477@yahoo.com; yangl-cf@nefu.edu.cn
Northeast Forestry University
Department of Silviculture, School of Forestry, No.26, Hexing-Lu, Harbin 150040, Heilongjiang Province

ZHANG, Jin Feng
zjf@bjfu.edu.cn
Beijing Forestry University
College of Biological Sciences and Technology, Beijing

ZHAO, Jian
zhaojian0703@bjfu.edu.cn
Beijing Forestry University
College of Biological Sciences and Technology, Beijing

ZHANG, Peng
zhangpeng@nefu.edu.cn
Northeast Forestry University
State Key Laboratory of Tree Genetics and Breeding, School of Forestry, No.26, Hexing-Lu, Harbin 150040, Heilongjiang Province

COSTA RICA (1)

HINE GOMEZ, Ana
ana.hine.gomez@una.cr
National University of Costa Rica
Institute of Forest Research and Services, Heredia

CUBA (1)

SILVA PUPO, Juan J.
jsilvap@udg.co.cu
Universidad de Granma
Centro de Estudios de Biotecnología Vegetal, Facultad de Ciencias Agrícolas, Bayamo, Granma

CZECH REPUBLIC (6)

CVRČKOVÁ, Helena
cvrckova@vulhm.cz
Forestry and Game Management Research Institute
Strnady, Jíloviště

DORDEVIĆ, Biljana
biljana.dordevic@mendelu.cz
Mendel University
Department of Forest Protection and Wildlife Management, Zemědělská 3, Brno

ELIÁŠOVÁ, Kateřina
eliasova@ueb.cas.cz
Institute of Experimental Botany of the Czech Academy of Sciences
Rozvojová 263, Praha 6-Lysolaje 16502

FISCHEROVÁ, Lucie
fischerova@ueb.cas.cz
Institute of Experimental Botany of the Czech Academy of Sciences
Rozvojová 263, Praha 6-Lysolaje 16502

KOMÁRKOVÁ, Martina
komarkova@vulhm.cz
Forestry and Game Management Research Institute
Strnady, Jíloviště

MÁCHOVÁ, Pavlína
machova@vulhm.cz
Forestry and Game Management Research Institute
Strnady, Jíloviště

DENMARK (1)

NIELSEN, Ulrik Bräuner
ubn@ign.ku.dk
University of Copenhagen
Dpt. of Geosciences and Natural Resource Management, Copenhagen.

DEMOCRATIC REPUBLIC OF THE CONGO (1)

MAZINGA KWEY, Michel
profmazingakwey@gmail.com
University of Lubumbashi
Faculty of Agronomy, Laboratory of Ecophysiology and In vitro Culture of plants, Lubumbashi

FINLAND (4)

ARONEN, Tuija
tuija.aronen@luke.fi
Natural Resources Institute Finland (LUKE)
Production Systems, Forest Tree Breeding, Finlandiantie 18, FI-58450 Punkaharju.

JAIN, Shri Mohan
mohan.jain@helsinki.fi
University of Helsinki
Department of Agricultural Sciences, PL-27, Helsinki

TIKKINEN, Mikko
mikko.tikkinen@luke.fi
Natural Resources Institute Finland (LUKE)
Production Systems, Forest Tree Breeding, Finlandiantie 18, FI-58450 Punkaharju.

VARIS, Saila
saila.varis@luke.fi
Natural Resources Institute Finland (LUKE)
Production Systems, Forest Tree Breeding, Finlandiantie 18, FI-58450 Punkaharju.

FRANCE (5)

AWADA, Rayan
rayan.awada@cirad.fr
Nestlé R&D Centre Tours
101 av. Gustave Eiffel, BP 49716, 37097 Tours Cedex 2
CIRAD
UMR IPME, F-34398 Montpellier
University of Monpellier, CIRAD, IRD
F-34394 Montpellier

FILLODEAU, Audrey
audrey.fillodeau@rdto.nestle.com
Nestlé R&D Centre Tours
Department of Plant Cell Biology and Molecular Biology department of Plant Science Research Unit, 101 av. Gustave Eiffel, BP 49716, 37097 Tours Cedex 2

MAURY, Stéphane
stephane.maury@univ-orleans.fr
University of Orléans
Laboratoire de Biologie des Ligneux et des Grandes Cultures (LBLGC), 1207 USC1328, INRA-Univ. Orléans, Orléans

TEYSSIER, Caroline
caroline.teyssier@inra.fr
INRA
UMR BioForA INRA-ONF
2163 Avenue de la pomme de pin,CS 4001, Ardon, F-45075, Orléans Cedex 2

TRONTIN, Jean-François
jean-francois.trontin@fcba.fr
FCBA Technological Institute
Biotechnology and Advanced Forestry Department
71 Route d'Arcachon, Pierroton, 33610 Cestas

GERMANY (6)

AURICH, Claudia
claudia.aurich@rz.hu-berlin.de
Humboldt University of Berlin
Institut für Biologie, AG Botanik & Arboretum, Invalidenstraße 42, 10115, Berlin

BENNECKENSTEIN, Tim
tbenneckenstein@gmx.de
Humboldt University of Berlin
Institut für Biologie, AG Botanik & Arboretum, Invalidenstraße 42, 10115, Berlin

RASCHKE, Juliane
Raschkej@hu-berlin.de
Humboldt University of Berlin
Institut für Biologie, AG Botanik & Arboretum, Invalidenstraße 42, 10115,Berlin

SEIFERT, Jana Yvonne
seifert.jana@gmx.de
Humboldt University of Berlin
Institut für Biologie, AG Botanik & Arboretum, Invalidenstraße 42, 10115, Berlin

WALTHER, Madlen
mwalther_dienstl@web.de
Humboldt University of Berlin
Institut für Biologie, AG Botanik & Arboretum, Invalidenstraße 42, 10115, Berlin

ZOGLAUER, Kurt
kurt.zoglauer@rz.hu-berlin.de
Humboldt University of Berlin
Institut für Biologie, AG Botanik & Arboretum, Invalidenstraße 42, 10115, Berlin

INDONESIA (1)

MOON, Heung-Kyu
jesusmhk@hanmail.net
PT. Korintiga Hutani
R&D Department, Camp Pellita, Ds. Nanga Mua, Kab. Kotawaringin Barat 74152, Kalimantan Tengah

IRAN (1)

YADOLLAHI, Abbas
yadollah@modares.ac.ir
Tarbiat Modares University
Faculty of Agriculture, Horticulture Dpt, Tehran

JAPAN (1)

MARUYAMA, Tsuyoshi
tsumaruy@ffpri.affrc.go.jp
Forestry & Forest Products Research Institute (FFPRI)
Department of Forest Molecular Genetics and Biotechnology, Matsunosato 1, Tsukuba, Ibaraki, 305-8687

LATVIA (2)

GIRGŽDE, Elva
elva.girgzde@silava.lv
Latvian State Forest Research Institute "Silava"
Rīgas 111, Salaspils LV–2169

SAMSONE, Ineta
ineta.samsone@silava.lv
Latvian State Forest Research Institute "Silava"
Rīgas 111, Salaspils LV–2169

NEW ZEALAND (3)

AITKEN, Jenny
jenny@thetreelab.com
The Tree Lab
PO Box 11236, Palm Beach, Papamoa 3151

KRAJŇÁKOVÁ, Jana
Jana.krajnakova@scionresearch.com
Scion
49 Sala Street, Private Bag 3020, Rotorua 3046

REEVES, Catherine
catherine.reeves@scionresearch.com
Scion
49 Sala Street, Private Bag 3020, Rotorua 3046

NORWAY (1)

LEE, YeonKyeong
Carl.Gunnar.Fossdal@nibio.no
Norwegian Univ. of Life Sciences
Dep. of plant sciences, P.O. Box 5003, N-1432 Ås
Centre of environmental radioactivity, P.O. Box 5003, N-1432 Ås

PHILIPPINES (1)

AGGANGAN, Nelly
nelly_aggangan@yahoo.com
University of the Philippines Los Banos
National Institute of Molecular Biology and Biotechnology (Biotech), Laguna 4031

PORTUGAL (18)

ALMEIDA, Tânia
info@klonbio.com
Klón, Innovative Technologies from Cloning
Biocant Park, Parque Tecnológico de Cantanhede, Núcleo 4, Lote 4A – Piso 1, Labs 6, 8, 10, 3060-197 Cantanhede

ALVES, Ana
acalves@itqb.unl.pt
Instituto de Biologia Experimental e Tecnológica (iBET)
Apartado 12, 2781-901, Oieras
Univ. Nove de Lisboa, Inst. Tec. Química e Biológica António Xavier (ITQB)
Av. República 2780-157, Oeiras
Universidade de Lisboa, Faculdade de Ciências (FCUL)
Biosystems &Integrative Sciences Institute, Lisboa

CAEIRO, André
afcaeiro91@gmail.com
University of Coimbra, Department of Life Sciences
Centre for Functional Ecology, Calçada Martim de Freitas, 3000-456 Coimbra

CANHOTO, Jorge
jorgecan@uc.pt
University of Coimbra
Centre for Functional Ecology (CFE), Department of Life Sciences, Calçada Martim de Freitas, 3000-456, Coimbra

CARDOSO, Arlindo
acdiana@bot.uc.pt
University of Coimbra, Department of Life Sciences
Centre for Functional Ecology, Calçada Martim de Freitas, 3000-456 Coimbra

CAPOTE, Tiago
tiago.capote@cebal.pt
Instituto Politécnico de Beja (IPBeja)
Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo (CEBAL), 7801-908 Beja

CORREIA, Sandra
sandraimc@ci.uc.pt
University of Coimbra
Centre for Functional Ecology (CFE), Department of Life Sciences, Calçada Martim de Freitas, 3000-456, Coimbra

COSTA, Joana
joana.costa@thenavigatorcompany.com
RAIZ, Instituto de Investigação da Floresta e do Papel / The Navigator Company
Herdade de Espirra, 2985-270, Pegões-Gare

COSTA, Ricardo
rmfdcosta@uc.pt
University of Coimbra
Centre for Functional Ecology (CFE), Department of Life Sciences, Calçada Martim de Freitas, 3000-456, Coimbra

COSTA, Rita Lourenço
rita.lcosta@iniav.pt
Instituto Nacional de Investigação Agrária e Veterinária
I.P., Avenida da República, 2780-159 Oeiras
Universidade de Lisboa
Centro de Estudos Florestais, Instituto Superior de Agronomia, Tapada da Ajuda, 1349-017 Lisboa

CURRAIS, Sandra
laracurrais@hotmail.com
University of Coimbra
Centre for Functional Ecology (CFE), Department of Life Sciences, Calçada Martim de Freitas, 3000-456, Coimbra

GOMES, Filomena
fgomes@esac.pt
Instituto Politécnico de Coimbra / Escola Superior Agrária, CERNAS
3045-601 Coimbra

MARQUES, Cristina
cristina.marques@thenavigatorcompany.com
RAIZ, Instituto de Investigação da Floresta e do Papel / The Navigator Company
Herdade de Espirra, 2985-270, Pegões-Gare

MARTINS, João F. (DA SILVA)
joao.martins@uc.pt
University of Coimbra
Centre for Functional Ecology (CFE), Department of Life Sciences, Calçada Martim de Freitas, 3000-456, Coimbra

MARUM, Liliana
liliana.marum@cebal.pt
Instituto Politécnico de Beja (IPBeja)
Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo (CEBAL), 7801-908 Beja
Universidade de Évora
Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Pólo da Mitra, 7002-554 Évora

MIGUEL, Celia María
cmiguel@itqb.unl.pt
Instituto de Biologia Experimental e technológica (iBET)
Apartado 12, 2780-901, Oeiras
Univ. Nove de Lisboa, Inst. Tec. Química e Biológica António Xavier (ITQB)
Av. República 2780-157, Oeiras
Universidade de Lisboa, Faculdade de Ciências
BioISI - Biosystems & Integrative Sciences Institute, Campo Grande, 1749-016, Lisboa

NEVES, Mariana
mariananevespt@gmail.com
University of Coimbra
Centre for Functional Ecology (CFE), Department of Life Sciences, Calçada Martim de Freitas, 3000-456, Coimbra

NORO, Jorge
jnoro@uc.pt
University of Coimbra
Centre for Functional Ecology (CFE), Department of Life Sciences, Calçada Martim de Freitas, 3000-456, Coimbra

REPUBLIC OF KOREA (4)

AHN, Chang-Ho
dosanahn@gmail.com
Kangwon National University
Department of Forest Resources, College of Forest and Environmental Sciences, Chuncheon 24341

KANG, Hae-In
kangks84@snu.ac.kr
Seoul National University
The Department of Forest Sciences, 08826 Seoul

KANG, Kyu-Suk
kangks84@snu.ac.kr
Seoul National University
The Department of Forest Sciences, 08826 Seoul

KIM, Yong Wook
bravekim@korea.kr
National Institute of Forest Science (NIFoS)
Department of Forest Genetics, 39 Onjeong Ro, Gwonseon-gu, 16631 Suwon

RUSSIA (1)

PARK, Maria E.
mtavi@bk.ru
Siberian Branch, Federal Research Center «Krasnoyarsk Science Center SB RAS»
Russian Academy of Sciences, V.N. Sukachev Institute of Forest, Laboratory of Forest Genetics and Breeding, Akademgorodok 50/28, Krasnoyarsk

SLOVAK REPUBLIC (2)

ĎURKOVIČ, Jaroslav
jaroslav.durkovic@tuzvo.sk
Technical University, T.G.
Phytology Department, Masaryka 24, 96053, Zvolen

KRAJMEROVÁ, Diana
diana.krajmerova@tuzvo.sk
Technical University, T.G.
Phytology Department, Masaryka 24, 96053, Zvolen

SPAIN (17)

ARRILLAGA, Isabel
isabel.arrillaga@uv.es
University of Valencia
Facultad de Farmacia, ISIC/ERI BiotecMed, Dept. of Plant Biology, Vicent Andrés Estellés, 46100 Burjassot, Valencia

CANO, Vanesa
vanesa.cano.lazaro@iiag.csic.es
Instituto de Investigaciones Agrobiológicas de Galicia (IIAG-CSIC)
Avda Vigo s/n, Campus Vida, Apartado 122, 15705, Santiago de Compostela, La Coruña.

CARNEROS, Elena
ecarneros@cib.csic.es
Biological Research Center, CIB-CSIC
Pollen Biotechnology of Crop Plants, Madrid

CORREDOIRA, Elena
elenac@iiag.csic.es
Instituto de Investigaciones Agrobiológicas de Galicia (IIAG-CSIC)
Avda Vigo s/n, Campus Vida, Apartado 122, 15705, Santiago de Compostela, La Coruña.

FERNÁNDEZ-LORENZO, Juan Luis
juanluis.fernandez@usc.es
University of Santiago de Compostela
Department of Crop Production and Engineering Projects, Santiago de Compostela

HERNÁNDEZ, Inmaculada
inmaher.sanchez@madrid.org
Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (IMIDRA)
Finca "El Encin". Apdo. Postal 127. 28800, Alcalá de Henares (Madrid)

LICEA-MORENO, Ricardo J.
ricardolicea@bosquesnaturales.com
Bosques Naturales S.A.
Department of Biotechnology, Micropropagation Unit, Avenida de la Vega 1, 28108 Alcobendas, Madrid

MONCALEÁN, Paloma
pmoncalean@neiker.eus
Neiker Tecnalia
Centro de Arkaute. Apdo. 46. 01080, Vitoria-Gasteiz

MORCILLO, Marian
angeles.morcillo@uv.es
University of Valencia
Facultad de Farmacia, ISIC/ERI BiotecMed, Dept. of Plant Biology, Vicent Andrés Estellés, 46100 Burjassot, Valencia

PEREIRA, Cátia
catiaslp9@gmail.com
Neiker-Tecnalia
Campus Agroalimentario de Arkaute, Apdo. 46, 01080, Vitoria-Gasteiz
University of Coimbra
Plant Biotechnology Laboratory, Dpt. Life Sciences. 3000-456 Coimbra, Portugal

PINTO, Glória C.
gpinto@ua.pt
University of Aveiro
Department of Biology, Centre for Environmental and Marine Studies, Aveiro

REY, Manuel
mrey@uvigo.es
University of Vigo
Departamento de Biología Vegetal y Ciencia del Suelo, Facultad de Biología, 36310 Vigo

RUIZ-GALEA, Maria del Mar
mdelmar.ruiz@madrid.org
Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (IMIDRA)
Departamento de Investigación Agroambiental, Finca "El Encin". Apdo. Postal 127. 28800, Alcalá de Henares (Madrid).

SANCHEZ, María Concepción
conchi@iiag.csic.es
Instituto de Investigaciones Agrobiológicas de Galicia (IIAG-CSIC)
Dpt. Fisiología Vegetal, Avda de Vigo s/n, Apart. de correos 122, 15705 Santiago de Compostela, La Coruña, Galicia

TESTILLANO, Pilar
testillano@cib.csic.es
Biological Research Center, CIB-CSIC
Pollen Biotechnology of Crop Plants, Madrid

TORIBIO, Mariano
mariano.toribio@madrid.org
Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (IMIDRA)
Finca "El Encin". Apdo. Postal 127. 28800 Alcalá de Henares (Madrid)

VIDAL, Nieves
nieves@iiag.csic.es
Instituto de Investigaciones Agrobiológicas de Galicia (IIAG-CSIC)
Dpt. Fisiología Vegetal, Avda de Vigo s/n, Apart. de correos 122, 15705 Santiago de Compostela, La Coruña, Galicia

SWEDEN (6)

ABRAHAMSSON, Malin
malin.abrahamsson@swetree.com
Swetree Technologies
Umeå

BUSINGE, Edward
Edward.Businge@swetree.com
Swetree Technologies
Umeå

EGERTSDOTTER, Ulrika
Ulrika.Egertsdotter@slu.se
Swedish University of Agricultural Sciences
Umeå Plant Science Center, Department of Forest Genetics and Plant Physiology, 901 83 Umeå
Georgia Institute of Technology
G. W. Woodruff School of Mechanical Engineering, 801 Ferst Drive, Atlanta, GA 30332, USA

PACURAR, Daniel
Daniel.Pacurar@swetree.com
Swetree Technologies
Umeå

SHAH, Ravi Jagdish
Ravi.Shah@SweTree.com
Swetree Technologies
Umeå

WU, Harry X.
Harry.wu@slu.se
Swedish University of Agricultural Sciences
Umeå Plant Science Centre, Dept. Forest Genetics and Plant Physiology, SE-90183 Umeå

SWITZERLAND (1)

MIGNON, Eline
emi@socfin.com
SOCFIN
Fribourg

TUNISIA (4)

BEN GHOZLEN, Hajer
hajer.benghozlen@gmail.com
University of Sousse
Research Unit on Conservation and Valorization of Plant Resources through the creation of Botanical Garden, Higher Agronomic Institute, Chott Mariem, IRESA
Ghent university
Laboratory for Applied In Vitro Plant Biotechnology, Faculty of Bioscience Engineering, Ghent, Belgium

BEN MAHMOUD, Kaouther
kaoutherbenmahmoud@gmail.com
National Institute of Agronomic Research of Tunisia
Laboratory of Plant Protection, Tunis

GRIRA, Maroua
grira.maroua@gmail.com
University of Sousse
Department of Horticultural Sciences, High Institute of Agronomy, 4042 Chott-Mariem, Sousse
Ghent University
Faculty of Bioscience Engineering, Dept. Plants and Crops, Laboratory for Applied In Vitro Plant Biotechnology, Ghent, Belgium

LOTFI, Mariem
mariemfejji@gmail.com
IRESA - University of Sousse
Research Unit on Agrobiodiversity, Department of Horticultural Sciences, Higher Agronomic Institute, 4042 Chott-Mariem, Sousse
Ghent University
Faculty of Bioscience Engineering, Dept. Plants and Crops, Laboratory for Applied In Vitro Plant Biotechnology, Ghent, Belgium

UNITED KINGDOM (1)

SAYYED, Imam
isayyed@maelor.co.uk
Maelor Forest Nurseries Limited
Bronington, Whitchurch, Shropshire. SY13 3HZ

USA (2)

GUPTA, Pramod
Pramodgupta.trees@gmail.com
Trees for the future LLC
29329 3rd Ave SW Federal Way WA 98023

MERKLE, Scott A.
smerkle@uga.edu
University of Georgia
Warnell School of Forestry and Natural Resources, Athens, GA 30602

VENEZUELA (1)

TRUJILLO, Iselen
iselen03@yahoo.com
Universidad Nacional Experimental Simón Rodríguez (UNESR)
Centro de Estudios para el Desarollo Agroecológico Tropical-CEDAT, Instituto de Estudios Científicos y Tecnológicos-IDECYT, Laboratorio de Biotecnología Agrícola, Altos de la Mariposa, sector El Cují, Caracas

Next IUFRO 2.09.02 conference

We will meet again in China for our 6th IUFRO 2.09.02 conference!

Pr. Hailong SHEN

Northeast Forestry University

School of Forestry

State Key Laboratory of
Tree Genetics and Breeding

Pr. Hailong SHEN (shenhl-cf@nefu.edu.cn) will organize our next conference in the megacity of Harbin located in the Southwest of the Heilongjiang Province, China.

Join us and discover the latest advances in vegetative propagation of trees and also the world-renowned "Ice City" with unique and exotic cityscape as well as beautiful natural scenery! *Harbin has rich historical and cultural heritages from northern ethnic minorities and has integrated both Chinese and foreign cultures.*

Harbin, Sept. 2020
People's Republic of China

A supportive message from the IUFRO 2.09.02 Unit

Our colleague and friend Mariano Toribio, a prominent figure of our IUFRO 2.09.02 group, was unable to attend Coimbra2018 due to personal reasons. He is now retired from IMIDRA, and we wish him all the very best. We all know Mariano's extraordinary human qualities and we all deeply missed that he could not be with us in Coimbra.

Mariano, we have been thinking a lot about you throughout this conference. We sincerely hope to see you again soon! Look how happy the young students are at the University of Coimbra. This student's Tuna is also for you!

Warmest regards on behalf of all IUFRO 2.09.02 members and participants to Coimbra2018!

April 5, 2019

Jean-François Trontin, Paloma Moncaleán, Yong-Wook Kim
Sandra Correia, Jorge Canhoto, Sandra Sharry, Jana Krajňáková
Jan Bonga, Yill-Sung Park

Summary report of Coimbra 2018

Clonal trees: an alternative to seed-based plantation forestry

Background information*:*

VEGETATIVE PROPAGATION OF TREES FOR COMPLEMENTING SEED-BASED STRATEGIES

Forests are a considerable source of biomass, wood and other products. Their rational management is therefore a key component of the "green economy" towards more efficient and sustainable use of resources. Forest regeneration is mostly achieved through seed-based propagation of wild or improved varieties. However, seed production is a critical and long process that can be severely impacted by environmental stresses, especially those associated with climate change. Efficient and flexible reproduction strategies based on clonal propagation may therefore become more essential for plantation forestry and conservation of genetic resources (see the Proceedings of the 4th IUFRO 2.09.02 conference, La Plata, Argentina, https://www.iufro.org/science/divisions/division-2/20000/20900/20902/publications/).

Key issues discussed/latest findings:

CLONAL VARIETIES FOR DYNAMIC ADAPTATION OF PLANTED FORESTS

Clonal forestry can complement seed-based forestry for sustainable wood production and restoration of endangered resources together with reducing the pressure on native forests. Bulk propagation of elite clones would mitigate perceived risks such as (bio)diversity losses. The long process of field-testing may be streamlined by early genomic selection followed by deployment of clones with greater flexibility and faster turnover.

TOWARDS COST-EFFECTIVE PRODUCTION OF CLONES

Vegetative propagation technologies are developed according to objective (conservation, sanitation, breeding) and economic potential. "Rejuvenation" through tissue culture can reactivate organogenetic capacities. Specialized platforms including mobile biofactories are key tools to design low-cost, automated and scaled-up production.

VEGETATIVE PROPAGATION TO ACCESS USEFUL COMPLEMENTARY TECHNOLOGIES

Vegetative propagation (especially somatic embryogenesis) enables technologies such as hybrid breeding, genotype screening for stress tolerance, cryopreservation and further variety improvement (gene editing, endophytes, priming for increased resilience).

(EPI)GENOMICS: TOWARDS TIGHT CONTROL OF SOMATIC EMBRYOGENESIS

Epigenetic changes are associated with phenotypic plasticity and considered as key factors of embryogenic competence. (Epi)genome-wide profiling produces valuable resources for refining somatic embryogenesis, including from mature tree explants. Automatic screening may support high-throughput optimization of the process.

CLONAL VARIETIES FOR DYNAMIC ADAPTATION OF PLANTED FORESTS

There are strong theoretical and practical justifications that clonal forestry can complement or even outperform seed-based forestry for sustainable production of wood products (H. Wu, I. Wendling, Y.S. Park, C. Marques, U. Nielsen), propagation of native resources (H. Shen, P. Zhang, C. Peng, M.P. Guerra) and restoration in degraded environments (S. Sharry, A. Mosseler). The main public concern about clonal forestry, i.e. the loss of (bio)diversity at the forest or landscape levels, is balanced by the perception that productive plantations could significantly reduce the pressure on native forests (S. Sharry, I. Trujillo). Diversity issues and other perceived risks from using clones (plantation failure, success rate of vegetative propagation (H. Wu) would be partly mitigated by bulk propagation strategies of registered, tested clones allowing the optimization of genetic gains at predefined levels of genetic diversity (Y.S. Park, T. Aronen). A combination of quantitative genetics and pedigree reconstruction using DNA markers could help identify superior clones to propagate (U. Nielsen, J.F. Trontin). In species with significant breeding programs, the long process of field-testing clones (Y.S. Park, A. Mosseler) may be streamlined or replaced by early genomic selection coupled with vegetative propagation to result in significant time and cost savings to identify and deploy new varieties with greater flexibility and faster turnover (Y.S. Park, H. Wu). This is a cornerstone for real-time, dynamic adaptation of planted forests to market and socio-environmental issues.

TOWARDS COST-EFFECTIVE PRODUCTION OF CLONES

Vegetative propagation technologies must be developed considering biological features, objectives (conservation, sanitation, breeding) and economic potential. “Rejuvenation” through tissue culture (micropropagation) usually reactivates organogenetic capacities compared to conventional methods (C. Marques, I. Wendling). Both approaches can be advantageously combined and cost-effective (P. Moncaleán, S. Merkle). Strikingly, different vegetative propagation methods can result in contrasted growth and wood traits (J. Ďurkovič). Trained people is a strong prerequisite for successful application (J. Aitken). Specialized platforms (T. Almeida, T. Aronen, Y.S. Park) including mobile biofactories (G. Salvatierra, J. Aitken) are therefore key tools for selection of clones with good propagation ability (T. Benneckenstein), to design low-cost protocols (C. Sanchez, M. Tikkinen, P. Moncaleán, H. Shen), and to develop automated (Y.S. Park) and scaled-up (M. Lotfi, U. Egertsdotter) production of high-quality plants at cost similar to reference seedlings (P. Gupta).

VEGETATIVE PROPAGATION TO ACCESS USEFUL COMPLEMENTARY TECHNOLOGIES

Vegetative propagation, especially somatic embryogenesis, the process of producing multiple embryos from vegetative cells, is not only amenable to industrial production but is also critical for implementing complementary technologies such as:

- ✓ hybrid breeding (S. Merkle, C. Reeves, J. Martins),
- ✓ genotype screening for tolerance to biotic (T. Maruyama) and abiotic stresses (B. Dordević, S.M. Jain, Y.K. Lee),
- ✓ long-term germplasm conservation (cryopreservation, P. Moncaleán),
- ✓ promising technologies for further improvement of selected varieties:
  - gene editing, although its scope still needs to be discussed (T. Maruyama, C. Reeves),
  - inoculation with mycorrhizae and other endophytes (J. Aitken, F. Gomes, H.K. Moon),
  - priming of initial explant for increased resilience (I. Arrillaga, C. Pereira, P. Moncaleán, J.F. Trontin).

(EPI)GENOMICS: TOWARDS TIGHT CONTROL OF SOMATIC EMBRYOGENESIS

Epigenetic changes with short (developmental plasticity), middle (epigenetic memory) or long-term (local adaptation) effects (especially DNA methylation and chromatin remodeling events) are associated with phenotypic plasticity in trees (S. Maury), opening huge perspectives for tree breeding and management in the context of climate change (especially priming). Interestingly, epigenetic regulation was already reported as a key factor for totipotent cell commitment to embryogenic competence (S. Correia, P. Testillano). The knowledge accumulating on epigenomic (S. Maury) but also on transcriptomic (C. Miguel, A. Alves, W.F. Li, S. Correia, H.I. Kang, T. Capote, J. Krajňáková,), proteomic (C. Teyssier) and metabolomic (B. Navarro,

A. Caeiro) profiles during embryogenesis are valuable resources (reliable markers) to support further improvements in somatic embryogenesis (C. Miguel, P. Testillano) including repeatable induction from mature tree explants (epicormic shoots, primordial shoots, inflorescences) a promising possibility for shortened breeding cycles, now demonstrated in both angiosperm (V. Cano, S. Merkle, M.P. Guerra) and conifer tree species (S. Varis). Automatic screening systems may soon support high-throughput optimization of somatic embryogenesis (R. Awada).

Conference Website: http://www.uc.pt/en/uid/biotec/events/iufro2018

Book of abstracts, 5th International Conference of the IUFRO Working Party 2.09.02, Somatic Embryogenesis and Other Vegetative Propagation Technologies:

Canhoto J.M., Correia S.I. (Eds). Clonal trees in the bioeconomy age: opportunities and challenges. September 10-15, 2018, Coimbra, Portugal. 162 pp. Legal Deposit 445367; ISBN 978-989-20-8651-4. *https://www.iufro.org/science/divisions/division-2/20000/20900/20902/publications/*

Your team at the Coordination of IUFRO 2.09.02 activities:

Jean-François Trontin (FCBA Technological Institute, France) - *jean-francois.trontin@fcba.fr*
Paloma Moncaleán (Neiker-Tecnalia, Spain) - *pmoncalean@neiker.eus*
Yong-Wook Kim (NIFoS, Republic of Korea) - *bravekim@korea.k*

And our honorary members!

Yill-Sung Park (NRC/Canadian Forest Service, Canada) - *yillsung.park@canada.ca*
Mariano Toribio (IMIDRA, Spain) - *mariano.toribio@madrid.org*
Heung-Kyu Moon (NIFoS, Republic of Korea) - *hkmoon@korindo.co.id*

Any inquiry/idea about vegetative propagation of trees? Please, contact us!
https://www.iufro.org/science/divisions/division-2/20000/20900/20902/

Clonal Trees in the Bioeconomy Age: Opportunities and Challenges

Rational management of forests is a cornerstone of the bioeconomy. Clonal plantation forestry can outperform seed-based forestry together with reducing pressure on native forests. Early genomic selection strategies coupled with clonal propagation have the potential for flexible deployment with faster turnover of new varieties for dynamic adaptation of planted forests. Somatic embryogenesis is particularly amenable to industrial clonal production provided that tight control can be achieved. Epigenomic changes associated with developmental and phenotype plasticity appear critical for expressing embryogenic competence.


IUFRO