



3<sup>rd</sup> International  
Conference of the

**IUFRO**

Working Party  
2.09.02

Somatic Embryogenesis and  
Other Vegetative Propagation Technologies

# BOOK OF ABSTRACTS

Woody Plant Production Integrating Genetic  
and Vegetative Propagation Technologies

September 8 - 12, 2014. Vitoria-Gasteiz, Spain

EUSKO JAURLARITZA



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**3<sup>rd</sup> International conference of the IUFRO WORKING PARTY 2.09.02**

# **Woody Plant Production Integrating Genetic and Vegetative Propagation Technologies**



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## Welcome Message

Welcome to “Vitoria-Gasteiz 2014”: the Third International Conference of the IUFRO unit 2.09.02: Somatic embryogenesis (SE) and other vegetative propagation (VP) technologies.

I am particularly pleased that we have this conference in Vitoria-Gasteiz, because some 15 years ago, Vitoria was my first impression of Spain and I have kept it very fondly in my heart ever since. I think this city is fantastic. Here we are, I am so happy that we are going to have a great meeting in this beautiful city.

This year’s meeting is co-hosted by Neiker-Tecnalia, a prestigious institution for Agricultural Research and Development operated by the Basque Government and by the Korea Forest Research Institute (KFRI) in the Ministry of Forestry of the Republic of Korea. We are so fortunate that we have such strong supporters of our work.

This IUFRO unit was launched in 2008 and, since then, we have had two successful international conferences: Suwon2010 in Korea and Brno2012 in Czech Republic. We started out as a small “Somatic Embryogenesis” interest group but, during Suwon2010, “Other Vegetative Propagation” technologies group joined in to make this group more complete. The main goal of this IUFRO unit is to foster the development and application of somatic embryogenesis (SE) and other vegetative propagation technologies in woody plants.

Of the various cloning methods, SE is the primary enabling technology for the development of tree biotechnology and research. Although SE is available for many tree species, they are at different stages of development: For some species, it is well refined and ready for industrial application but for others it is still difficult to obtain. So, an aim of this conference is to progress together by sharing successes, exchanging knowledge, collaborating, and networking.

The most important aspect of vegetative propagation technologies is their integration in tree breeding, and successful integration requires the collaboration of related disciplines. We have a collection of expertise in this conference, including tree breeders, geneticists, tissue culturists, physiologists, biotechnologists, molecular biologists, etc. We believe this is an excellent opportunity to connect various disciplines. So, we wish you a productive conference and happy networking.

On behalf of the organizing committee, I would like to thank Mr. Josu Ezcurdia, Director of Neiker Tecnalia and Dr. Young-Kyoon Yoon, Director General of KFRI for supporting and hosting this conference. Without their support, having this conference would not be possible. Thank you.

I also would like to take this opportunity to thank the organizing committee, particularly, Paloma; she worked tirelessly for the conference. Also, thanks to Ms. Iranzu Telletxea who looked after the local arrangements. As a result, we have a good balance of scientific and social programs.

Once again, welcome and enjoy the conference!

Yill-Sung Park  
Coordinator, IUFRO 2,09.02







## **ABSTRACTS FOR ORAL PRESENTATIONS**



3<sup>rd</sup> International Conference of the  
**IUFRO Working Party 2.09.02**

Somatic Embryogenesis and Other  
Vegetative Propagation Technologies

September 8 - 12, 2014. Vitoria-Gasteiz, Spain.



**ORAL - SESSION 1:**

**APPLICATION OF BIOTECHNOLOGIES  
INTO TREE BREEDING AND  
DEPLOYMENT**

## **Integration of tree biotechnologies into multi-varietal forestry**

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Multi-Varietal Forestry (MVF) is defined as the use of a range of genetically tested tree varieties in commercial plantation forestry. Somatic embryogenesis (SE) and cryopreservation are the enabling technologies for the implementation of MVF. Recently, it has been shown that genomic selection (GS) has a great potential to be incorporated with MVF. In this presentation, we review the achievements, issues, and outlook for implementing SE and other tree biotechnologies in integrated MVF. MVF is well suited for intensively managed, high-productivity sites. MVF integrated with GS offers a drastically faster and much greater genetic gain than that from traditional tree breeding. In white spruce breeding in eastern Canada, for example, the gain is delivered 15 years sooner than the traditional seed orchard breeding. Moreover, GS will make the testing and selection efforts more efficient and streamlined through pre-screening. Sufficiently refined and efficient SE protocols for commercial MVF are available for a number of conifers, primarily the spruces, some pines, and a few larches, but more refinements are required for several economically important conifers. The main challenge for implementing commercial MVF, however, is the high cost of SE seedling production due to manual handling of embryos, both pre- and post-germination. In order to be cost effective, it requires the development of a mechanized embryo handling system for transplanting into mini-plugs for greenhouse culture. With the lack of an automated transplanting system, complementary serial rooting of cuttings may be used as a mass propagation tool once the suitable varieties are developed from the SE-based system. In addition to obtaining a large genetic gain at significantly reduced time, MVF offers flexibility to adapt to changing breeding goals, environment and disease and insect conditions, and this flexibility is provided by the up-to-date information obtained from varietal field testing. Furthermore, in MVF, plantation diversity is dynamically managed over time by carefully balancing genetic gain and diversity based on the best available varietal field test data.

**Keywords:** Cryopreservation, deployment strategy, genomic selection (GS), multi-varietal forestry (MVF), somatic embryogenesis (SE), varietal field testing.





## Genomic Selection and Clonal Forestry Revival

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The recent developments in both genomics (Next Generation Sequencing) and quantitative genetics (methods that incorporate dense sequencing information in evaluation and selection (i.e., genomic selection)) have created a paradigm shift where the long-term phenotype-dependent selection is distant to be replaced by genotype-dependent selection, thus reducing the time for economic traits' genetic evaluation to virtually days or weeks. Shortening economic and complex traits' long-term genetic evaluation will result in: 1) speeding breeding generations' turnover and 2) considerable increase in the selection differential, thus yielding substantial genetic gain increase and programs' flexibility for coping with unperceived contingencies (e.g., climate change or resistance to pests). The combined effect of early selection and greater selection differential calls for appropriate tree improvement delivery systems that by-pass sexual reproduction and maintain the genetic integrity of the selected elite genotypes in congealed state. Vegetative propagation and somatic embryogenesis, in particular, offers unprecedented opportunity to the delivery of the genetic gain attained through conventional and marker-based selection programs. In this presentation, an overview of genomic selection covering various pedigree scenarios and the integration of cloning with modern selection methods will be presented.







## Biotechnological Approaches for Walnut Wood Production

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The use of biotechnological tools can improve virtually any experimental procedure aimed at increasing the productivity of long-lived woody species. More efforts are needed, however, to take advantage of the opportunities offered by this technology, especially for economically important species. We present in this communication some recent advances of our team regarding wood production in walnut. They have focused on (i) the experimental protocols for micro-propagating selected high-yield *Juglans major* x *J. regia* hybrids; and (ii) the use of reliable molecular markers to selectively genotype such elite material. Regarding in vitro micro-propagation, key modifications have been introduced in the standard protocols to improve walnut rooting ability as well as to increase survival during the ex vitro phase. Our modifications have mostly targeted culture conditions and the use of temporary immersion systems, with the final goal of optimizing rhizogenesis and improve overall plant performance. As a result, we have been able to produce and plant in the field several thousands of *vitroplants* from nine different elite genotypes. Regarding molecular markers, a specific set of genomic microsatellites has been evaluated, with the long term goal of using them to identify valuable genotypes and assist selection. The transfer of these markers to other walnut species and hybrids was analyzed as well. Finally, a selection of stress tolerant material based on the natural expression of molecular chaperones is under study at present.

Keywords: Recalcitrant, Acclimatization, Mycorrhization, Microsatellites, Chaperone.





## Possibilities of somatic embryogenesis for production of Scots pine trees with improved heartwood quality

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Stilbenes, phenolic compounds rich in Scots pine (*Pinus sylvestris* L.) heartwood, have strong fungicidal properties. Accumulation of stilbenes in heartwood is correlated with wood decay resistance. Concentration of the stilbenes varies among the trees and is highly inherited. Hence, it is possible to breed for reforestation materials for the future use, which could produce resistant heartwood when grown up. Vegetative propagation of Scots pine provides a way to produce the plant material with certain characters; in this study we studied potential of somatic embryogenesis (SE) for production of Scots pine trees with potential for improved heartwood quality.

Our aim was to use donor trees having high stilbene concentration in their heartwood to initiate embryogenic cultures, and screen for the progenies with high stilbene content. The effect of stilbene concentration in the donor tree on the somatic embryogenesis in Scots pine is not known. Hence, six mother trees with high or low phenolic content were selected to produce embryogenic cultures from the open pollinated and control-pollinated immature cones during 2011-2013. In 2011, the immature cones were picked 4 times from the open-pollinated donor trees. 1800 megagametophytes with zygotic embryos were used for initiation. In total, 67 lines survived. In 2012 and 2013, the initiation was made from the control-pollinated donor trees. All together 3000 megagametophyte were used and 125 lines initiated. Response of the embryogenic cultures having either the high or low stilbene concentration to UV irradiation and fungal induction was also studied. Phenolic concentration has been analyzed by HPLC/DAD from the donor trees, responding and non-responding megagametophytes, embryogenic lines with higher embryo production capacity, and germinated emblings.

Results showed that embryogenic lines having high phenolic content could be initiated from all the donor trees (both the high and the low phenolic contained mother tree) following both the open-pollination and the controlled-crossings. Phenolic compounds in megagametophyte did not show the inhibitory effect on the initiation rate. Stilbene concentration (mainly pinosylvin and monomethylpinosylvin) in the proliferating embryogenic cultures, without any induction treatment, varied from 0.002-1.5 mg/g, DryW, which is rather low in comparison with the donor tree range from 5-23 mg/g in heartwood. Response of the embryogenic cultures having either the high or low stilbene concentration to UV irradiation and fungal induction showed that the cultures with low stilbene content had stronger response to these stresses. The result indicated that SE provides an option for clonal testing and accelerated production of genetically improved materials in Scots pine.

This work belongs to an EU (ERDF)-funded project “Vegetative propagation – knowhow and technology for enhancing bioeconomy” carried out at Finnish Forest Research Institute during 2011-2014.

Keywords: somatic embryogenesis, Scots pine, stilbenes and wood quality.





## “So who needs a megagametophyte? - Initiation study with a promising dry-country *Pinus* species hybrid for New Zealand and Spain”

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Recent research in both Spain and New Zealand has shown that a *Pinus radiata* × *Pinus attenuata* hybrid may be better suited to dry and/or high altitude sites than *P. radiata*. Currently, *P. radiata* is the dominant production species in these countries and both changing land use and climate means that sites now under consideration for planting may not be suitable for the pure species. Propagation methodologies, including methods that start with immature seed, facilitating the amplification and storage of embryogenic tissue while field testing takes place, are well established for *P. radiata*. Field proven *P. radiata* varieties with known site tolerances, disease and wood quality characteristics are available for forestation. These propagation methods have not been developed for the *Pinus radiata* × *Pinus attenuata* hybrid and the primary focus of this work was to initiate embryogenic cultures, test and modify existing *P. radiata* protocols to produce plants for field trials.

In the work presented here, we pose several questions pertinent to the production of hybrid pines; how effective is embryo rescue? Are different media needed for initiation and proliferation? Does the presence of the megagametophyte at initiation inhibit or enhance success? Could it be that the megagametophyte is a buffer against unfavourable conditions in the medium and/or does it offer nutritional benefit? Can we mimic the megagametophyte environment with the use of nurse tissue for the dissected zygotic embryos? What is the effect of the mother (i.e. *P. radiata* or *P. attenuata* as the female parent)? What is the effect of the father? What if the same parents are used in reciprocal crosses?.

With an eye on the future, and the likely need for the rapid propagation of more recalcitrant hybrid combinations in both New Zealand and Spain, we are using the *P. attenuata* × *P. radiata* hybrids as a test case. Our findings will be presented at this 3rd International Conference of the IUFRO Working Party 2.09.02-Somatic Embryogenesis and Other Vegetative Propagation Technologies to be held in Vitoria-Gasteiz, Spain September 8-12, 2014.

Acknowledgements: Shaf van Ballekom of Proseed Ltd New Zealand is thanked for the provision of seed and warm support of this research. We also 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.

Keywords: somatic embryogenesis, *Pinus radiata*, *Pinus attenuata*, hybrid, megagametophyte.





## Pine tree biotechnology for high value forestry

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Nowadays, a growing demand for high quality forestry products reinforces the need to establish new methodologies for genotyping, clonal propagation and preservation of high value genotypes that retain traits of interest.

In this context, **Klón - Innovative Technologies from Cloning** invests in research activities related to molecular marker-assisted selection and mass propagation techniques, via somatic embryogenesis and micropropagation of mature trees. This allows to obtain *Pinus* spp clones from elite trees with industrial interest providing higher frequencies of homogeneous plants and enabling a large genetic gain.

Micropropagation is one the most suitable method for large-scale production of superior genotypes in tissue culture. Our research team has been dedicated to elite tropical *pinus* tree species multiplication namely by application of meristem isolation techniques from mature tree shoots. We have been developing a reproducible tissue culture introducing variations in culture media composition and conditions during induction, elongation and rooting phases. Different basal macro and micronutrients and concentrations of cytokinins, auxins and carbohydrates have been tested with continuous increments in the multiplication rate.

The somatic embryogenesis (SE) is also one of the most promising techniques for large-scale propagation of elite genotypes. However few works have been described within tropical pine species. The somatic embryogenesis in hybrid pine has been one of the procedures we followed to propagate elite plants. Initiation and maturation of embryogenic tissue can be critical steps for the commercial application of SE in *Pinus* species. The effect of collection time or developmental stage of the zygotic embryos have been studied simultaneously with the application of different basal media and PGR combinations to overcome these bottlenecks.

Preservation of selected plant material with important forestry features is also one of **Klón's** objectives. We have been developing systems for long term storage of plant material at low temperature in liquid nitrogen for cryopreservation of somatic embryogenesis tissue, ensuring the availability of juvenile material, avoiding losses of embryogenic capacity and providing biological material genetically stable. Protocols of cryopreservation were developed successfully from selected genotypes of tropical pine from a breeding program established since 1991. **Klón** has structural capacity to cryopreserve about 35 800 samples of important germoplasm.

In long-term growing species such as forest trees, the conventional breeding programs can take a long time to provide returns. Hence, nowadays, the molecular marker-assisted selection is a powerful tool to accelerate and become breeding programs more efficient and productive. In this sense, **Klón** has been investing on the identification and selection of new molecular markers for high throughput genotyping of pine trees with economic traits. The research has been focused mainly in two types of molecular markers, namely, Single Sequence Repeats (SSRs) and Single Nucleotide Polymorphism (SNP). A set of 9 SSRs markers, previously described for *Pinus* spp., is being validated in populations of tropical *pinus* tree species. In parallel, based on sequencing data generated by transcriptomic analysis of selected pine trees, we are also developing new SNPs and SSRs markers associated with genes controlling traits with high economic interest (resin and wood production).

Keywords: *Pinus*, micropropagation, somatic embryogenesis, cryopreservation, genotyping.







## Applying SE technology for mass-propagation of high-quality Norway spruce in Finland

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Irregular flowering of seed orchards and problems caused by pests have caused periodic shortages in availability of genetically improved Norway spruce (*Picea abies* (L.) Karst.) seed over the past decade in Finland. Vegetative propagation based on somatic embryogenesis (SE) is expected to become a remedy to the supply problems and an effective way to produce high-quality clonal material for forest reforestation. The SE laboratory of the Punkaharju Unit, the Finnish Forest Research Institute, is currently making rapid progress in producing SE clones of Norway spruce for genetic field testing.

Seed embryos used as a starting material in SE originate from controlled crosses between superior plus trees. The embryos are derived from immature seeds and used as explants for producing the SE clones. Suitability of the SE clones for mass-propagation is evaluated in multiple-stage tests carried out in the laboratory. The viable SE clones with the highest embryo producing capacity will be selected for clonal field testing and cryopreservation.

The first field trials with SE clones will be planted in 2014. Two-year-old emblings are used for the trials. The trees will be evaluated, e.g., for growth, phenology, Lammas growth and resistance to various pathogens. Each set of clones will be tested in at least four sites with ten emblings per clone and site. Following the completion of field testing, the selected clones will be taken from the cryopreservation and delivered to commercial plant producers for large-scale multiplication.

In the future, emblings may be utilised in reforestation either as mixtures of tested clones or as untested full-sib families. However, dissemination of the new material requires implementation of the SE-technology in commercial plant production. Transferring the know-how and technology from research to practice has already begun in a pilot with Norway spruce special forms selected for ornamental use.

Keywords: vegetative propagation, somatic embryogenesis, Norway spruce, *Picea abies*.





# Dealing with expected and unexpected human errors: development of a quality control SNP array for to the clonal production of white spruce (*Picea glauca*) using somatic embryogenesis

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Somatic embryogenesis, when integrated into a large-scale production process, allows to produce elite materials at the desired time and in the desired quantities. If this technique allows reproducing *ad infinitum* an individual with known characteristics, its very nature also means that if mistakes are made during the process, they will be, in the same way, multiplied.

In order to track down such expected human errors, the objective of this project was to develop a traceability method that could be applied to the clonal production of white spruce (*Picea glauca*) using somatic embryogenesis in tree nurseries in the province of Quebec (Canada). This method would use a minimum number of DNA markers (so as to limit costs) that would allow to distinguish between the different clonal varieties (using a unique genetic fingerprint) and to verify their breed of origin.

For several decades, white spruce has been at the heart of a vast genetic improvement program which, in recent years, has also benefited from the contribution of genomics. Large amounts of genomic data (mostly SNP genotypes) therefore were already available, from which we selected the most informative SNP markers. The selection process was performed using the Random Forest classification method on *in silico* datasets. Those datasets were generated using in-house bioinformatics tools that simulated crosses between the elite parents involved in the improvement program and for which genotypes from hundreds of SNP markers were available. Once a subset of informative markers had been selected, their capacity and robustness to correctly assign offspring to their breed of origin were assessed, first on *in silico* progeny trees, then on real individuals, using a Bayesian clustering method and a parental allocation software.

If, theoretically, the strategy considered appeared to be straightforward and supported by large amounts of genomic data that were already available, it also had some glitches. Some of them were caused by a modification of the genotyping technique while others, ironically, were associated with the presence of unexpected errors in the original dataset. This presentation will thus detail the different steps leading to the development of such a quality-control method applied to tree production. It will also discuss the practical aspects associated with its implementation at the production scale. More importantly, it will demonstrate its capacity to effectively detect errors in the material produced by tree nurseries, such errors that were, this time, expected.

Keywords: traceability, SNP, quality control, genetic fingerprint, spruce.





## Argentine science policies in forest biotechnology

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We analysed the present status of forest biotechnology in Argentina and some countries in Latin America, in the light of information being collected within the framework of the Strategic guidelines 2012-2015-Ministry of Science, Technology and Productive Innovation. This plan includes several components. One of them is strengthening the scientific and technological system. This strengthening was imperative to address actions in which knowledge generation is coupled with solving needs and increasing the productivity of different chains. This plan has incorporated the concept of Strategic Socio Productive Cores (NSPE), that explains the close association that must exist between the scientific system and local needs. The planning was developed through the Implementation Tables (IT) of the 34 NSPE identified so far. These tables work in establishing consensus to achieve the desired scenarios. The courses of action will be reflected in operational plans that will guide the funding implementation, define new lines of scientific research and allow tracking and evaluation of the undertaken actions. One of the IT was “**Production and processing of forest resources**”. The aim is strengthening the timber industry and related activities, including the production of pulp, laminates, chemicals and new materials for packaging, and the generation of bioenergy from waste-use timber. The strategy must combine a key enabling technology (such as biotechnology, nanotechnology, or information and communications) with a strategic area. These developments are crucial because biotechnology is increasingly being used on trees and in forests in the world. We identified six priority areas in an attempt to meet global demand for forest products, biofuels, to restore threatened species, and to protect future forests from invasive pests and climate change. Biotechnology was identified as a tool to grow trees with special characteristics, characterize biodiversity and for energy. Breeding technologies like organogenesis, somatic embryogenesis, marker-assisted selection and genetic engineering have to be developed through new human resources and capacity in order to enhance bio-based products. Other specific areas were the combat of invasive threats, mainly in *Populus* and *Salix* and addressing forest health challenges. In the world, most forest biotechnology activities are carried out in developed countries, the most active U.S., France and Canada, but India and China are developing and transition countries reported a lot of activities to. In Latin America Brazil, Chile and Argentina are leading this development. Undoubtedly, the most commonly used biotechnology is the application of molecular markers, followed by tissue culture. The genus *Populus*, *Pinus* and *Eucalyptus* are the most used in biotechnology research in general, and the unique in terms of genetic transformation. In Brazil and Argentina, unfortunately, the global economy places a higher premium on meat and soya beans than on forests. Creating a new economic model and addressing I+D, forest will therefore take transformations that require science. In conclusion, in Argentina and other Latin-American countries, the use of biotech in forest trees is not yet well developed. Clear, strong and addressed policies in science are executing.

Keywords: capacity development, tree biotechnology, strategic planning, MERCOSUR.





# Genomic selection in spruces for multiclonal forestry by somatic embryogenesis

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Genomics has revolutionized several aspects of animal and plant breeding by providing information tools at the genetic level to speed up the evaluation and decision-making process. With the advent of complete genome sequencing, the making of large repertoires of DNA polymorphisms and access to high-throughput genotyping platforms, it is now possible to saturate the genome with SNPs and predict quite accurately the future value of candidate trees for selection by virtue of linkage between markers and quantitative trait loci and by capturing relatedness. The approach, called genomic selection (GS), has been recently developed for key forest tree species. We will show the recent progress in this area by focusing on boreal spruces, which are essentially undomesticated and characterized by long breeding cycles. We will show how GS can drastically shorten these cycles so to provide quicker response in the context of climate change, simplify breeding operations, and how it can be integrated into forward selection schemes involving somatic embryogenesis as a deployment mean of improved spruce varieties.







## Endophytes - a new “breeding” technology or not?

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Bioprotection of plants from diseases has many commercial examples in 2014. Use of natural endophytes and other bio-control agents are playing a key role for improving plant health and enabling plants to become more disease-tolerant. Two projects The Tree Lab has been involved in for the past two years are the PSA-V (a virulent form of *Pseudomonas syringae* pv “Actinidiae”) disease in kiwifruit (*Actinidia deliciosa* and *Actinidia chinensis*) and red needle cast (*Phytophthora pluvialis*) in radiata pine. Tissue cultured sterile plants were inoculated with selected endophytes, and confirmation of the presence of that same endophyte was achieved in the sterile plant parts and in field plants. This technology allows the introduction of a potential new “trait” to the plant. Latest results with kiwifruit and radiata pine and a discussion of how endophytes can impart “new traits” to plants will be presented in Vitoria.





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**ORAL - SESSION 2:**

**PHYSIOLOGY AND (EPI) GENETICS  
OF SE AND OTHER VP  
TECHNOLOGIES**

## **Cellular levels of ATP, are they important for success of somatic embryogenesis?**

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ATP, as a main product of respiration, represents the universal energy currency of living cells. ATP and other nucleotide triphosphates not only drive energy-dependent reactions inside a cell, but can also function in the extracellular matrix, where they function as antagonists that can induce diverse physiological responses without being hydrolyzed. The energetic metabolism occurring during somatic embryogenesis of cell cultures has been neglected for quite a long time, although it can be crucial for a correct development of somatic embryos where life and death processes co-exist. An overview of our research activities focused on bioenergetic aspects of somatic embryogenesis (proliferation and maturation stages) and cryopreservation will be discussed.

Keywords: bioenergetics, somatic embryogenesis, *Abies alba*, *Abies cephalonica*, cryopreservation, heavy metals





## **Multi-scale analysis of early molecular events during *Pinus pinaster* Ait. somatic embryo development under reduced water availability**

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Significant progress has been made in the development of maritime pine somatic embryogenesis but there are still technical issues precluding full integration of this powerful vegetative propagation system into the French breeding programme. Maritime pine somatic embryos (SEs) require a reduction in water availability (high gellan gum concentration in the maturation medium) to reach the cotyledonary stage. This key switch, reported specifically for pine species, is not yet well understood. To facilitate the use of somatic embryogenesis for mass propagation of conifers, we need a better understanding of embryo development.

Comparison of the transcriptome (Illumina RNA sequencing) and proteome (2D-SDS-PAGE with MS identification) of immature SEs, cultured on either high (9 g l<sup>-1</sup>, 9G) or low (4 g l<sup>-1</sup>, 4G) gellan gum concentration, was performed, together with analysis of water content, fresh and dry mass, endogenous ABA (GC-MS), soluble sugars (HPLC), starch, and confocal laser microscope observations. This multi-scale, integrated analysis was used to unravel early molecular and physiological events involved in SE development. Under conditions unfavorable for SE maturation (4G) both transcriptomic and proteomic profiling indicate enhanced glycolysis leading to proliferation of embryonal masses (EMs) which may be antagonistic to SE maturation. Under favorable conditions (9G), we observed adaptive, ABA-mediated molecular and physiological responses to reduced water availability resulting in early transition of EMs from proliferation to the SE developmental pathway (indicated by active protein synthesis, and overexpression of proteins involved in cell division, embryogenesis and starch synthesis). Specific pathways (synthesis of protective secondary metabolites, regulation of oxidative stress) are also activated, apparently to overcome constraints due to culture conditions.

This is the first study on the early molecular mechanisms involved in somatic embryogenesis of pine following an increase in gellan gum concentration in the maturation medium, and it is also the first report combining transcriptomic and proteomic data analysis during somatic embryogenesis in conifers. We have found novel candidate predictive markers for conifer SE development (germin-like protein and ubiquitin-protein ligase) as well as for adaptive responses (protein phosphatase 2C), which may facilitate practical application of the knowledge gained in this study to monitor early responses of embryogenic tissue to maturation conditions.

**Acknowledgements:** This work was supported by grants from the French “Conseil Régional de la Région Centre” (EMBRYOME project, contract 33639), the “Conseil Régional de la Région Aquitaine” (Embryo2011, contract 09012579-045), and the French Ministry of Foreign Affairs and the French Ministry of Higher Education and Research through the France/Czech Republic Science Cooperation BARRANDE Programme. Data analysis was made possible through the involvement of INRA's GenoToul bioinformatics platform in Toulouse (France).

**Keywords:** somatic embryo development, germin-like protein, glycolysis, transcriptomics, proteomics.







## The effect of tissue culture and cryopreservation on tree telomeres

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Eukaryotic chromosomes are formed of a single DNA molecule, which terminates in specialized heterochromatin called telomeres. The function of telomeres is to protect chromosomes from degradation and fusion during DNA replication. Shortening of telomeres has been connected with ageing and loss of cell replication or regeneration capacity. The information on the role of telomeres in ageing of plants is limited, and telomeres of long-living trees have only been studied in a few species. In addition, biotechnological methods are applied more and more also in tree species. Transgenic trees are studied, while clonal propagation by tissue culture is already a standard practice in many species, and clones can be maintained under tissue culture conditions or cryopreserved for long times. There is, however, no data existing on the effects of tissue culture and its duration, or stress factors involved in biotechnologies, on telomere length in long-living trees.

The aim of the present study was to examine potential effect of tissue culture and cryopreservation on tree telomeres. Tissue culture effects were studied in silver birch (*Betula pendula*) using clonal materials consisting of different-aged outdoor trees and tissue cultures of seven genotypes. In addition, the effect of cryopreservation and long-term culture was examined by comparing 12 embryogenic cultures of Norway spruce (*Picea abies*) prior to and following cryostorage.

In silver birch, no correlation of ageing and the length of telomeric repeats was found when germinated seeds, and leaf and cambium samples from 15- and 80-year-old trees were compared. Positional variation in the telomere length was, however, observed in the cambium of mature trees, the stem base having longer repeats than the upper parts of the tree. Tissue cultures were found to have shorter telomeres than outdoor trees: prolonged culture, callus culture and stressful conditions were all observed to shorten telomeric repeats and should thus be avoided in birch micropropagation. There were significant differences among the studied silver birch genotypes in their telomere length, and these differences were consistent over the sample types.

In Norway spruce, successful cryopreservation was not found to affect the length of telomeres in the embryogenic cultures. In non-regenerating cultures, however, telomeric DNA was observed to be severely damaged. Significant genotypic differences among the SE lines in their telomere length were found also in Norway spruce.

Reference: Aronen & Rynänen 2014. Silver birch telomeres shorten in tissue culture. *Tree Genetics & Genomes* (2014) 10:67–74

Keywords: *Betula pendula*, cryopreservation, genotypic variation, *Picea abies*, telomeric repeats, tissue culture.





## Are cotyledonary somatic embryos of *Pinus pinaster* Ait. equivalent to the zygotic counterpart?

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In maritime pine, improved protocols are now available for the whole somatic embryogenesis process i.e. from initiation of embryogenic tissue to somatic plant regeneration. However, field trials established in France from somatic plant material have consistently revealed that somatic embryos (SEs) developed at a lower initial growth rate compared to control seedlings. A better understanding of SEs maturation is therefore required in order to produce high-quality, vigorous somatic plants. SEs are currently matured for 12 weeks to reach the cotyledonary stage before being germinated and converted to plantlets. Although regeneration success is highly dependent on SEs quality, the harvesting date is still determined from morphological features. This empirical method does not provide any accurate information about embryo quality with respect to storage compounds (proteins, carbohydrates). We first analyzed SEs matured for 10, 12 and 14 weeks by carrying out biological (dry weight, water content) and biochemical measurements (total protein and carbohydrate contents). No significant difference could be found between collection dates, suggesting that SE harvesting after 12 weeks maturation is appropriate. Cotyledonary SEs were then compared to various stages, from fresh to fully desiccated, in the development of cotyledonary zygotic embryos (ZEs). Using hierarchical ascendant cluster analysis, we demonstrated that cotyledonary SEs matured for 12 weeks are most similar to fresh cotyledonary ZEs sampled from late July to early August (as indicated by dry weight, water content, sucrose, RFOs content, RFO/Sucrose ratio) or at any time up to October (with respect to total protein). Both types of embryo exhibited similar carbohydrate and protein content and signatures. This high level of similarity (94.5%) was further supported by proteome profiling. Highly expressed proteins included storage, stress-related, late embryogenesis abundant (LEA) and energy metabolism proteins. By comparing overexpressed proteins in developing and cotyledonary SEs or ZEs, some (23 proteins) could be identified as candidate biomarkers for the late, cotyledonary stage. Of these, 18 belonged to five large families of proteins including five HSPs, four LEAs and two other stress-related proteins (aldose reductase, 6-phosphogluconate dehydrogenase), five storage proteins and two proteins involved in purine metabolism (adenosine kinase 2, SAM synthase).

This is the first report of useful generic protein markers for monitoring embryo development in maritime pine. Our results also suggest that improvements of SEs quality may be achieved if the current maturation conditions are refined.

**Acknowledgements:** This work was supported by grants from the French “Conseil Régional de la Région Centre” (EMBRYOME project, contract 33639) and “Conseil Régional de la Région Aquitaine” (EMBRYO2011 project, contract 09012579-045). Some equipment used at FCBA to get results presented in this work was funded by ANR-10-EQPX-16 XYLOFOREST. We gratefully acknowledge Gilles Bruneau, Francis Canlet, Sandrine Debille, Karine Durandau, Séverine Quoniou and Françoise Wan-Fung (FCBA) for their valuable contribution to the collection of somatic and zygotic embryos and Pierre Alazard (FCBA) for cone sampling in the field. We acknowledge Marlène Bailly for her technical assistance in electrophoresis.

**Keywords:** carbohydrates, embryo quality, maturation, proteomic, somatic embryogenesis, zygotic embryogenesis.





## Somatic embryogenesis: what plant biologists can learn from animal cells

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The totipotency of plant cells has been known for many years and, in the last three decades, has been widely exploited to obtain somatic and pollen embryos in hundreds of species. The ability to produce an organism from a differentiated somatic cell was never achieved in animals, which makes cloning of animal organisms much more complex. Nonetheless, in recent years, very effective protocols for the manipulation of pluripotency in animals have been developed, and genes involved in the expression of pluripotency and in the induction of stem cells are well known. Protocols are now available to obtain what are called induced *pluripotent stem cells* (iPS) from adult cells by the simple manipulation of a very limited number of genes, suggesting that the triggering mechanisms to reverse cells to an undifferentiated state are under the control of a few genes. In a similar way, great achievements were made on the induction of pollen and somatic embryogenesis in plants, but few progresses concerning the molecular mechanisms and genes controlling the induction of totipotency were obtained. It is true that genes involved on somatic embryo formation, such as *SERK* and *LEC* have been identified and characterised in *Arabidopsis* and other few models. However, for most of the species in which somatic embryogenesis induction has been achieved, the induction process remains dependent on the application of auxins or stress conditions and the molecular mechanisms relating these stimuli to somatic embryo formation are poorly understood. One of the reasons that can explain the difficulties to understand and manipulate somatic embryogenesis at will is related with the type of experimental systems used. Usually, an explant (e.g. young leaves, zygotic embryos, floral organs), possessing thousands of cells is submitted to a stimulus and, among that large cell population, a few are stimulated to express their totipotency. In recent years, we have developed very effective protocols for the induction of somatic embryogenesis in several woody species such as bay laurel (*Laurus nobilis*), carob (*Ceratonia siliqua*), pineapple guava (*Feijoa sellowiana*), strawberry tree (*Arbutus unedo*) and tamarillo (*Cyphomandra betacea*). Among them, tamarillo is particularly interesting for somatic embryogenesis studies since embryogenic cells can be induced from different tissues and in large amounts. Moreover, embryogenic and non-embryogenic calli are formed from the same tissue, making easier to compare macromolecule profiles in both types opening the way for the identification of genes related with the somatic embryogenesis process. Using this strategy we were able to identify a putative somatic embryogenesis inhibitor protein NEPTC – (GenBank, accession number JQ766254.1) that seems to block somatic embryo formation in non-embryogenic cells of tamarillo. In this presentation we will discuss the role of this protein on the expression of totipotency and show the potential of this embryogenic system when compared with animal iPS systems.

Keywords: auxins, pluripotency, stem cells, stress, totipotency.





# Influence of ethylene modulators on the expression of *QsERF1* during secondary embryogenesis of *Quercus suber*

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Ethylene production and signaling play an important role in somatic embryogenesis, especially for species that are recalcitrant to *in vitro* culture. Precursors to ethylene, like 1-Amino-1-cyclohexane carboxylic acid (ACC), and inhibitors of ethylene synthesis or perception like silver thiosulphate (STS), have been reported to have different effects on the induction and development of somatic embryos depending on the species and on their developmental stages.

In the present study we investigated the effect of different concentrations of ACC and STS on the morphological features and the proliferation levels of *Quercus suber* somatic embryos. On the other hand we studied the transcript accumulation of *QsERF1* using qPCR in embryos collected at different times after ACC and STS treatments.

*QsERF1* (*Q. suber ethylene response factor 1*), isolated by our research group, encodes a member of the ethylene response factor (ERF) transcription factors. ERFs belong to the APETALA2/Ethylene Responsive Factor (AP2/ERF) family, whose members are conservatively widespread in the plant kingdom. *QsERF1* is related to *Medicago truncatula somatic embryo-related factor1* (*MtSERF1*), which has been shown to be required for somatic embryogenesis.

*Quercus suber* somatic embryos were initiated from expanding leaves of epicormic shoots forced to flush in branch segments of a centenarian tree. Embryos were subcultured monthly by secondary embryogenesis in RITA® vessels with Murashige and Skoog medium supplemented with 0.1 mg L<sup>-1</sup> benzyladenine, 0.05 mg L<sup>-1</sup> naphthalene-1-acetic acid and 3% sucrose. For ACC and STS experiments, embryos were cultured in Petri dishes in the same medium described above but gelled with 0.6% Vitroagar. This medium was supplemented with 0, 10, 50 or 100 μM of ACC or STS. Two types of explants were used: 1) embryo clumps at globular/torpedo stages, and 2) isolated cotyledonary embryos (6-10 mm). The effect of ACC and STS on the morphological features of *Q. suber* embryos was monitored weekly and the proliferation levels were recorded after 4 weeks of culture. Both embryo clumps and isolated cotyledonary embryos treated with ACC showed good performance without significant differences with the control treatment. However, the ability of the cork oak embryogenic cultures to produce secondary embryos was severely affected by the presence of 50–100 μM STS, and a significant increase in percentage necrosis was also observed. The relative transcript abundance of *QsERF1* was analysed during somatic embryogenesis by qPCR in samples of embryo clumps of immature stages, as well as in isolated cotyledonary embryos collected at 0, 2, 4, 7, 14, 21 and 28 days after plating.

This work was funded by the Xunta de Galicia (10MRU400033PR).

Keywords: ACC, *ethylene response factor 1* (*ERF1*), qPCR; *Quercus suber*, somatic embryogenesis, STS







## Characterization and expression analysis of *QsTCTP* and *QsADF* during *Quercus suber* somatic embryogenesis

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Somatic embryos develop through a sequence of stages associated with morphological and biochemical changes related to genomic activity and can be used to identify the genes that may be involved in the process. The different developmental stages of somatic embryos are controlled by the temporal expression of specific genes; characterization and expression analysis of these genes are required to gain a better understanding of the mechanism of somatic embryogenesis. Two genes, *TCTP* (*translationally controlled tumor protein*) and *ADF* (*Actin-depolymerizing factor*) were isolated from *Quercus suber* in order to study if they are involved in histodifferentiation processes during the development of cork oak somatic embryos.

Translationally controlled tumor protein (TCTP) is a highly conserved protein that is widely expressed in all eukaryotic organisms including plants, yeast and mammals, and his expression found to be controlled post transcriptionally. The actin-depolymerizing factors (ADFs) are ubiquitous low-molecular-mass actin-binding proteins that are important for actin filament assembly in eukaryotes facilitating dynamic remodeling of the actin cytoskeleton. They are both known to be involved in different developmental processes such as morphogenesis, plant cell division and growth.

The objectives of this work were 1) to isolate and identify homologous genes to *TCTP* and *ADF* in *Quercus suber* and 2) to study the expression pattern of *QsTCTP* and *QsADF* by *in situ* hybridization and qPCR during somatic embryogenesis. For that purpose, *Q. suber* somatic embryos were initiated from expanding leaves of epicormic shoots forced to flush in branch segments of a centenarian tree. Embryos were subcultured monthly by secondary embryogenesis in RITA® vessels with Murashige and Skoog medium supplemented with 0.1 mg L<sup>-1</sup> benzyladenine, 0.05 mg L<sup>-1</sup> naphthalene-1-acetic acid and 3% sucrose.

To identify homologous genes to *TCTP* and *ADF*, RNA was isolated from *Q. suber* somatic embryos and EST databases sequences were used to design specific primers. Reverse transcription polymerase chain reaction (RT-PCR) and 3'-5' Rapid Amplification of cDNA Ends (RACE) were performed and the fragments obtained were cloned and sequenced. In order to study the expression of *QsTCTP* and *QsADF* during somatic embryogenesis, embryos at globular, torpedo and cotyledonary stages were collected from proliferating cultures.

Analysis of sequences reveals that *QsADF* and *QsTCTP* encode proteins of 143 and 168 aa, respectively, that belong to the ADF/cofilin and TCTP families, respectively. Previous results showed an increase of relative transcript abundance of *QsTCTP* as long as the embryo develops to more advanced stages, suggesting that the gene is involved in the histodifferentiation of somatic embryos. A more detailed data of the expression patterns of both genes will be presented.

This work was funded by the Xunta de Galicia (10MRU400033PR).

Keywords: *Actin-depolymerizing factor* (*ADF*), gene expression, *Quercus suber*, somatic embryogenesis; *translationally controlled tumor protein* (*TCTP*)





## **Anti-actin drugs latrunculin B and cytochalasin D in somatic embryogenesis of Norway spruce: diverse effects due to different mode of action**

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The role of the actin cytoskeleton in different stages of Norway spruce somatic embryo development was investigated using anti-actin drugs latrunculin B and cytochalasin D. Both of these compounds changed the state of actin polymerization and the organization of actin filaments in cells, but targeted cells and mode of action of both drugs were different. Cytochalasin D (0.5 or 5  $\mu$ M) damaged both suspensor and meristematic cells, latrunculin B (50-100 nM) primarily affected the suspensor cells. Lethal damage to the meristematic and suspensor cells was observed when the drugs were applied throughout the maturation period, although the severity of this effect depended on their concentrations. The drugs were applied to ESM for one week at three different points in the maturation process: 1) immediately prior to the start of maturation, 2) during the first week of maturation, and 3) during the fourth week of maturation. The strongest effects were observed when the drugs were applied at the start of maturation. Under these conditions, cytochalasin D treatment strongly inhibited further development of embryos. On the other hand, latrunculin B destroyed the suspensors and did not affect actin filaments in meristems of embryos. Chemical cleavage of suspensors in this stage accelerated the subsequent development of embryos that were already capable of separating from the suspensors. Thus, while the total number of embryos at the end of the maturation period was lower than in untreated control cultures, the surviving mature embryos were of higher quality compared to control. Moreover, the development of embryos was more synchronized. Drug treatment at the end of the maturation period did not significantly affect embryo development: latrunculin B caused no change in the yield of somatic embryos, but cytochalasin D treatment increased the number of malformed embryos compared to untreated controls.

Different sensitivity of actin in suspensor and meristematic cells of ESM was explained by the existence of four spruce actin genes. Analysis of their expression during embryo maturation revealed that one actin isoform was expressed constitutively in both cell types, whereas three actin isoforms were expressed predominantly in suspensor cells and their expression declined during the maturation. The expression decline was greatly enhanced by Lat B treatment. Sequence analysis revealed amino-acid substitutions in the Lat B-binding site in one of the suspensor-specific actin isoforms, which may result in a different binding affinity for Lat B.

Keywords: somatic embryogenesis, Norway spruce, actin, latrunculin B, cytochalasin D, embryo.





## **Time is of the essence - Cotyledon formation in somatic embryos of *Larix decidua* requires proper auxin efflux for the right period and for the right point in time**

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The development of a proper mature embryo is closely linked to the formation of a fully functional shoot apical meristem (SAM). This process largely depends on a precise timely interaction of plant growth regulators (PGR) such as auxin with genetic regulators. The auxin level and distribution during embryogenesis is a key factor to trigger cell differentiation and thus initiate cotyledon development and separation. Essential studies on the transferability of functions known from angiosperms to conifers have been initiated on *Picea abies*. Further analyses are necessary for detailed comparisons regarding conserved developmental strategies.

We used somatic embryos (sE) of *Larix decidua* as an experimental system, because these reach maturity within a month and yield up to 100 sE per 0.1 g FW. It has been the object to investigate the establishment of a proper meristem and cotyledon development depending on auxin distribution. To this end the polar auxin transport (PAT) has successively been disrupted or was restored with 1-N-Naphthylphthalamic acid (NPA) during embryogenesis. Thus a time frame which is crucial for correct auxin transport was defined at which sE are less affected by auxin flow disruption.

In order to find a way of measure, the embryos were grouped into several categories regarding cotyledon morphology using light and RE microscopy techniques:

NPA treatment caused two major types of embryos with distinct morphological aberrations - either forming embryos with a closed cotyledon ring – “Cups”, or a “Cucumber”-like embryo, with a pin-formed apex. Yet the later NPA was applied to the embryos, the lesser was the effect on its morphology. Though embryos are to some extent always affected by an NPA application and the restoration to a normal morphology can hardly be observed, yet it was observed that the NPA affected morphology is adjusted to transitional phenotypes. In an attempt to predict cotyledon development in early stages, a sample of sE has been tracked. Subsequent analyses were conducted to demonstrate the effect of NPA on the auxin distribution. Therefore the activity of the reporter gene GUS, which was controlled by an auxin inducible promoter (GH3::GUS) was studied in mature sE, which were continuously treated with NPA. The results indicated that NPA reduces the auxin concentration.

Furthermore changes in expression levels in embryos were analysed with the intention to correlate Auxin efflux to genes with relevance to embryogenesis and patterning [*PINFORMED (PIN)* and *SHOOT MERI-STEMLESS (STM)*]. First results suggest an effect of auxin inhibition on the gene regulation.

We have demonstrated that the formation of a proper embryo apex depends on a regulated auxin flux, which is most important before cotyledon appearance.

Keywords: *Larix decidua*, Embryogenesis, Auxin inhibition, NPA, Cotyledon formation.





## An overview of the current achievements in Holm oak (*Quercus ilex* L.) somatic embryogenesis

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Holm oak (*Quercus ilex* L.) is one of the main evergreen species that conform the Mediterranean forests in the Iberian Peninsula. It is commonly found with the cork oak in the dehesas or montados, which have historical importance in social and rural development. Besides its ecological importance, the main economic interest of the holm oak relies on its acorn production mostly to feed the cattle, and the mycorrhizal relationships that establishes with several edible fungi, being the most profitable species the black truffle (*Tuber melanosporum*). There are several problems that menace the holm oak survival such as variable climate conditions, wildfires, the oak decline syndrome and a low natural regeneration. However, developing genetic amelioration and breeding programs can improve heritable factors that influence pests and diseases resistance and the acorn production. Oaks species are considered recalcitrant species in terms of seed conservation and their organogenic capacities are limited up to a few months after germination. Among vegetative propagation techniques, somatic embryogenesis (SE) enables mass propagation and the implementation of multivarietal forestry. SE has been defined before as a fine method for clonal regeneration of adult trees in other *Quercus* species<sup>(1)</sup>, which has allowed the establishment of highly efficient clonal tests of phenotypic selected material. Contrary to other oak species, the induction of SE from leaves of mature holm oak trees was unsuccessful, but catkins and the integuments of developing acorns were found to be suitable explants depending (mainly) on their developmental degree<sup>(3,4)</sup>. Several embryogenic lines were established and during the culture maintenance, cotyledonary embryos differentiated spontaneously in the proliferation medium. Maturation, germination and conversion of those embryos were successfully attempted<sup>(3)</sup>.

In order to improve both efficiency and quality of the embryogenic cultures maintenance, the influence of light and the presence of plant growth regulators during the secondary embryogenesis was approached, concluding that both factors reduced the quality of the cultures, but had no remarked effects over the productivity. Secondly, liquid cultures for scaling-up were also studied: Temporary immersion systems (TIS) and the influence of glutamine on the growth dynamics of an established embryogenic suspension were also addressed. When comparing to the gelled cultures, no significant differences were observed in the cotyledonary embryo production of TIS with the tested conditions. The addition of glutamine to the proliferation medium significantly improved the growth, although no morphological differences were observed. Cryopreservation methods are commonly used in order to prevent the loss of embryogenic competence or contamination problems, and also decrease the costs of the maintenance of the lines. Some of the holm oak embryogenic lines were successfully cryopreserved using the protocol developed for the cork oak<sup>(4)</sup>.

Clonal regeneration of mature holm oak trees was successfully achieved although there are some bottlenecks to be improve in order to secure a high productivity of the process, as the differentiation and maturation processes and also the ex vitro acclimatisation method.

Acknowledgements: A Barra-Jiménez has PhD-fellowship of the IMIDRA. This work was funded by the Spanish National Project AGL 2010-22292-C03-01 and the European Program Trees4Future.

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Key words: plant regeneration, secondary embryogenesis, liquid medium, cryopreservation.







## Regulators of gene expression in pine embryogenesis

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The elucidation of the molecular mechanisms governing conifer embryogenesis may contribute valuable information to understand the basis for the distinct features observed during embryo development in angiosperms and gymnosperms. Additional insight into the molecular regulation of embryo development in conifers will have great utility for the improvement of these species and their vegetative propagation through somatic embryogenesis.

We used a transcriptomic approach to identify mechanisms of epigenetic and transcriptional control by transcription factors of the gene expression program during pine embryogenesis. Microarray analysis of *P. pinaster* zygotic embryos at five stages of development showed that most changes in transcript levels occurred in the transitions from early to pre-cotyledonary embryo and cotyledonary to mature embryo (de Vega-Bartol *et al.*, 2013). Several epigenetic regulation mechanisms were highlighted by the analysis of functional categories of the differentially expressed genes through embryogenesis. In early embryogenesis several putative orthologs of transcripts associated with mechanisms that target transposable elements and repetitive sequences were strongly expressed while PRC2-mediated repression of genes seemed more relevant during late embryogenesis. Interestingly, functions related to sRNA pathways appeared differentially regulated across all stages of embryo development with a prevalence of miRNA functions in mid to late embryogenesis. Transcriptional control by transcription factors putatively related to auxin transport and response appeared critical during early to mid stages of embryogenesis and are probably related embryo patterning establishment. Later in development, transcripts with homology to genes acting on modulation of auxin flow and determination of adaxial-abaxial polarity were up-regulated, as were putative orthologs of genes required for meristem formation and function as well as establishment of organ boundaries. Comparative analysis with *A. thaliana* embryogenesis indicated highly correlated transcript profiles between the two species.

The profiling of non-coding smallRNAs involved in the regulation of different stages of embryo development is currently in progress and, together with the gathered information on the coding transcriptome analysis will help to elucidate the regulatory network involved in the coordination of gene expression during pine embryogenesis.

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Keywords: Conifer, epigenetics, *Pinus pinaster*, small RNAs, transcription factor, transcriptomics





## **Molecular and functional approaches to enhance biomass production in forest trees**

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Forest trees comprise a large group of angiosperm and gymnosperm species that play a crucial role in global carbon fixation, and maintenance of biodiversity. Forest trees are also of great economic importance since they provide a wide range of products of commercial interest, including wood, pulp, biomass and important secondary metabolites. A sustainable management of forest resources is needed to preserve natural forests and to meet the increasing international demands in the production of wood and other forest-derived products. New advances and developments in biotechnology may contribute to accelerate the domestication of important traits for forest productivity. Nitrogen assimilation and recycling play a key role in tree growth and biomass production and we firmly believe that knowledge on nitrogen metabolism will lead to approaches aimed at increasing forest productivity. We are interested in studying nitrogen metabolism and its regulation in maritime pine (*Pinus pinaster* Aiton), a forest tree species of great economic and ecological importance in the Mediterranean area and a relevant model for conifer genomic research in Europe. An overview of our research programme will be presented and discussed. Research supported by Spanish Ministry of Economy and Competitiveness and Junta de Andalucía (Grants BIO2012-33797, PLE2009-0016 and research group BIO-114).





3<sup>rd</sup> International Conference of the  
**IUFRO Working Party 2.09.02**

Somatic Embryogenesis and Other  
Vegetative Propagation Technologies

September 8 - 12, 2014. Vitoria-Gasteiz, Spain.



**ORAL - SESSION 3:**

**DEVELOPMENT AND APPLICATION  
OF COMPLEMENTARY TECHNOLOGIES  
BASED ON SE/VP**

## Cryopreservation of embryogenic callus of trees: an overview

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The application of tissue culture technology to the preservation of plant genetic resources has greatly evolved in recent years with the conservation of plant tissues and organs both at low and cryogenic temperatures (cryopreservation). The latter approach refers to the conservation of explants at the ultra-low temperature of liquid nitrogen (-196°C), being the only sound alternative to clonal orchards for the *ex situ* long-term storage of germplasm of vegetatively propagated trees. At a cryogenic temperature, the rate of chemical and biophysical reactions is so slow that the biological growth and development of the frozen organ/tissue are hampered, while, if a proper technology of ultra-rapid freezing is applied, cell survival is not affected. Hence, in theory, germplasm cryopreservation can be considered unlimited in terms of time. First attempt to cryopreserve woody plant material dates back to 1960, when Akira Sakai showed that one-year-old twigs of several *Populus* species were not injured when cooled to -196°C, if they were first held at low temperatures for 6-24 hours. Thirty years later, Sakai and co-workers developed the Plant Vitrification Solution n° 2 (PVS2) which showed to be very effective for the induction of vitrification in nucellar cells of *Citrus sinensis* during ultra-rapid freezing in liquid nitrogen. Since then, the number of PVS2-based protocols, developed for the cryopreservation of tree shoot tips, increased yearly, while at the same time new and effective slow-cooling and one-step freezing methods were also proposed.

Today, a range of cryo-techniques are available for the conservation of cells, tissues and organs. Among them, the cryopreservation of embryogenic callus is a tool of strategic importance for the safe long-term conservation of cell lines which are used in programs of propagation and genetic transformation of trees. The slow-cooling technique is still the most common approach for the cryopreservation of cells (or clumps of cells) from embryogenic callus cultures of softwood trees (conifers), while both the slow-cooling and the one-step freezing methods are applied when the conservation of embryogenic lines from hardwood trees is required. Examples of procedures developed at the CNR-IVALSA of Florence, Italy, for the cryopreservation of embryogenic lines from *Fraxinus* spp., *Aesculus hippocastanum* and *Cupressus sempervirens* are described.

Keywords: cryopreservation, embryogenic callus, liquid nitrogen, conifers, hardwood trees.





## Factors affecting somatic embryogenesis of Norway spruce - endophytes and cryopreservation

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In several countries including Finland, somatic embryogenesis (SE) systems are currently developed for vegetative propagation of selected spruce materials for future reforestation. From the sustainability and biological diversity points of view, it is important to know the properties of SE materials well prior to applications. In practical work with Norway spruce (*Picea abies*), cryopreservation step has proved to be the bottleneck for the applications of the SE. Thus, several pretreatment and slow-cooling methods are being tested to enhance the survival of embryogenic lines stored in liquid nitrogen.

Endophytes are micro-organisms which live inside plant tissue or cells at least part of their life cycle without causing any harm to host plant, but may also be beneficial for the host. The most common microbes occurring as endophytes are bacteria and fungi, but also mycoplasmas, rickettsia and archaebacteria have been found. In Norway spruce, numerous endophytes have been found, both in the needles of mature trees, but also in the seed embryos. The aim of the study was to find out if endophytes are present in embryogenic cultures of Norway spruce, and if so - what kind of endophytes they are, and where do they come from.

For detection of endophytes, molecular methods were used. For molecular screening, five PCR primer pairs specific for known endophyte groups were used to samples consisting of embryogenic cultures, mature somatic embryos, regenerated emblings, and seeds used as explants for the cultures; as well as pollen used in the controlled crossings from which the seed originated.

Primer pairs specific towards fungi and *Mycobacteria* were able to amplify right-sized fragments from the samples. Sequencing and subsequent BLAST analyses of the obtained PCR-products identified several different fungi belonging to two out of the five fungal phyla, *Ascomycota* and *Basidiomycota*. This study indicates that endophytes, especially fungal endophytes, are present in embryogenic cultures of Norway spruce, and they are transferred through the tissue culture process to regenerated emblings. It also seems that fungal endophytes could be transmitted to embryogenic cultures from seed explants used for initiation of cultures.

Keywords: somatic embryogenesis, Norway spruce, cryopreservation, endophytes.







## Synthetic Seeds for the production of disease-free cassava plants

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Cassava (*Manihot esculenta* Crantz) is a multipurpose crop important for food security in developing countries. Its heterozygous nature is the main reason to propagate it clonally, with the concomitant spreading of systemic phytopathogens such as ACMV (African cassava mosaic virus) and CBSV (cassava brown streak disease), causing considerable losses. The goal of this project was to establish protocols for the production of Synthetic Seeds using Somatic Embryos (SEs) or Shoot Tips (STs) from cassava cvs TMS60444 (African), Tai16 (Asian) and SM1219-9 (South American). The methodological approach considered two possibilities for the massive production of disease-free planting material: encapsulated SEs and STs, and non-encapsulated SEs. Encapsulation experiments evaluated the effects of variables like desiccation, cold storage, embryo morphology, and composition of artificial endosperm on the conversion of encapsulated SEs/ST into plants. Furthermore, to help the establishment of plantlets directly from encapsulated SEs and STs in the greenhouse, we supplemented beads with antibiotics and fungicides, and germinated them *in vitro* on Petri plates with MS medium, or directly on artificial rooting substrates (i.e., Ultrafoam<sup>®</sup> growing medium). Experiments with non-encapsulated SEs focused on their germination *in vitro* on artificial substrates for rooting. Encapsulated SEs germinated at different rates depending on their genotype and developmental stage. The best germination rate (up to 93%) was obtained with early-cotyledon stage embryos of cv SM1219-9. For cv Tai16, the average was 53%, while TMS60444 was the lowest at 19%. Storing synthetic seeds at 5°C for 20 days reduced germination rates of cv SM1219-9 (maximum 33%), although plants were still recovered. Antibiotics didn't have detrimental effects on germination of encapsulated SEs or STs *in vitro*. Encapsulated STs germinated at rates closer to 100%, while germination of encapsulated SEs on Ultrafoam<sup>®</sup> was moderate (<10%). For embryo maturation, non-encapsulated SEs with two cotyledons were transferred to solid MS medium with NAA for one week, then to Ultrafoam<sup>®</sup> in Microboxes<sup>®</sup> containing liquid MS medium. Results showed that after two weeks, germination rate reached 74%. All germinated/converted embryos were successfully transferred to pots in the greenhouse and then to the field.

Our results indicate that Synthetic Seed is an alternative to other vegetative propagation techniques for producing pathogen-free, certified cassava plants. We are currently planning further work to optimize and scale-up the production of somatic embryos and Synthetic Seeds using liquid media in bioreactors. This work was funded by the Bill and Melinda Gates Foundation, Grand Challenges Explorations Phase I.

Keywords: clonal propagation, manihoc, artificial seeds, synseeds, certified planting material.





## Somatic embryogenesis of *Abies alba* Mill. and *Picea abies* (L.) H. Karst. under effect of heavy metal ions

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Pollutants from various sources have become a very serious threat to environmental degradation and caused extreme disruption of ecological balance. According to EEA (2013) emission of three most harmful metals – cadmium, lead and mercury extremely decreased over the past years but still there are many areas where their concentrations in the environment are still too high. Phytoremediation technologies can help us to understand which plants can be used in detoxification processes. But, the question, how heavy metals act at the cellular level and how plants may defend themselves against these pollutants are still receiving increasing attention. Somatic embryogenesis can be used as a model system for studying the biochemical, physiological and molecular processes during embryogenesis. The aim of the present study was to examine the effect of cadmium (Cd<sup>2+</sup>) and lead (Pb<sup>2+</sup>) ions on the development of Norway spruce and Silver fir somatic embryos during proliferation and maturation stages. The effect of heavy metal ions were evaluated on: proliferation rate, capability of embryogenic cell lines to form somatic embryos, and on morphology of developed somatic embryos. Although, both heavy metals involved in this research are very toxic, response were different. Ions of Cd<sup>2+</sup> decreased the proliferation rate compared to control variants and inhibited further growth in both, spruce and fir. Embryogenic cell masses (ECMs) of Norway spruce show higher resistance to these ions than Silver fir. Unlike Cd<sup>2+</sup>, Pb<sup>2+</sup> in higher concentrations (500 µM) in both species had very simulative effect on growth of ECMs. Furthermore, from the examination of the effect of heavy metal ions on maturation process, it was shown that both heavy metals influenced formation, morphology and further development of somatic embryos.

Keywords: somatic embryogenesis, *Abies alba*, *Picea abies*, heavy metals.





## Maritime pine haploid cell lines for massive sequencing of the species

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The potential of haploid tissues for genetic studies in conifers is hampered by the lack of abundant and homogeneous plant material suitable for DNA isolation. In this work we have studied factors promoting haploid callus induction and proliferation from megagametophytes of Oría 6, a genotype of *Pinus pinaster* Aiton (maritime pine), selected based on its response to extreme drought conditions.

Factors tested were developmental stage of the megagametophyte, culture medium and plant growth regulators. Mature cones, dark-brown in color, with dehydrated seeds, were collected in February; pre-fertilized immature cones in May, mid June, and July whereas light brown cones, with no dehydrated seeds, enclosing a mature zygotic embryo, were collected in mid September. Three nutrient media containing different concentrations of 2,4-D and BA were tested: MS medium (Murashige and Skoog, Plant Physiol 15:473-497, 1962) supplemented with 2,4-D (4.4, 8.8, 22.0 and 44.4  $\mu\text{M}$ ) alone or with 2.2  $\mu\text{M}$  BA; a modified Litvay's medium containing 9.0  $\mu\text{M}$  2-4-D and 4.4  $\mu\text{M}$  BA as described by Lelu-Walter et al (Plant Cell Rep 25:767-776, 2006) for somatic embryogenesis of the species; and a modified MS medium (MSD) supplemented with 3% glucose, 5 mg l<sup>-1</sup> dithiothreitol, 0.1 g l<sup>-1</sup> myoinositol, 4.4  $\mu\text{M}$  BA and 0.5  $\mu\text{M}$  2,4-D, as described in Azevedo et al (Plant Cell Tiss Org Cult 93:115-121, 2008). Developmental stage of the megagametophyte was determined by microscopy and histological techniques. Although initial cell proliferation was achieved in megagametophytes at every developmental stage, the generation of haploid callus was restricted to megagametophytes isolated from light-brown cones with no dehydrated seeds collected in September.

Culture medium composition did not significantly affect callus induction, but a modified Murashige and Skoog medium with 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyladenine (BA) favored further multiplication. The ploidy status of the callus lines was determined by chromosome counting, flow cytometry and seven polymorphic microsatellites.

A total of sixteen haploid callus lines were established and one of these is being used as a source of DNA for massive sequencing of maritime pine genome.

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Keywords: chromosome counting, flow cytometry, genome sequencing, haploid callus, microsatellites, *Pinus pinaster*.





# Regulation of embryo development, germination and plant growth in Norway spruce (*Picea abies*)

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The demand for higher production yields and better quality materials from the forests is increasing globally. In northern Europe, the vast majority of forest products are from coniferous species, mainly Norway spruce (*Picea abies*). Tree breeding programs are directed to meet the future demands on forests through seed production. Today, Norway spruce plants for reforestation are primarily produced from seed orchard seeds. Due to the long time from establishing a seed orchard to the seed is produced, about 15 years, the seedlings are genetically far behind the edge of breeding. Thus, in order to support the reforestation efforts, there is increasing need to complement the existing seed production with more efficient techniques for large scale propagation of trees with elite traits. Somatic embryogenesis (SE) is a technique that can be implemented in order to achieve those goals.

Somatic embryogenesis is a well-established *in vitro* method for experimental studies on embryo development. In addition, somatic embryogenesis has the potential to be used for large scale propagation of conifers.

We are using somatic embryogenesis as a model system to study nitrogen utilization during embryogenesis, from early stage embryos to plants. In particular, we are studying: (a) nitrogen sources required during embryo development; (b) how the nitrogen sources may affect embryo development and plant regeneration; (c) and the link between embryo development and enzymes involved in the nitrogen metabolism. To achieve the above mentioned aims, we are using embryogenic cell lines of Norway spruce with different capabilities of embryo development and plant regeneration. The results from these studies will contribute to our understanding of the regulation of embryo development in conifers. In addition, the results could be used to optimize the existing protocols for propagation of Norway spruce plant by somatic embryogenesis. The results from this project also have the potential to advance the protocols used in the automated system process for large scale industrial production of Norway spruce plants.

Key words: somatic embryogenesis, Norway spruce (*Picea abies*), plant regeneration, nitrogen source, nitrogen metabolism, enzymatic activity.







# Towards the acquisition of competence for the induction of adventitious roots: from the effect of the juvenile-adult transition to cell fate switch and resetting of gene expression patterns

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Cellular plasticity enables differentiated cells to switch their developmental fate to acquire a new determination program. One way plant cell plasticity is manifested is through *de novo* regeneration of organs from somatic differentiated cells in an ectopic location. However, switching the developmental program of adult cells prior to organ regeneration is difficult in many plant species, especially in forest tree species. In these species, a decline in the capacity to regenerate shoots, roots or embryos from somatic differentiated cells is associated with tree age and maturation. This raises the question of whether trees maintain certain cells, which have not been determined to develop a specific organ, in a differentiated state that can easily access pluripotent or totipotent properties at a mature stage of development. Both adventitious rooting and somatic embryogenesis are highly maturation-dependent programs. Therefore, the effect of the developmental transitions and the age of the tree on the capacity of cells to be reprogrammed, and to induce a new developmental program poses a major question. In general, the bulk of evidence indicates that the ease in propagation capacity of juvenile tissues of some trees is a function of the ease in maintaining or inducing cell initials. Cell fate switches are characterized by remarkable changes in the pattern of gene expression. Therefore, determining how cells reset their gene expression pattern is crucial to understand cellular plasticity. The most promising approach to investigate the temporal distribution of specifically regulated transcripts associated with the induction of an adventitious program is the use of techniques detecting genes differentially expressed in the different stages of the adventitious program in competent and non-competent tissues. In general, simple and synchronized experimental systems have been exploited for this purpose. The sequencing of various tree genomes has led to the development of high-throughput technologies used to follow the changes of expression of thousands of genes simultaneously. Transcriptome analysis revealed significant shifts during the very earliest stages of the adventitious processes. Hormonal signalling pathways, the recruitment of meristem programs or cell-specific factors could be key elements for switching cell fate and for the control of maturation-dependent cellular plasticity.

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Keywords: age, developmental plasticity, epigenetics, pluripotency, transcription factors.





## Micropropagation of *Calophyllum brasiliense* (Cambess.) from nodal segments

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Micropropagation may represent a way to overcome difficulties in achieving large-scale plant production of *Calophyllum brasiliense* Cambess. (Clusiaceae), given the recalcitrant nature of the seeds, the irregular fructification and absence of natural vegetative propagation of this species. Cultures were established using nodal segments 2 cm in length, obtained from 1-2 year old seedlings, maintained in a greenhouse. Mercury chloride and PPM™ (Plant Preservative Mixture) were used in the surface sterilizing stage, the better results being achieved with PPM™ incorporation in culture medium, at any concentration between 0.2 and 0.8%. Polyvinylpyrrolidone (PVP), activated charcoal (AC), cysteine (Cys), ascorbic acid (AA) or citric acid (CA) were added to the culture medium to avoid oxidation. After 30 days of culture in a WPM medium supplemented with PVP (0.1%) and AA (200 mg.L<sup>-1</sup>), the oxidation was avoided in most explants. For shoot multiplication, benzylaminopurine (BA) was used at concentrations of 4.4 and 8.8 μM in WPM medium, resulting in an average of 4.43 and 4.68 shoots per explant, respectively, after 90 days. For elongation of shoots with less than 3 cm, low concentrations of BA (1.1 and 2.2 μM) and addition of 2.89, 5.77 or 11.15 μM gibberellic acid (GA<sub>3</sub>) were tested. No elongation was observed after 60 days and most explants in culture medium supplemented with GA<sub>3</sub> suffered necrosis. Indole-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA) were used for producing roots, reaching a maximum rooting rate of 24% with 20μM NAA. For acclimatization, the rooted plants were transferred to Plantmax® substrate and cultured in a greenhouse, reaching 79% of survival after 30 days and 60% after one year.

Keywords: benzylaminopurine, Clusiaceae, gibberellin, oxidation.





## **Somatic embryogenesis and plant regeneration of 700-year-old *Kalopanax septemlobus* tree by stress treatments**

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Asexual propagation technique via somatic embryogenesis is a feasible method for supporting tree breeding and tree improvement program. However, the induction of somatic embryogenesis from mature trees is still recalcitrant in many woody species except a few conifer and oak species. Previously, we developed somatic embryogenesis system for mature *Kalopanax septemlobus* tree (40-year-old) using rejuvenated tissues obtained by serial grafting. In the present study, we examined somatic embryogenesis and plant regeneration of 700-year-old *K. septemlobus* tree. Callus was induced from young leaf and petiole of grafted plants by treating with high osmoticum (1M sucrose) and 2,4-D (100  $\mu$ M), although embryogenic callus (EC) induction rate was less than 0.5%. The EC was proliferated well and easily formed somatic embryos (SE), which were then converted to plantlets via normal process of somatic embryogenesis. The EC did not show any differences in terms of its capability of SE formation, germination and plant conversion, compared with zygotic embryo derived EC. Finally the genetic identities of regenerated plantlets were compared with a 700-year-old mother tree by chromosome counting, cytometry analysis and ISSR marker analysis.





# Strawberry tree (*Arbutus unedo* L.) micropropagation: shoot proliferation, organogenesis and somatic embryogenesis

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Strawberry tree (*Arbutus unedo*) is a shrub or small tree growing around the Mediterranean basin. It is well adapted to stress conditions, showing a large potential for fruit production. However, this potential has not been fully exploited and the species is even considered a Neglected or Underutilized Crop (NUC). Trying to make this species more attractive for producers and stakeholders an intensive propagation and breeding program is being carried out.

Several genotypes with interesting characteristics have been selected and micropropagation protocols were developed. A shoot proliferation protocol was established, using a culture medium supplemented with 2 mg/L BAP. Using this protocol a maximum of  $10.8 \pm 1.3$  shoots per explant were obtained. In order to reduce production costs, a very efficient and easy to apply micropropagation protocol in liquid medium was also developed. Moreover, a protocol for organogenesis induction was established using 1 mg/LTDZ. When leaves were used as explant, the maximum induction rate obtained was 60%, while a 100% induction rate was achieved with shoots. When compared to others, this method is very effective in terms of the number of plantlets produced. However, the plantlets obtained from organogenesis tend to be smaller than those obtained from shoot proliferation, due to the high number of shoots formed in the calli. The rooting and acclimatization rates of the plants obtained from the three methods were compared, and no significant differences were found. In most cases induction rates higher than 90% were achieved.

Regarding somatic embryogenesis, a one-step induction protocol was established. Although strawberry tree is a very recalcitrant species, calli formation was found in some of the tested genotypes when the leaves were cultured on a medium containing 2 mg/L BAP and 5 mg/L NAA. The induction rates and number of formed embryos per explant showed to be genotype-dependent. Both the induction and formation of somatic embryos occurred on the same medium and embryo development was not synchronized. When the quality of the somatic embryos was accessed, several types of abnormal embryos were found. However, some of those embryos were able to germinate and originate phenotypically normal plants. An anatomical analysis was carried out to compare tissue organization between somatic and zygotic embryos. The anatomy of the somatic and zygotic embryos is quite similar. However, the size of the somatic embryos is quite variable, depending on the number of embryos formed per explant. The amount of reserve substances increased progressively with the maturation of the embryos, and a large amount of proteins and starch could be found at the cotyledonary stage. Nevertheless, the amount of these substances is considerably high on zygotic embryos than in somatic embryos.

The propagation protocols that have been developed are very effective especially shoot proliferation in liquid medium, that allows the production of a large amount of plants, consuming less resources and time than other techniques. Although the results obtained on the induction and conversion of somatic embryos are very promising, the all processes must be optimized in order to apply it for mass propagation purposes. Acknowledgments: studies were funded by Fundação para a Ciência e Tecnologia, research contract PTDC/AGR-FOR/3746/2012.

Keywords: liquid medium, micropropagation, organogenesis, somatic embryogenesis.







# Mastic Gum Tree (*Pistacia lentiscus* var. *chia*) Vegetational Propagation by Grafting

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Among Türkiye's rich biodiversity, mastic tree is a species with economic worth. Because of the restriction in its cultivation area and decrease in mastic trees numbers, it can not make a production potential at the present time. Whole 250 tons world's yearly production is supplied by Greece. Although the wild species (*P. lentiscus*) can be observed along all Mediterranean and Aegean coasts, *P. lentiscus* var. *chia*, from which the mastic is produced, is spread only in Çeşme coast. For a productive potential, first of all, clonal plantations should be formed in the area with appropriate ecological conditions to the plant species. In this work, for establishing clonal breeding possibilities by budding and grafting, the most suitable rootstock, grafting time and grafting method have been searched. The work has been achieved in glass greenhouse of Karşıyaka Forestry nursery-garden with controlled heat and humidity. In grafting, 2+0 aged *P. lentiscus* and 3+0 aged *P. atlantica* have been used as rootstocks. Since grafting method affects the results, split grafting, tonque budding and T budding methods have been used. In grafting, time affects plant's physiological status, consequently, grafting method and the success in grafting. So, started by 15 February, different grafting methods have been applied till 15 October. The success ratio in grafting changes according to the grafting time, grafting method and the rootstock used. Grafting has been observed to be more successfully in spring, when cambium is more active.

According to statically analysis results, when applied in different times, grafting success shows differences conditional to grafting method. In graftings applied on 15th Feb., tonque budding method, on 1st and 15th March and 1st April, split grafting method; 15th April and 1st May, T budding method showed better results. Except 1st September, the graftings applied during summer and autumn weren't successful. So, the graftings from 15 Feb. till 1 Apr. is approved. The success ratio of the graftings after this date is very low. According to statistical results, on both rootstocks, split grafting appears in first rank, followed by T budding and tonque budding. The bandage on graftings should not be solved even if the mastic graftings start growing and it is better to wait for 2.5-3 months. No incompatibility observed in graftings. At the time grafting success is in its highest level, fungus *Pestalotiopsis* is also very active and results to graftings die when contaminated.

Key Words: Mastic, *Pistacia lentiscus* var. *chia*, grafting.





## **Sensitivity of *Populus deltoides* clones with gamma rays and its synergistic effects with the growth regulators during dormant phase**

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The stem cutting of *Populus deltoides* having 12 months age have been treated with Continuous and Fractionated (C&F) doses of gamma rays to analyze its sensitivity .The mother plant has been growing at approximately 4000ft height (msl).The size of the cuttings were 18cm long and diameter were 2.5cm.The clones have been irradiated to gamma rays at FRI Dehradun. The cuttings were irradiated with gamma rays at 500R, 1KR, 2KR and 4KR dose level. The doses were given in continuous and fractionated pattern.The cuttings were irradiated in the first week of January when the mother plant was in dormant phase of its growth .For analyzing the synergistic effects irradiated cuttings were treated with Kinetin and STIK. The sprouting was slow initially and bud started to sprout after 40 days of potting. The fractionated dose of gamma rays favoured better sprouting. Combination treatments with continuous doses with Kinetin 500ppm showed better sprouting than fractionated doses .The lateral growth were inhibited by both C&F treatments. Most of the C&F treatments induce leaf number per cuttings.Root formation in the cuttings were favoured by continuous doses of gamma rays in *Populus deltoides* but maximum number of primary roots per cutting have been observed at 500RF dose level. It is interesting to note that most of combination treatments with STIK 500ppm enhanced the primary, secondary and tertiary root formation per cutting.This experimentation results will help to manipulate the sprouting and rooting pattern in vegetative propagated tree species.

Keywords: Irradiation, Gamma rays, Sensitivity, Clones, Synergistic effects.





## Challenges for the large-scale propagation of forest trees by somatic embryogenesis

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The large number of plants produced by somatic embryogenesis has the potential to be the most effective way to produce large numbers of forest trees for commercial use. However, in reality the number of forest trees actually produced by somatic embryogenesis is relatively small. This is due to several biological, logistical and economic challenges. The main biological challenges include the efficiency and effectiveness of rates of initiation, maturation, conversion, establishment *ex vitro* and cryopreservation across a range of genotypes. The production of large numbers of plants in equal numbers across a range of genotypes necessary for commercial application presents major logistical challenges. Some deployment strategies, especially the large-scale planting of monoclonal blocks may face the challenge of public acceptance in some situations. However, it is the current high cost of producing somatic embryos that presents the greatest challenge to the large-scale use of this technology. Unless greater attention is paid to these challenges the large-scale use of somatic embryogenesis in the propagation of forest trees will remain limited.





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SCALE-UP APPLICATIONS**



## **Responding to stress found to be a critical factor for success: propagation of mature spruce trees through somatic embryogenesis appears to require cultural adaptation**

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Adult conifers are recalcitrant to vegetative propagation. The repeatable induction of somatic embryogenesis (SE) in primordial shoot explants of somatic white spruce from the ages of 2 to 14 years (in 2014), reproductive stage trees provided a unique opportunity to study the molecular aspects underlying SE response from aged trees (Klimaszewska et al. 2011). Transcriptome analysis of two contrasting genotypes (G6 responsive vs G12 non-responsive) led to the identification of four candidate genes within these two genotypes.

Further investigation established that all four of the G12 candidate genes are homologs of stress response genes described in angiosperms, and that expression profiling using real-time qPCR demonstrated that all four genes were induced to high and persistent levels. This in turn suggested that induction of a defense response could contribute to the non-responsiveness of this genotype.

While the four G6 candidate genes were less informative, one was found to encode a specialized dehydrin, which in combination with transient expression of apoplastic peroxidase (Prx52) and in combination with a much lower activation of the G12 candidate genes, suggested an adaptive response to the induction treatment that may underpin the SE induction responsiveness of this genotype.

To further investigate the potential role of defense induction in SE induction, the primordial shoot explants from G6 were cultured on a medium containing jasmonic acid (JA 10  $\mu$ M) in 2013. Although SE was inhibited in some clonal trees by JA the results were inconclusive. In an attempt to exploit germinating somatic embryos as an alternative to primordial shoots, somatic seedlings of G6, G12 and G653 at age 2 weeks were placed onto induction media containing JA at 10 and 20  $\mu$ M or salicylic acid (SA) at 100 and 500  $\mu$ M. The seedling shoots of all three genotypes responded on control and the other tested media by initiating SE; strong reduction in the response was evident only with 500  $\mu$ M SA.

Expression profiling was also expanded to include genes reflective of metabolism rate, cell division and oxidative stress, in an attempt to identify markers of biotic/abiotic stress induction. However, no substantive differences were observed between genotypes or these various stress hormone treatments, whether within primordial buds or somatic seedlings.

Keywords: biotic defense, jasmonic acid, *Picea glauca*, primordial shoots, salicylic acid, somatic seedling shoots





# Somatic embryogenesis optimization in tamarillo through the use of cell suspension cultures and manipulation of culture conditions

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*Cyphomandra betacea* (syn. *Solanum betaceum*) is a solanaceous tree, commonly known as tamarillo, which produces edible high nutritional fruits. Originated from the Andean regions, this species is mainly cultivated in Colombia, USA and New Zealand, which are the main producers of this still underutilised crop. *In vitro* propagation and genetic transformation are valuable biotechnology tools that can be applied to tamarillo breeding, and several lines of research have shown the interest of this species to analyse particular aspects of somatic embryogenesis (SE), such as the recalcitrance of explants of adult origin and the efficiency of embryo maturation. Tamarillo SE system is a two-step process in which embryogenic (EC) and non-embryogenic cells (NEC) are induced from the same cultured explants, being easily separated and independently established. Somatic embryos arose after transferring EC clusters into an auxin-free medium. Using EC with different ages and origins, it was found that by manipulating the maturation media composition with the addition of various osmotic agents, ABA and activated charcoal, higher yields and better quality embryos were obtained. Even though a high number of anomalous embryos was still formed, plant conversion was not significantly affected, indicating that shoot apical meristem (SAM) development is not always affected in anomalous somatic embryos. Efficient protocols for the proliferation of EC are essential for large scale cloning. To achieve this goal the multiplication of EC in cell suspension cultures was achieved. Growth measurements of EC showed optimal values of growth (3363mg of increment per 100mg of initial cell mass) when 40mg of cells were cultured in 20ml of culture medium. Preliminary assays showed differences in the proteins secreted to the culture medium by EC and NEC cells in suspension cultures suggesting that these proteins may play an important role on the embryogenic process. All the analysis and optimizations carried out in tamarillo SE system are strong contributions for a better knowledge of this process in a woody species, which can be applied for large-scale propagation and breeding programs of several other relevant trees.

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Keywords: *Cyphomandra betacea*, embryogenic competence, embryo maturation, model system, secreted proteins.





# Current situation and prospect of somatic embryogenesis for *Fraxinus mandshurica*, an ecologically and economically important hardwood species in China

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In the current context of climate change, drought and increased temperature are the most remarkable consequences that will affect plant growth and distribution. In most cases, the plant response to stress has clearly defined metabolic pathways. But epigenetic variation can also contribute to the phenotypic plasticity of plants, which can be especially important for the adaptation of forest trees to changing environmental conditions. In this sense, somatic plantlets can offer an added value, such as tolerance to stressful conditions with no detriment in the growth rate.

In the last years, our research team has optimized different stages of somatic embryogenesis process in radiata pine such as initiation and proliferation, and maturation. In the latter, we have obtained high amounts of somatic embryos from low amounts of embryogenic tissue in most of the embryogenic lines tested till date. Furthermore, we have developed somatic embryogenesis in other species (*Pinus halepensis*), and combined this propagation technology with organogenesis. Nowadays, this knowledge provides us a biotechnological tool to manipulate the physical and chemical conditions at different stages of somatic embryogenesis process. As a result, a great amount of clonal material is obtained and then, an *ex vitro* analysis of this clonal material can be carried out to assess what level of stress tolerance they have acquired along the first phases of their development. Furthermore, the analysis of physiological parameters in plants with different tolerance to abiotic stress could give valuable information about the mechanisms used by plants to survive under adverse environmental conditions.

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Keywords: conifer, genetic improve programs, micropropagation, radiata pine, somatic embryogenesis.





# Enhancing the efficiency of somatic embryo formation from leaf explants of *Eucalyptus grandis*

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At present, the propagation of commercially-important *Eucalyptus* genotypes by somatic embryogenesis (SE) is only successful if the explants are sourced from germinated seeds, flowers, lignotubers or zygotic embryos. Whilst leaf material does offer a viable and sustainable source of explants, the low yield of somatic embryos thus far achieved from leaves has hampered progress from this route of embryogenesis. However, since somatic embryogenesis is under the influence of exogenous plant growth regulators (PGRs), this difficulty can potentially be overcome by considering the properties and interaction effects of various PGR combinations on somatic embryo formation. Most of the reports on somatic embryogenesis in eucalypts have been empirical in the selection of the PGRs used, for various types of explants. The present study, however, investigated the choice of PGRs used for somatic embryo induction of leaf explants, based on their specific interactions in plant tissues during embryo formation. Explants were cultured on callus induction medium supplemented with, in addition to *trans*-zeatin, various types and concentrations of auxins: indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D). The influence of the auxin polar transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) on embryo formation was also investigated. After 8 weeks in callus induction medium, callus formation was recorded at 100%, 90% and 98% for 2,4-D, IBA and IAA, respectively. Moreover, the natural auxin IAA was found to favour the formation of somatic embryos significantly more than the other tested auxins. Compared with an average of 4±0.6 embryos per leaf explant resulting from the more stable IBA + *trans*-zeatin treatments, IAA + *trans*-zeatin treatment resulted in a mean of 23 embryos per leaf explant. Maintaining an auxin maximum through the restriction of auxin efflux by TIBA did not significantly improve embryogenesis. These results indicated that the interaction of PGRs is a determining factor in the success of their desired influence, since both IAA and *trans*-zeatin are natural PGRs and hence exhibit greater synergy than empirical combinations of synthetic and natural PGRs. Additionally, the relative stability of the auxin used is instrumental if the varying sensitivity of plant tissues to auxin during the somatic embryo formation process is considered. This yield of somatic embryos, using IAA coupled with *trans*-zeatin, represents a significant improvement over previously published findings, for leaf explants of *E. grandis*. In addition, implementing both stereo and scanning electron microscopy, an identification key was developed in order to relate the gross visual appearance of different types of calli formed *in vitro*, from leaf explants, and the likelihood that somatic embryos would be present in such calli. Having established an improved protocol for somatic embryo production from leaf material, the potential to exploit this readily available explant source for clonal forestry, whilst maintaining the advantages of vegetative propagation through embryogenesis, is closer to being achieved.

Keywords: *Eucalyptus*, embryogenesis, leaf explants, plant growth regulator interactions.







## Megagametophyte *in vitro* tissue culture of *Pinus sibirica* and *Larix sibirica* and somaclonal variation

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The main objectives of the study were to 1) develop the biotechnological procedure for obtaining a haploid embryogenic callus in a megagametophyte *in vitro* tissue culture of *Pinus sibirica* and *Larix sibirica*, 2) to study the characteristic features of embryogenesis *in vitro*, and 3) conduct the molecular genetic studies of gynogenic culture of *L. sibirica*. Megagametophytes of *P. sibirica* and *L. sibirica* were cultured *in vitro* on 1/2 LV and AI medium (patented), supplemented with sucrose (30 g l<sup>-1</sup>), L-glutamine (1 g l<sup>-1</sup>), casein hydrolysate (0.5 g l<sup>-1</sup>), mesoinositol (1 g l<sup>-1</sup>), and ascorbic acid (0.3 g l<sup>-1</sup>). 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (6-BAP) were at concentrations of 2 and 1 mg l<sup>-1</sup>, respectively. Callus formation began on the cut surface of megagametophytes on the 7<sup>th</sup> day of cultivation. By the 14<sup>th</sup> day, callus growth was observed in the apical region of the megagametophytes. The first stage of embryogenic competence of explants in the megagametophyte culture consisted of the formation of long vacuolated cells (300-500 µm long). Then, the formation of a nuclear coenocyte was observed. Free nuclei moved to either cell pole to form embryoids. In the proliferating megagametophyte culture embryogenic cells (initials) experienced multiple divisions and formed embryo globules, at the distal ends of which embryonic tubes were developed and formed the suspensor. The embryoid production was exhibited at this stage.

Eleven nuclear simple sequence repeat (SSR) microsatellite markers were genotyped in calli obtained from *L. sibirica* megagametophytes to check the calli's stability. Microsatellite genotypes demonstrated high frequency of mutations in the obtained megagametophyte-derived callus cultures. All cultures contained new mutations in one or more microsatellite loci. But some (four) line were haploid and can potentially be used for whole genome sequencing.

The somatic embryogenesis in gymnosperms can serve as a model system to study the molecular and genetic events at the very early stages of plant development.

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Keywords: *Pinus sibirica*, *Larix sibirica*, megagametophyte culture, embryoids, microsatellite loci, somaclonal variation.





## **Somatic embryogenesis in Nordmanns fir (*Abies nordmanniana*). Results, problems and some solutions in relation to scale up from basic research to commercial production.**

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Nordmanns fir (*Abies nordmanniana*) is grown in Danish forestry as an ornamental for the production of Christmas trees. During the last decades, the species has become the economically most important tree species in Danish forestry. It amounts for approximately 90 % of the income, even though it only covers 10 % of the forest area. Breeding is focussed on traits such as: form, growth, cold hardiness, pest resistance. Lately, serious attacks by the fungi *neonectria* has been observed in Christmas tree plantations, and *neonectria* resistance is now included in breeding programmes. In addition to production of elite trees with identified traits, clonal propagation offers standardised plant material which reduces production costs and rotation time.

After almost 20 years of research there has been developed a stable and functional system for clonal propagation by means of somatic embryogenesis (SE). The presentation will report on the first results from clonal field tests and furthermore on results on production of transgenic plants from the embryogenic cultures. Problems and some solutions in relation to scale up of the methods from basic research to establishment of a commercial set up will be discussed. This comprises biological and technical problems, but also includes cultural differences, such as expectations and traditions from scientific researchers, production engineers, and commercial partners. It furthermore includes: the integration of SE into the existing nursery production of seedlings, expanding the production of SE plants from a few well tested clones to 400-600 untested cell lines for production of about 100 plants from each clone/cell line for field testing, and the establishment of a large scale gene bank. At present the production from a few selected cell lines are expanding to 25-50.000 plants per year, and tested for scale up to the expected goal of 5 million plants per year. By this numbers the cost price per plant is critical and the first results from test of a prototype for automated production are reported.

Keywords: Somatic embryogenesis, *Abies nordmanniana*, transgenic plants, scale up, clonal test.





# Use of a Continuous Immersion System (CIS) for micropropagation of chestnut in photoautotrophic and photomixotrophic conditions

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Conventional micropropagation of chestnut is expensive and labour intensive. The use of liquid culture systems in bioreactors may reduce labour costs and allow for the automation of the process. In this study, a continuous immersion system (CIS) for micropropagation of axillary shoots of European chestnut was evaluated, in order to improve productivity and allow large scale plant propagation. Also, the feasibility of culturing chestnut explants in photoautotrophic conditions was explored.

Explants from six chestnut genotypes were cultured in different bioreactors with Murashige and Skoog medium with half strength nitrates supplemented with 0.05 mg/L N<sup>6</sup>-benzyladenine (BA) and 30 g/L sucrose. Parameters such as the vessel size (1, 6, 8 or 10 L), volume of medium per explant, explant type (apical or basal explants), explant size (1-1.5 or 2-3 cm), supporting material (rockwool cubes, perlite, glass beads, vermiculite) and air exchange were evaluated on the basis of shoot quality and proliferation rates after 5 weeks of culture. Also, three different growth conditions (heterotrophic, photoautotrophic or photomixotrophic) were applied by varying sucrose, light intensity and CO<sub>2</sub> concentration inside the bioreactors.

Successful propagation in CIS was achieved for all genotypes tested, although genotypical differences, as well as interactions between some of the studied parameters, were detected. The use of inert supporting materials such as rockwool cubes, together with increasing air exchange by applying forced aerations prevented hyperhydricity, a disorder that can be considered one of the major problems of axillary culture in liquid medium. Basal explants rendered higher proliferation rates than apical explants, but the latter could be propagated successfully when initial explant size was increased to 2-3 cm (instead of 1-1.5 cm used in semisolid cultures). Explants cultured in vessels of 6, 8 or 10 L showed longer shoots and higher proliferation rates than those cultured in 1 L vessels.

In order to propagate European chestnut in photoautotrophic conditions, an experimental unit equipped with light-emitting diodes (LEDs) of different wavelengths, as well as a forced ventilation unit for supplying CO<sub>2</sub>-enriched air was used. Explants were cultured in 10 L bioreactors with Murashige and Skoog medium with half-strength nitrates supplemented with 0.05 mg/L N<sup>6</sup>-benzyladenine (BA), and rockwool cubes were used as inert supporting material. Three levels of sucrose (0, 10 and 30 g/L) and photosynthetic photon flux densities of 50, 100 or 150 μmol m<sup>2</sup> s<sup>-1</sup> were tested. CO<sub>2</sub> levels in culture vessels were manipulated by applying forced ventilation with or without CO<sub>2</sub>-enriched air.

This work was partially funded through the program FEDER INNTERCONECTA 2013/2014 (project INTEGRACASTANEA EXP00064828/ITC-20133040).

Keywords: chestnut, continuous immersion, LEDs, liquid culture, photoautotrophic.





# Induction of somatic embryogenesis in leaf and shoot apex explants from adult *Eucalyptus* trees

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*Eucalyptus globulus* is one of the most widely planted trees in the world, mainly because the wood is of suitable quality for paper pulp production. Despite the important advantages of the somatic embryogenesis (SE) procedure, including clonal mass propagation, cryostorage of valuable germoplasm and genetic transformation, induction of SE in *Eucalyptus* has been reported in very few species of the genus. Specifically in *E. globulus*, SE has only been induced from immature and mature zygotic embryos (Pintos et al. 2013). The main goal of the present study was to develop an efficient protocol for SE induction in material derived from mature *Eucalyptus globulus* and the hybrid *E. saligna* x *E. maidenii* trees.

Axillary shoot proliferation cultures, previously established from two *E. globulus* trees and one *E. saligna* x *E. maidenii* hybrid tree (all 12-year-old elite trees), were used as sources of initial explants for induction of SE. Shoot apex explants (1-2 mm long) and leaf explants (the two most apical expanding leaves from the apex) were cultured on medium consisting of MS mineral salts and vitamins, casein hydrolysate (500 mg/L), sucrose (30 g/L) and Vitroagar (6 g/L) (basal induction medium) and supplemented with different concentrations of naphthaleneacetic acid (10.74, 16.11, and 21.48  $\mu$ M) in combination with either 2 mg/L arabinogalactan from larch wood or 40 mg/L arabic gum (AG). In a second series of experiments, basal induction medium was supplemented with different concentrations of picloram (20, 30, and 40  $\mu$ M) and 40 mg/L AG. All cultures were kept in darkness at 25°C for 8 weeks.

Somatic embryogenesis was induced in the shoot apex as well as in leaf explants of the three genotypes evaluated, although embryogenic frequencies were significantly influenced by the genotype, auxin and explant type. Picloram was more efficient for embryo formation than NAA. The highest frequencies of induction were obtained in medium containing 40  $\mu$ M picloram and 40 mg/L AG, in which 64.0% of the shoot apex explants and 68.8% of the leaf explants yielded somatic embryos.

For somatic embryo proliferation, individual cotyledonary-stage embryos or small clusters of globular and torpedo-stage embryos generated on initial explants were isolated and transferred to basal induction medium containing NAA (16.11  $\mu$ M), yielding new cycles of somatic embryos every 4-5 weeks. Secondary embryogenesis competence was maintained under these conditions for more than 2 years, despite the relatively low capacity of eucalypt somatic embryos for secondary SE. Somatic embryo differentiation process was short, and intermediate embryo development stages were rarely observed, because transition to the cotyledonary stage occurred rapidly. Histological analysis showed that secondary embryos exhibited bipolar organization with root and shoot apex meristems and differentiation of closed procambial tissue that bifurcate into small cotyledons. The root pole is apparently more developed than the shoot pole, and the latter comprises two meristematic cell layers.

To prevent the rapid drying and browning of isolated embryos that occurred on semisolid germination medium, the use of liquid germination medium was evaluated. Cotyledonary-stage embryos (with a well-defined root) were isolated from embryogenic clusters and placed on two filter paper discs (Whatman grade 181) in Petri dishes containing 10 mL of liquid germination medium consisting of MS medium supplemented with 0.44  $\mu$ M 6-benzylaminopurine and 1.44  $\mu$ M gibberelic acid. Embryo conversion into plantlets was observed under these conditions.

The protocol for SE induction in mature *Eucalyptus globulus* trees described here provides new opportunities for the development of improvement programs for this economically important species.

Pintos G, Araújo C, Santos C, Neves L (2013) Southern Forest 76:59-69.

Acknowledgments: This research was funded by Foresta Mantenimiento de Plantaciones (S.L.).

Keywords: arabic gum, *Eucalyptus globulus*, *Eucalyptus saligna* x *E. maidenii*, larch wood, NAA, picloram.







## **Nestle Cocoa plan: Cocoa propagation by somatic embryogenesis**

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Nestlé is transforming about 12% of the world cocoa production. This production is threatened by many factors such as aging trees and increasing exposure to biotic and abiotic stresses. As a consequence world average yields are very low (about 400kg of dry beans /ha) and it is very difficult for cocoa farmers to run profitable farms. Surfaces grown with cocoa trees tend to decrease while the world cocoa consumption trend is increase, raising an issue of sustainable supply.

To tackle this situation, Nestlé launched in 2009 The Nestlé Cocoa Plan (NCP). This Plan has 3 pillars:

1. Enabling farmers to run profitable farms
2. Improving social conditions
3. Sourcing sustainable, good quality cocoa

Today it is deployed in many cocoa producing countries including the 1st producer, Ivory Coast. The 1st pillar focuses on teaching good agricultural practices and distributing high yielding trees. To support the tree distribution, Nestlé decided to promote the development of clonal material via somatic embryogenesis and orthotropic rooted cuttings.

Nestlé R&D Center at Tours has acquired expertise in cocoa somatic embryogenesis over the last 20 years. Nevertheless, the response to somatic embryogenesis is very variable (genotype dependent and no repeatable experiment) resulting in unpredictable and low conversion rate (5 to 20% of embryos give plants). One of the particularities of R&D Tours is to have the unique collection of SE trees in liquid nitrogen, in the greenhouse in Tours and in fields in Ecuador. It has around 140 accessions.

We will describe the propagation process that we have implemented. It is mainly based on the multiplication of embryogenic callus developing from secondary embryos and on the use of liquid medium for the production of the embryos.

Keywords: Cocoa, Somatic embryogenesis, Nestle Cocoa Plan.





## Plant production with the SE-Fluidics System

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High cost of production due to tedious manual handling is the major obstacle preventing large-scale commercial plant production through somatic embryogenesis (SE). Previously, we have presented a ‘fluidics-base’ approach to automating the SE plant production process. We now present the first preliminary results from early test production of Norway spruce somatic embryo plants using the 2-Line Pilot System (model number U-P2) at SweTree Technology’s facilities in Uppsala, Sweden. We show high yields of mature embryos from the partially immersed bioreactors designed for rapid attachment and extraction into U-P2, and subsequent development of embryos and germinants developing into plants ready for the nursery greenhouses. Tests at the forestry nurseries show that the SE plants develop equally or better depending on cell line compared to the control seedlings.

Keywords: automation, somatic embryogenesis, plant production.





## **Advancement of somatic embryogenesis of conifers at Weyerhaeuser**

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Clonal forestry offers very significant advantages for forest productivity due to the genetic gain (volume and quality improvement), which can be realized through selection and mass propagation of elite clones. These benefits are already being realized in some hardwood species but not yet to a significant extent with conifers. The main limitation with conifers is the phenomenon of physiological maturation which prevents sustained clonal propagation through cuttings, due to (a) decreased rooting and (b) increasing occurrence of plagiotrophic growth problems as the donor plants age. The establishment of embryogenic cultures that are capable of both long-term cryostorage and sustained production has effectively overcome these limitations. Clonal propagation via somatic embryogenesis offers an inexpensive and efficient way to produce an unlimited supply of genetically uniform selected clones for reforestation. In forestry, the production of somatic embryos throughout the year provides a complementary technology to reduce the risk where seed production is limited or uncertain. Considerable progress has been made over the last 25 years in the development of somatic embryogenesis system for large-scale clonal propagation of conifers. We have established several clonal field tests including both Douglas- fir and Loblolly Pine using Weyerhaeuser NR Company patented somatic embryogenesis protocols. Somatic embryo quality has been improved by pretreatment before plating using abscisic acid and activated charcoal (US patent 7888099) and by manipulating the sugars at different stages of embryo development ( US patent 7521237, 7598073 ). ESM cultures have been scaled-up using Wave bag bioreactors (US Patent 7625754) and somatic embryos have been produced in large quantities with—large food serving boxes (Forestry Solution October 2003 P 28). Storage of embryos has been applied to smooth out the labor needs to process the material and synchronize germination timing (US 8216840) and manufactured seed. Weyerhaeuser NR Company also has numerous patents on the development of and the scale up of manufactured seed construction (**US20130167437A1, US20120000125A1, US8466086B2, US2011072716A1, US8375630B2** ). Here we will present the current status of Weyerhaeuser technology for large-scale clonal production of conifer trees for reforestation.







## **ABSTRACTS FOR POSTER PRESENTATIONS**





3<sup>rd</sup> International Conference of the  
**IUFRO Working Party 2.09.02**

Somatic Embryogenesis and Other  
Vegetative Propagation Technologies

September 8 - 12, 2014. Vitoria-Gasteiz, Spain.



**POSTERS - SESSION 1:**

**APPLICATION OF BIOTECHNOLOGIES  
INTO TREE BREEDING AND  
DEPLOYMENT**

## ***Agrobacterium tumefaciens*-mediated transformation of *Melia volkensii* leaf explants using kanamycin selection**

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*Melia volkensii* (melia or mukau) is an indigenous multipurpose dryland tree species commonly grown in Kenya. Reforestry programs promote this fast growing tree for its valuable termite-resistant timber, firewood and animal fodder. Micropropagation is hampered by irregular root induction. To study these rooting process is necessary to provide genetic transformed lines with marker genes, controlled by root related promoters. Here we report how the *Agrobacterium tumefaciens*-mediated transformation for *Melia volkensii* was established. Leaf explants were cocultivated with a disarmed *A. tumefaciens* strain harboring the binary vector p35S::GUS reporter gene construct and the selectable marker pNOS::nptII. Regeneration took place on MS medium with 10  $\mu$ M of thidiazuron, 50 mg/l ticarcillin and 25 mg/l kanamycin. Transgenic plants were regenerated from the leaf explants. The total time required 2.5 to 3 months. The transgenic nature of several shoots was also confirmed by the GUS assay and PCR analysis. This is the first step in producing a transgenic model of melia to study the rooting process.

Keywords: Transformation, *Melia*, *Agrobacterium*.





## ***Agrobacterium*-mediated transformation of olive (*Olea europaea* L.) with an antifungal protein from *Aspergillus giganteus*.**

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Broad-spectrum resistance to pests and diseases is difficult to obtain through classical breeding programs, hence, this is a targeted trait for accelerating the development of major olive cultivars using plant transformation technologies. Olive Verticillium wilt, caused by *Verticillium dahliae*, is considered to be an important constraint for cultivation of olive trees (López-Escudero and Mercado-Blanco 2010). Different transgenic approaches have been proposed to engineer plants for resistance to fungal diseases, including production of antifungal proteins (Gurr and Rushton 2005). Regarding this approach, among different antifungal compounds, the antifungal protein (AFP) from *Aspergillus giganteus* can be considered a promising candidate for practical applications in crop protection (Meyer 2007). AFP is a defensin-like protein that belongs to a group of small-sized secretory proteins rich in cysteine residues. The protein possesses *in vitro* antifungal activity inhibiting the growth of several fungal pathogens. Previous work has already shown that *afp* gene can be expressed in transgenic rice plants inducing resistance to the fungus *Magnaporthe grisea* and indicating the usefulness of such approach for protection against rice blast. (Coca et al. 2004).

In this work, transgenic olive plants were generated by *Agrobacterium*-mediated transformation as described by Torreblanca et al. (2010). The AGL-1 strain containing the pBIN61-afp binary vector was used. This plasmid contains the *nptII* gene for paromomycin selection and a chemically synthesized codon-optimized *afp* gene under the control of the 35S CaMV promoter. Globular somatic embryos derived from a mature seed of cultivar ‘Picual’ were transformed obtaining an average success rate around 2%. Plants were regenerated from six independent lines and transgenic nature was confirmed by PCR studying *nptII* and *afp* insertion. With the aim of studying whether the *afp* gene can be used to induce resistance against fungal diseases in olive, susceptibility to the fungal pathogens *Rosellinia necatrix* and *Verticillium dahliae* will be evaluated. In addition, the inhibitory effect of proteins extracts from transgenic leaves on the *in vitro* growth of these fungal pathogens will also be examined.

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Keywords: *Olea europaea*, Antifungal protein, *Aspergillus giganteus*, transgenic olive.





# Genetic transformation of *Pinus pinaster* embryogenic lines: molecular characterization, optimization of embryo maturation-germination and plantlet micropropagation

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The increasing world demand for forest products is generating an intense pressure on forest resources. One way of meeting this high demand is to increase the productivity by establishing plantations of genetically improved varieties. Innovative biotechnologies like genetic engineering may provide a powerful tool to accelerate the identification and validation of relevant genes that could be used as targets for implementation of marker-based strategies in breeding programs. Genetic engineering depends on the availability of both tissue culture techniques allowing plant regeneration and efficient DNA transfer methods. Somatic embryogenesis has been frequently used to obtain transgenic plants in recalcitrant or slow-growing species. The advantages of SE for transformation include, among others, rapid tissue proliferation and the possibility to cryopreserve individual transformed lines while transgenic plants are regenerated and tested. Stable *Agrobacterium*-mediated transformation of maritime pine embryogenic cell cultures had been previously obtained by our group using three constitutive overexpression and three RNAi constructs of candidate genes related to biomass production or stress tolerance (Trontin *et al.*, 2013). Quantitative PCR and RT-PCR were used to analyse transgene copy number and gene expression of the target genes in the PCR-positive lines. Phenotypic characterization of transformed embryogenic lines was carried out through microscopic analysis. Cotyledonary somatic embryos showing different morphologies were successfully regenerated from the different transformed lines. However, maturation rates were highly variable. Germination of mature embryos became a limiting factor for plant regeneration and, hence, for the success of the whole process. A pre-germination treatment involving mature embryo slow desiccation at high relative humidity improved the rate of germination. In order to propagate transgenic plants for phenotype analysis avoiding acclimatization, transfer to soil conditions and long generation times, a micropropagation procedure via axillary organogenesis from transformed epicotyls was developed. Factors affecting the success of the process and key points for the optimization will be discussed.

**Acknowledgments:** Sustainpine (2010-2013 Genomic tools in maritime *PINE* for enhanced biomass production and *SUSTAINable* forest management, PLE2009-0013). The Sustainpine research was supported within the framework of the Transnational (France, Germany, Spain and Portugal) Cooperation within the 2009 PLANT-KBBE Initiative, with funding from the Agence Nationale pour la Recherche (ANR), Projektträger Jülich (PtJ) on behalf of the Federal Ministry of Education and Research (BMBF), Ministerio de Ciencia e Innovación (MICINN) and Fundação para a Ciência e a Tecnologia (FCT). We would like to acknowledge Dr. Mariano Toribio's research group from IMIDRA for the technical assistance in the microscopic analysis.

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Keywords: Candidate genes, embryo micropropagation, embryo regeneration, maritime pine, transgenesis.





# Public attitude towards adoption of transgenic forest crops in students from La Plata University

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A pilot KAP (Knowledge Attitude Perception) survey was conducted in graduate and students from Agriculture and Forestry Faculty, La Plata University, to explore public awareness and acceptance of use of transgenic forest trees in the country. More specifically, it was attempted to identify: whether people know about transgenic forest trees, whether people would accept adoption of transgenic forest plantations, which issues are of specific concern to the public in case of adoption of transgenic forest plantations. Purposive sampling with specifically designed questionnaires was used to target groups consisting of students of two disciplines, namely: forestry and agriculture. The questionnaires were handed in to the students, who completed them on site. A total of 15 questionnaires were collected and subject to statistical analysis. The sample consisted of 15 students and graduates. The universe of students coming to complete the degree is low (from 1-10 each year). Therefore, the number of surveys is representative of the existing student population in the career. All the students/graduates (100%) knew what the forest transgenic trees are, but only 46% knew whether final products of transgenic plantations are sold in the market. However, the majority (73%) did know whether transgenic forest plantations are grown in the world. Most of the respondents (93%) would agree with forest transgenic crops to be approved for commercial planting and would be willing (93%) to purchase products originating from transgenic forest plantations. However, the great majority, almost 80%, agreed that it should be legally mandatory these products to be labeled in order to indicate that they originate from genetically modified trees. The majority of the respondents considered that use of less pesticides in forest plantations (modification for insect resistance), restoration of contaminated soils (modification for tree stress tolerance) and better timber productivity are among the most important benefits that may accrue if transgenic forest crops will be adopted. Potential loss of biodiversity due to genetic flows between transgenic and wild trees was the main concern (60% of respondents) regarding safety of adoption of transgenic forest crops. Also, loss of biodiversity followed by more use of pesticides if new genetic traits enter these ecosystems were considered by most respondents as the most potentially serious hazards in students/graduates from La Plata University regarding adoption of transgenic forest crops.







## **ProCoGen: Promoting a functional and comparative understanding of the conifer genome - implementing applied aspects for more productive and adapted forests**

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Conifers are one of the oldest, largest and most long-lived species in the plant kingdom. They have a great economic value, primarily for timber and paper, and a high ecological importance, representing the largest terrestrial carbon sink. They are also recognized as an important feedstock in current and future green energy production. In the midst of a climatic change scenario, the study of genetic control of productive and adaptive responses in conifers becomes essential to ensure a sustainable management of genetic resources and an effective breeding. Conifers diverged from angiosperms 300 million years ago, and the study of their genome, seven times larger than the human genome, is revealing unique information which cannot be inferred from currently sequenced angiosperm genomes. Application of genomics plays an important role in understanding genome evolution, epigenetic/genetic variation and the molecular basis of productive and adaptive traits. The main objective of the ProCoGen project (<http://www.procogen.eu>) is to develop an integrative and multidisciplinary genomic research in conifers, using high-throughput platforms for sequencing, genotyping and conducting functional analysis. We aim to unravel genome organization and identify genes and gene networks controlling important ecological and economic traits, such as those related to the reduction of climate change impact in relation to growth, propagation, drought and cold stress with the final goal to provide tree breeders with tools for precise selection. ProCoGen has brought together European and North American experts working at the cutting edge of Forest Genomics from a wide and complementary range of disciplines to encourage multi- and interdisciplinary co-operation. **ProCoGen structure** is organized in five research and technological work packages (WPs) with the aim to conduct an integrative genomic research program in conifer model species. These WPs involve the sequencing of two complete conifer genomes and the analysis of their genetic diversity, functional genomics and epigenetics as well as comparative, computational and translational genomics. Another work package is devoted to integration with other worldwide conifer genomics initiatives, dissemination and training. **ProCoGen partners** involve institutions from eleven EU countries and Canada. USA and Brazil also collaborate in the project.

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Keywords: adaptation, breeding, forest reproduction, next generation sequencing, pine.





## Gene expression in relation to growth traits in *Pinus pinaster* Ait.

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Selection of elite tree individuals for superior biomass production is a labour- and time-consuming slow process. Phenotypic traits as plant diameter and height are regulated by a number of genes, what constrained the application of biotechnological tools to the selection of elite genotypes. Understanding the processes of plant growth through the study of the underlying gene expression patterns can contribute to the improvement of plant production.

The goal of this study was the identification of candidate genes in relation to phenotypes of differential growth in diameter and height in *Pinus pinaster* Ait.

To achieve this goal, we analyzed the relative expression levels of 12 genes potentially related to growth in height and diameter using quantitative PCR (qPCR). These genes were previously selected after comparative transcriptomic analysis among individuals showing contrasted phenotypes. Total RNA was extracted from needles of pines vegetatively propagated from progenies of superior pine trees obtained by Tragsa. According to the phenotypes of the selected individuals, five groups were defined as a function of the diameter and height of the plants after one year of growth: Group 1, plants with high diameter and high height; Group 2, plants with high diameter and low height; Group 3, plants with low diameter and high height; Group 4, plants with low diameter and low height; and Control Group, plants with intermediate values for the traits examined. The relative expression of the candidate genes in the different phenotypic groups in respect to the control group was analyzed, and the results were normalized using two reference genes (Actin and Ubiquitin). In addition, the relative expression of each individual sample was also calculated, and the obtained data were correlated with those of growth in diameter and height of each plant.

From the 12 analyzed genes, 10 showed significant differences in expression in some of the phenotypic groups in respect to the control one. From all analyzed genes, only prokaryotic-AAT and DAHP showed a linear expression pattern among different phenotypic groups, having groups 1 and 4 antagonistic expression, whereas the groups 2 and 3 showed intermediate expression. These results suggest that the prediction of elite individuals (both in diameter and height) would be possible by using these genes.

Correlation analyses showed that the expression of 6 genes was correlated with growth in diameter, whereas the expression of other 2 was correlated with growth in height. None of the analyzed genes showed a statistically significant correlation for both traits at the same time. The results of the correlation analyses allowed us to complement the data of comparison among phenotypic groups for gene expression, helping us to understand them and showing synergistic and antagonistic features between both traits of diameter and height of the plants.

Acknowledgements: Sustainpine (Genomic tools in maritime PINE for enhanced biomass production and SUSTAINable forest management) 2010-2013, 2009 Plant KBBE initiative supported by transnational cooperation with funding from FCT (Portugal), MICINN (Spain), ANR (France), and BMBF (Germany). Empresa de Transformación Agraria SA co-financed their own tasks within this project.

Keywords: *Pinus pinaster*, growth, gene expression, qPCR.





## Generation of transgenic maritime pine somatic embryos with altered expression of genes involved in Nitrogen metabolism and Wood formation

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This study was initiated in the frame of the Sustainpine project (2009 PLANT-KBBE, (<http://www.scbi.uma.es/sustainpine/>) and aimed at connecting expression of candidate genes involved in nitrogen metabolism and wood formation with phenotype. The targeted genes were *asparagine synthase (ASN1)*; *arginase (ARS20)*; *ornithine d-aminotransferase (dOAT)*; *PII* (a nitrogen sensor protein) and *LIM* (a transcription factor involved in wood formation).

The five candidate genes were overexpressed and/or knockout (RNAi strategy) in a reference embryogenic line (PN519) using an *Agrobacterium tumefaciens* coculture method initially developed in both France (FCBA, INRA) and Portugal (IBET) (see Trontin et al. 2013, Proceedings of the IUFRO Working Party 2.09.02, Brno conference, pp 184-187) with the following small modifications: increased bacterial density (up to an O.D<sub>600</sub> 1.2-1.4), acetosyringone concentration (200 µM) during infection and coculture; and the use of 0.5 mg/L phosphinothricin during eight weeks for selection of putative transgenic embryogenic tissue. Transgenic embryogenic tissues were tested for the presence of the *bar* gene (PCR), and amplified for production of cotyledonary somatic embryos. Aliquots of the PCR-positive lines were cryopreserved or used for expression analysis of each targeted candidate gene by RT-qPCR compared to a PN519 non-transformed control.

Under these conditions we were able to obtain between 4-20 PCR-positive lines depending on the gene. For most of the genes, at least three of the lines were significantly up- or down-regulated compared to the non-transgenic control.

The number of mature cotyledonary embryos obtained in maturation experiments was lower in transgenic lines than in the controls, and most SEs were arrested at the precotyledonary stage. Plants obtained had no well-developed roots.

To facilitate further SE development we assayed the effect of cellulose and dialysis membranes as cell physical support but none of them favored further maturation. Ongoing experiments included addition of active charcoal (AC) and pulses of ABA. Results of these experiments will be presented and the possible relation between gene expression and maturation process will be discussed.

In order to induce root development in the germination period, mature somatic embryos were transferred to 1/4 DCR medium with AC as described by Tereso et al. (2006, Plant Growth Regul. 50:57-68). This treatment allowed the recovery of plantlets that will be acclimatized and used for metabolic assays.

Acknowledgements: The Sustainpine research was achieved within the framework of the Transnational (France, Germany, Spain and Portugal) Cooperation (PLANT-KBBE 2009, PLE2009-0016). Specifically, the work presented here was funded by the Agence Nationale pour la Recherche (ANR) and the Ministerio de Ciencia e Innovación (MICINN-MINECO).

Keywords: germination, maturation, nitrogen metabolism, *Pinus pinaster*, reverse genetics, somatic embryos.





## **Multivariate analysis of selected factors contributing to successful Induction of somatic embryogenesis *Larix x eurolepis* – Establishing a tool to evaluate large data sets**

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The large scale biomass production of fast growing and economically important species is still an issue of applied forestry sciences. The hybrid larch (*Larix x eurolepis*) has shown potential to fit those demands. Unfortunately it also suffers from infrequent and unreliable mast. Therefore somatic embryogenesis is a useful tool to generate material from directed crossing continuously available in large quantities. There are several protocols for the induction of somatic embryogenesis to which we referred in order to establish a clone collection. Furthermore we gathered extensive data resulting from induction experiments and correlated them with relevant parameters. Based on our studies the possibility to predict the suitability of starting material and its proper treatment to generate superior clones will be envisaged. To this end we studied the influence of several selected factors to improve induction rates and quality of maturation and plantlets. Plant growth regulators (PGR), genetic background and developmental stages of the primary material were analyzed regarding induction rates and further development. The assessment of quality was based on induction events of somatic embryogenesis and on respective numbers and traits of mature embryos and characteristics of resulting plantlets. These evaluations of the newly induced somatic material were based on repeated maturations, conversions and subsequent field studies. Preliminary data confirmed the influence of PGR, parental background and age of the zygotic material. Furthermore we conducted a subsequent Multivariate-analysis of variance to highlight underlying correlations between the factors and analyzed quality criteria.

Our work illustrates an approach to assess a complex data set and delivered a tool for multi layered analysis that can be extended and transferred to alternative study designs. Combining information such as the genotypical backgrounds with their specific preferences will enhance induction efficiency as well as safe time and resources.

Keywords: *Larix x eurolepis*, induction, plant growth regulators, genetic background, Multivariate-analysis.







3<sup>rd</sup> International Conference of the  
**IUFRO Working Party 2.09.02**

Somatic Embryogenesis and Other  
Vegetative Propagation Technologies

September 8 - 12, 2014. Vitoria-Gasteiz, Spain.



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**OF SE AND OTHER VP**  
**TECHNOLOGIES**

## Indirect organogenesis of *Populus x canadensis* in order to genetic transformation

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Forest biotechnology, and more specifically genetic transformation of trees, is an option for achieving some genetic improvement. The next steps in transgenic trees are aimed at enhancing traits related to biotic and abiotic stress, wood quality, CO<sub>2</sub> capture capability, productivity, rapid growth, production of compounds of pharmacological interest and phytoremediation. Success in forest species is, so far, limited by the problems associated with plants regeneration, especially considering that many species are still considered recalcitrant to *in vitro* culture. The genus *Populus* has become a model organism for tree biotechnology. There are several reports of genetic transformation of poplar, but the clones of Argentina's interest have not been genetically transformed yet; thus, the optimization of a protocol for *in vitro* regeneration and its subsequent genetic transformation is a challenge for research groups and a topic of great interest among Salicaceae wood producers in Argentina. Therefore, the aim of this work was to induce indirect organogenesis *in vitro* in some clones of *Populus x Canadensis* (hibryds of *P. deltoids* x *P. nigra*). We worked with leaves peaces about 1cm x 1 cm of *P. x canadensis* cv. Ragonesse 22 INTA from Argentina. The explants were surface-sterilized by Benlate (1000 mg/l) for 3 hours and immersion in 10% sodium hypochlorite for 10 minutes. The basal medium used was 1/2 MS (Murashige-Skoog) and several concentrations of growth regulators were tested in order to search for shoot proliferation. The environmental conditions of cultivation were obtained in environmental chambers. The callus were originated at various concentration of ANA (Naphtaleneacetic acid) (1, 2, 3 y 4 mg/l) after 20 days. The shoots appeared from the callus formed with 2 and 3 mg/l of ANA after putting in a 1/2MS with TDZ (Tidiazuron) 0,25 mg/l after 8 weeks. The knowledge gained on an efficient and reproducible transformation method will make it possible to generate transgenic plants with technology aimed at solving the current problems of the forestry sector of Argentina and the world.

Keywords: micropropagation, poplar, genetic modification.





## ***In vitro* organogenesis of *Prosopis alpataco***

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*Prosopis alpataco* (Fabaceae, Mimosoideae) is a good example of a shrub adapted to highly stressed environments in Patagonia, Argentina. The present work was undertaken to develop a basic and simple protocol for micropropagation of *P.alpataco*. *In vitro* organogenesis was studied with the aim to select the best explant and plant growth regulators (PGRs) for multiplication of shoots. Different types of explants were isolated from seedlings germinated under *in vitro* conditions. Shoot tips, cotyledons, hypocotyl segments, internodal sections and roots were stimulated on MS supplemented with 6.62µM BA, 9.05µM 2,4D, 1% sucrose and 1.0 g/L activated charcoal. The explants were cultured on MS without PGRs and 3% sucrose. All the explants formed callus on both media tested. The best results were obtained in apical meristems, from indirect differentiation of shoot buds on MS medium without PGRs. Significant differences in proliferation, multiplication and callus formation due to the type of explant and to the presence of growth regulators were found.

Keywords: bud sprouting, multiplication, organogenesis, patagonian shrubs, vegetative propagation.





# Induction of somatic embryogenesis from leaf and shoot apex explants derived from red oak trees. Effect of explant type, silver thiosulphate and activated charcoal for improving the embryogenic system

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The genus *Quercus* includes some of the most commercially important hardwood species in the world. Among North American oak species, *Q. rubra* (red oak) is of significant ecological and economic relevance. Application of biotechnological tools, such as somatic embryogenesis (SE), would be of great interest for accelerating red oak improvement programs. Induction of SE in *Q. rubra* has been only reported from very juvenile material such as immature zygotic embryos and seedling (Rancillac et al. 1996; Vengadesan and Pijut 2009). Furthermore, in these studies no clear patterns of development for repetitive embryogenesis have been described. The aims of the present work are: 1) to induce SE from leaf and shoot apex explants from young trees of *Q. rubra*; 2) to define the culture conditions for reliable embryo proliferation of embryogenic lines by secondary embryogenesis in order to obtain suitable material for plant regeneration.

Axillary shoot proliferation cultures, established from three 6 to 7 year-old *Q. rubra* trees, have been used as source of both leaf explants (the two most apical expanding leaves from the shoot apex) and shoot apex explants (1.5-2.0 mm long). The initiation of somatic embryos was achieved by a three-step culture procedure which involves successive culturing of explants in Murashige and Skoog medium (MS, 1962), supplemented with naphthalenetic acid (21.48  $\mu$ M, NAA) and 6-benzyladenine (2.22  $\mu$ M, BA) for 8 weeks (M1 medium), then transferred onto fresh medium of the same composition except that the NAA and BA concentrations were reduced to 0.54  $\mu$ M and 0.44  $\mu$ M, respectively (medium M2) for further 4 weeks, followed by transfer of explants into plant growth regulator (PGRs)-free medium for another 8 weeks (medium M3). Induction of SE has been achieved in the three red oak genotypes with frequencies ranging from 2.0 to 3.3%. In spite of the relatively low induction frequencies, embryogenic lines have been established and maintained by repetitive embryogenesis in the three genotypes evaluated.

To determine the better conditions for somatic embryo proliferation, nodular embryogenic structures (0.8-1.0 mm and averaging 0.2 mg fresh weight) and torpedo stage embryos (2.0-2.5 mm and averaging 0.8 mg fresh weight) were used as embryogenic explants to generate secondary embryogenesis. These explants were subcultured on three different media 1) MS + 0.44  $\mu$ M BA; 2) MS devoid of PGRs; 3) MS without PGRs + 0.4% activated charcoal (AC). For each proliferation media, sucrose was included at 3% and 6% for a total of six culture treatments. All these media were supplemented with STS at 20  $\mu$ M. The best results in terms of frequency of secondary embryogenesis were obtained when nodular structures were used as embryogenic explants cultured in MS + AC proliferation medium giving rises to 79.2% response in contrast to 48.8% response achieved with torpedo- embryos. Regarding secondary embryo productivity, medium containing AC and 3% sucrose was deemed the most appropriate combination for development of nodular structures to be used for maintenance of embryogenic lines as well as for production of well-formed and easily detachable cotyledonary-shaped embryos to be used in maturation and germination experiments. After maturation in sorbitol containing medium and 2-months chilling (4°C) treatments, somatic embryos (6-8 mm) were germinated on Gresshoff and Doy (1972) medium supplemented with 0.44  $\mu$ M BA and 20  $\mu$ M STS. These studies represent a significant advancement of SE in oaks, due to the recalcitrant character of this species for regeneration.

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Keywords: activated charcoal, nodular embryogenic structures, *Quercus rubra*, secondary embryogenesis, STS, sucrose.







## **Plant regeneration from somatic embryos of Japanese black pine (*Pinus thunbergii*) after maturation with polyethylene glycol or a high concentration of gellan gum**

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Japanese black pine (*Pinus thunbergii* Parl.) is an important forest tree species used in reforestation and landscaping in Japan. However, *P. thunbergii* populations have decreased dramatically as a result of pine wilt disease, caused by the pinewood nematode (*Bursaphelenchus xylophilus*). Therefore, the development of an efficient and stable plant regeneration system is essential for the large-scale propagation of resistant clones. Somatic embryogenesis is the most promising technique for mass propagation of clones and for plant regeneration in genetic transformation.

Somatic embryogenesis was initiated from megagametophytes containing immature zygotic embryos. Embryogenic cultures were maintained and proliferated in a medium supplemented with 3  $\mu\text{M}$  2,4-dichlorophenoxyacetic acid, 1  $\mu\text{M}$  6-benzylaminopurine, 30 g l<sup>-1</sup> sucrose, and 1.5 g l<sup>-1</sup> L-glutamine. The somatic embryo maturation experiments were performed in darkness at 25°C. Embryogenic tissues were cultured on maturation media containing 50 g l<sup>-1</sup> maltose, 2 g l<sup>-1</sup> activated charcoal, 100  $\mu\text{M}$  abscisic acid, and 100 g l<sup>-1</sup> polyethylene glycol or a high concentration of gellan gum (Gelrite 10 g l<sup>-1</sup> without polyethylene glycol). After maturation, somatic embryos were transferred to a germination medium containing the formulation needed for maintenance and proliferation, but without plant growth regulators, and supplemented with 30 g l<sup>-1</sup> glucose (instead of sucrose) and 2 g l<sup>-1</sup> activated charcoal. Cultures were kept at 25°C under a photon flux density of approximately 65  $\mu\text{mol m}^{-2} \text{s}^{-1}$  provided by cool, white fluorescent lamps for 16 h. The numbers of somatic embryos that germinated and developed into plantlets were recorded after 6 and 12 weeks, respectively.

Low germination and conversion frequencies (16% and 12%, respectively) were achieved from somatic embryos after maturation on medium supplemented with polyethylene glycol. In contrast, when somatic embryos were matured on medium containing a high concentration of gellan gum without polyethylene glycol, the germination frequency recorded was 80%, and then 78% of somatic embryos developed into plantlets. Somatic embryos matured with polyethylene glycol were desiccated to improve both germination and plant conversion frequencies. Slow desiccation of somatic embryos at a high relative humidity resulted not only in a marked increment in germination frequency (from 16 to 81%) but also a subsequent improvement in plant conversion rate (from 12 to 78%). Somatic plants were acclimatized, and their growth was monitored in the field.

Keywords: Japanese black pine, somatic embryogenesis, germination, plant regeneration, polyethylene glycol, gellan gum.





## Differentiation of cork oak somatic embryos in liquid medium

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Several protocols have been used to clone select adult cork oak (*Quercus suber* L.) trees by somatic embryogenesis using semisolid medium. However, cultures in liquid medium need to be developed for economically viable mass propagation. Embryogenic lines of cork oak were established in Schenk and Hildebrandt's (SH) liquid medium without plant growth regulators (PGRs). Three ways of differentiation of somatic embryos were observed in these cultures (Alegre et al., 2010). In the first way, embryos developed from embryogenic tissues following the pattern usually observed in cultures on semisolid medium, beginning with the formation of shoot apex and cotyledons. The second way of differentiation was observed in differentiated embryos or in organized structures; in this case new embryos differentiated as structures that looks like globular embryos loosing from their surface. In the third way, small non-organized aggregates of few embryogenic cells in suspension cultures were able to develop globular embryos. The second way of differentiation happened when we cultured differentiated tissues at low density of inoculation and low speed. Then, globular structures arose on their surfaces that developed into free and well-formed cotyledonary embryos. The main purpose of this study was to characterize and optimize the production of secondary somatic embryos in liquid medium by this way.

Somatic embryos from genotype ALM80 picked from liquid proliferation medium were used. They were cultured in Erlenmeyer flasks with 50 ml of liquid medium composed of macronutrients of SH medium, micronutrients, cofactors and Fe-EDTA of MS medium, 30 g l<sup>-1</sup> sucrose, and lacking PGRs. The flasks were placed in a growth chamber at 25 ± 2 °C and a 16-h photoperiod (180 μmol m<sup>-2</sup> s<sup>-1</sup>) for 60 days. The effects of type of initial structure (single embryos and embryo clusters), of flask size (100 and 150 ml), of density of inoculation (1, 2, 3 or 4 embryos/flask), of culture under light or in darkness, of stationary culture vs. shaken at 40 rpm, and the effect of subculture number on the number of differentiated embryos were assayed. Data were recorded after 60 days in culture.

Single embryos produced more embryos per structure than embryo clusters. There was an important effect of subculture number: the production from the first embryos picked from proliferation cultures was 30 ± 16 embryos/embryo. The embryos of the second generation produced 14 ± 2 embryos/embryo, and those of the third generation 5 ± 1 embryos/embryo. The best density of inoculation was two embryos per flask. In this case embryos of the third cycle produced 8 ± 2 embryos/embryo. Cultures in 150 ml size flasks shaken at 40 rpm under light rendered the largest number of differentiated embryos, but no significant differences were observed. This way of embryo differentiation was tested with five additional genotypes. There was high variability in embryo production among genotypes. This differentiation pathway confirms the possibility of producing isolated and well-developed somatic embryos up to the cotyledonary stage from embryos and embryogenic clusters produced in liquid medium.

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Key words: Forest biotechnology, micropropagation, *Quercus suber*, secondary embryogenesis.





# Formation of embryogenic-like tissues from mature zygotic embryos of stone pine

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The stone pine (*Pinus pinea* L.) is an important economical forest species in the Mediterranean ecosystem, considered as a “fruit tree” for edible pine-kernel production. Besides its agronomical value, it is also used for ecological (forestation of coast and continental dunes) and ornamental purposes. The Spain’s breeding program is mainly focused on the improving of seed yield in grafted orchards. Therefore the production of clonal rootstocks is desired. The induction of somatic embryogenesis (SE) in several *Pinus* species has been investigated dealing with factors as the choice of the initial explant, basal medium components, growth regulator additions and environmental conditions. The main limitations when immature zygotic embryos are used as explants are the low initiation frequency, the restriction to a certain time window for initiation, the impossibility of long-term storage of immature seeds, and the genetic specificity of explants. Some of these problems can be solved and availability of suitable explants can be increased if mature zygotic embryos (ZE) are used. Embryogenic tissue was obtained from mature ZE in some *Pinus* spp but at low frequency for large-scale production. Although the induction of embryogenic lines from immature zygotic embryos of stone pine has previously been reported (Carneros et al., 2009), this is the first study on induction of SE from mature zygotic embryos.

The effects of family, culture medium and collection data on the cell mass induction and proliferation were investigated. Cones from selected trees were collected in two clonal banks on three years. Several developmental stages of ZE enclosed in the megagametophytes were observed at the same data collection, that consisted of the dominant (cotyledonary) and subordinate embryos (both cotyledonary and aborted). Isolated mature ZE were cultured on several nutrient medium supplemented with different PGRs (NAA, 2,4-D, BA, TDZ, Brassinolide) in darkness. Responsive tissues were found in both cotyledons and hypocotyls of ZE, and also in remains of suspensor cells. The best response was obtained on medium with both TDZ and Brassinolide. Although induction of embryogenic-like cultures from mature ZE was obtained, cell masses failed to grow further by continuous subcultures and the proembryogenic structures could not be maintained. These results provide the first information on embryogenic potential of mature embryos and may be useful to the optimization of the somatic embryogenesis protocol in *Pinus pinea*.

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Keywords: Agroforestry, embryogenic cultures, forest biotechnology, *Pinus pinea*, somatic embryogenesis.





## Control of shoot necrosis during micropropagation of *Platanus x hispanica*

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London plane trees are hybrids of *Platanus orientalis* and *Platanus occidentalis*. The most planted plane tree in Spain is *P. acerifolia* (Ait.) Willd, also known as *P. hispanica*. Currently many Spanish plane trees suffer of anthracnose, a disease caused by the fungus *Apiognomonina veneta* (Tello et al., 2000; Alfonso Corzo et al., 2009). The severity of the disease under common environmental conditions is influenced by the genotype (Tello et al., 2000), which allows to select the most tolerant individuals. A micropropagation protocol was started in order to clone some individuals of *P. hispanica* tolerant to anthracnose using the culture medium described by Liu and Bao (1993) supplemented with 1.33  $\mu$ M BAP and 0.27  $\mu$ M NAA. Three genotypes potentially tolerant to anthracnose (A, B and D), and one of high ornamental value (S), were successfully established in culture (Alegre et al., 2013). However, high endophyte proliferation and shoot necrosis occurred during multiplication restricting the protocol applicability. This work focus on alleviating shoot leaf necrosis from established organogenic cultures of these four genotypes.

Shoots from established organogenic cultures of the four genotypes were cultured on the multiplication medium described above. The effect of several factors on the percentage of shoots showing necrosis, on multiplication rates and other parameters was tested. The factors were: decrease of the concentration of ammonium nitrate of MS medium at half (LB<sub>AL</sub>); use of glutamine as a reduced nitrogen source instead of ammonium nitrate (LB<sub>GL</sub>); use of Silver Thiosulfate (STS) as ethylene inhibitor (LB<sub>STS</sub>); use of two different types of antibiotics (Augmentine® and streptomycin sulfate at different concentrations) to reduce the endophyte proliferation (LB<sub>A50</sub>, LB<sub>A150</sub>, LB<sub>S50</sub>, and LB<sub>S150</sub>); and culture on medium with higher calcium content (DKW). Explants were subcultured to fresh medium after 4 weeks, and data were recorded after 8 weeks. Differences in the frequencies of necrosis among media were observed. Culture on medium with reduced ammonium nitrate content or with increased calcium proved effective in reducing shoot necrosis. In contrast, the use of antibiotics did not solve the problem of necrosis, and shoot multiplication was negatively influenced. There were also differences in survival rate. The use of glutamine and STS caused the death of most explants. While survival rate, biomass and axillary multiplication rate were higher when the ammonium nitrate reduced medium was used, shoot leaf necrosis although alleviated remained in cultures.

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Keywords: Forest biotechnology, London plane, organogenesis, vegetative propagation.







## Conversion of Douglas-fir somatic embryos to organogenic shoot cultures and the development of a rooting protocol

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The potential to use micropropagation protocols in combination with a nursery production system via stool beds is attractive. Somatic embryogenesis protocols would amplify high value control-pollinated seed and organogenesis of the resulting mature somatic embryos (SE) would provide a large number of uniform shoots for initial production of stool beds. SE material held in liquid nitrogen could then be used to provide a supply of juvenile stool beds over a number of years.

Successful results have been obtained with initiation and proliferation of Douglas-fir embryogenic cultures at Scion. These protocols have been developed using modified *Pinus radiata* media and methods and provide Scion with protocols for the micropropagation of Douglas-fir.

Mature somatic embryos (from 5 cell lines) had their bases removed and were placed on *Pinus radiata* organogenic media (LPch) to establish shoot cultures. Shoots were sub-cultured every 4-6 weeks, transferring to jars when elongated. Whenever possible, stem segments were isolated from the elongated shoots and transferred to petri dishes for multiplication. Multiplication data was collected for 30 weeks.

Thirty organogenic shoots from each of the five SE cell lines were used to test four different pre-rooting treatments. Shoots were transferred to the nursery propagation house after 11 days. The results of these pre-rooting treatments on the ability of the shoots to produce roots will be presented and future work discussed.

Acknowledgements: This work was supported by grants from the French Ministry of Foreign Affairs and the French Ministry of Higher Education and Research through the France/New Zealand Science Cooperation Dumont D'Urville Programme. Financial support was also obtained from Scion Core Funding and the MBIE CO4X805 "Diverse Forests" initiatives Thanks to Shaf van Ballekom of Proseed New Zealand Ltd and Mark Dean of Ernslaw One Ltd for supply of cones used to produce SE lines used in this work.

Keywords: Douglas-fir, organogenesis, somatic embryogenesis, rooting.





## Differential protein profile in *Pinus radiata* cell lines with high productivity of embryos

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Improving the rate of success at initiation stage of somatic embryogenesis is critical for large-scale commercial use of this propagation tool. The aim of this work was to analyse the effect of physical and chemical environments at initiation stage of radiata pine somatic embryogenesis. In this sense, the first objective was to assess if different temperatures and/or water availability conditions during initiation of embryogenic tissue affected the success rate of the process. Our second objective was to test if embryogenic lines from different environmental conditions that lead to high and low initiation rates produced somatic embryos with different protein profiles. Moreover, we wanted to correlate the success at initiation stage with the expression of certain proteins in the somatic embryos obtained. Some treatments (18°C, 4 gL<sup>-1</sup> gellan gum) produced high initiation percentages and others produced low initiation percentages (28°C, 2 gL<sup>-1</sup> gellan gum). Then, somatic embryos were obtained and 2-D electrophoresis was performed to obtain the proteomic profiles of the embryos coming from cell lines initiated under different conditions. Our results showed differences between the profiles of the groups tested, with 139 differentially expressed proteins between high initiation and low initiation group samples, when compared to the control group (23°C, 2 gL<sup>-1</sup>). Between those, 26 proteins were significantly ( $p < 0,05$ ) overexpressed in all the high initiation groups and 10 others in the low initiation groups. An approach based on liquid chromatography together with tandem mass spectrometry (LC-MS/MS) will allow quantifying and identifying differentially expressed proteins.

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Keywords: conifer, micropropagation, radiata pine, somatic embryogenesis, tissue culture, protein profile.





## Development of somatic embryogenesis in Loblolly Pine (*Pinus taeda* L.): Advances in the induction

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*Pinus taeda* is the most widely planted tree species at Mesopotamia region in Argentina. Mass vegetative propagation has become a valuable tool to be more competitive forestry based industry. Nowadays, the used planting material includes seedlings (coming from seeds produced in Clonal Seed Orchards) or rooted cutting from superior full-sib families of tree breeding programs.

One focus of the breeding program that is carrying out INTA for *Pinus taeda* is to establish a somatic embryogenesis (SE) protocol using immature zygotic embryos as explants. Several factors could affect induction of somatic embryogenesis, then, the aims of this study were to determine the optimal period to megagametophyte cultivation (window of embryonic development) and to reach induction of embryogenic cell masses (EM).

Immature cones were collected from ten clones in the clonal seed orchard located at San Antonio, Misiones, Argentina from the first week of December to the first week of January in 2011. Megagametophytes containing immature zygotic embryos were excised from seeds and they were cultivated in 1253 induction medium. An average of 2% of cultivated megagametophytes generated proliferating embryogenic cell masses. The status of zygotic embryo development is directly related to the state of cone maturity. This was confirmed by observing that 11.5% of EM were induced from the mega gametophytes collected and cultivated in the first week of sampling, 75.3% in the second and third weeks, 11.5% during the fourth week and 1.7% during the fifth week. The low percentage of EM generated from embryos gathered at the beginning and the end of assessed period restricts the window to collect and cultivate the mega gametophytes. Most of the zygotic embryos cultivated during developmental stages 2 to 4 had the best responses. Induction and maturation phases were carried out encouraging a successful optimization of these processes and the progress on next phases of the SE including cryopreservation.

Keywords: Embryogenic mass induction, Somatic embryogenesis; *Pinus taeda*.





## **The effects of double-phase culture and light intensity on emblings growth process**

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One of the main objective for the Biotechnology laboratory of Arauco is the production of embryogenic clones of *Pinus radiata* (Monterey pine). The current challenge is to obtain homogeneous plant morphology for all mature embryos, in order to obtain high quality plants from selected clones. The goal of this study was to improve the morphology of emblings through the evaluation of the double-phase culture and light intensity effects. The study was initiated with five embryogenic clones of *Pinus radiata*. The experiment was established using emblings with active shoot apex growth and at least 1 cm length. To create the double-phase culture, a 3 to 4 mm film of sterile water was left on the solid culture media and the container was closed using a vented filter cap. Solid culture media and non-vented caps were used as control treatments. Three light intensities were compared (25, 45 and 90  $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ ) and evaluated using a PAR light meter. Growing conditions were set to 16 hours photoperiod and controlled temperature (22 to 24 °C). Measurements of total plants length were made 30 days after the establishment. The results showed that double-phase culture improved plant morphology and also reduced hyperhydration. An increase of 25% on of standard plants was obtained, without affecting the survival rate measured on the greenhouse, during the acclimatization stage. Otherwise, light intensity of 90  $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$  reduced the amount of standard plants by 18%. The study allowed to identify the factors affecting the variability in plant's morphology and to confirm the utility of double-phase culture as a relevant stage in maturation. Further experiments will be planned for continuous adjustment of emblings growth process and the achievement of additional advantages into the Arauco's clonal forest program.

Keywords: Somatic embryogenesis, light intensity, double-phase culture, morphology, *Pinus radiata*, emblings.







## Analyses of storage compounds during somatic embryo development in *Quercus suber* L.

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Somatic embryogenesis induction and plant regeneration have been reported in *Quercus suber* L. (cork oak) from explants of both adult and juvenile origin. However, the quality of the embryos is somewhat poor which may be related with a deficient accumulation of reserve compounds in the developing somatic embryos. To better understand the somatic embryogenic process in this species a set of assays has been carried out to analyse the levels of storage materials in somatic embryos at different developmental phases. Somatic embryos were obtained according a previous established protocol in which leaf segments were first cultured on an auxin rich medium (induction phase) followed by somatic embryo development on an auxin-free medium. In this last medium repetitive somatic embryogenesis occurs from previously induced somatic embryos. An embryogenic line (TRG3) subcultured at 6-week intervals was used in all experiments. Somatic embryo were divided in four development stages from globular to well developed cotyledonary embryos. The levels of storage compounds were compared with those of zygotic embryos. The results showed an increase in starch content from 2.5 mg/g fresh weight in the first stage to 8.7 mg/g in mature somatic embryos, values considerably lower than in zygotic embryonary tissues (13.9 mg/g). Lipid content showed to be higher in the first stage (52.4 mg/g) and significantly dropped in the following stages to a value of 35 mg/g, also a lower value than in their zygotic counterparts (82.8 mg/g). A maximum protein content was observed on the later stages of somatic embryo development (4.7 mg/g) an amount about four times lower than in the embryo axis of mature zygotic embryos (18.6 mg/g). Histochemical studies performed on previously fixed material confirmed the biochemical analyses. SDS-PAGE profiles from the different developmental stages showed an increase in the expression of three protein bands (60 kDa, 46 kDa and 26 kDa) whereas another three sets of protein bands (30 kDa, 40 kDa and 50 kDa) decreased during embryo development and maturation. Protein profiles of mature somatic embryos and zygotic embryos showed also important differences. The results show that the patterns of storage compound accumulation are quite different between zygotic and somatic embryos. Efforts must be pursued to achieve a better maturation of somatic embryos in order to increase the efficiency of the somatic embryogenesis protocol.

Keywords: cork oak, lipids, proteins, SDS-PAGE, starch, somatic embryogenesis.





## Effect of temperature on growth and maturation of stone pine embryogenic lines

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The temperature at which seeds develop was reported to influence height growth and bud phenology of plants (1). *Picea abies* plants regenerated from somatic embryos were also influenced by temperature during somatic embryo development (2). Regeneration of plants by somatic embryogenesis was achieved in Stone pine (*Pinus pinea*) (3). In that study, proliferation and maturation of somatic embryos was carried out at 23 °C. In the present study the effect of colder (18 °C) and warmer (28 °C) temperatures, besides the usual temperature (23 °C), during proliferation of embryonal masses (EM) and embryo maturation steps on the production of somatic embryos and conversion was assessed. Growth was measured by weighing (fresh weight and dry weight) individual clumps from EM of four genotypes at the beginning and at the end of a period of 10 weeks in culture. Eight individual clumps (60 mg fresh weight) per treatment were biweekly subcultured on proliferation medium (M-mLV) at the tested temperatures (P18, P23 and P28) in darkness. After each subculture, the individual clumps were divided into two to four pieces, depending on the attained size, and placed again onto fresh medium. The experiment was performed twice. Before maturation, individual clumps (100 mg fresh weight) collected at the EM periphery from the tested embryogenic lines, were preconditioned by biweekly subculturing on the same proliferation medium but with reduced nutrient and PGR concentrations (UL-½ mLV) and cultured at each tested temperature. After 4 weeks, samples of the EM periphery (50 mg fresh weight) were dispersed and poured onto filter paper disks, and then placed on maturation medium supplemented with high concentration of ABA, maltose and Gelrite (mLV2). EM from cultures at each one of the proliferation temperatures were incubated at 18, 23 and 28 °C for differentiation-maturation. The number of aberrant and normal cotyledonary somatic embryos was recorded for each of the nine temperature combination treatments (e.g. P18M18, P18M23, P18M28) after 4 months in culture. Normal somatic embryos produced were germinated according to Carneros et al. (2009).

The growth of embryogenic lines was less at 18 and 28 °C than at 23 °C. Proliferation of EM at 28 °C was less than at 18 °C, and most samples showed necrosis. Although early somatic embryos were formed in all embryogenic lines, embryos of three of them did not completed the developmental embryo patterning. Normal cotyledonary somatic embryos (20 embryos per gram) were obtained in one of the embryogenic lines. Production of normal embryos was promoted by increasing temperatures. These results are in accordance with those reported in *Picea abies* (2). The best results were obtained when temperatures during maturation were higher than those during proliferation (e.g. P18M23, P18M28, P23M28). Some of the normal embryos germinated.

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- (1) Johnsen et al. (2005) *Plant Cell Environ* 28:1090-1102
- (2) Kvaalen and Johnsen (2008) *New Phytologist* 177:49-59
- (3) Carneros et al. (2009) *Plant Cell, Tiss. Org. Cult.* 98: 165-178.

Key words: Agroforestry, embryogenic cultures, forest biotechnology, *Pinus pinea*, somatic embryogenesis.





## Vegetative propagation of *Pinus maximinoi* H. E. Moore with different sizes of cuttings and substrates in South of Brazil

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The species *Pinus maximinoi* is a pine tree from Central America and it has been showing a good growth in some parts of Brazil, especially in the north of Parana State, a transition zone between subtropical to tropical climate, where it is growing, in some trials, up to 20% more in volume than *Pinus taeda*, the most planted pine in Brazil. The breeding of this species is in an initial stage, so the availability of good genetic seeds is very low, therefore the propagation by cutting of progenies with good performance is an alternative to produce plants in large scale. The aim of this work was to test the influence of three sizes of cuttings and four different substrates in *Pinus maximinoi* rooting. Cutting sizes tested: 3 cm, 5 cm and 7 cm. Substrates tested: (a) 100% composted pine bark; (b) 50% composted pine bark, 50% carbonized rice bark; (c) 25% composted pine bark, 25% carbonized rice bark and 50% coconut fiber; (d) 25% vermiculite, 25% carbonized rice bark and 50% coconut fiber. The genetic material used was a bulk of five elite progeny planted in hydroponic sand beds. The cuttings were collected, planted in 55 cm<sup>3</sup> containers with the respective substrate treatments, placed in a greenhouse with approximately 85% relative humidity and 25°C for 60 days, and after this period the plants were reallocated to a shadow house. The statistic design used was randomized complete block with 24 plants per plot and 6 replications. The location of the trial was in Telêmaco Borba City, Paraná State, south of Brazil. After 150 days of the planting it was measured the percentage of cuttings with roots, the diameter and height of the cuttings, weight of the roots and weight of shoots. The data were analyzed with the ANOVA and Tukey tests in the SAS program. Statistically there were no differences between any treatments and any parameter analyzed. The parameters general means were: 90% of rooting, 15.6 cm of height, 3.4 mm of diameter, 0.9 g of shoots and 0.3 g of the roots. The results of this work shows that is possible to produce plants by rooting cutting of young *Pinus maximinoi* progenies with relatively facility using different sizes of cuttings and different kinds of substrates.

Keywords: rooting, size of cuttings, substrates, *Pinus maximinoi*





## **Somatic embryogenesis and growth retardation of acclimatized plantlets by paclobutrazol (PBZ) in yellow poplar (*Liriodendron tulipifera*)**

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Clonal propagation of high-value forest trees through somatic embryogenesis has the potential to rapidly capture the benefits of breeding or genetic engineering programs and to improve the uniformity and quality of the nursery stock. Experiments were conducted to study the growth effect of the paclobutrazol (PBZ), a plant growth retardant, and its impact on the growth of acclimatized somatic plantlets (emblings) in yellow poplar (*Liriodendron tulipifera*). The somatic plantlets were treated with foliar applications of PBZ at various concentrations of 0 to 500mg/L. The height and leaf area of the acclimatized plantlets were significantly decreased, but the root collar was increased with concentrations of PBZ. Observance in lignin formation by phloroglucinol staining, no remarkable evidence of secondary xylem or lignin formation was found in the acclimatized plants which PBZ was applied.

Keywords: acclimatized plantlets, *Liriodendron tulipifera*, paclobutrazol, somatic embryogenesis







## Embryogenic tissue initiation and somatic embryogenesis in Fraser fir (*Abies fraseri* [Pursh]Poir.)

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The ESM initiation potential was higher with seeds collected in late June (16-273, 4.7%) or early July (16-45, 2.2 %) and decreased as the zygotic embryos matured. Excised proembryo stage of zygotic embryos was most appropriate to initiation of ESM. From the four different culture media we compared, seven ESM lines were obtained: two lines from Murashige and Skoog (MS) medium with 4.4 µM benzyladenine (BA), one from Schenk and Hildebrandt (SH) medium with 4.5 µM thidiazuron (TDZ), and four from SH with 4.4 µM 6-benzyladenine (BA). However, only one ESM line from 16-273 (June 24, SH+TDZ) could be proliferated in subsequent culture. The highest ESM proliferation rate (1.16 fold) was obtained from SH medium supplemented with 250mg/L CH and 3.42 mM L-glutamine. As for somatic embryo maturation, the highest number of mature precotyledonary (100.1/g-1FW) or cotyledonary (64.3/g-1FW) somatic embryos was obtained on a medium containing 20 or 80 µM abscisic acid (ABA), 10% polyethyleneglycol- (PEG), 4% maltose and 0.3% gellan gum. For germination, the cotyledonary somatic embryos derived from maturation medium were transferred to ½ LM containing 0.3% gellan gum.

Keywords: embryo maturation; immature seeds, megametophyte, proembryo, regeneration.





# Mass Propagation of *Acer* spp. by Green-Wood Cutting: Effects of Cutting Time, Cuttings Position on Shoots, and Auxin on Rooting Characteristics

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This study was conducted to develop an efficient mass propagation method for *Acer mono*, *A. mono* for. *rubripes*, *A. okamotoanum* seedlings (6 years old). For each species, categories of cutting time, cuttings position on shoots, and auxin treatments were tested. Green-wood cutting was carried out in an unheated greenhouse equipped with fog system. The dates of commencement of rooting after taking cuttings were 20 days for *Acer mono* and 30 days for *Acer mono* for. *rubripes* and *Acer okamotoanum*, respectively. The dates of completion of rooting after taking cutting were 45 days for *Acer mono* and 60 days for *Acer mono* for. *rubripes* and *Acer okamotoanum*, respectively.

For the 3 species, according to the categories of cutting time, cuttings position on shoots, and auxin treatments, highly significant difference ( $P < 0.0001$ ) was observed in rooting rate. However, depending on different auxin treatment, highly significant difference ( $P < 0.0001$ ) was recognized only in the number of roots. The highest average rooting rate was observed in cutting time of July 1st (73.0%) which was 21.4% higher than that of June 1st (51.6%). The development of roots and root length was also best in cutting time of July 1st. For cuttings position, rooting rate of the lower part (72.4%) was 20.2% higher than the upper part but there was no significant difference in the number of roots and root length. For each species, commonly applicable auxin treatment was turned out to be IBA 100ppm and control (no auxin treatment) where high rooting rate and poor root development was observed. For interaction effect of rooting for each treatment, high significance ( $P < 0.0001$ ) was recognized on the rooting rate in all three species. However, each species showed different interaction effect on the number of roots. Best combination for rooting rate was composed of cutting time of July 1st, the lower part, and control for *Acer mono* (100%). For *Acer mono* for. *rubripes*, that was composed of cutting time of July 1st, the lower part, and IBA 100ppm (96.7%). For *Acer okamotoanum*, that was composed of cutting time of July 1, the lower part, and control (88.7%).

Key words - fog system, interaction effect, rooting completion date, rooting rate.





## Clonal propagation of superior individual in *Aralia elata* via somatic embryogenesis

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*Aralia elata* is a perennial deciduous small tree or shrub, native to eastern Russia, China, Korea, and Japan. Its new shoots have long been used as edible vegetables due to their flavor and nutritional value, and so are being used as a valuable crop to increase agricultural income sources. *A. elata* vegetable cultivation has broad market prospects and higher economic efficiency.

*A. elate* 'Haetteulnal 3ho' is a selected, genetically superior individual in Sunrise Chamdureub Guild of Farmer Co. Ltd. Its shoots are bigger and thicker than the others, and have delicate flavor and good texture. The continuous supply of its propagules is necessary in meeting the increasing commercial demand. Therefore, the aim of the current study was to establish a somatic embryogenesis protocol for clonal mass propagation of the superior *A. elate* 'Haetteulnal 3ho'.

Leaf blades and petioles from *A. elate* 'Haetteulnal 3ho' provided by Sunrise Chamdureub Guild of Farmer Co. Ltd were used for embryogenic cell induction. As a result, embryogenic cells were obtained from petiole explants cultured on MS medium supplemented with 1.0 mg/L 2,4-D and 0.2 mg/L BA. The proliferated embryogenic cells were transferred to hormone-free 1/2 MS basal medium and then developed into somatic embryos after 8 weeks of culture. About 80% of mature somatic embryos were successfully converted into plantlets. In addition, Approximately 90% of the converted plantlets were successfully acclimatized in artificial soil.

Keywords: *Aralia elata* , Haetteulnal 3ho, somatic embryogenesis, clonal propagation.





## Embryogenic cultures of eggplant (*Solanum melongena*) and tamarillo (*Cyphomandra betacea*): towards new systems to understand somatic embryogenesis

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The availability of somatic embryogenesis (SE) model systems has created effective tools for studying cell differentiation processes in plants and increasing our understanding about the functional aspects of genes implicated in SE. Over the years several models such as *Daucus carota* and *Arabidopsis thaliana* have been widely used to characterize SE. However, the effectiveness of the discoveries based on such plants is often difficult to be verified in other systems. In recent years, other species have demonstrated an emerging role for the understanding of various *in vitro* induced morphological processes. This is the case of eggplant and tamarillo, two members of Solanaceae, a large, diverse plant family whose economic importance and biotechnological interest are well known. Eggplant is very responsive to numerous tissue culture techniques due to a high morphogenic potential that is particularly useful for SE induction. The availability of several efficient SE protocols, combined with an increasing number of identified *loci* available on databases, makes this solanaceous an alternative model to be considered for studies of *in vitro* morphogenesis. Another promising solanaceous is tamarillo a tree species in which effective SE protocols have been developed. One of the main advantages of tamarillo is the simultaneous formation of embryogenic (EC) and non-embryogenic (NEC) tissues from the same explant (leaves or mature zygotic embryos) under the same culture conditions. Comparative proteomic analysis of EC and NEC allowed the identification of proteins differentially expressed during the acquisition of SE competence including *NEP-TC* (GenBank, accession number JQ766254.1), a 26.5 kDa protein consistently present in NEC, suggesting its putative role as an inhibitor of tamarillo's SE induction. In the present study eggplant and tamarillo SE induction systems were analyzed in a combined approach to testify their role as alternative model systems for SE studies. For this purpose SE was induced in both species using leaf explants from several genotypes. The assays involved SE induction on media containing different concentrations of the auxins 2,4-D or NAA and different sucrose levels, 3% and 9% (w/v) for eggplant, and 9% (w/v) for tamarillo. The results showed distinct responses however, in both species, responsive and non-responsive *calli* could be obtained and analyzed in relation with the expression of specific genes like *NEP-TC*. The abundance of *NEP-TC* transcripts in several tissues of eggplant and tamarillo was determined through RT-PCR analyses. The results showed that *NEP-TC* transcripts levels vary in a similar way in tamarillo and eggplant tissues, with high abundance in NEC, but also in EC and non-induced leaf samples, which is in accordance with previous results obtained with tamarillo. Further studies will be focused on the functional role of *NEP-TC* on both tamarillo and eggplant.

Keywords: embryogenic *calli*, model systems, non-embryogenic *calli*, Solanaceae,







## **Somatic embryogenesis induction on vegetative buds explants from a female tree of *Pistacia vera L***

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The pistachio tree is big interesting due to its soil and climatic conditions requirements, which allow them to adapt to various locations. In North Africa, it is grown in Tunisia, Morocco and Algeria. In Algeria is grown mainly in Sidi Bel Abbès, Saida, Tlemcen and the region of Aures. From the perspective of re-  
newing and expansion of pistachio orchards, there is a big interest in clonal propagation of *Pistacia vera*. Somatic embryogenesis would help to rejuvenate and subsequent micropropagate mature female elite lines. Buds were isolated from a mature mother tree. Thin cell layers were inoculated on B5 medium enriched with 1 mg / l 2,4-D. The proliferation of embryogenic callus has been optimized under light-emitting diode (LED). 30 Days after transfer of embryogenic callus on hormone-free MS medium, globular stage was obtained. On these structures cotyledonary axes were developed in the presence of ABA and BA. Somatic embryos were transferred on germination medium to obtain juvenile female trees.

Keywords: somatic embryo, rejuvenation, female tree.





## **Origin of embryogenic callus and somatic embryos on immature secondary seed integument of *Hevea brasiliensis***

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Traditional grafting techniques remains the most common way of vegetative propagation of natural rubber in commercial rubber plantations. Heterogeneous seedling rootstocks induce a large variation in field growth and latex yield between trees within a clone. Planting full clones, growing on their own roots could overcome this heterogeneity. In vitro microcutting is hampered by the recalcitrant reaction of shoot explants of adult trees. Rejuvenation by somatic embryogenesis is the key for success of in vitro mass propagation. Propagation of germinated somatic embryos by microcutting is preferred, to reduce the risk of somaclonal variation as faced by secondary somatic embryogenesis. Although the rejuvenation process by somatic embryogenesis on secondary integument tissue from immature rubber seeds is well described, the success rate is rather low. The somatic embryo induction is not only genotype dependent, but even within a clone, tree, fruit or seed, explants react differently. To reveal the origin of this variation and increase the success rate, the start of callus induction was localized in detail. It originates at a the border zone between integument and endosperm. Within the embryogenic callus, there are cell clusters of meristematic cells surrounded by parenchymatic callus cells. Somatic embryos were clearly distinguished histologically by their unique shape. The spherical callus type yielded most of the somatic embryos. The ploidy of callus and embryos was always diploid, indicating that they didn't originate from the endosperm.





## High content of oleic acid in lipids of morphogenic calluses of Siberian larch as a marker of early somatic embryogenesis

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Current rate of Siberia's post-industrial development entails dramatic reduction of forest lands causing irreversible damage to genetic diversity of valuable forest-forming species. Approaches based on biotechnological methods aimed at the acquisition of cells cultivar and study of their potential vegetative propagation play an important role in preservation of conifers biodiversity. Clonal propagation of rare genotypes with unique characteristics via somatic embryogenesis and organogenesis may be regarded as the most promising strategy of preserving these genotypes. However, biotechnology of acquiring somatic embryos for most coniferous species remains a challenge. The difficulties are caused by the lack of knowledge on molecular mechanisms of somatic embryogenesis in gymnosperms. One of the key peculiarities of higher plants is variability of qualitative and quantitative composition of cell lipids and fatty acids resulting from changes in external (environment) and internal (growth and development processes) factors. No detailed study of peculiarities of fatty acids (FA) and lipids composition throughout individual stages of somatic embryogenesis has been accomplished so far. The work was aimed at the study of cell lipids fatty acid composition *in vitro* at the early stages of somatic embryogenesis of Siberian larch. Four *Larix sibirica* callus lines of different origin were used, including two lines of non-morphogenic calli and two lines of calli at the stage of embryonal-suspensor masses proliferation. FAME (methyl ethers) were analyzed by gas-liquid chromatography. Total content of saturated FA in lipids of non-morphogenic lines equaled on average 33% of the fatty acids sum. Within morphogenic calli saturated FA content amounted to 20% on average, which is about 1.5 times lower than the content in non-morphogenic calli. The content of unsaturated fatty acids amounted on average to 66% for the lines of non-embryogenic calli and was much higher for embryogenic lines - 80%. Differences in the content of mono-unsaturated and poly-unsaturated FA were identified between morphogenic and non-morphogenic calli. The content of mono-unsaturated fatty acids is significantly higher in the case of somatic embryogenesis. For oleic acid it averaged to 50%, for taxoleic acid - to 7%. The content of these fatty acids in non-morphogenic calli was approximately 3 times lower. At the same time, morphogenic calli manifested reduction in the content of linoleic and pinolenic acids by 3.5 and 5 times respectively as compared to non-morphogenic calli. On the whole, considerable differences in lipids fatty acid composition were found between the groups of non-embryogenic and embryogenic callus lines of Siberian larch. Such parameter as the content of oleic and linoleic acids is proposed to be taken into account for differentiation of morphogenic and non-morphogenic lines of Siberian larch at the stage of early embryogenesis. A more profound analysis of changes in lipids composition during morphogenesis *in vitro* cultivar will reveal biological function of lipid metabolism restructuring at the initial stages of somatic embryos formation. Results of this study may contribute to the development of conifers' clonal propagation technique.

Keywords: Siberian larch, embryogenic callus, oleic acid.





## **Influence of megagametophyte free amino acids and polyamines profile in the induction of embryogenic cell lines in Brazilian pine (*Araucaria angustifolia* Bertol. Kuntze)**

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Somatic embryogenesis (SE) associated to cryopreservation can represent a useful strategy for *ex situ* conservation of endangered conifers. However, to reach this purpose is important that most genotypes of a population can be propagated using SE. In order to improve the efficiency of *in vitro* embryo development, a comprehensive understanding of the biochemical and molecular events in somatic and zygotic embryo development is required. In gymnosperms zygotic embryo development is highly dependent on nutrients and hormones signals provided from the megagametophyte. Despite this importance, there is little information to what extent megagametophyte physiological status can influence zygotic embryo capacity to induce embryogenic cell lines (ECs). Induction and proliferation of ECs in Brazilian pine, a native endangered conifer of South America, is carried out in culture medium without auxins and cytokinins. In Brazilian pine, polyamines (PAs) and amino acids, seem to be involved in the regulatory mechanisms responsible for embryogenic tissue induction and early somatic embryo formation. In the present study, we characterize levels of free amino acids and PAs in the megagametophyte of four mother trees for the following purposes: a) to identify if free amino acids and PAs megagametophyte profiles can influence zygotic embryo capacity for SE induction, and b) to gain a better knowledge about the chemical environment in Brazilian pine megagametophyte for refining artificial media for *in vitro* embryogenesis. Immature seeds are collected in December 2013 from two natural populations of Brazilian pine (22°44'20"S 45°35'27"W and 26°15'16"S 49°31'06"W). SE induction was carried out according to dos Santos *et al.* (2008). Free amino acids and PAs profile were determined according to Astarita *et al.* (2004) and Jo *et al.* (2014). Principal component analysis (PCA) was employed to discriminate four Brazilian pine mother trees according to embryogenic tissue initiation based on megagametophyte free amino acid and PAs contents. Aspartic acid, glutamic acid, ornithine and lysine were the main free amino acids observed in megagametophytes, whereas free spermidine and spermine were the main polyamines present followed by putrescine and cadaverine. Initiation frequencies varied significantly among mother trees, ranging from 7 to 14%. Three principal components accounted for 79.6% of the total variance in the data. Based on the results observed so far (one induction season), PCA analyses of megagametophyte free amino acids and PAs contents were useful to discriminate mother trees according to its induction rate. The obtained data improve our understanding about conifer embryogenesis and bring insights for the development of new artificial media conditions aiming to increase the somatic embryogenesis efficiency.

Keywords: endangered conifer, megagametophyte physiological status, principal component analysis, somatic embryo







## **Embryo maturation ability is subjected to line ageing – a way to assure the quality of somatic embryos of Nordmann fir**

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Nordmann fir (*Abies nordmanniana*) has an enormous commercial importance for Christmas tree production in Europe. It is exclusively grown from seeds harvested from natural populations in the Caucasian mountains. Clonal varieties would help to considerably improve the quality of trees and the cultivation characteristics. Methods based on somatic embryogenesis are expected to become a realistic possibility to solve these problems in the near future.

The biotechnical procedure for clonal mass propagation of Nordmann fir has been studied for more than 10 years and was improved with regards to critical steps such as long-term propagation of embryogenic cultures and the subsequent loss of maturation ability. As a result of high sub-cultivation numbers, the quantity of normally developed mature embryos per gram fresh weight decreases.

Yet the main concern remains with the loss of maturation ability after longer propagation times which has been solved by an early cryopreservation to produce stable quantities of somatic embryos over time. To this end critical steps of the preservation protocol have been determined and a user-optimized procedure to manage the high number of clones have been established.

The increasing inability a prolonged period of sub-cultivation has let to the development of an improved culture scheme. Reasons, conclusions and therefore possible solutions will be discussed.

Keywords: Nordmann fir, somatic embryogenesis, clonal varieties, cryo preservation, maturation ability.





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## ***WUSCHEL-RELATED HOMEobox 8/9* regulates cell division patterns during embryo development in the gymnosperm Norway spruce**

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Proper embryo development is crucial as it is when the primary body axes are established. In Arabidopsis, *AtWOX8* and *AtWOX9*, members of the *WUSCHEL-RELATED HOMEobox* (*WOX*) gene family, are crucial for embryo development. Previous studies have shown that in Norway spruce there is a single *PaWOX8/9* gene, which is expressed in embryos. In this work we show that the expression of *PaWOX8/9* is high during early and late embryogeny and that the expression decreases when the maturation phase starts. To address the function of *PaWOX8/9* during embryo development, we established RNAi lines to knock-down the expression of *PaWOX8/9*. Using both constitutive and inducible promoters, we could show that down-regulation of *PaWOX8/9* during early and late embryogeny disturbs the orientation of cell division plane at the basal part of the embryonal mass resulting in aberrant morphology of the embryos. In addition, the expression of several key cell cycle regulating genes, for example *PaE2FAB-LIKE* genes and *PaCYCLIN B-LIKE* genes, are affected by the down-regulation of *PaWOX8/9*. Taking these together we show that *PaWOX8/9* performs an evolutionarily conserved function as an essential regulator of the apical-basal embryo pattern establishment.

### *Abbreviations:*

PEM, Proembryogenic mass

EE, Early embryo

LE, Late embryo

ME, Mature embryo

PGR, Plant growth regulator

Keywords: Apical-basal, cell cycle, development, embryogenesis, polarity, spruce, *WUSCHEL-RELATED HOMEobox*





## Timing as critical factor in cryopreservation of *in vivo*-buds of hybrid aspen

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Hybrid aspen is an artificial cross between European aspen (*Populus tremula* L.) and American aspen (*Populus tremuloides* Minchx.). Most of the aspen cultivation in Finland has been with hybrid aspen; due to the heterosis the growth rate of the best hybrids has been 2- to 3-fold that of domestic aspen, with the best volume growth reaching 24 m<sup>3</sup>/year/ha. For the conservation of hybrid aspen germplasm, cryostorage of dormant *in vivo* buds is a convenient back-up method for field collections. When needed, the stored material is regenerated using micropropagation. In practice in Finland, bud collection for cryopreservation has been performed from February to March, as a compromise between the still remaining cold hardiness and the increasing bud burst ability. The aim of this study was to assess how this time schedule can be extended without compromising regeneration. In addition, an easily measurable marker for successful cryopreservation was examined.

Timing of cryopreservation by slow-cooling method was tested from August to February, using dormant buds from both outdoor and indoor plants. To find a marker, water content and gene expression (dehydrin, *dhn*) of hybrid aspens, as well as environmental factors such as temperature, development of temperature sum (day degrees, d.d., i.e. the temperature sum with a threshold of 5°C), and light period were followed. Cryopreservation was successful from October to February, when, on an average, at least 75% of the buds regenerated through micropropagation, and there was no difference to non-frozen controls. Significant genotypic variation was observed in October and February, with regeneration rates of 61-100% and 37-98%, respectively. The overall contamination rate of the present material was relatively low: 4.6 ± 0.6% had a fungal contamination and 6.8 ± 0.8% a bacterial contamination in micropropagation. No marker for successful cryopreservation was found among the studied factors. The results provide flexibility for the undertaking of practical work, with a recommendation that cryopreservation can be carried out from November to January – earlier than the current practice.

Reference: Aronen & Rynänen 2014. Cryopreservation of dormant *in vivo*-buds of hybrid aspen: Timing as critical factor. Cryoletters, in press.

Keywords: cryopreservation, dormant bud, micropropagation, *Populus*, timing.





## Can we induce tolerance to stress in *Pinus radiata* somatic trees?

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In the current context of climate change, drought and increased temperature are the most remarkable consequences that will affect plant growth and distribution. In most cases, the plant response to stress has clearly defined metabolic pathways. But epigenetic variation can also contribute to the phenotypic plasticity of plants, which can be especially important for the adaptation of forest trees to changing environmental conditions. In this sense, somatic plantlets can offer an added value, such as tolerance to stressful conditions with no detriment in the growth rate.

In the last years, our research team has optimized different stages of somatic embryogenesis process in radiata pine such as initiation and proliferation, and maturation. In the latter, we have obtained high amounts of somatic embryos from low amounts of embryogenic tissue in most of the embryogenic lines tested till date. Furthermore, we have developed somatic embryogenesis in other species (*Pinus halepensis*), and combined this propagation technology with organogenesis. Nowadays, this knowledge provides us a biotechnological tool to manipulate the physical and chemical conditions at different stages of somatic embryogenesis process. As a result, a great amount of clonal material is obtained and then, an *ex vitro* analysis of this clonal material can be carried out to assess what level of stress tolerance they have acquired along the first phases of their development. Furthermore, the analysis of physiological parameters in plants with different tolerance to abiotic stress could give valuable information about the mechanisms used by plants to survive under adverse environmental conditions.

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Keywords: conifer, genetic improve programs, micropropagation, radiata pine, somatic embryogenesis.







## Productivity of *Larix sibirica* embryogenic lines *in vitro*

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Immature isolated zygotic embryos of *Larix sibirica* were experimentally cultured on AI medium added by L-glutamine, casein hydrolysate, ascorbic acid, and hormones (2,4-Dichlorophenoxyacetic acid and 6-benzyladenine). The embryonal suspensor mass exhibited active proliferation on this medium under a reduced concentration of cytokinins. The somatic embryos matured on the basal medium with abscisic acid (32 mg l<sup>-1</sup>) and polyethylene glycol (100 mg l<sup>-1</sup>). The 14 long-term proliferative embryogenic lines (ELs) were derived from three genotypes, which resulted from open pollination and one (EL5) from the genotype of *Larix sibirica* controlled pollination by *L. sukaszewii* pollen. ELs differed in somatic embryo production, more specifically, in embryo quantity, size, and capability to mature, to germinate, and to form viable plantlets. While for young (1-year-old) ELs embryo numbers ranged from 2040 (EL6) to 7655 (EL9), EL10 contained 11103 embryos per 1 gram of fresh weight of embryonal suspensor mass (ESM). Somatic embryos reached maturity with the highest number (1220.6) in EL4. Small somatic embryos of EL5 did not mature on the medium supplemented with ABA. During the somatic embryo maturation the formation of embryo axis, cotyledons, apical and root meristems occurred. In highly embryogenic ELs embryos were formed, which were normal in terms of morphogenesis. The anomalies occurred in greater numbers in lines of low embryogenic productivity. The developmental anomalies covered mainly the basal areas (roots). It should be noted that embryo apical meristems and cotyledon disturbances resulted in production of deficient plantlets.

In ELs number of healthy plantlets ranged from 11.1 % (EL 7) to 82.4 % (EL 2). After acclimatization in sterile soil emblings were transferred to a soil substrate in a growing vessel and then planted in a greenhouse.

Therefore, we have been the first to observe proliferating cell lines, somatic embryogenesis and plantlets in Siberian larch and the hybrid of this species with Sukachev larch. Somatic embryos of Siberian larch underwent a successful maturation on AI medium supplemented with ABA, IBA, PEG, and Gelrite. The embryonal lines we obtained are capable of long-term (over four years) self-maintenance and mass production of somatic embryos and plantlets, which will become a highly promising material for establishing conifer forest plantations in Siberia.

Keywords: embryogenic lines, somatic embryogenesis, *Larix sibirica*, proliferation, maturation, germination.





## Molecular phenotyping of Maritime pine somatic plants transformed with an RNAi construct targeting cinnamyl alcohol dehydrogenase

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As a major conifer species for genomic research with great ecological and socio-economical interests in Southern Europe, various biotechnological approaches are concurrently developed in *Pinus pinaster* towards enhanced selection efficiency and deployment of improved varieties. Accumulation in recent years of information about expressed genes (e.g. reference transcriptome), genomic areas, and gene pathways controlling breeding traits paved the way for designing practical, marker-based strategies to improve biomass and wood productivity. Functional genomics revealed a wealth of candidate genes to be validated at the population scale (allelic variation, expression and association studies) or more readily by establishing direct association between genes and adaptively significant phenotypes through loss-of-function techniques such as RNAi. For this specific purpose we developed a post-transcriptional gene silencing method based on stable constitutive expression of intron-spliced hairpin RNA constructs in embryogenic tissue and derived somatic plants. To circumvent the lack of full genome sequence, we elaborated a strategy based on 4 constructs per gene involving gene sequence tags (GSTs) designed to address RNAi efficiency vs. specificity. Three independent, tandemly arranged GSTs (a, b, c) and one overlapping GST (f) were selected in the variable EST regions of 21 candidate genes involved in wood formation. We specifically reported here on data obtained during pilot experiments with cinnamyl alcohol dehydrogenase (*CAD*), a gene involved in the last step of monolignols biosynthesis whose silencing is expected to result in decreased lignin content, increased free phenolic groups and pulping efficiency. RT-qPCR analysis of *CAD* expression in needles of up to 16-month-old somatic plants regenerated from 7 transgenic lines revealed a strong GST effect. *CAD* expression was significantly downregulated only in independent lines (*CADf5*, *CADfG*) transformed with the GSTf-derived construct. Additional analyses of 2.5 years old transgenic plants from these lines and corresponding non-transgenic controls grown in the greenhouse confirmed the strong reduction in *CAD* expression with concomitant decreased level of *CAD* protein and activity in stem. The same samples were used to explore putative genome-wide effects of *CAD* downregulation at both transcriptomic and proteomic levels. Strikingly, numerous genes (including *CAD*) were found differentially expressed in stem of *CADf5* and *CADfG* samples. Proteomic profiling further revealed that the abundance of more than 100 proteins was modified in stem and/or needles (including proteins from the lignin biosynthesis pathway) with large differences in significant proteins between lines. The implication for functional gene studies of putative large off target, pleiotropic effect is discussed.

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Key words: Transgenic plant, *CAD* gene, silencing, transcriptomics, proteomics, off target effect.





## Impletation of micropropagation techniques to enhance the Spanish Elm Breeding and Conservation Programme

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The second pandemic of Dutch elm disease (DED), caused by *Ophiostoma novo-ulmi*, had devastating effects on Iberian field elm (*Ulmus minor* Mill.) stands. In order to mitigate these effects, the Technical University of Madrid and the Spanish Environmental Administration initiated the Spanish Elm Breeding and Conservation Programme (SEBCP) 27 years ago. Since then, research performed by the SEBCP has obtained many results that have aided the protection and recovery of this emblematic and cultural species. Among the most important achievements we highlight the discovery of seven Iberian *U. minor* clones with high degree of resistance to DED. These clones have recently been registered in the Forest Reproductive Material National Catalogue as “qualified clones” for their use and marketing. The breeding programme has also obtained other genotypes with promising degrees of tolerance to DED in their initial susceptibility trials. The SEBCP obtained low rooting success rates (10-20 %) when trying to propagate elm clones with traditional vegetative propagation techniques. Since this limited the production and distribution of valuable elm genotypes, the programme has now focussed on micropropagation. This technique enables increasing elm clones’ multiplication rates and achieving rooting rates of over 60 %, with the additional advantage of only requiring small quantities of initial material.

Currently, efforts of the SEBCP concentrate on three major work lines. First, the propagation of 9,000 plants of the seven DED resistant clones for the project *Restoration of Iberian elms (Ulmus minor and U. laevis) in the Tajo River basin* (LIFE13 BIO/ES/000556). The main objective of this project is to restore elm groves in Madrid’s Southeast Regional Park and in the San Sebastián de los Reyes and Aranjuez municipalities. The long term purpose is to boost the species resistance to DED when these clones breed with the local genotypes. The seven clones have been introduced successfully into *in vitro* culture and are in the multiplication stage, **producing between 5-10 shoots per explant and month. Rooting has been induced in some of these shoots, and as a result, 500 plants of clone MDV2.3 and 50 of MMJ1 have already been grown.**

Second, each new genotype that shows a high degree of resistance to DED when tested as a seedling in progeny trials will be propagated by *in vitro* culture, aiming for a minimum of 50 copies. This will allow testing these genotypes as clones in several research plots under different environments, shortening the period required for determining their DED resistance level. **Clones from 10 new genotypes, that showed a high tolerance to DED after being inoculated twice with *O. novo-ulmi* as seedlings,** have already been planted in research plots.

Finally, DED resistant and susceptible elms are being propagated by *in vitro* culture since for investigating their biochemical responses to *O. ulmi* and *Ophiostoma novo-ulmi*, as well as, the response in the presence of other fungal *endophytes that could provide susceptible elms a greater tolerance to DED.*

Keywords: *Ulmus minor*, micropropagation, DED, elm breeding, restoration, tolerant genotypes





## Characterization of the embryogenic capacity of *Pinus pinea* genotypes by genome-wide transcript profiling

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Somatic embryogenesis has become the leading biotechnology for forest tree improvement and for the implementation of multi-varietal forestry. Despite the major advances in clonal propagation by this technology, many forest tree species, such as conifers, show a low regeneration capacity, especially in the adult phase of development. Somatic embryogenesis is a developmental process by which somatic cells can dedifferentiate and reorganize into an embryo. The identification of candidate genes involved in the regulation of key steps of the process is essential to develop tools and strategies to improve clonal propagation programs in forest tree species. Somatic embryogenesis is also a genotype-dependent program in conifers. To characterize the molecular events of the genotype-dependent embryogenic capacity of *Pinus pinea*, transcriptome analysis, in combination with expression analysis by quantitative real-time PCR (qRT-PCR) were conducted in genotypes with low and high embryogenic capacity. *Embryogenic masses from eight genotypes, maintained by bi-weekly subculture on proliferation medium and collected at the end of subculture*, somatic embryos at different developmental transitions and adult tissues were used as plant material for transcriptome and expression analysis. We have *de novo* assembled 1,000,000 GLX-454 reads from two libraries, one from a pool of genotypes with low embryogenic capacity and one from a pool of genotypes with high embryogenic capacity, to build a *Pinus pinea* gene index associated with the embryogenic capacity. This index contains 29.509 unique contig/isotig sequences, 15.070 sequences from genotypes with high capacity and 14.439 sequences from the genotypes with low capacity, and includes genes coding for cell cycle proteins, auxin transporters, transcriptional regulators, including chromatin remodeling factors, or cell signaling proteins that were differentially expressed in different genotypes with low or high embryogenic capacity.

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**Keywords: epigenetics, somatic embryogenesis, stone pine, transcriptome.**







## **Establishment of a Protoplast-System for Nordmann Fir (*Abies nordmanniana*) - a necessity for reembryonalizing adult cells through electric fusion of protoplasts**

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Nordmann fir is the most important Christmas tree in Europe and its clonal mass propagation a major target in the Christmas tree industry. One approach to this problem is reembryonalizing cells from adult trees by transferring embryogenetic factors from the cytoplasm of embryogenic protoplasts by means of protoplast fusion. A working Protoplast-system is a must for this target, but has not been reported yet.

Through somatic embryogenesis, it is possible to generate embryogenic tissue-cultures (ESM) from juvenile material that can be used to isolate embryogenic protoplasts by applying an enzyme solution consisting mainly of Cellulase, Mazerzyme and Driselase. Isolating Protoplasts from adult material is much more difficult. It was necessary to establish callus-cultures by applying high auxin-concentrations to winter-buds (10µmol/l 2,4-D) and to transfer the callus to suspension culture. Protoplasts then were immobilized in an alginate-dish and put into culture medium with adjusted auxin and cytokinin concentrations. For protoplasts derived from adult material, it was necessary to add at least 5-10µmol/l 2,4-D to the culture-medium. Embryogenic protoplasts only grow and divide in absence of Auxin, but in presence of 5µmol BAP. Also, adding conditioned medium derived from embryogenic suspension cultures proved to be necessary. With this improved culture-medium, it was possible to regenerate 39% of the protoplasts derived from adult material to callus-colonies and up to 21% of the Protoplasts derived from embryogenic tissue to embryogenic colonies that could be matured to somatic embryos. Morphogenesis was direct, forming phase-I Embryos from single protoplasts. After 5 days, first cell-divisions were observed that formed a dense clump of cytoplasmic cells in the following 2 weeks. After 3 weeks, suspensor-like cells were formed. After 4 weeks, fully developed phase-1 embryos were present. In a second step, callus derived from winter-buds was transformed with *Agrobacterim*-mediated gene-transfer to obtain hygromycine-tolerance. Protoplasts from these transgenic cultures can now be fused with embryogenic protoplasts. Fusion products will be cultured on a selective culture-medium containing hygromycine and no Auxin, so only embryogenic cultures containing the hygromycine-resistance from the adult protoplast-partner are able to grow. This would be the first proof for the possibility to transfer embryogenic competence through cell-fusion in conifers.

Keywords: *Abies nordmanniana*, protoplast, culture, electric fusion.





## Isolation and culture of olive (*Olea europaea*) protoplasts

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In Algeria, the olive potential is concentrated mainly in the mountainous regions characterized by orchards aging. Despite the importance of this species, very few studies of micropropagation have been realized, where the use of modern technology is still limited. Many different olive cultivars are cultivated in Algeria such as 'Azradj', 'Sigoise' and 'Chemlal'. Protoplast culture and somatic hybridization with the rootstock species *Olea europaea* subsp var *sylvestris* could be an alternative for classical breeding. In this research, factors affecting protoplast isolation, viability, fusion and regeneration are studied. As for different explants were used, high yield of protoplasts was obtained using callus compared with mesophyl material. Many types and combinations of enzymes were tested, and showed that driselase gave the highest yield.

The standard protoplast culture medium was based on a modified MS medium ( $\frac{1}{2}\text{KNO}_3$  and  $\frac{1}{2}\text{NH}_4\text{NO}_3$ ) and supplemented with sucrose, mannitol, NAA and different cytokinins. Different culture systems were used to test protoplast regeneration capacity (liquid culture, semi-solid/liquid culture and agarose beads in liquid media). Protoplasts isolated from leaves of 'Chemlal' and cultured in agitated MS mod liquid medium supplemented with Z + TDZ, and 10g/l sucrose + 90 g/l mannitol yielded most callus colonies. The same was observed for protoplasts isolated from callus of *Olea europaea* subsp. *europaea* var. *sylvestris*. On two phase semi-solid/liquid system, only cell divisions were noted without colony formation, agarose bead culture didn't show any cell division.

Keywords: protoplast, callus, *Olea*.





# Caulogenic induction in cotyledons of stone pine (*Pinus pinea*): an experimental system for functional validation of candidate genes involved in bud formation by qRT-PCR

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In the recent years, a model integrating the mechanism of cytokinin signal transduction and meristem formation in *Arabidopsis thaliana* has been proposed. However, there is still little understanding about the underlying molecular mechanisms of this process in conifers. Conifers are important forest components in many areas of the world, especially in the Northern Hemisphere, and have a great environmental and economic importance, but their extraordinary large genomes with high heterozygosity levels and the lack of reliable experimental systems have made difficult the study of the molecular basis of processes and traits that are important for conservation and breeding, such as growth, wood formation and stress tolerance.

During the last years, several ESTs databases have been published and recently it was reported the draft assembly of the 20-gigabase genome of *Picea abies* (Conifer Genome Integrative Explorer, ConGenIE). In order to achieve a better understanding about the mechanisms that control meristem formation in conifers, we conducted the isolation of candidate genes in *Pinus pinaster* through the search in available public databases, the use of bioinformatics tools and the design of specific primers. A genomic sequence whose derived peptide sequence showed the characteristic homeodomain and motifs of the *Arabidopsis WUSCHEL (WUS)* gene was obtained and designated as *PipsWUSCHEL-LIKE*. We also obtained the full-length cDNA sequence of four Class I *knox* genes, here named *PipsKNI* to *PipsKN4*. Studies in *Arabidopsis* and other angiosperms have shown that *WUS* is a central regulator of meristem cell identity whose expression is induced by cytokinins. *WUS* in turn is thought to stimulate the expression of several cytokinin A-type response regulators, negative regulators of cytokinin signal transduction, creating a region of high cytokinin sensitivity in the center of the meristem. *WOX5* gene plays a similar role in the root apical meristem. Furthermore, class I *knox* genes induce cytokinin biosynthesis in the meristem, and their expression is also induced by high cytokinin levels.

Adventitious bud formation in pine cotyledons cultured in the presence of the cytokinin benzyladenine (BA) has been proposed as a model for the study of the caulogenesis and the involvement of cytokinins in this process in conifers. This experimental system is very repetitive and it has several advantages, including the need of only one plant growth regulator in the induction medium. Furthermore, cotyledons have a low degree of differentiation and are competent to form buds *per se*, without a callus phase, responding in a very synchronous fashion to the induction. In order to assess the role of the isolated genes, their expression profiles during *in vitro* adventitious caulogenesis induced by BA were analyzed by quantitative real-time PCR (qRT-PCR).

Keywords: caulogenesis, benzyladenine, *WUSCHEL*, *KNOX*, qRT-PCR.





## Genetic transformation of *Pinus pinaster* Ait. with genes involved in water stress response

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Current climate change is specially affecting the Mediterranean region imposing strong restrictions in forest tree species. Some Mediterranean species are already suffering a severe decline, since their adaptation rate cannot keep pace with this fast change. Even pine species, considered as drought stress tolerant, are suffering important damages in the Iberian Peninsula. In such scenario it is of utmost importance to obtain further knowledge of the molecular mechanisms by which forest tree species face drought, as well as to consider potential applications of this knowledge in breeding programs to catalyse the production of individuals highly resistant to water stress.

An exhaustive analysis using *Pinus pinaster* as model species has enabled us to identify an important number of reliable drought candidate genes for conifers. Three genes were selected due to their remarkable up-regulation during a prolonged drought stress in soil, and presumably they are involved in different steps of the water stress response. These genes putatively encode (1) a dehydrin (DH2) which would be involved in protecting cells from structural collapse suffered during dehydration; (2) a nodulin (NOD) presumably involved in sugar transport through membranes and (3) an AP2 transcription factor which may control the activation of new pathways during dehydration process. For all of them, the exon/intron structure was established and their promoter region was sequenced.

An ubiquitin promoter-based binary vector (pMBb7Fm21GW-UBIL) was used to prepare four plasmid constructs for constitutive overexpression of these genes (NOD, AP2, DH2 and DH2-GFP as fusion protein). A selected embryogenic line of *Pinus pinaster* (PN519) provided by FCBA (France) was transformed by *Agrobacterium* with the four gene constructs and an empty vector as positive control. The transformation protocol was based on phosphinothricin (PPT) selection and on previously described procedures [1]. After 12 weeks, a high percentage of tissue clumps showed PPT resistant lines (from 37.8% for AP2 to 87.2% for NOD) corresponding to a high number of putative transformation events per gram of embryogenic tissue (from 34.5 for AP2 to 74.5 for NOD). All analysed events were PCR-positive and were able to proliferate both in selective medium as well as in proliferation medium. Transgene expression in the embryogenic tissues was highly variable among lines transformed with the same vector reaching, in 2 transgenic lines over-expressing *NOD*, expression levels approximately 100-times higher than in the control. Three selected lines from every construct were subjected to culture conditions for promoting embryo maturation. After three months in maturation medium high numbers of normal embryos were isolated (from 70 to 370 embryos per gram of mass) and transferred to germination medium to obtain complete plants. Rooting and acclimation were unsuccessful and only a few well developed plants were obtained. These plant regeneration experiments will be repeated using transgenic lines re-established from cryopreserved stocks in order to obtain enough transformed plants to analyse their performance under different conditions.

[1] Trontin *et al.* (2013) Somatic embryogenesis as an effective regeneration support for reverse genetics in maritime pine: the Sustainpine collaborative project as a case study. In Park Y, Bonga J (eds) Proceeding of IUFRO Conference 25-28 Jun 2012 (Brno, Czech Republic). Pp 184-187

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Keywords: *Pinus pinaster*, drought, somatic embryogenesis, transgenesis, overexpression.







# Transcriptional regulation of auxin signaling transduction pathway in *Araucaria angustifolia* (Bertol.) Kuntze: a comparative analysis between early somatic (SEM) and globular zygotic (GZE) embryos

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Brazilian pine, *Araucaria angustifolia*, is the only native conifer species in Brazil with economic importance. The Araucaria moist forest is within the Atlantic Forest Biome, which is recognized as a biodiversity hotspot. Due to intense exploitation of wood resources and the lack of reforestation programmes, this species is classified as critically endangered, according to the International Union of Conservation of Nature Red List of Threatened Species (2013). Somatic embryogenesis (SE) is a method for producing embryos *in vitro* and is considered a highly promising approach for efficient micropropagation and germplasm conservation. However, the application of SE for genetic breeding and *ex situ* conservation of certain species, such as *A. angustifolia*, faces several technical challenges, including the difficulty of inducing embryogenic cultures using mature tissues, the rapid loss of embryogenic competence of cell cultures and incomplete development of somatic embryos at early stage. The difficulties in successfully establishing artificial conditions allowing continuous embryo development to maturity are associated with the fragmented knowledge concerning the genetic programmes that regulate zygotic embryogenesis. Recently, based on a comprehensive transcriptome analysis of *A. angustifolia* embryogenesis, alterations in the expression of auxin-related genes were observed between early somatic and zygotic embryos. Aiming to improve our understanding about the origin of the developmental blockage observed in *A. angustifolia* somatic embryos a curated identification and annotation of auxin metabolism-related genes was performed within the transcriptome. The survey retrieved, 8, 13, 11 and 3 transcripts homologous to genes functionally characterized as involved in biosynthesis, perception, signaling transduction pathway and catabolism, respectively. The differential expression profile of the thirty-six identified genes between early somatic (SEM) and globular zygotic embryos (GZE) was assessed by RNAseq analysis. Three mRNA homologous to *ARFs* (auxin responsive factors), four *AAI* (auxin inducible protein) and two *PAT* (polar auxin transport) genes were up-regulated in GZE. *WUSCHEL* transcription factor (*WUS*) was highly represented in the SEM transcriptome. The crosstalk between *ARFs* and *WUS* is crucial for normal embryo patterning restricting *WUS* expression to the center zone. The overexpression of *WUS* results in abnormal shoot apical meristem morphology. The results suggest that SEM fails to establish the correct auxin distribution and signaling, consequently the embryo development is blocked at early stage. *WUS* and *ARF* expression analyses are being performed in somatic embryos grown under different auxin concentrations aiming to reproduce the zygotic auxin distribution allowing the development progress of the *in vitro* embryos.

Keywords: auxin responsive factors, auxin signaling, polar auxin transport, somatic embryogenesis and *WUSCHEL*.





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Vegetative Propagation Technologies

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**POSTERS - SESSION 4:**  
**DEVELOPMENT OF SE**  
**AND VP TECHNOLOGIES AND THEIR**  
**SCALE-UP APPLICATIONS**

## **Industrial implementation of multi-varietal forestry using somatic embryogenesis and genomic selection**

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Natural Resources Canada (NRCan) has been involved in research related to conifer somatic embryogenesis since the mid 1980's. JD Irving, Limited began investigating the potential for industrial implementation of multi-varietal forestry (MVF), in collaboration with NRCan, using somatic embryogenesis in the mid-1990s. Over this period, JD Irving (JDI) has established 45 varietal tests (VT) using over 2,000 candidate varietal lines derived from the crossing of the selected individuals from the company's breeding program. The current MVF deployment is based on the growth, branching traits and insect resistance using the VT information. The lack of mechanized embryo handling systems has limited large scale production with current levels of approximately 300,000 per year. Production is planned to be increased to 3 million by 2017 when the construction of new laboratory with semi-automated embryo handling systems is completed. Recent advances in genomic selection are showing potential and providing optimism that application in MVF programs will be possible for wood quality, growth, insect resistance and adaptive traits.

Keywords: tree breeding, somatic embryogenesis, multi-varietal forestry, genomic selection





## Improvement and Clonal Propagation Process Development in Espirra Nursery

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The Espirra Nursery is currently the largest forest nursery plants in Europe, with an area of 22 hectares, fully equipped with advanced and innovative technology. Recently on Espirra held an investment for modernization and doubling production capacity Nursery. Now has 250,000 stock plants, an innovative system that involves the elevation of the donor plants by installing cement rings, so that, from the point of view of work ergonomics, more comfortable for the workers that are collecting the shoots. After the collection, the cuttings are quickly transported to the work area, designated by Biofactory, thus avoiding moisture stress and loss of quality of the cuttings by evapotranspiration and wilting. The nursery gathers a lot of 6 'clones' that are carefully selected, contributing to the production of 6 million clonal eucalyptus plants for macro-cuttings. In this nursery are achieved the best results of rooting and the highest success rates in the world (by comparison with Chile, Spain, Uruguay and Australia) due to experience and accumulated knowledge, the existing technology and the clones produced. The optimization process led to operating earnings by increasing spaces, mechanization of procedures and improvement of hygiene conditions of production, while ensuring better standards of ergonomics, health and safety at work. The Espirra Nursery became the world's largest producer of *Eucalyptus globulus* by vegetative propagation (clonal plant), which would lead to a real improvement in the national forest of Eucalyptus, with substantial increases in productivity, integrating the gains achieved with the Genetic Improvement.

Keywords: Clone, Vegetative Propagation, *Eucalyptus globulus*, Biofactory.







## **Cocoa propagation by somatic embryogenesis at Nestlé R&D-Tours**

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Mass propagation of selected clones of cocoa by somatic embryogenesis requires the following steps:

- Induction of primary somatic embryos (SE) from bud flowers ,
- Induction of secondary SE,
- Multiplication of embryogenic callus,
- Production of the SE in liquid medium
- Maturation step on ABA-containing solid medium
- Embryo-to plantlet conversion on solid medium

As for many other species, the efficiency of SE is hampered by a genotype-dependent response for primary SE induction and by low embryo-to-plantlet conversion rates (5 to 20%). Furthermore, we observe a large variability of the SE quality according to the production batches. As an example of optimization trials that we conducted, we show our study on visual criteria for the selection of SE able to regenerate plantlets, particularly the criteria of opacity.

Keywords: Cocoa, Somatic embryogenesis, genotype dependent, opacity.





## Direct shoot regeneration from mature zygotic embryos of *Pinus elliotii* x *P. caribaea* hybrids and plantlets production assisted by temporary immersion bioreactors

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In order to develop biotechnological tools to produce *in vitro* plantlets of *Pinus elliotii* var. *elliottii* x *P. caribaea* var. *hondurensis*, vegetative buds were induced from mature zygotic embryos isolated from sterile seeds. The regenerative explants were transferred to a temporary immersion system for the elongation phase. Finally, adventitious rooting was induced and whole plants were obtained.

Vegetative buds were induced through a direct pathway from cotyledons cultured on half strength Muras-hige and Skoog (MS $\frac{1}{2}$ ) semisolid medium (plus sucrose 15 g·L $^{-1}$ ) containing different combinations of 6-benzyladenine (BA 0.1 mg·L $^{-1}$ ) and thidiazuron (TDZ 0.1-5 mg·L $^{-1}$ ). The cultures were incubated in a growth room at 27 $\pm$ 2°C with a 14-h photoperiod (180  $\mu$ mol·m $^{-2}$ ·s $^{-1}$ , from fluorescent lamps) for 30 days. Consequently, regenerative explants from BA 0.1 mg·L $^{-1}$  + TDZ 0.1 mg·L $^{-1}$  were transferred to bioreactors containing 200 mL MS $\frac{1}{2}$  plus sucrose 15 g·L $^{-1}$  and indole-3-acetic acid (IAA, 0.1 mg·L $^{-1}$ ) for the elongation phase. For the temporary immersion program, the explants were in contact with the medium for 1 min every 4 h. Finally, shoots were rooted by partial immersion in an aqueous solution of indole-3-butyric acid (IBA) 500 mg·L $^{-1}$  for 1 h and transfer to either a semisolid medium (Phytigel $^{\text{®}}$  3.5 g·L $^{-1}$ ) or vermiculite added with MS $\frac{1}{2}$  (sucrose 15 g·L $^{-1}$ ) and IBA 0.5 mg·L $^{-1}$ .

The regeneration frequency (93 $\pm$ 3.3%) and the number of buds formed per responsive explants (6 $\pm$ 0.8) was greater with explants cultured in MS $\frac{1}{2}$  with BA 0.1 mg·L $^{-1}$  and TDZ 0.1 mg·L $^{-1}$ . Rooting of regenerated shoots was observed in MS $\frac{1}{2}$  medium with vermiculite as the substrate and supplemented with IBA 0.5mg·L $^{-1}$ . All plants raised *in vitro* were phenotypically normal and successfully hardened to *ex vitro* conditions.

These methodologies enable obtaining a great quantity of propagated material, which can be used in breeding programs and forestation planes according to market.

Keywords: *Pinus elliotii* x *Pinus caribaea*, bud regeneration, bioreactors, plantlet production.





## Mastic Gum Tree (*Pistacia lentiscus* var. *chia*) Vegetational Propagation by Grafting

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The large number of plants produced by somatic embryogenesis has the potential to be the most effective way to produce large numbers of forest trees for commercial use. However, in reality the number of forest trees actually produced by somatic embryogenesis is relatively small. This is due to several biological, logistical and economic challenges. The main biological challenges include the efficiency and effectiveness of rates of initiation, maturation, conversion, establishment *ex vitro* and cryopreservation across a range of genotypes. The production of large numbers of plants in equal numbers across a range of genotypes necessary for commercial application presents major logistical challenges. Some deployment strategies, especially the large-scale planting of monoclonal blocks may face the challenge of public acceptance in some situations. However, it is the current high cost of producing somatic embryos that presents the greatest challenge to the large-scale use of this technology. Unless greater attention is paid to these challenges the large-scale use of somatic embryogenesis in the propagation of forest trees will remain limited.





## Gene expression analysis during in vitro shoot development and root morphogenesis in chestnut

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The axillary bud micropropagation system is one of the simplest and most applied methods for true-to-type in vitro propagation of elite genotypes. Protocols for in vitro cloning and production of mature trees of chestnut via axillary shoots have been defined when physiologically juvenile-like material is used as initial explants. Shoot proliferation and adventitious root formation are hampered by the low morphogenetic capacity of many forest species and also by the loss of morphogenetic competence associated with maturation. The identification of molecular players involved in these developmental processes and their regulatory mechanisms will help to the practical improvement of the in vitro production of important genotypes. Two experimental systems, chestnut microshoots and leaves excised from these microshoots are used to study genes involved in shoot development as well as to investigate how differentiated cells acquire the developmental plasticity to form a new root.

In this study we evaluated the expression of four genes, *CsERF1* (*Ethylene-responsive factor*), *CsGH3* (*auxin-inducible GH3-like*), *CsLRR-RLK* (*leucine-rich repeat receptor-like kinase*) and *CsSCL1* (*Scarecrow-like-1*), in different organs from chestnut microshoots by qPCR and in situ hybridization. Organs were excised from microshoots at the end of the proliferation cycle: shoot apex, axillary buds, internodes, and leaves. Adventitious roots developed from indol-3-butyric acid (IBA)-treated shoots were also harvested and divided in three different zones designated apical, media and basal. The expression pattern of these genes was also analyzed during the induction and development of adventitious roots. Furthermore, we also evaluated by qPCR the responses to auxin treatment and the effect of NPA (N-1-naphthylphthalamic acid) on the relative expression levels of *CsGH3* and *CsSCL1* genes in IBA-treated leaves during the early steps of adventitious rooting. Samples were harvested at different times (6, 12 and 24h) after treatments.

The results showed that *CsERF1* and *CsLRR-RLK* transcripts were highly abundant in axillary buds but they were also localized during root development. The expression patterns of both genes suggest that they may play a role in the development or maintenance of meristems. The highest levels of *CsGH3* and *CsSCL1* transcripts were detected in the medium and basal parts of the root, that correspond to the maturation zone of the root where lateral root primordia are formed. NPA treatment reduced not only the rooting ability but also the expression of both genes during the induction of adventitious rooting in IBA treated samples. This data together with the specific location of transcripts in the root primordia of lateral and adventitious roots indicate that *CsGH3* and *CsSCL1* are involved in root meristem initiation.

This work was funded by the Xunta de Galicia (10MRU400033PR).

Key words: adventitious roots, axillary bud, chestnut, gene expression, morphogenesis.







## **iTRAQ-based comparative proteomic analysis of embryogenic and non-embryogenic tissues of Prince Rupprecht's larch (*Larix principis-rupprechtii* Mayr)**

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Prince Rupprecht's larch (*Larix principis-rupprechtii* Mayr) is an important native coniferous tree species with major ecological and commercial value in China. In larch, somatic embryogenesis (SE) occurs through an indirect process beginning with the formation of embryogenic tissue on the auxin-rich medium and further development of embryos. During the induction stage, we observed embryogenic calli (EC) and non-embryogenic calli (NEC) sharing the same origin arise in the presence of both 2,4-D and 6-BA. However, the precise molecular mechanisms during SE induction in larch were unclear, and a comparative proteomic analysis of EC and NEC was performed to examine somatic embryogenic-specific proteins. Isobaric tags for relative and absolute quantitation (iTRAQ) combined with LC-MS/MS were employed to detect differences between EC and NEC proteomes. In total, 503 proteins were detected, revealing 71 differentially expressed proteins. Obtained proteins were further analyzed to determine their potential functions in the induction of SE. Several proteins involved in the development metabolic processes, such as ADP-ribosylation factor GTPase-activating proteins (ARF-GAPs), triosephosphate isomerase, and proliferating cell nuclear antigen (PCNA), were significantly upregulated in EC, indicating candidate protein markers of SE in larch. The results provide new insights on the characterization and understanding SE at the proteome level.

**Keywords:** Prince Rupprecht's larch, Embryogenic calli, Non-embryogenic calli, Comparative proteome, iTRAQ





# Cupressus sempervirens and allergy: genetic improvement to reduce pollen production

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Common cypress (*Cupressus sempervirens*) is one of the most widespread species in the Mediterranean area. It has been traditionally cultivated for its ornamental value becoming a typical feature of urban and rural landscapes, and for its high timber quality. Cypresses, however, release a huge amount of allergenic airborne pollen, and the prevalence of winter pollinosis due to cypress has reached more than 20% in the past decade.

The main goal of the present study is the selection of cypress plants producing null or low amount of allergenic pollen to reduce the disease effects on the population and to increase the market of cypress plants. The selection of clones suitable for ornamental purposes and resistant to bark canker, the main disease due to the fungal pathogen *Seiridium cardinale*, has been conducted in the past decades on trees growing at the IPSP-CNR experimental fields located in Tuscany. However, the combination of desirable characters through traditional breeding requires a huge amount of time. Somatic embryogenesis (SE) revealed a suitable *in vitro* regeneration technique for conifer cloning and for achieving genetic transformation: with this attractive method one can perform the improvement of desirable traits in clones selected for their superior quality.

Protocols for SE in cypress has been initiated several years ago by Lambardi et al. (1995), however the conversion of immature embryos to emblings was never achieved. During the preliminary phase of the study a protocol for maturation and conversion of embryogenic lines derived from the patented clone “Mediterraneo” was developed by changing the osmotic potential of maturation medium and through the mechanical isolation of immature embryos at the cotyledonary stage. Cryopreservation experiments are in progress for the long-term conservation of the selected lines.

Immature clumps of somatic embryos are a suitable material for genetic transformation in order to introduce novel traits in plants. The main aim is to obtain male sterile cypress plants (to prevent production and release of allergenic pollen) through the expression of cytotoxic genes involved in male flower development, under the control of a conifer tapetum-specific promoter. Currently, some technical variables during immature embryos transformation with GUS reporter gene, using a particle-gun approach, are tested.

**Keywords:** Cypress, Somatic embryogenesis, Genetic transformation, Pollinosis, Conifer, Particle gun.

**Theme:** Application of somatic embryogenesis and other vegetative propagation technologies in tree breeding and biotechnology

**Post doctoral project presentation**





## Regulation of embryo development, germination and plant growth in Norway spruce (*Picea abies*)

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The demand for higher production yields and better quality materials from the forests is increasing globally. In northern Europe, the vast majority of forest products are from coniferous species, mainly Norway spruce (*Picea abies*). Tree breeding programs are directed to meet the future demands on forests through seed production. Today, Norway spruce plants for reforestation are primarily produced from seed orchard seeds. Due to the long time from establishing a seed orchard to the seed is produced, about 15 years, the seedlings are genetically far behind the edge of breeding. Thus, in order to support the reforestation efforts, there is increasing need to complement the existing seed production with more efficient techniques for large scale propagation of trees with elite traits. Somatic embryogenesis (SE) is a technique that can be implemented in order to achieve those goals.

Somatic embryogenesis is a well-established *in vitro* method for experimental studies on embryo development. In addition, somatic embryogenesis has the potential to be used for large scale propagation of conifers.

We are using somatic embryogenesis as a model system to study nitrogen utilization during embryogenesis, from early stage embryos to plants. In particular, we are studying: (a) nitrogen sources required during embryo development; (b) how the nitrogen sources may affect embryo development and plant regeneration; (c) and the link between embryo development and enzymes involved in the nitrogen metabolism. To achieve the above mentioned aims, we are using embryogenic cell lines of Norway spruce with different capabilities of embryo development and plant regeneration. The results from these studies will contribute to our understanding of the regulation of embryo development in conifers. In addition, the results could be used to optimize the existing protocols for propagation of Norway spruce plant by somatic embryogenesis.

The results from this project also have the potential to advance the protocols used in the automated system process for large scale industrial production of Norway spruce plants.

Keywords: somatic embryogenesis, Norway spruce (*Picea abies*), plant regeneration, nitrogen source, nitrogen metabolism, enzymatic activity.





# Direct organogenesis from internodal explants of *Parkinsonia aculeata* L.

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## Summary

This report describes an in-vitro propagation protocol through direct organogenesis in *Parkinsonia aculeata* L., a native leguminose tree of wide distribution in Argentina. Internodal sections were cut from orthotropic-growing juvenile stems, collected from stock plants held under controlled conditions in a greenhouse.

Explants were surface disinfested, washed and inoculated in vertical position onto agar solidified Murashige & Skoog (MS) medium (1962) containing 3.0% sucrose supplemented with 1.5 ppm 6-bencil amino purine. The cultures were maintained at 25 +/- 2°C and a 16-h photoperiod (60mmol m<sup>-2</sup> s<sup>-1</sup>) using cool fluorescent lights. Adventitious shoot proliferation was observed throughout the cambium tissues on the distal end of the explants without formation of callus, suggesting the occurrence of direct organogenesis; Microscopical examination revealed vascular connection between adventitious shoots and maternal tissues.

## Conclusions:

- 1.- The experiment proved the positive effect of pruning in the rejuvenilization of stock plants in order to obtain juvenile explants suitable for *in vitro* propagation.
- 2.- The ontogenic stage of the plant used as source of explants represents a restriction to direct organogenesis in *Parkinsonia aculeata*. Juvenile internodal sections have proved to be an appropriate material for *in vitro* shoot formation.
- 3.- In internodal sections of *Parkinsonia aculeata*, direct organogenesis occurs from cambium tissues on the distal end of the explant if inoculated vertically in solid medium.

Keywords: in vitro culture, micropropagation, woody plant tissue culture, direct organogenesis







## **Non-freezing storage of initial explants affects positively somatic embryogenesis in *Pinus radiata***

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Traditionally, many efforts have been carried out in order to improve the success of somatic embryogenesis process in conifers, but little attention has been paid to the influence of the plant material storage conditions in the subsequent phases of the somatic embryogenesis process.

In this work our objective was to study the feasibility of storing *Pinus radiata* plant material at 4°C for a period longer than four weeks in order to make easier the initiation with high amount of plant material. The effect of cold storage on the different stages of somatic embryogenesis process has been evaluated. Storage periods of one to three months enhanced initiation rates and the number of somatic embryos obtained. These results demonstrate the beneficial effect of cold storage and open the possibility of considering a cold preconditioning of plant material as a good alternative to improve the somatic embryogenesis process in conifers.

Keywords: conifer, *in vitro*, micropropagation, radiata pine, somatic embryo.





## Analysing effects of an inhibited auxin transport on morphology and gene expression in somatic embryos of *Larix decidua* Mill

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The development of a proper mature embryo is closely linked to the formation of a fully functional shoot apical meristem (SAM). This process largely depends on a precise timely interaction of phytohormones such as auxin with genetic regulators. The auxin level and distribution during embryogenesis is a key factor to trigger cell differentiation and thus initiate cotyledon development and separation. Essential studies on the transferability of functions known from angiosperms to conifers have been initiated on *Picea abies*. Further analyses are necessary for detailed comparisons regarding conserved developmental strategies.

We used somatic embryos (sE) of *Larix decidua* as an experimental system, because these reach maturity within a month and yield up to 100 sE per 0.1 g FW. It is the object of this study to investigate the establishment of a proper meristem and cotyledon development depending on auxin distribution. To this end the polar auxin transport (PAT) has successively been disrupted or was restored with 1-N-Naphthylphthalamic acid (NPA) during embryogenesis. Thus a time frame which is crucial for correct auxin transport was defined at which sE are less affected by auxin flow disruption.

In order to find a way of measure, the embryos were grouped into several categories regarding cotyledon morphology using light and RE microscopy techniques:

NPA treatment caused two major types of embryos with distinct morphological aberrations - either forming embryos with a closed cotyledon ring – “Cups”, or a “Cucumber”-like embryo, with a pin-formed apex. Yet the later NPA was applied to the embryos, the lesser was the effect on its morphology. Though embryos are to some extent always affected by an NPA application and the restoration to a normal morphology can hardly be observed, yet it was observed that the NPA affected morphology is adjusted to transitional phenotypes. In an attempt to predict cotyledon development in early stages a sample of sE will be tracked throughout its maturation every other day.

Subsequent analyses were conducted to demonstrate the effect of NPA on the auxin distribution. Therefore the activity of the reporter gene GUS, which was controlled by an auxin inducible promoter (GH3::GUS) was studied in mature sE, which were continuously treated with NPA. The results indicated that NPA reduces the auxin concentration.

Furthermore changes in expression levels in embryos will be analysed with the intention to combine quantitative and spatial expression data for genes with relevance to embryogenesis and further apical-basal patterning [*LEAFY COTYLEDON1 (LEC1)*, *PINFORMED (PIN)*, *WUSCHEL-related HOMEODOMAIN 2 (WOX2)*].

Additionally a homologue to *SHOOT MERISTEMLESS (STM)*, which is known to regulate cell differentiation and establishes meristem identity in Arabidopsis, has been identified [*LdKNOX1-similar to STM (LdKN-sSTM)*]. Future functional analysis will establish if *LdKN-sSTM* holds marker characteristics to aid studies on meristem development in *L. decidua*.

We have demonstrated that the formation of a proper embryo apex depends on a regulated auxin flux, as the inhibition of the auxin transport affects embryo development.

Keywords: *Larix decidua*, Embryogenesis, Auxin, Cotyledon formation, NPA, Meristematic marker







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## **Participants and contact list**

