Casuarinas for Green Economy and Environmental Sustainability

Proceedings of the Sixth International Casuarina Workshop
Krabi, Thailand
21-25 October 2019

Editors
Maliwan Haruthaithanasan
Khongsak Pinyopusarerk
Abel Nicodemus
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IUFRO Taskforce Forest Biomass Network

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Front cover: *Casuarina equisetifolia* trees on a beach in South Thailand (K. Pinyopusarerk)
Back cover: An 18-month-old *Casuarina* hybrid clonal plantation in Tamil Nadu, South India (A. Nicodemus)

Foreword

The substantial socio-economic importance of casuarinas (several members of the family Casuarinaceae) has ensured ongoing international interest in research and development of this group of nitrogen fixing trees. Plantings are invariably associated with agricultural systems and there are more than two million hectares planted in the tropics and sub-tropics, offering stability to fragile sandy coastal ecosystems and offering services of protection, soil organic matter, poles, fuelwood and commercial wood fibre.

Following the previous five international casuarina meetings, the most recent one in Chennai, India in 2014, Kasetsart University is pleased to host the Sixth International Casuarina Workshop in Krabi province, Thailand during 21-25 October 2019 in collaboration with the IUFRO Working Party-Improvement and Culture of Nitrogen Fixing Trees, and the IUFRO Taskforce-Forest Biomass Network. The theme of this sixth workshop, Casuarinas for Green Economy and Environmental Sustainability, reflects in the current relevance of casuarina trees in the world. A total of 79 participants from ten countries attended the Workshop and delivered 43 oral presentations under six sessions.

Outputs from the Sixth International Casuarina Workshop are synthesized in these proceedings. I hope this publication is valuable for all stakeholders involved in casuarina research and development.

Dr Chongrak Wachrinrat
President
Kasetsart University

Preface

Extensive plantings of casuarinas have been developed throughout the tropics, including highlands, humid and semi-arid zones, by smallholders and Governments and increasingly by private forestry corporations. The continued successful development of casuarina forests and agroforests is dependent on research and development to provide improved and diverse germplasm, including for improved agroforestry systems, integrated pest and disease management, understanding of their tolerance to physiological stresses and climate change, and their wood properties including for pulpwood, in biorefineries and as biofuels.

International meetings of casuarina scientists are vital to develop a strong network of researchers in order to share ideas, information, germplasm and new technologies. Previous and highly successful international casuarina meetings have been held in Canberra, Australia in 1981; Cairo, Egypt in 1990; Danang, Vietnam in 1996; Hainan, China in 2010 and Chennai, India in 2014.

These proceedings report on the sixth international casuarina workshop held at Krabi, Thailand during 21-25 October 2019. In line with the theme of the Workshop, 'Casuarinas for green economy and environmental sustainability' the papers and discussions during the meeting were focused on assessing the impact of casuarina planting in meeting the industrial biomass raw material demand and improving edaphic and climatic conditions. In particular the additional benefits derived due to the research and development work carried out so far and increasing the accessibility of such new findings to all those involved in casuarina planting were reviewed.

The workshop was supported by Kasetsart University and the International Union of Forest Research Organizations (IUFRO), with crucial organizational support provided by the Kasetsart Agricultural and Agro-Industrial Product Improvement Institute (KAPI). Key international partners were the CSIRO (Australia) and IRD (France), and key national partners in Thailand were the Department of Marine and Coastal Resources, Royal Forest Department, and Forest Industry Organization.

Regional and international collaborations were proposed to share the expertise and germplasm from countries that have already made substantial genetic and economic gains through long-term research programmes with those that are beginning or in early stages of improving the species. Strategies for conservation of available genetic resources in the natural range and broadening of the genetic base and overcoming the biotic and climate change induced threats in the cultivated stands were proposed for long term securing of casuarina utilization.

Maliwan Haruthaithanasan
Khongsak Pinyopusarerk
Abel Nicodemus
David Bush
Lex Thomson

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Summary of discussions and recommendations

David Bush[1], Stephen Midgley[2] and Khongsak Pinyopusarerk[1]

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Summary

We report on the plenary session held on 25 October 2019 at the conclusion of the Sixth International Casuarina Workshop held at Krabi, Thailand. *Casuarina* continues to be a genus of considerable social, environmental and economic importance and considerable progress has been made over 40 years since the First International Casuarina Workshop was held in Canberra in 1981. Despite the acknowledged importance of the genus, complacency is challenging progress and there remains a need to quantify and share publicly the overall socio-economic benefits offered by this group of trees in terms of employment, livelihoods and provision of wood fibre for industry.

Several important issues were raised. These included:

- Concerns about pests and diseases
- Germplasm access and exchange
- Genetic diversity and clonal plantations
- Focus on industrial utilisation
- Expansion of casuarina plantations to areas beyond China and India

Some highlights of the technical discussions are synthesised below:

I Pests and diseases

Concerns about pests and diseases were raised throughout the conference. *Ralstonia* bacterial wilt has become a very serious concern in coastal protection forests in southern China (Zhang et al., these proceedings) and is an ongoing major concern in Guam (Schlub et al., these proceedings). Dr Schlub (Guam) commented that it is possible that the outbreaks in China and Guam may be different diseases, and this needs to be looked at more closely. Dr Kennedy (Australia) expressed interest in trying to get a better understanding of the interaction of the disease with casuarina root systems, as well as possible interacting edaphic factors including aluminium or other metal toxicity and soil decline. These factors have not been widely studied to date. The impact of disease in China and Guam is certainly very concerning. An increased understanding of the role of interacting factors including genetics, especially given the narrow genetic base of many plantations, and environmental factors with the disease should be an international priority. Other methods of control (see Karthikeyan et al. these proceedings) are also starting to show some promise.

II Germplasm

The need for new germplasm is ongoing. Firstly, even for species that have been widely tested and for which domestication is well underway (*C. equisetifolia* and *C. junghuhniana*), there is a need for infusion material. For *C. equisetifolia,* extensive collections across the species natural and planted ranges had been made by the CSIRO Australian Tree Seed Centre (ATSC) in the 1990s and thereafter. However, there are still some gaps in the collected range including in Indonesia. Many of the Pacific Island nations also

have geographically extensive populations that have been minimally sampled (for example there is quite unique inland population of *C. equisetifolia* in Fiji; many of the other countries have only had one or a few collected accessions). Drs Zhang and Zhong (China) commented that accession and testing of a wider range of *C. equisetifolia* germplasm for *Ralstonia* bacterial wilt resistance was a high priority for Chinese coastal plantings (see also Zhang et al. these proceedings).

A second area of interest raised by Dr Nicodemus (India) is potential for tropical provenances of *C. cunninghamiana* for hybrid work in India. The ATSC has only collected a few provenances of tropical *C. cunninghamiana,* though it has a very extensive range throughout the tropics. This would be another species of interest.

A third area of discussion was previously untested or minimally tested *Casuarina* species from the Pacific. Dr Thomson (Australia/Fiji) and SM mentioned *Casuarina oligodon, C. grandis, C.* 'Santo' and *C. junghuhniana* from Timor Leste. Some of these genetic resources have not been widely accessible and may have promise either as stand-alone species or in hybrid combinations. Dr Hardiyanto (Indonesia) mentioned that species and/or provenances that are well-adapted to peatland, saline and waterlogged conditions was a priority for their plantation programmes. Dr Thomson suggested that *C. collina* (New Caledonia) and certain *Gymnostoma* sp. would be worth testing, though neither have readily available sources of germplasm at present.

KP reminded conference delegates that import and export of forest genetic resources is not as easy as it once was: the process is now often lengthy, expensive and subject to both reasonable and unreasonable bureaucratic controls. The result is that both lead times and costs of germplasm need to be considered as major factors for prospective tree breeders. Dr Kien Nguyen (Vietnam) mentioned that international exchange of germplasm within and among species is a very useful way to broaden germplasm accessions, especially of already-improved species. DB said that the ATSC would be happy to facilitate exchange of germplasm among nations and would also look at collecting new material from the wild where possible as long as there was a firm commitment from potential users to help meet the costs of collections.

III Clonal diversity

A keenly discussed topic linked closely with germplasm acquisition and testing was the role of clonal forestry and the importance of clonal diversity in casuarina plantations. Clonal forestry is the norm in both India and China (see Zhang et al., Sakthivel, Seenivasan et al. these proceedings). Dr Nicodemus (India) said that low clonal diversity is a real worry in the context of widespread exposure to diseases. He argued that developing and planting a wider range of species and hybrids would be a complementary part of the solution, additional to ensuring that a sufficient selection of clones of the main species (*C. junghuhniana, C. equisetifolia*) are made available. Dr Zhong said that in China, while there is selection of clones for disease resistance, only one or a few clones are typically planted over extensive areas and that these clones tend not to be useful for more than one or two rotations. It is a priority in China to develop *C. equisetifolia* clones that have better disease resistance, and also to understand the interactions between crops and forest plantations. A further consideration there is that plantations immediately adjacent to the coast are often left solely for environmental purposes, so clones for these littoral belts need not be selected for especially fast growth rates: mixed clones with the highest levels of disease resistance could be deployed. Dr Ashok Kulkarni asked whether seedlings would be a better prospect than clones for diversity and potential resistance to diseases – the consensus was that though there may be a growth performance penalty, this may be a good strategy in many circumstances.

IV Industrial utilisation

Industrial use of casuarinas remains substantial and the species and hybrids remain particularly important for construction and pulp industries, however industry commitment to ongoing research and development

remains limited. There is an obvious need to reinforce the importance of the genus to the private sector and a need to quantify the benefits the genus brings.

Industrial utilisation was not a major focus of this workshop (though see overview – Midgley; biorefinery opportunities – Jahan et al., wood properties – Sompoh et al., these proceedings), but discussion ensued about rotation times and potential products. SM said that a difficulty in producing sawn timber is that growers typically want to cut early to realise cashflow, but sawn timber requires a much longer rotation time. Another product that would be higher value than pulpwood is veneer: this can be produced from logs that are smaller than sawlog size and the wait is not so long. The returns can be quite attractive, particularly if appearance grade products can be targeted. The Kenyan delegation agreed that industrial utilisation is of interest to them and that this would be a good focus topic for the next conference.

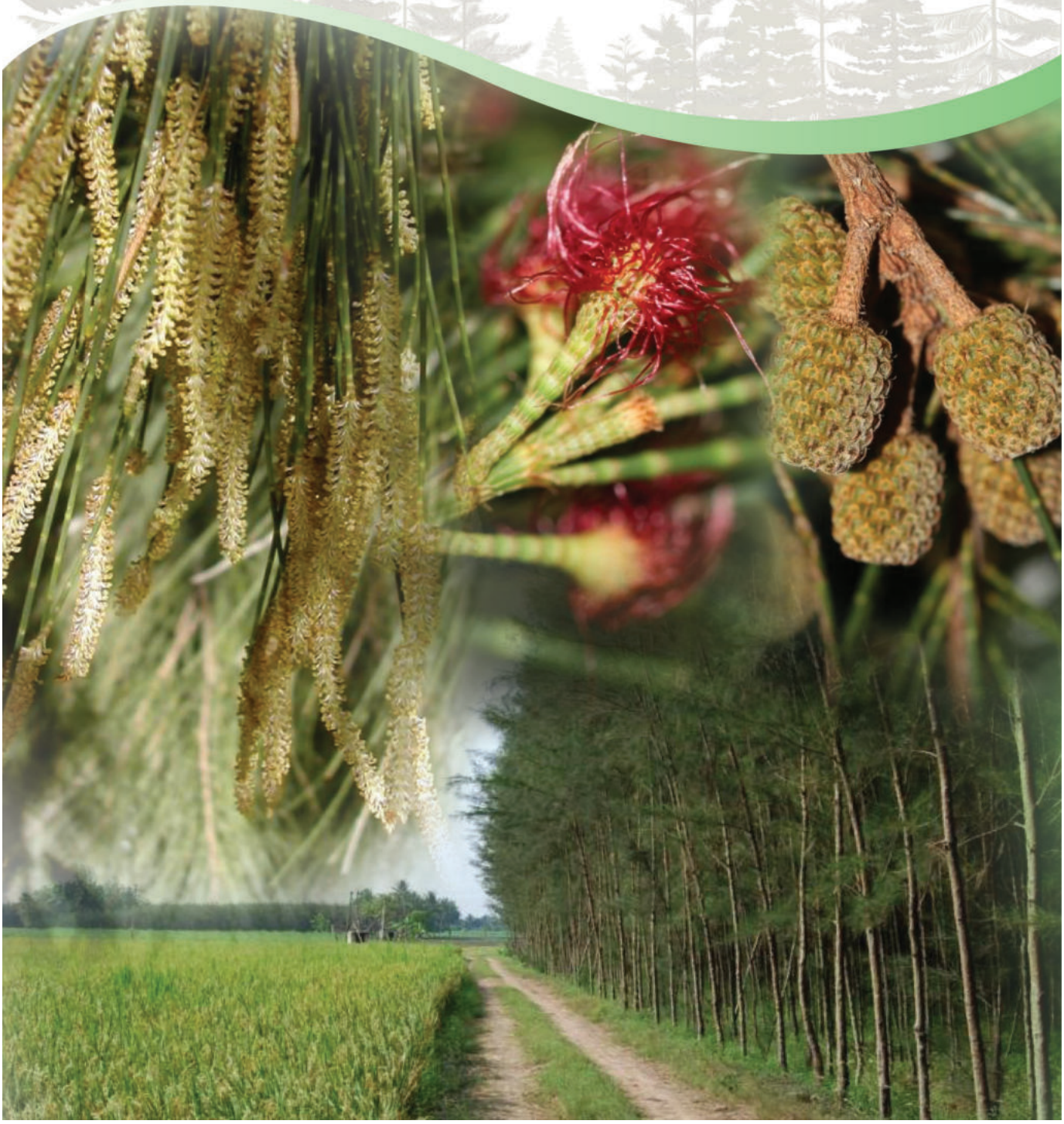
V Expansion of plantations

The topic of expansion of widespread casuarina plantations outside India and China was discussed with inputs from Kenya and Indonesia. Both countries are examining options and either establishing new tests (see Hardiyanto et al. these proceedings) or re-examining the results of existing ones (see Kimondo et al., Kimani et al., – these proceedings). In both cases, accessing existing knowledge and potentially, genetically-improved germplasm, would provide a boost to industry establishment. There may also be a need for novel research as environments are quite different to those typical in either China or India (for example waterlogged and peat soils in Indonesia as discussed by Dr Hardiyanto).

VI Summary remarks

The workshop, and its plenary discussion, encompassed a wide range of topics covering many aspects of casuarina ecology, pests, diseases and their management, genetics and industrial utilisation. Inputs from researchers representing well-established research and development programmes in India and China were complemented by a number of excellent contributions from parties with emerging and growing interest in casuarina. Though disease is a threat to casuarina plantations, new strategies involving use of a greater diversity of clonal material, seedling-based germplasm and tapping the wider genetic base of casuarina pure species and hybrids are all strategies that hold promise. Further development of casuarina-based products and industries is also an area that is likely to see continuing growth in the next decade and beyond.

Status reports



Proceedings of the Sixth International Casuarina Workshop

Four decades of international research and development in casuarinas

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Abstract

Compared to other more commercially important forest tree species such as eucalypts and pines, research into casuarinas is relatively recent. The discovery of *Casuarina/Frankia* association in the 1960s marked the beginning of research interest in casuarinas. However, major interest in casuarina research began as a result of the first international casuarina workshop in Australia in 1981 which near-coincided and preceded several international development projects on fuelwood species for rural community in developing countries.

The primary role of casuarina plantings is still often perceived as for environmental protection or ecological restoration, not for industrial wood production. Since the mid 1990s, paper mills in India started using casuarina wood as pulpwood raw material. In southern China, casuarina wood has been processed by woodchip mills and veneer factories since the early 1990s. The development in these countries has generated continuing interest in casuarina species.

This paper presents an overview of research and development in casuarinas over the past four decades providing a platform for discussion on future research and development directions in casuarinas.

Keywords: Casuarinaceae, taxonomy, *Casuarina-Frankia* associations, genetic improvement, pests and diseases, utilization, international cooperation

1 Introduction

Many species of the Casuarinaceae family have been planted for wood production, shelterbelts, environmental protection, and ecological restoration dating back for more than a century. Extensive planting of casuarinas has been undertaken in countries outside the species' natural distribution range (Midgley et al.,1983; El-Lakany et al., 1990; Pinyopusarerk et al., 1996). Some species, especially *Casuarina equisetifolia* and *C. glauca* have become naturalized in countries that introduced them.

It was not until the early 1960s when the biological nitrogen fixation ability of casuarinas was realized, followed soon after by the discovery of *Casuarina/Frankia* associations. By the late 1960s, introduction and large-scale planting of casuarinas had become extensive around the world from Asia (e.g. China, India and Vietnam) to the Middle East and East and West Africa, as well as to a smaller extent in South

America and southern United States. Casuarinas were further promoted throughout the 1980s as a major firewood crop by international development agencies such as the Food and Agriculture Organization of the United Nations (FAO), the US National Research Council and the Australian Centre for International Agricultural Research (ACIAR).

Wide interest in research in casuarinas did not begin until the first international casuarina workshop held in Australia in 1981. Subsequently, four more international casuarina workshops have taken place in Africa and Asia, the most recent one being in India in 2014. These workshops have been vital for fostering and catalyzing research and development in casuarina ecology, genetic resources, biotechnology, *Casuarina-Frankia* association, plantation silviculture, tolerance to adverse conditions, susceptibility to biotic and abiotic factors, and wood properties. This paper summarizes casuarina research and development activities carried out over the past four decades. However, the paper by no means attempts to cover all aspects of casuarina research.

2 *Casuarina-Frankia* associations

The ability of casuarinas to thrive on nitrogen-deficient soils is due to their symbiosis with the actinomycete *Frankia* which results in the fixation of atmospheric nitrogen. Such beneficial symbiotic relationships were recognized in the early 1900s. Various aspects of the associations between casuarinas and root nodule *Frankia* were widely studied during the 1970s and 1980s when potential of casuarinas as multipurpose species was being investigated and widely promoted. Successful application of *Frankia* inoculation was further enhanced because the effectiveness of *Frankia* strains is not necessarily species-specific (Sellstedt, 1995; Yang et al., 2007; Hoang and Dommergues, 2011).

More recently, the three-way symbiotic associations of *Casuarina-Frankia*-endomycorrhizal fungi was found to further enhance biomass productivity of casuarinas. Dual inoculation with *Frankia* and mycorrhizal fungi helps in adaptability under stress and nutrient-limiting environments and in turn increases productivity. Advances in genetics and molecular biology has enabled an improved understanding of the molecular basis underlying the specificity of symbiotic interactions.

Although the benefits of *Casuarina-Frankia* symbiotic association are clearly demonstrated, inoculation is still not widely practiced either at nursery seedling stage or in the field. This is attributable to a perception that inoculation is not necessary in areas where casuarinas have already been planted.

3 Taxonomy and distribution

Casuarinaceae family is unique among the angiosperms, having been assigned to an order of its own, i.e. the Casuarinales (Beadle, 1981). This family extends from Australia to the islands of Pacific and South-east Asia (Turnbull, 1990). Until 1980, all 96 tree and shrub species of the family belonged to a single *Casuarina* genus. Accumulated evidence from morphology, anatomy and cytology led to the current recognition of four genera: *Gymnostoma* (Johnson, 1980), *Allocasuarina* (Johnson, 1982), *Casuarina* and *Ceuthostoma* (Johnson, 1988). The number of leaf teeth in a whorl, size and shape of fruiting body (cone) and colour of seed are useful characteristics for differentiating genera and species.

Gymnostoma is a genus of 18 tropical tree and shrub species distributed in the Philippines, Malaysia, Indonesia/New Guinea, Solomon Islands, Fiji, New Caledonia and northeastern Australia. Species of this genus have four leaf teeth.

Allocasuarina consists of 59 species all distributed in Australia. The number of leaf teeth ranges from 4 to 15.

Ceuthostoma, the only genus not represented in Australia, consists of two species in Malaysia and the Philippines. A distinctive feature is that they have four leaf teeth like *Gymnostoma* species.

Casuarina genus consists of the remaining 17 species (Wilson and Johnson, 1989) distributed widely in Australia, Southeast Asia and Pacific island countries. The number of leaf teeth per whorl ranges from 4 to 20.

4 Breeding system

Casuarinas are predominantly dioecious with male and female inflorescences on different trees but monoecious reproductive biology, with male and female inflorescences on the same tree, has been reported for two species in the genus *Casuarina,* i.e.

C. equisetifolia and *C. junghuhniana*. Casuarinas are wind-pollinated and mostly dioecious, and it is reasonable to consider they are obligate outbreeders.

The sex ratio in *C. equisetifolia* trees was studied during 1980s and 1990s with varying findings. An earlier report in India showed that male and female trees in a given stand occurred at 56% and 42% respectively with monoecious trees accounting for 2-3% (Dorairaj and Wilson, 1981). In contrast, Le and Phi (1993) reported 20% male trees and 5% monoecious trees in casuarina plantations in Vietnam, while in Thailand Luechanimitchit and Luangviriyasaeng (1996) found 48% male and 35% female trees in a mature stand with up to 8% monoecious trees. In a more recent study, Schlub (2011) reported that *C. equisetifolia* population on Guam Island was comprised of 80% monoecious and 13% dioecious trees (3 % male).

Published information on chromosome numbers in Casuarinaceae appears to be limited to those reported in Barlow (1959). The number differed among the genera. *Gymnostoma* and *Casuarina* have small chromosomes with n = 8 and n = 9, respectively, and with no reported polyploidy. In *Allocasuarina,* the number of chromosomes is larger, ranging from n = 10 to n = 14, and with polyploidy frequent. There is no information on ploidy in *Ceuthostoma* species.

5 Genetic improvement and breeding programmes

Research on genetic improvement and breeding in casuarinas is recent compared to other commercially planted forest tree species. During the 1980s and 1990s, much of the work was limited to provenance trials involving four species: *C. cunninghamiana, C. equisetifolia, C. glauca* and *C. junghuhniana*. Systematic genetic improvement programmes for *C. equisetifolia* started in India and China towards the end of 1990s (Zhong et al., 2011; Rawat et al., 2011), and for *C. junghuhniana* in India and Thailand in the early 2000s (Luechanimitchit et al., 2011; Nicodemus et al., 2011). These programmes were made possible through range-wide seed collections, from the species' natural distribution range and landraces, coordinated by CSIRO Australian Tree Seed Centre. Genetically improved seed and clones are now available. In addition, hybrids of *C. equisetifolia* and *C. junghuhniana* were successfully developed through artificial pollination (Nicodemus et al., 2011; Pauldasan et al., 2016). The current improvement programmes have advanced to second generation populations with more of a focus on hybrid breeding involving the four *Casuarina* species.

Morphological and molecular markers have been used successfully to determine genetic diversity and genetic relationship among species within the genus *Casuarina* and between *Casuarina* and *Allocasuarina* (Moran et al., 1989; Kamalakannan et al., 2006; Hu et al., 2016). More recently, morphological and microsatellite-based nuclear DNA have further elucidated population structure and provided evidence for the origins of landraces in *C. equisetifolia*.

6 Propagation

Casuarinas are normally propagated by seed which is abundant and easy to handle. Seed of casuarinas is reported to be short-lived, 1-2 years. However, germination tests at CSIRO Australian Tree Seed Centre revealed that seed of *C. junghuhniana* and *C. equisetifolia* stored air-tight in cool room (3-5 °C) for 22 years still maintained viability close to or slightly less than the initial germination rates (CSIRO Australian Tree Seed Centre, unpublished data).

As genetically improved clones from breeding programmes are increasingly being planted in large-scale—notably *C. equisetifolia, C. junghuhniana* and their hybrids in China and India—vegetative propagation by cuttings taken from young shoots has expanded rapidly. The cuttings propagation is able to be carried out in greenhouses or mist chambers. Commercially available root hormones are suitable for speeding up and improving rooting percentage.

In China, water culture is now the common method for cutting propagation of casuarinas (Liang and Chen, 1982). Young branches 8-10 cm in length preferably less than 3 months old are taken from stock plants and soaked in 50-100 ppm of NAA (naphthalene-acetic acid) or IBA (indole-butryic acid) solution for 24 hours. They are then washed and soaked in clean water which must be changed daily. Temperature of the water needs to be maintained at 25-32°C to obtain optimum results. The cuttings develop roots within two weeks, and thereafter are transplanted into containers and grown in open nursery beds. Plants are ready for planting out after a further 10-12 weeks in the nursery.

7 Utilization

Casuarinas are truly multipurpose trees with a wide range of applications in the form of wood and non-wood products and services. The wood is renowned for excellent firewood qualities with high calorific value of 20.9 Mj kg^{-1} (Pinyopusarerk and Midgley, 2018).

More recently, the wood of *C. equisetifolia* and *C. junghuhniana* is being increasingly used to produce paper pulp, especially in India. Numerous studies confirmed that the wood of both species has the pulping properties similar to those of *Eucalyptus* and bamboo species which are widely used by the paper industry (Kumar et al., 1996; Paul and Rao, 2001; Ramadevi et al., 2011; Satishchandra and Kuila 2012; Warrier et al., 2012; Nicodemus et al., 2015).

The most common use of casuarinas for environmental protection is in stabilization of coastal sand dunes. Research in China found that sand dunes on Hainan Island moved inward 1.6 km during 1749-1949, an average of 8 m $year^{-1}$ (Li and Huang, 2011). Wind protection efficiency of casuarinas was a research focus in China in the 1990s and 2000s, and effective structure of shelterbelts recommended (Huang et al., 2003; Wang et al., 2008; Zheng et al., 2008). Casuarina shelterbelts have stopped or slowed the moving sand to an insignificant level. In Vietnam, study showed that casuarina shelterbelts effectively stabilize moving sands in the central coast after just three growing seasons (Ha and Le, 1996).

8 Pests and diseases

Pests and diseases affecting casuarina productivity have been documented but there has been no coordinated research on management of the impacts. The two most serious and widely documented diseases are blister bark, *Trichosporium vesiculosum* and bacterial wilt, *Ralstonia* (*Pseudomonas*) *solanacearum*.

Blister bark disease, associated with the fungus *T. vesiculosum*, is the most destructive disease of *C. equisetifolia* in India (Mohanan and Sharma, 1993; Karthikeyan et al., 2011) and to a lesser extent in China, Kenya, Thailand and Vietnam (Narayanan et al., 2003). This disease is characterised by initial foliage yellowing, rapid wilting, then desiccation, browning and dieback of trees, either singly or in group. The blistering symptom is the longitudinal crack of the bark exposing the black sooty mass. There are no effective preventative or control measures other than removal and burning of dead or dying trees.

Bacterial wilt *R. solanacearum* is the most serious disease killing casuarina trees In China and less so in India. In China it was first observed in casuarina plantation in Guangdong province in 1964 (Liang and Wang, 1982), and later extended to other southeast coast provinces including Guangxi, Fujian, and Hainan after a typhoon in 1969 (Sun et al., 2013). In the past, disease-affected trees were often scattered and in small numbers, sometimes just a few individuals. In recent years, the mortality has occurred in large plots of 7-15 ha. At present, total area of casuarina plantations affected by bacterial wilt is estimated to be around 10,000 ha mostly in the western part of Guangdong (Zhang Yong 2020, pers. comm.).

Decline of *C. equisetifolia* associated with *Ralstonia* bacterial wilt is currently a major research subject on Guam in the northern Pacific (Schlub et al., 2011; Ayin et al., 2019). The decline was first noticed in 2002 in a single windbreak row of casuarina trees less than 10 years old. Initially five trees were characterized as wilted with the following symptoms: acropetal chlorosis, tip-burn of lower branches giving the tree a singed appearance, and tree death within 6 months. Soon after dieback of casuarina trees was rapidly spreading to other areas on the island such that by 2008, half of Guam's 115,000 casuarina trees were affected. Wood samples of the dead trees have always tested positive for bacterial wilt, *R. solanacearum* although other wetwood bacteria e.g. *Klebsiella* sp. have also been present in some instances. There are striking similarities among cross-sections of logs affected by the bacteria wilt in China and those of trees in Guam.

9 Negative impacts of casuarinas

Casuarinas can pose unintended negative impacts if their planting is not properly managed. Three *Casuarina* species are known to be planted in the USA for ornamental and roadside planting and as windbreaks. *Casuarina equisetifolia* was the first casuarina species to be introduced to Hawaii and Florida in the 1890s, followed by *C. glauca* and *C. cunninghamiana* in 1924 (Morton, 1980; Wheeler et al., 2011). *C. equisetifolia* (known as Australian pine) and *C. glauca* are highly salt tolerant and widespread in coastal areas, while

C. cunninghamiana invades riverine habitats.

The fast growth, prolific seeding and thick litter accumulation of casuarinas impedes the establishment of native plant species and their associated herbivores, disrupting natural processes. The invasiveness has become so problematic that possession, collection, cultivation, transportation and importation of casuarinas are not permitted in Florida. In 2013, an 'Australian Pine' task force was set up to develop state-wide management plan to protect native flora and fauna (Pernas et al., 2013).

10 Concluding remarks

Considerable progress has been made in casuarina research and development over the past four decades. Species such as *C. equisetifolia* and *C. junghuhniana* have become commercially important plantation species for the wood-based industry, in particular the paper industry in India. There is evidence of application of research and development results especially in a wide adoption of improved genetic materials by farmers and wood industries in India.

There has also been a shift in species focus over the past four decades. *Casuarina cunninghamiana* and *C. glauca* were studied more extensively during the 1970s-1980s. In the 1990s and 2000s, the majority of research publications were related to *C. equisetifolia.* Since 2010s, *C. junghuhniana* has been the focal point because of its fast growth and good adaptability.

The sixth casuarina workshop in Thailand, nearly four decades after the first, reflects the continuing interest in casuarina research and development. It provides an excellent opportunity to prioritise research directions and discuss implementation strategies.

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Contributions of casuarina planting to India's green economy and environmental sustainability

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Abstract

Casuarina has a high level of socio-economic and environmental significance in India. The diverse attributes of the tree are aptly utilized for various services and end uses. Shelterbelts benefit from the ability to withstand salt spray, afforestation in nutrient-poor areas is helped by the nitrogen-fixing ability, amenability to grow in high density plantations facilitates short rotations and the minimal requirement for cultivation inputs engenders farmers' acceptance. The current estate of *Casuarina* (*C. equisetifolia* and *C. junghuhniana*) plantations is estimated to be around half a million hectares and fulfils around 15% of pulpwood requirement (around 1.65 million tonnes per year) of paper industries in India. The market for poles, the other major use of casuarina, is estimated to be about twice that of pulpwood and the overall annual turnover of casuarina wood is around USD 471 million. Considering that the average landholding in India is around two hectares, at least 250,000 farmers are involved in casuarina cultivation indicating the extent of its socio-economic importance. In the context of scanty and erratic rainfall, unstable prices for agricultural products and acute shortage of farm labour in the rural areas, tree cultivation functions as insurance for farmers and provides stability to the farm economy. Casuarina has been the most preferred alternative to eucalypts, especially when the latter suffered heavily from *Leptocybe* gall insect and in areas where its planting is restricted. The plywood industry, which heavily relies on eucalypt wood, has also started exploring the possibilities of using casuarina wood for manufacturing particle and MDF board. Pilot plantations raised with drought tolerant species like *C. junghuhniana* are promising for cultivation under low rainfall (600 to 800 mm) regions where the soil is generally degraded. The other emerging trend is to integrate casuarina trees with agricultural and other tree crops. Windbreak agroforestry with specially selected casuarina clones offers both protection from wind damage and improvement in microclimate to increase yield in crops like banana. Casuarina is also extensively used as a nurse plant for the sandalwood tree (*Santalum album*) which is a hemi-parasite. The outlook for casuarina cultivation in the next decade seems promising both for the farmers and the industries. Twenty years

of breeding and development has resulted in planting stock with significantly improved growth and wood properties, boosting the yield (and/or shortening the rotation period) substantially. The additional wood production expected in the near future is likely to be absorbed by the emerging new areas of usage.

Keywords: plantation, poles, pulpwood, shelterbelt, socio-economics, windbreak

1 Introduction

India is a wood-deficient country. Although the per capita consumption of wood and wood products is among the lowest in the country, the demand is high due to a large population and the rapid economic progress made during the last two decades. The domestic production is unable to fulfil the need which is limited by many factors. India's forests have the lowest productivity of 0.45 m^3 ha^{-1} $year^{-1}$ compared to the world average of 2.1 m^3 ha^{-1} $year^{-1}$. Similarly forest plantations also have low productivity of 0.77 m^3 ha^{-1} $year^{-1}$. The per capita forest area is 0.06 ha which is just one-tenth of world average. The current policy of the government is not to use the natural forests for any commercial extraction of wood and timber and their demand to be entirely produced outside the forests. The total wood production in the country during 2015 is 456 million m^3 of which wood produced from natural forests is a meagre 1.25 million m^3 only. Around 85% of wood produced is used as fuelwood for meeting domestic energy needs (60%). The total timber and allied (pulp, ply) wood production is 71 million m^3 and around 18 million m^3 is imported (25%) which is worth USD 6.7 billon to meet the gap in the demand. The import projection for 2030 is 31.5 million m^3 (Shrivastava and Saxena, 2017).

Trees outside Forests (ToF) which constitute all trees grown outside the natural forest area under different systems like agroforestry, social forestry and farm forestry are the main source of wood and timber in India. During 2015, these tree cultivation systems produced 44.3 million m^3 of wood and timber coming mostly from the species, *Casuarina, Eucalyptus, Leucaena,* Poplar and Teak. These sources meet about 80% of raw material needs of plywood industries and about 60% of pulpwood need. In this context, the contribution of casuarina to India's green economy and environmental sustainability is substantial.

2 Casuarina in India

Casuarina is grown extensively throughout India as a multipurpose tree for many products of economic importance and environmental services. Casuarina cultivation in India has a high socio-economic importance as it is closely connected with livelihood opportunities of smallholding farmers and landless agricultural labour. *Casuarina equisetifolia* was first introduced into India in Karwar, Karnataka during 1868 (Kaikini, 1937) to fulfil the increasing need for fuel wood, preventing sand movement in the coast and to enhance aesthetics through beach and avenue planting. Since then this versatile tree has had a remarkable journey of nearly 150 years evolving into a dependable crop for wood production, environmental services and livelihood improvement.

3 Extent of plantations

India is the largest casuarina-growing country in the world with an estimated half a million hectares of plantations mainly concentrated in the States of Andhra Pradesh, Odisha, Tamil Nadu, Telangana and Union Territory of Puducherry in the east coast. It is also cultivated to a lesser extent in the States of Karnataka, Maharashtra, Gujarat and West Bengal. It is one of the five most extensively planted tree crops in the country and is harvested in short rotations of two to five years.

4 Major uses and services

The principal uses of the wood include pulpwood, poles and fuelwood. It is being considered by the plywood industry for the manufacture of plywood and MDF boards due to shortage of wood raw material. Casuarina is also preferred for environmental services like shelterbelts, wind breaks and reclamation of mined areas. Casuarina is a nitrogen-fixing tree through symbiotic relationship with the actinomycete bacteria called *Frankia*. This ability helps the tree to establish well even in nutrient-deficient sites and also to maintain nutrient balance in high density plantations. It is planted as an alternative to eucalyptus where the latter was heavily infested with the gall insect, *Leptocybe invasa*. It is also a preferred host plant for the high value Indian sandalwood tree which is a hemi-parasite. The market demand for casuarina wood has steadily increased over the years mainly due to expansion of production capacity of paper industries.

5 Indian paper industry and casuarina

India has more than 800 paper mills of which 32 are considered to be large units with a production capacity of 100,000 tonnes or more per annum. These mills contribute 28% of installed capacity followed by medium-sized mills (63%) and small sized ones (9%). The compounded annual growth rate (CAGR) is around 8% which is considered one of the fastest industrial growth. With an installed capacity of 4.66 million tonnes per annum, the Indian paper industry accounts for about 4% of global paper production and have an annual turnover of USD 8 billion. Although the share of wood as raw material for paper making is 11.6 million tonnes per year it is only about 25% of total raw material used. Agro-residues and waste paper are the other raw materials used. In consistent with the government policy, use of bamboo harvested from natural forests which used to meet around 30% of wood raw material need has gradually declined to just 5% today that too mostly coming from farmlands. The increased investment of mills on farm forestry programmes on farmers' land to meet their pulpwood needs has helped to meet nearly 60% of their demand from them. On the other hand the reliance on government supplies has been brought down from 40% to 13% in a span of two decades. India's per capita paper consumption is among the lowest in the word (13 kg) which is expected to become 17 kg by the year 2025. It is estimated that every one kg increase in per capita paper use will require an additional one million tonnes of pulpwood to meet the demand for paper. Accordingly the projected pulpwood need in 2025 is 15 million tonnes (IPMA, 2020).

Casuarina is an important source of pulpwood raw material for papermaking in India. The annual consumption of casuarina wood for papermaking is around 1.65 million tonnes which is 15% of total pulpwood used in India. It is the main pulpwood raw material for five major paper mills constituting 20-50% of their total pulpwood need (Table 1). The other major market for casuarina in India is poles for construction, scaffolding and agricultural use. Since the pole market is unorganized, precise data on the amount of wood used is not available. But it is generally estimated to be twice that of pulpwood and the price of poles is roughly 50% higher than that of pulpwood. Considering these facts, the total value of casuarina wood produced per year is around USD 471 million which indicates the substantial socio-economic importance of casuarina to a large number of smallholding farmers who raise the casuarina plantations.

6 Traditional cultivation practices

Traditionally casuarina is considered to be a tree of coastal areas. It was originally introduced in the coastal region and its cultivation expanded along the coast as wood-producing plantations as well as shelterbelts protecting human habitations from cyclones and preventing formation and movement of sand dunes. Over a period of time and with increasing demand for pulpwood for papermaking, farmers started cultivating casuarina in areas away from the coast (inland) as well (Lal et al., 1996; Paul and Rao, 2001). It is common to find casuarina plantations and agricultural crops like paddy are grown alongside each other in South India. Apart from the traditional sandy soils it is now cultivated in all types of soils including clayey soils experiencing a short duration of water-logging. Only in soils extremely saline or alkaline that it fails to establish altogether or put up poor growth. Generally casuarina needs irrigation support at least during the first year for good survival and growth.

Table 1. Average annual pulpwood consumption in major paper mills of south India.

Paper mill	Annual pulpwood consumption (10^3 tonnes)	Casuarina pulpwood (10^3 tonnes)	Share of casuarina wood (%)
Andhra Paper	800	320	40
JK Paper	900	450	50
Seshasayee Paper and Board	600	300	50
Tamil Nadu Newsprint and Papers	800	400	50
West Coast Paper Mills	900	180	20
All Mills in India	11,000	1,600	15

Casuarina farmers normally adopt a narrow spacing of 1x1 m with a stocking of around 10,000 trees ha^{-1}. Such a high density planting is preferred mainly due to large growth variation among the trees raised from unimproved seed sources. Bare-rooted seedlings produced in local nurseries are planted in the well-ploughed fields during the rainy season. Under irrigated conditions, an intercrop is grown during the first three to four months after planting when there is sufficient light penetration in the plantation due to small size of the trees. Intercropping also helps in managing weeds. Pruning of side branches is carried out first and second year after planting. Fertilizers are also applied during this period. The usual rotation period is 4 to 5 years under irrigation and 6-7 years if grown as a rainfed crop.

7 Productivity

The average yield from a 4-year old plantation raised with unimproved bare-root seedlings is 100 tonnes of pulpwood ha^{-1} (25 tonnes ha^{-1} $year^{-1}$). This yield is obtained from the original stocking of about 10,000 trees planted at a spacing of 1 m. The average yield per tree is only 10 kg which is far below the potential of the land and the species. The main reason for this low yield is the tree-to-tree variation found in such high-density plantations. More than a 10-fold difference in wood production is observed between the smallest and the largest tree. Further the proportion of small trees is far greater than that of large-sized trees which produce a major portion of merchantable wood (ICFRE, 1994). Till recently clonal plantations of casuarina has been practiced in a limited area by planting only a clone imported from Thailand commonly referred as 'Marakkanam clone' or CJ-1 (Thirawat, 1953). This clone yielded around 125 tonnes of pulpwood ha^{-1} in a rotation period of four years under irrigated conditions (31 tonnes ha^{-1} $year^{-1}$).

8 New varieties released

There are two species of *Casuarina* grown in India. *Casuarina equisetifolia* is under cultivation for over a century and is generally regarded as a coastal species. Plantations are mainly raised through seedlings grown from seeds. Genetically improved seed varieties produced from seed orchards are supplied by the Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore. The other species of *Casuarina* currently under cultivation is *C. junghuhniana* introduced by IFGTB about 20 years ago from Indonesia in collaboration with the CSIRO Australian Tree Seed Centre. It is generally faster growing and more drought and disease tolerant than *C. equisetifolia*. It is also a wind-hardy species. The bark of *C. junghuhniana* is thicker and rougher than that of *C. equisetifolia* providing protection from moisture stress. IFGTB has been implementing systematic breeding programmes for the two *Casuarina* species and established breeding populations, seed orchards and clonal tests in different locations. Clones were assessed periodically for fast growth, stem straightness, incidence of pest and disease attack, wind-hardiness, drought tolerance and ability to grow in sodic soils and the best performance across different test locations were shortlisted for public release. The details of high-yielding clones of *Casuarina* released by IFGTB are given in Table 2.

The new clones on an average yield 20 tonnes more pulp wood per hectare than the currently cultivated clone (CJ-1) in a rotation period of four years (5 tonnes ha^{-1} $year^{-1}$). At the prevailing price of pulpwood (USD 57 per metric tonne), a farmer gets an additional income of around USD 1140 ha^{-1} in a rotation period of four years (USD 285 ha^{-1} $year^{-1}$). Till July 2018, nearly 16,500 ha of plantation raised through genetically improved seeds and clones were harvested. The value of additional wood produced from planting superior stock is estimated to be USD 16.22 million (Table 3). These clones also increase the availability of pulpwood raw material to paper industries with 1 to 2 percent more pulp yield than the benchmark clone.

The new clones are popularized among farmers through establishing on-farm demonstration plots in locations where casuarina is widely grown. Farmer visits are arranged to these plots through various training and awareness programmes to facilitate farmer-farmer interaction on the cultivation of new high-yielding clones. In order to make these clones easily accessible to farmers in different parts of the country, licenses have been issued to paper industries and private nurseries for commercial propagation and supply. The licensed nurseries produce around 45 million plants in a year and supply to farmers for raising plantations.

9 Improved cultivation practices

Casuarina cultivation in India has undergone major changes during the last decade particularly when genetically improved planting material is used. A comparison of cultivation techniques for seedling and clonal plantations is given in Table 4. The major change that happened recently is reduction in the number of trees grown per unit area. With the development of clones possessing uniform growth and opportunities to sell the wood on the basis of weight has removed the need for high density plantations. The widely practiced spacing in clonal plantations is 1.5 m (roughly 5 feet) between trees which works out to be 2.25 m^2 $tree^{-1}$. At this spacing about 4,500 trees are planted in a hectare. If the plantations are intended to be harvested

Table 2. End-use and adaptability features of casuarina clones planted in India.

Taxa	Clone name	Salient end-use features	Special adaptability features
C. equisetifolia	IFGTB-CH-5 IFGTB-CH-6 IFGTB-CH-7	Fast-growth and straight stems; thin and smooth bark	High adaptability to sodic sites (pH 9 and above)
C. junghuhniana	IFGTB-CJ-WB-1 IFGTB-CJ-WB-2 IFGTB-CJ-WB-3 IFGTB-CJ-WB-4 IFGTB-CJ-WB-5	Ideotype suitable for windbreak function; fast growth and straight stems.	Suitable for windbreak agroforestry systems.
C. junghuhniana	IFGTB-CJ-9 IFGTB-CJ-10	Fast-growth; straight stem; high-pulp content; thick and fissured bark	Drought-tolerant; suitable for Inland area and all soil types.
Casuarina hybrid (*C. equisetifolia* x *C. junghuhniana)*	IFGTB-CH-1 IFGTB-CH-2 IFGTB-CH-3 IFGTB-CH-4 IFGTB-CH-5	Fast-growth; straight stem; high-pulp content; thin and smooth bark	Suitable for both coastal and inland area except clayey soils

Table 3. Gains from increased wood production by genetically improved planting material of casuarina in India.

Particular	Seedling-raised plantations	Clonal plantations
Area harvested till July 2018 (ha)	9,000	7,480
Minimum gain (tonnes ha^{-1})	15	20
Value of additional wood (USD ha^{-1})	855	1,140
Total additional income to farmers (USD million)	7.69	8.53

Table 4. Cultural practices adopted for casuarina plantations raised from seedlings and clones in India.

Cultivation technique	Unimproved seedling plantation	Clonal plantations
Spacing (m)	1 x 1	1.5 x 1.5
Number of trees ha^{-1}	10,000	4,500
Rotation age (years)	4–5	2.5–4
Irrigation method	Flooding	Flooding or trickle
Number of pruning of side branches	1–2	3–4
Fertilizer application	Broadcasting	Targeted
Average survival at the time of harvest (%)	75	90

at the age of 3 years or less, the spacing can be even 1.2 m x 1.2 m. With 10% additional plants for casualty replacement, nearly 5,000 plants are needed per hectare of plantation. Some farmers use a slightly modified spacing of 2 m x 1.25 m or 2 m x 1.5 m spacing to facilitate mechanized weeding and ploughing between rows. The wide spacing between rows also facilitates cultivating an intercrop during the first six months.

Casuarina trees respond well to fertilizer application especially when cultivated under irrigated conditions. Timely application of specific nutrients as fertilizers has been found to significantly increase wood production. Casuarina is cultivated in a range of soil types in various agro-climatic conditions. Specific recommendation of fertilizer application can only be made based on the results obtained from a soil test. Casuarina trees receive their nitrogen requirement through the help of *Frankia*, a nitrogen-fixing bacteria living in the root nodules. Hence they do not need a large amount of nitrogen fertilizer. Adding phosphorous fertilizer (10 g $plant^{-1}$) and organic fertilizers like farm yard manure or vermicompost as a basal dose helps the nitrogen-fixing bacteria to synthesize more nitrogen for the tree. Timely application of specific nutrients as fertilizers has been found to significantly increase wood production. Casuarina is cultivated in a range of soil types in various agro-climatic conditions. Specific recommendation of fertilizer application can only be made based on the results obtained from a soil test.

Manual weeding at least four times during the first year is necessary for the uniform establishment of trees. Intercropping during the first six months also helps in weed management. Where labour is scarce for manual weeding, farmers adopt wider spacing between rows and take up mechanical weeding through power tillers or mini tractors. Weeding is usually not necessary beyond the first year during which time the canopy of trees closes-in and suppresses weed growth. Pruning of side branches up to one-third height of the tree is needed at the age of 6, 12 and 18 months. Pruning improves the growth and straightness of the main stem and also promotes penetration of air and light inside the plantation helping in improving the overall

hygiene and health of the plantation.

10 Current research focus

The outlook for casuarina cultivation in the next decade seems promising both for the farmers and the industries. Twenty years of breeding and development has resulted in planting stock with significantly improved growth and wood properties, boosting the yield (and/or shortening the rotation period) substantially. The additional wood production expected in the near future is likely to be absorbed by the emerging new areas of usage. The area under cultivation of high-yielding clones of casuarina has to be expanded to benefit a large number of farmers and adopted in new areas having potential for casuarina cultivation. The current levels of genetic diversity and gain from breeding populations have to be sustained in future through systematic infusion of new germplasm. Industrial end-use related characters have to be further improved to keep the market for the casuarina wood in the long-term.

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Past and current casuarina research and development in China

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Abstract

Casuarina trees are planted along the coastal area of southern China as windbreaks, in agroforestry systems and for wood and fuel wood production. At present, casuarina plantations cover about 300,000 hectares. *Casuarina equisetifolia, C. cunninghamiana, C. glauca* and *C. junghuhniana* are the most commonly planted species. Series of field trials have been carried out using different *Casuarina* species, provenances and clones to screen for adaptability to biotic and abiotic stress in different areas, and in the glasshouse. The role of *Frankia* and mycorrhizal fungus inoculation in casuarina plantations in China was reviewed. Results showed casuarina-symbiotic *Frankia* strains had been widely collected and studied in China. In forestry practices, *Frankia* inoculum, also including in combination with mycorrhizal fungus, can be used to improve casuarina seedling growth and biomass in nursery and in field. With an increase in ecological uses or environmental amelioration of casuarina trees, not just focused on tree growth and productivity, e.g., breeding to ecological purposes, typhoon-resistance, disease-resistance, salt-resistance, cold-resistance, wood property, casuarina mixed plantations (mixed model, species), genetic diversity and molecular biology become much more important. Accordingly, impacts of climate change on casuarina cultivation in China are discussed in this paper.

Keywords: casuarina tree, ecological use, resistance, genetic diversity

1 Introduction

The family Casuarinaceae includes 4 genera and 99 species and subspecies of trees and shrubs that have the capacity to enter different symbiotic interactions with soil microbes including the nitrogen-fixing actinomycete *Frankia*, ectomycorrhizal and endomycorrhizal fungi (El-Lakany et al., 1990; Pinyopusarerk and House, 1993; Zhong et al., 2011). Many species in Casuarinaceae are economically and ecologically important as they provide a wide range of goods and services.

Casuarinas were first introduced to China in 1897 (Yang et al., 1995), and currently cover about 300,000 hectares mostly in the coastal areas. Large-scale shelterbelt planting commenced in the 1950s with the objectives of stabilizing moving sands, providing windbreaks for protection of agricultural

lands and water resources, and acting as a source of fuelwood and timber. The three key species are *Casuarina equisetifolia, C. cunninghamiana* and *C. glauca,* but two other species *C. junghuhniana* and *Allocasurina littoralis,* are also planted in both coastal and inland regions (Zhong, 2000). However, the growth of some provenances of *C. junghuhniana* originating from high altitudes has not been good on sandy soil. *C. glauca* and *C. cunninghamiana* can grow in cooler regions. *C. glauca* grows well along the coasts of Zhoushan (northernmost point on the coast, 30° 44′ N) in Zhejiang province, and *C. cunninghamiana* grows in Hangzhou (northernmost point inland, 33° 20′ N) in Shanxi province.

Casuarina trees develop symbioses with the soil bacteria *Frankia,* as well as ectomycorrhizal and endomycorrhizal fungi (Zhong et al., 1995; Franche et al., 1998). Casuarina trees are widely planted as windbreaks, in agroforestry, and in general rehabilitation programs, and currently account for about 300,000 hectares in China, mostly in coastal areas (Zhong et al., 2011). Chinese researchers have been studying casuarina-*Frankia* symbioses since 1984 (Zhang et al., 1984). *Frankia* research has been conducted in different fields, including isolate technology, application technology, the interaction of casuarina between different genotypes and *Frankia,* genetic biodiversity. For the present time, only degraded lands can be used for plantations of casuarina tree species in China, as fertile areas are already being used for production of agricultural crops. Consequently biological measurements are needed to improve the survival and growth of casuarina trees in the field.

There is an increasing use of *Casuarina* for ecological purposes and environmental amelioration, whereas in earlier times the focus was on tree growth and productivity. Accordingly breeding for ecological purposes, typhoon resistance, disease resistance, salt tolerance, cold tolerance, wood properties, casuarina mixed plantations (mixed model, species), genetic diversity and molecular biology have become much more important. As an example, saline soils bordering the South China Sea occupy over 20,000 ha and hence understaning and improving salt tolerance in *Casuarina* is a vital R&D topic. This paper reviews the use of casuarina trees for rehabilitation and environmental improvement in China.

2 Changes in the function and utilization of casuarina plantations in China

From 1954 to 1965, casuarina plantations were mainly for ecological uses along the South China Sea coastline, e.g. sand-fixation and shelterbelts. From 1965 to 1985, casuarina trees were used for ecological purposes, and production of fuelwood, board, timber and roundwood. From 1986 to 2010, casuarina wood had multiple end uses such as woodchips, plywood, construction, sawnwood and fuelwood, but ecological values were not considered. From 2010 to the present, casuarina plantations were used for multiple wood purposes, coupled with more attention on environmental amelioration through coastal shelter forests as directed by national policy. China's mainland coastline extends about 18,000 km, with over 6,000 km have casuarina plantations as windbreak forests. Nowadays the main use for casuarinas in China is as windbreak forest.

From 1996 to 2009, around 30,000 to 70,000 MT of casuarina woodchips were annually exported from Hainan, China to South Korea, and indicated changes in use of casuarina wood in Hainan (Table 1) (Li and Huang, 2011). Export to Korea has declined since 2008 because of local supply to Asia Pulp and Paper company (APP).

3 Challenges for casuarina plantations in China

Once a clone has been used for more than 2-3 generations on the same sites in Guangdong, there will usually be a decline in productivity. More than 80% of shelterbelt plantations are pure, even-age forests of a single clone. The simple structure, low biodiversity, and lack of natural regeneration have contributed to low protection efficiency against typhoons and storms.

Today, most of the lands for casuarina planting are degraded seasdide lands, where trees are at risk from salt, drought and cold wind damage, e.g. (1) high salt levels make young casuarina branchlets increase in diameter compared with normal branchlets , and salt sources are not only in underground soil but also from sea water salt; (2) 5-yr-old casuarina trees on dry sandy soil are stunted (< 1 m tall) in southeastern Hainan;

Table 1. Utilization of casuarina wood (in percentage) in Hainan.

Type of uses	1980	1990	2000	2010	2018
Firewood and charcoal	80	60	20	15	15
Sawn wood	10	20	10	10	10
Construction timber	10	20	10	5	5
Woodchips	-	-	5	60	50
			export	local	local
Veneer	-	-	10	10	20

(3) on front line casuarina plantation faced to the sea, casuarina trees are often damaged by salt and cold wind; (4) bacterial wilt by *Ralstonia solanacearum* (formerly *Pseudomonas solancearum*) is a very serious disease originating on agricultural crop plants including egg plant, sweet potato and chilli,. Bacterial wilt sites are difficult to rehabilitate.

4 Collection and selection of provenances, families and clones in Casuarinaceae

Since 1897, three genera, 25 species, >200 seedlots and >460 families and >200 clones had been tested in field trials (Table 2). The 25 species (or subspecies) introduced into China are as follows: *Casuarina collina, C. cristata, C. cunninghamiana, C. equisetifolia* ssp. *equisetifolia, C. equisetifolia* ssp. *incana, C. glauca, C. grandis, C. junghuhniana, C. obesa, C. oligodon, Allocasuarina campestris, A. decaisneana, A. dielsiana, A. fraseriana, A. huegeliana, A. humilis, A. littoralis, A. luehmannii, A. nana, A. paludosa, A. pinaster, A. torulosa, A.verticillata, Gymnostoma deplancheanum,* and *G. papuanum*.

5 Increasing resistance to biotic, chemical and physical stresses

Improvement work to increase the hardiness of *Casuarina* in China focuses on typhoon resistance at provenance, family and clone levels; disease resistance at species, provenance, family and clone levels, salt tolerance-species/provenance/clone levels, cold tolerance in species and at clone level in *C. glauca,* and drought tolerance in species and clone levels.

During strong typhoons, with wind speeds of from 32.7 to 50.9 m s^{-1}, superior provenance, family and plus tree clones have been recorded in tree typhoon resistance performance from field sites along the South China Seaboard.

On wilt disease, a series of experiments in field trails, nursery and glasshouse had been done at seedlot and family levels (Table 3-5), which included 346 sources in 4 species (Table 2), 54 provenances and 145 families of *C. equisetifolia,* and 147 clones. Over 80 clones were tested in nursery and glasshouse in fields, and selected 40 clones were tested in fields.

Table 2. Information on trials of the four most widely planted *Casuarina* species in China.

Species	No. of provenances	No. of families	Field experiments
C. equisetifolia	76	361	>15 trials
C. cunninghamiana	35	-	6 trials
C. glauca	28	-	6 trials
C. junghuhniana	36	101	9 trials

Table 3. Wilt disease damage on 21 provenances of *C. equisetifolia* at aged 4 years in Xuwen, Guangdong.

No.	Seedlot	Country	Disease rate (%)	DBH (cm)	Tree height (m)	Survival (%)	SFA*	SFS*
1	18013	India	42.5	9.4	11.1	78.1	4.4	2.3
2	18312	Vanuatu	43.4	4.7	6.0	83.3	3.9	1.8
3	18269	China	47.7	5.2	6.9	43.9	4.6	2.5
4	18403	Solomon Is.	44.6	8.7	10.2	64.4	3.7	2.3
5	18402	Solomon Is.	47.9	7.9	8.9	60.0	4.0	2.3
6	18298	Thailand	40.0	12.6	13.8	36.5	4.9	2.4
7	21199	Thailand	45.9	11.5	12.3	49.2	4.9	2.2
8	18268	China	49.7	9.9	12.1	67.6	4.8	2.5
9	18586	China	47.8	9.2	12.4	68.1	4.2	2.5
10	18119	India	54.7	9.9	11.7	70.3	4.3	2.3
11	18357	Philippines	47.5	5.8	7.9	37.5	4.6	2.9
12	18267	China	54.3	9.6	10.8	77.1	4.9	2.6
13	18040	India	58.2	7.2	9.0	41.7	5.1	2.3
14	18142	Kenya	60.6	6.1	7.6	46.5	4.9	2.3
15	18297	Thailand	56.8	8.8	9.4	39.5	3.7	1.8
16	18135	Kenya	58.3	6.1	8.6	52.8	5.4	2.5
17	18128	Vietnam	57.8	10.1	12.3	66.8	5.1	3.0
18	18122	Egypt	65.0	4.7	7.1	62.5	5.1	2.3
19	18565	Mauritius	66.9	6.2	7.8	52.8	4.8	1.9
20	18144	Kenya	70.0	4.1	6.5	75.6	5.3	2.5
21	18355	Benin	75.0	7.6	10.1	62.5	4.3	2.5
Overall Mean			54.0	7.9	9.6	58.9	4.6	2.4

*SFA=Stem Form Axis persistance in 6 scores from 1 (worst) to 6 (best); SFS=Stem Form Straightness in 6 scores from 1 (worst) to 6 (best) (Pinyopusarerk et al., 2004); Same as in following tables.

The order of disease resistance in nursery evaluations was *C. glauca* and *C. cunninghamiana* > *C. equisetifolia* > *C. junghuhniana*. Meanwhile, *C. equisetifolia* ssp. *incana* would be a potential source of resistance against wilt disease, based on field observation.

Casuarina equisetifolia from 16 countries (21 seedlots and 84 families) were planted in 2014 in three provinces: the objective was to select single plus trees from provenance/family trials after three strong typhoons (because high levels of disease occurs after typhoon). It is hoped to be able to select some higher resistant individual trees against wilt disease. At one trial in Xuwen, Guangdong, disease rates for *C. equisetifolia* were from 40% to 75% among 21 provenances, first 10 lower damaged seedlots were 41-50% (Table 3), of which seven seedlots were from Thailand, Solomon Islands, India, Vanuatu and the Philippines (over 50% of the most resistant seedlots), indicated international provenance introductions are important to develop new sources of disease resistance. The level of disease ranged from 21% to 86% among

84 families. The 10 seedlots and 20 families (selected rate around 20%, over 50% from Thailand, Solomon Islands and India), 21-40% were selected as superior genetic resources in field test (Table 4).

The total 65 clones, screened out from 94 clones with higher typhoon resistance, had been further selected for disease resistance in the 12 field clonal trails on serious wilt disease sites, or/and tested, inoculated with *Ralstonia solanacearum* strains, in glasshouse or nursery, of which six-year-old trees of 28 clones were evaluated for disease resistance in field trials in Guangdong, China: wilt disease in nine of the clones ranged from 0.2% to 11.5%, and the first 5 clones were less than 3.0%, compared with control local clone A13 which had 42% disease (Table 5).

Table 4. Disease rate (%) of 84 families of *C. equisetifolia* trees at 4 years old.

No.	Family No.	Disease rate (%)	DBH (cm)	Height (m)	SFA	SFS
1	219	20.9	5.7	7.0	4.5	3.1
2	202	23.4	8.3	8.3	4.1	2.7
3	283	24.1	13.9	14.2	4.8	3.4
4	378	25.0	8.6	8.6	4.0	3.1
5	211	26.0	7.4	10.2	4.6	2.3
6	221	26.6	7.6	9.8	4.4	3.1
7	437	30.9	12.5	14.1	4.5	3.1
8	32	32.5	6.2	7.6	3.5	3.0
9	208	32.5	10.1	12.5	5.1	3.3
10	226	32.5	4.9	6.5	4.1	3.0
11	441	32.5	10.9	10.1	4.9	2.9
12	446	32.5	11.7	15.0	4.3	2.5
13	381	35.9	10.7	12.8	4.9	2.7
14	227	37.5	4.8	5.8	5.2	3.2
15	442	37.5	12.6	14.1	4.9	3.0
16	287	38.4	11.4	13.3	4.4	3.2
17	389	38.4	8.4	8.5	4.0	3.1
18	201	39.1	9.9	12.3	5.3	2.3
19	388	40.0	10.4	10.6	3.0	2.3
20	449	40.0	4.9	5.1	4.6	3.5
21	213	40.9	11.0	13.6	4.8	2.8
80	223	77.5	6.5	8.5	4.0	3.1
81	393	79.1	6.1	7.8	4.7	2.1
82	206	80.0	10.2	11.8	4.4	2.4
83	148	85.0	4.8	5.9	5.3	3.4
84	341	85.9	6.3	8.3	4.3	2.8
Overall mean		51.8	8.6	10.2	4.5	2.9

Table 5. Clonal disease rate at 6 years old in Wuchuan, Guangdong.

No.	Clone ID	DBH (cm)	Height (m)	Disease rate (%)
1	16	10.5	15.2	9.5
2	20	9.6	15.2	6.8
3	21	9.2	14.9	1.3
4	27	5.2	12.3	2.5
5	34	9.0	14.9	0.6
6	7	6.9	14.9	0.2
7	BF3	9.0	15.4	1.1
8	Bao9	10.9	15.8	6.4
9	Dong2	8.86	14.6	11.5
10	A13 (control)	8.6	14.6	42.1

A mixed selection of four clones performed better than a single clonal plantation against wilt disease. Clonal tests on former wilt disease lands showed that slower-growing clones performed better than fast-growing clones against wilt disease.

Wilt disease typically becomes heavy after typhoon damage, and there is a need to select genetic sources which are resistant to both typhoons and wilt disease. There needs to be a balance between the growth and resistance, as normally faster growing sources will have poor wilt disease resistance.

The salt tolerance, and physiological and morphological responses under NaCl salt stress of 10 seedlots of *C. equisetifolia* seedlings was investigated by Ma (2014). The ranking of salt tolerance in the ten chosen provenances was: 18402>18128>18586>18015>18142>18355>17862>18297>18268>18244.

In relation to drought resistance, planting *Casuarina* species/clones on poor sites in the hot-dry Yuanmou river valley, with dry red soil and where evaporation is more than 6 times greater than rainfall, the percentage survival of seedlings inoculated with five strains of *Frankia* increased by 10% to 20.6% compared with uninoculated seedlings (Yang et al., 2007). This result indicates that *Frankia* can significantly improve plant survival on highly degraded sites.

There has been over 30 years of casuarina improvement in China, involving provenance/family/plus tree selection and breeding: this has been followed by selection and rapid adoption and wide use of superior clonal sources. Plus trees can be produced either through grafting or from young shoot cuttings, which can be used for seed orchards, clonal gene bank, clone test, then establish clonal plantation. Cuttings are taken from branchlets from wounded bottom or branches of trunk to produce cutting seedlings by water culture. However, cuttings propagation of selected clones is less successful for several reasons: (1) plants do not develop good root system making them less resistance to typhoon winds, compared with plants raised from seed; (2) high resistant clones tend to be more difficult to propagate by cuttings; and (3) genetic diversity of clonal plantations reduces in long term. So it is necessary to enrich casuarina genetic diversity by introducing and testing new genetic resources for wilt disease and environmental stresses.

6 Genetic diversity and molecular assisted breeding

The genetic structure and diversity of *C. equisetifolia* provenances were revealed by RAPD, AFLP and EST-SSR markers (Guo et al., 2003; Huang et al., 2009; Hu, 2015). The 220 trees in 18 *C. equisetifolia* populations were analyzed by eight primer combinations of AFLP markers. The total gene diversity (0.4126), the coefficient of population differentiation (0.4878) and the low gene flow (0.5250) between populations indicated that *C. equisetifolia* is a highly differentiated species and with levels of genetic diversity. An AMOVA analysis showed that 45.7% genetic variance is distributed between populations and 54.3% within populations: when the 18 populations were divided into five groups, the result indicated that the variance components between groups, among populations within groups, and among individuals within populations were 2.8, 43.3 and 53.9%, respectively. Possible reasons for the genetic differentiation and diversity of *C. equisetifolia* are discussed below along with possible conservation and breeding strategies (Huang et al., 2009). Another study using eight EST-SSR markers rvealed the main variation of 29 *C. equisetifolia* populations in AMOVA analysis were from the individuals within provenances, which accounted for 70.1% of total variance and 28.3% among provenances, and 1.6% among regions. On region level, the order of variance was: AN (Asia natural,

81.2%) >AI (Asia landraces, 74.6%) >CI (Central America landrace, 72.3%) > AF (Africa landraces, 68.4%) >AP (Australia natural, 61.5%). A Mantel test showed that the variation of genetic differentiation coefficient F_{ST} of *C. equisetifolia* did not follow the distance isolation patterns. Changeable environment and effects of gene drift may be the main reasons that bring out the genetic differentiation condition. In addition, the serious inbreeding identified in the populations of *C. equisetifolia* may cause the genetic diversity to decrease in the offspring. According to the seedling phenotype traits clustering and Nei's genetic distance UPGMA clustering of EST-SSR genetic diversity analysis, landraces of China might derive from Asia natural provenances, while landraces of Kenya, India and Vietnam might originate from Australian natural provenances (Hu, 2015).

In vitro and genetic transformation of *C. equisetifolia* and *C. cunninghamiana* have been studied in China (Zhong et al, 2011; Jiang et al., 2015): this work included shoot regeneration on *LEA* gene transgenic callus and showed that transgenic plants can nodulate after *Frankia* inoculation of *C. cunninghamiana* seedlings.

These studies have provided basic molecular assisted selection and breeding data for improvements of this species in China.

7 Improve eco-function of casuarina plantations

Management techniques that have been investigated include mixed plantation with species or/and clones, pruning, fertilizing, biological method, *Frankia* + mycorrhizal fungus application.

(1) Mixed casuarina plantations can retard wilt disease occurrence

Compared with casuarina, *Acacia auriculiformis* has lower typhoon resistance. In addition, *Acacia, Eucalyptus* and *Pinus* trees can not be planted too close to seaside (front line of coast).

A good planting model is mixed plantations that can effectively improve eco-function.

Mixed plantations of casuarina, coconut tree and other species (e.g. *Calophyllum inophyllum* and *Pandanus tectorius*) along coastal sides in South China, which is one of better models to increase resistance of tree plantings to typhoons. High eco-function plantation-mixed relative stable structure plantation decreased wind velocity by 88 - 98% (Li, 2007).

(2) *Frankia* isolates, infection and application of inoculums

In order to guide casuarina plantation management in future an investigation (Liu and Zhang, 2002) was conducted to understand *Frankia* nodule distribution, morphology, density and biomass: this study found there were 7.3 -124 nodules m^{-2} and 15-858 kg live nodules ha^{-1} in 19 -26 year-old *C. equisetifolia* plantations in Haikou. *Frankia* development in *Casuarina* plantations aged between 4.5 years and 26 years old was investigated in 12 locations in four provinces along the south China Seaboard (Kang and Zhong, 1999). This study found more young nodules less than 1 cm in diameter in younger forest plantations (less than seven years old) than in mature forest (over 20 years old). Most nodules were distributed between 0 and 30 cm depth in sandy soils, and between 0 and 10 cm in red earth, with young nodule biomass in the plantation significantly positively correlated with concentrations of soil phosphorus and cobalt.

It is vital to select "tree genotype-*Frankia* and mycorrhizal fungus combinations", if casuarinas are used for ecological purposes. More than 120 *Frankia* isolates have been collected from casuarina plantations along the coast in the southeastern and southern regions of Hainan, Guangxi, Guangdong, Fujian and Zhejiang provinces since 1985 (Zhong et al., 2019). Nitrogenase activity (C_2H_4) of the 26 *Frankia* strains from casuarina trees in Fujian and Zhejiang provinces ranged from 0.045 to 2.90 μmol mg^{-1} h^{-1} in the laboratory (Li, 2016; Zhang et al., 2012).

On poor sites, the *Casuarina-Frankia* symbiosis is vital to improve tree performance, particularly on new sites for casuarina planting.

In the nursery, *Frankia* innocualtion was shown to effectively improve seedling growth and biomass, compared with uninoculated seedlings. Four months after inoculation the height of seedlings of *C. equisetifolia* clones increased from 31% to 245% while biomass increased by 34% to 110% (Li and Wu, 1995). Six months after inoculation with the two strains ($Cc38_A$ and U_4), the height of *C. cunninghamiana* seedlings increased by 20 to 23%, biomass increased by 233 to 276% (Li et al., 1998).

Several field trails have also shown the effectiveness

of *Frankia* inoculation (Li et al., 1998; Kang et al., 2000; Yang et al., 2007; Zhang et al., 2012) (Table 6). It is consequently important to screen for suitable *Frankia* strains for use with different species on different sites (Zhong et al., 2010).

(3) Host casuarina tree species and interaction between casuarina genotype and *Frankia* strain

Chinese researchers have been working on the selection of casuarina symbiotic genotypes for many years (Zhong, 1993), and these studies have revealed interactions between *Frankia* isolates and the provenance and/or family or clone of *C. equisetifolia* and *C. junghuhniana* (Zhong, 1993; Zhong et al., 1995; Kang, 1997; Zhong, 2000). Up to now, more than nine *Casuarina* and *Allocasuarina* species (and numerous provenances / families) have been inoculated with *Frankia* or with native inoculation in the laboratory, the nursery, or in the field in China (Table 7).

8 Conclusions

China has been successful in planting casuarinas to protect and improve its environments and coastal ecosystems. However, this success has been built on a considerable body of research and development, and faced with many obstacles. Casuarinas will continue to be an important part of the landscape in the southern coastal regions of China. Technical support from national research institutes and provincial agencies is necessary in order for county and local governments to achieve their goals of protection and improvement of coastal environments.

Casuarina *Frankia* strains have been widely collected and studied in China. In forestry practices, *Frankia* inocula can be used to improve tree seedling survival and growth, biomass, and disease resistance, as well as soil fertility, using an environmentally friendly inoculant. The appropriate *Frankia* strains need to be selected in the nursery and in the field. Most research results obtained with casuarina seedlings inoculated with *Frankia* isolates on degraded lands in China are very encouraging.

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Table 6. Effectiveness of *Frankia* strains in enhancing casuarina tree growth 1-7 years after inoculation in fields trials in south and southwest China*.

Location	Soil type	Species	Age (yr)	Number of *Frankia* strains used	Effectiveness** (%)	
					Height (m)	DBH (cm)
Yangxi, Guangdong	red earth	*C. equisetifolia*	1	4	6.6-22.0	-
			2	4	40.2-53.1	16.8-45.9
			3	4	29.0-39.0	23.5-27.2
			4	4	25.3-37.0	23.0-26.1
Pingtan, Fujian	Sandy soil	*C. glauca*	7	2	12.5-15.1	10.3-22.1
		C. cunninghamiana	7	2	NE***	6.3-9.4
Yuanmou,Yunnan	Dry red soil	*C. cunninghamiana*	1	5	2.8-32.2	-
			2	5	2.4-22.3	-

Note: * A randomized complete block (RCB) design was used with 20-36 trees per block and 3-4 replicates; ** compared with uninoculated controls, data ranges differed among strains; *** NE means no positive effectiveness

Table 7. Some *Casuarina* species inoculated with *Frankia* in China.

Species	Location	Plant genotype*	*Frankia* strain	Reference
C. equisetifolia	Nursery	provenance, clone	1	Zhong, 1993; Zhong et al., 1995; Zhong 2000
C. equisetifolia	Nursery	species	25	Kang, 1997
C. cunninghamiana	Nursery, field	species	1	Zhong et al., 1995
C. cunninghamiana	Nursery	species	25	Kang, 1997
C. cunninghamiana	Nursery, field	species	5	Yang et al., 2007; Zhong et al., 2010
C. glauca	Nursery	species	25	Kang, 1997
C. glauca	Nursery	species	7	Kang et al., 1997
C. junghuhaniana	Nursery	species	25	Kang, 1997
C. junghuhaniana	Nursery	family	1	Zhong, 1993; Zhong, 2000
C. collina, C. cristata and C. obesa	Nursery	species	-	Li, 2002
C. cristata	Growth chamber	species	4	Qin et al., 1990
C. grandis	Nursery	species	-	Zhong et al., 2019
Allocasuarina littoralis	Chamber	species	4	Qin et al., 1990
A. littoralis	Nursery	species	25	Kang, 1997

*provenance= seeds from different locations or seedlots; family=seeds from a single tree. clone= seedlings propagated by cuttings.

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Casuarinaceae genetic resources in the Pacific Islands: enhancing their contributions to the green economy

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Abstract

Australia and the Pacific Islands are a centre of diversity for Casuarinaceae, with currently four accepted genera: *Allocasuarina, Casuarina, Ceuthostoma* and *Gymnostoma* encompassing almost 100 species. The main two genera of interest for development in the Pacific Islands are *Casuarina* and *Gymnostoma* with 14 currently accepted species in each genus. The most widespread *Casuarina* species, *C. equisetifolia* L., is an important timber, fuelwood, medicine and windbreak, and essential for coastal protection. In New Caledonia, the endemic *C. collina* J. Poiss. ex Pancher & Sebert was extensively used in the restoration of nickel mine sites, while *C. oligodon* L.A.S. Johnson is a tree component of ancient agroforestry systems in the New Guinea highlands. *Casuarina grandis* L.A.S. Johnson from south-eastern Papua New Guinea is a tall tree to 50 m with forestry/agroforestry potential. A putative new undescribed species (*C. paracletica* sp. nov. mss in herb.) from Santo (Vanuatu) which is related to the Australian riverine-distributed *C. cunninghamiana* Miq. and the PNG *C. grandis*, has yet to be evaluated for development purposes. *Gymnostoma papuanum* (S. Moore) L.A.S. Johnson, a fast-growing and well-formed large tree species from New Guinea and Solomon Islands, has outstanding, untapped forestry and agroforestry potential, as well as for stabilization of degraded soils. Fiji's endemic *G. vitiense* L.A.S. Johnson also has potential in agroforestry and landscaping. There are eight endemic *Gymnostoma* species in New Caledonia: the ones growing on ultramafic outcrops are relatively slower growing compared to their non-ultramafic relatives, but well-adapted to difficult or extreme ultramafic edaphic environments and with potential for mine site reclamation, revegetation of degraded sites and as ornamentals. The genetic resources of Pacific Casuarinaceae have considerable capacity to contribute to the green economies in the tropics, through production of timber, pulp, fuelwood and stabilization of infertile and degraded soils. It is recommended that the appropriate agencies assist Pacific Islands Forestry Departments to undertake research on the most promising species of Casuarinaceae especially for agroforestry and landscape restoration.

Keywords: *Casuarina, Allocasuarina, Gymnostoma,* Pacific Islands, taxonomy, natural distribution

1 *Casuarina* species in Pacific Islands

There are eight native species of *Casuarina* in the Pacific Islands: *C. collina, C. equisetifolia, C. grandis, C. oligodon, C. orophila, C. teres*, including two undescribed native species in Vanuatu, viz *Casuarina* sp. 'Aneityum' and *Casuarina* sp. 'Espiritu Santo' (K.L. Wilson, pers. comm.) (see Table 1).There is also at least one introduced species, viz. *C. glauca* in Tonga and Vanuatu. *Casuarina potamophila* Schltr. and *C. tenella* Schltr. are regarded either as doubtful species (Munzinger et al., 2020), as synonymous respectively with *C. collina* and *Gymnostoma chamaecyparis* (J.Poiss.) L.A.S. Johnson (Johnson mss in herb., Munzinger et al., 2020) or accepted species (POWO, 2020). Key references to the uses of Pacific *Casuarina* species include Cambie and Ash (1994), Gâteblé (2015), Jaffré et al. (1994), Keppel and Ghazanfar (2011), Pinyopusarerk and Midgley (2018), and Smith (1981).

1.1 *Casuarina collina* (creek ironwood, adapted from Gâteblé 2015)

Casuarina collina is considered as endemic to New Caledonia and very common on the main island of Grande Terre, especially in lower rainfall zones (900–1,100 mm annual rainfall) on the west coast: it occurs at lower frequency in other parts of the archipelago. Creek ironwood can form monospecific stands in diverse habitats, including secondary and ruderal vegetation units, "*maquis minier*" (scrub on ultramafic substrates) and dry forests, but has a clear preference for lowland, riparian sites (5–350 m elevation). It reaches 15–20 m tall in favourable habitats, but could be reduced to a shrub on difficult sites. *Casuarina collina* is widely planted as an ornamental and windbreak in New Caledonia: its attributes include a moderately fast early growth rate (c. 1 m $year^{-1}$), an upright habit and fine foliage along with the white male flowers and the red female flowers. The male flowers are visited by honey bees (*Apis mellifera*). It was formerly widely used in nickel mine site revegetation—in the 1970s and 1980s—but when planted too densely it tends to block the regeneration of other endemic species. Its very hard wood can be used in for poles and it is a highly regarded fuelwood.

1.2 *Casuarina equisetifolia* (Beach sheoak)

Casuarina equisetifolia is small to medium tree found throughout the South Pacific Islands including—PNG, Solomon Islands, Vanuatu, New Caledonia, Fiji, Tonga—as well as eastern Polynesia, where it may sometimes be a Polynesian or more recent introduction (Smith, 1981; Whistler, 2000). Usually it grows in beach/strandline communities, but may extend to inland locations in some countries. The latter include on recent

Fig. 1. *Casuarina collina* windbreak, St-Louis, Nouméa, New Caledonia (Photo: G. Gâteblé).

Fig. 2. *Casuarina equisetifolia* being used as a host for sandalwood (*Santalum yasi*) in Nausori Highlands, Fiji (Photo: L.Thomson).

volcanic ash deposits around Mt Yasur, Tanna, Vanuatu; *talasiga* ('sunburnt land')—droughty/ regularly burnt habitats usually associated with infertile, often stony, acidic red clays—in western parts of Viti Levu and Vanua Levu in Fiji; and *makatea* (raised coral limestone) in Cook Islands, e.g. Mangaia, and Tonga, e.g. 'Eua. The species exhibits complex and incompletely understood patterns of genetic variation (Bush et al., these proceedings). Beach sheoak is the most widely utilized and grown member of Casuarinaceae in the Pacific Islands. It is used in building construction, mainly in the round form as posts and poles. It is also a highly regarded fuelwood and sometimes used for charcoal production. The species has been planted as a coastal windbreak, e.g. Houma, Tonga to protect crops from salt-laden winds, as well as planted in agroforestry systems including as a host for sandalwood (*Santalum* species). In the role of sandalwood host it is best planted as scattered trees, as dense formations of *Casuarina* can suppress sandalwood growth as observed in Vanuatu (The Summit, Efate) and in Cook Islands (Mangaia). Individual trees can be planted adjacent to sandalwood due to their light canopy, which can provide protection for sandalwood trees during cyclonic-force winds. *C. equisetifolia* has a wide climatic and edaphic range and is exceptionally well adapted to cope with future predicted climate change in the Pacific Islands (Thomson and Thaman, 2016). The potential of *C. equisetifolia* to contribute to Pacific Islands green economies has been grossly underestimated, to the extent that previously stands of the species were removed in Fiji in the 1970s and 1980s to make way for *Pinus caribaea* Morelet plantations which have further degraded and acidified *talasiga* soils. Fast-growing, well-formed clones and hybrids of *C. equisetifolia* which have been developed in India (Nicodemus, these proceedings) ought to be trialled in South Pacific, especially on degraded grasslands in PNG, Solomon Islands and Fiji for site rehabilitation, production of building timbers, fuelwood and woodchips/pulpwood.

1.3 *Casuarina grandis*

Casuarina grandis is endemic to south-eastern Papua New Guinea, extending from near Tufi in northern Province to the Gwariu River in Milne Bay Province (Johnson, 1982). It is one of tallest Casuarinas reportedly reaching up to 50–60 m tall, but typically 35–40 m tall. It occurs in dense stands in lowland riverine locations up to 600 m elevation. Its habitats include gravel banks and small gullies in eroded volcanic debris. The species is reportedly very fast-growing and most

closely related to *C. cunninghamiana* and the Timor form of *C. junghuhniana* Miq. which occurs on the lowland alluvial riparian sites. The economic potential of *C. grandis*, including the possibility of making hybrids with related species, was recognised in the paper describing the species (Johnson, 1982), but no seed has ever been collected and the species is yet to be included in field trials to assess and confirm its potential.

1.4 *Casuarina oligodon*

Casuarina oligodon is endemic to islands of New Guinea where two subspecies are recognised, viz. subsp. *oligodon* (PNG highlands) and subsp. *abbreviata* L.A.S. Johnson (Bele and Upper Balim Valleys at least as far west as the Wissel Lake region, Papua, Indonesia), the latter having shorter leaf teeth (≤0.5 mm) than the type subspecies leaf teeth which are ≥ 8 mm. Ancient agroforestry systems based on the two subspecies of *C. oligodon* developed independently in PNG and Indonesia: in these systems the casuarinas are vital as windbreaks and soil improvement (cycling nutrients/fixing N), as well as providing roundwood/posts and fuelwood. The biology, attributes, distribution, environments and uses of the New Guinea highlands casuarinas have been well documented previously (Ataia, 1983; Askin et al., 1990; Bourke, 1985).

1.5 *Casuarina orophila*

Casuarina orophila is endemic to the Mt Doorman region of Western New Guinea (Indonesia), where it forms dense stands at elevations of 2,700–4,000 m on ultramafic substrates (Lam, 1945 as cited in Johnson, 1983). It is a shrubby tree to 8 m tall, with erect cladodes c. 0.7 to 1 mm in diameter (Johnson, 1983). Its potential for planting in other tropical highlands environments has yet to be investigated but likely to be limited to soil protection and improvement plantings in otherwise challenging environments for tree growth in tropical/sub-tropical, sub-alpine zones.

1.6 *Casuarina teres* (adapted from Gâteblé, 2015)

Casuarina teres is a rare, micro-endemic extending from the Vavouto peninsula, Voh in the North Province of Grande Terre, New Caledonia. It is restricted to low elevation (<200 m) in a kind of dry forest on ultramafic substrates. *Casuarina teres* is morphologically close to *C. collina*, from which it is distinguished by its larger, stiffer, longer leafy branches, and a denser and more erect habit (up to 7 m tall). The species has potential in bank and soil stabilisation in part due to its strong root suckering habit. However, its root suckering nature often reduces its utility in general landscaping plantings. *C. teres* is a drought hardy small tree, but relatively slow growing, c. 0.5 m year^{-1}, which might restricts its wider use in land rehabilitation and planting in agroforestry systems.

1.7 *Casuarina* spp. (undescribed species from Vanuatu)

There are two species of *Casuarina* in Vanuatu which are yet to be formally described, viz. *Casuarina* sp. 'Aneityum' (*C. elassodonta* L.A.S. Johnson & K.L. Wilson mss in herb.) and *Casuarina* sp. 'Espiritu Santo' (*C. paracletica* L.A.S .Johnson & K.L. Wilson mss in herb.). Until recently the 'Aneityum' species was known only from two specimens (*Bernardi 13045*; *Cabalion, 1961*), both on the northern section of Aneityum at elevation 50–100 m. Both specimens are female, so the species may be dioecious (K.L. Wilson, pers. comm.). Additional specimens were collected in late 2019 by Plunkett, Munzinger and Bruy. The 'Espiritu Santo' species is endemic to the northern parts of Santo. It is restricted to lowland (5–150 m), riverine gallery forests in the vicinity of Big Bay, including the Apouna River and a tributary of the Malao River—where it grows in alluvium, including sandy, gravelly and pebbly deposits. It is a slender tree to c. 15 m tall and 30 cm dbh, and known only from three specimens (*MacKee 24301, Veillon 4525, Raynal 16284*).

2 *Gymnostoma* species in New Caledonia

New Caledonia is a centre of species diversity for *Gymnostoma* with eight endemic species: *G. chamaecyparis*, *G. deplancheanum* (Miq.) L.A.S. Johnson, *G. glaucescens* (Schltr.) L.A.S. Johnson, *G. intermedium* (J. Poiss.) L.A.S. Johnson, *G. leucodon* (J. Poiss.) L.A.S. Johnson, *G. nodiflorum* (Thunb.) L.A.S. Johnson, *G. poissonianum* (Schltr.) L.A.S. Johnson and *G. webbianum* (Miq.) L.A.S. Johnson (Table 1). The following brief treatments for six of these *Gymnostoma* species are adapted from Gâteblé (2015).

2.1 *Gymnostoma chamaecyparis*

Gymnostoma chamaecyparis is a dioecious, rounded shrub to small tree (2–15 m tall) growing at low altitude

Fig. 3. Wild stand of *C. elassodonta* sp. nov. mss. In herb. in north Aneityum, Vanuatu (Photo: G. Plunkett).

(< 600m) mainly near the base of the ultramafic massifson serpentines or brown hypermagnesian soils of Grande Terre. In cultivation, the plant habit is generally conical and semi-pendulous, with rather fine bright green foliage not arranged in distinct whorls. The species has potential as an ornamental, particularly in pot culture and landscaping, and is used in rehabilitation of degraded, nickel-mining sites. It is readily propagated from seed and cuttings and its growth rate is relatively good when established. It has been infrequently been used in traditional medicines and for fuelwood.

2.2 *Gymnostoma deplancheanum*

Gymnostoma deplancheanum is a shrub to small tree (2-15 m tall) found on the southern ultra-basic massif of Grand Terre, at elevations between 200 and 300 m (rarely to 900 m). It grows on acidic (pH 4.7), ferralitic or ferritic desaturated hardpan or gravelly soils. *Gymnostoma deplancheanum* has an attractive form with branches arranged in pagoda-like, pseudo-whorls (Fig. 4). It has a similar utilization potential in landscaping and uses in mine-site rehabilitation as for *G. chamaecyparis.*

Fig. 4. *Gymnostoma deplancheanum*, Ile Ouen (S. of Grand Terre), New Caledonia (Photo: G. Gâteblé).

Fig. 5. Immature fruit of *Gymnostoma deplancheanum* (Photo: G. Gâteblé).

2.3 *Gymnostoma intermedium*

Gymnostoma intermedium is a shrub to small tree of 2 to 15 m tall that is generally found in higher altitudes ultramafic substrates mountains (Kouakoué, Montagne des Sources, Mé Ori, Dzumac) of Grande Terre. It has usually a parasol shape, with thick branchlets and swollen nodes. Like the other species its foliage and habit are of potential interest for landscaping and ornamental horticulture and it might also be used in revegetation programmes.

2.4 *Gymnostoma leucodon*

Gymnostoma leucodon is a dioecious shrub up to 4–5 m tall. It is found in lowland (5–200 m elevation), riparian habitats from southern Grande Terre, east of a Thio–Mt. Dore line, where it is restricted to ultramafic substrates. Its erect, ± fastigiate form and fine green to grey-green foliage make it an attractive ornamental, however it has an extremely low rate of growth, c. 10–15 cm year^{-1}, which might limit its use in landscaping and mine site restoration.

2.5 *Gymnostoma nodiflorum*

Gymnostoma nodiflorum grows usually in riverine habitats in central and northern parts of Grande Terre. It mainly occurs on non-ultramafic substrates, including in mixed alluvial soils. It is a bushy, dioecious, medium sized tree sometimes planted as an ornamental plant in some gardens e.g. in Ponérihouen (*MacKee 40824*). It has erect branchlets compared to *G. webbianum* with which it is has been often confused (Johnson, 1982).

2.6 *Gymnostoma poissonianum*

Gymnostoma poissonianum is a bushy, often wide spreading, small to medium tree to 15 m tall growing at low-mid altitudes (200–700 m elevation) on eroded slopes and in rainforest on Grand Terre. It occurs exclusively on ferralitic soils over ultramafic rock massifs: planting potential is limited by its slow growth rate (L'Huillier et al., 2010). *Gymnostoma* may be keystone ecological species in disturbed and degraded environments and the recent discovery of a new genus and species of felt scale (*Dzumacoccus baylaci*) feeding on *G. poissonianum* (Hodgson et al., 2018) is indicative of their important role in building forest biodiversity.

2.7 *Gymnostoma webbianum*

Gymnostoma webbianum is a dioecious tree found in humid and riverine forests on Grande Terre. It occurs in the south (Mt Dore), most commonly on the east coast especially in the north-east between Hienghène and Pouébo, but also on the west coast. It is moderately common on volcano-sedimentary substrates, and also found on ultramafic substrates. It attains 10–15 m tall in favourable sites with pendulous branchlets. It is traditionally utilised as an ornamental hedge or shrub around Hienghène. *Gymnostoma webbianum* has potential as a large potted plant and for river bank stabilization. Its growth rate is quite slow at the beginning (c. 20 cm height increment per year) but increases considerably after field planting.

3 *Gymnostoma* from New Guinea, Solomon Islands and Fiji

Outside of New Caledonia there are six *Gymnostoma* species in Melanesia (New Guinea, Solomon Islands and Fiji) including two which have been formally described: *G. papuanum* and *G. vitiense* and another four undescribed species: three in New Guinea (viz. *G. gracillimum* L.A.S. Johnson & K.L. Wilson sp. nov. ined., *G. mesostrobilum* L.A.S. Johnson & K.L. Wilson sp. nov. ined., *G. spicigerum* L.A.S. Johnson & K.L. Wilson sp. nov. ined.) and one in Solomon Islands (from Beaufort Bay, Guadalcanal; K.L. Wilson pers. comm.) (Table 1). The two most promising species, *G. papuanum* and *G. vitiensis*, are discussed below.

3.1 *Gymnostoma vitiense*

Gymnostoma vitiense is endemic to five high islands in Fiji—occurring from near sea level to 900 m—and typically on infertile acidic red clays. It is a canopy emergent, gregarious and increasing after logging. *G. vitiense* is a monoecious, small to large tree 5–25 m tall and up to 2 m dbh, with the ultimate size depending on local site fertility and rainfall regime. It produces a very dense wood (1070 kg m^{-3} air dry density), one of the hardest timbers known, but with low durability in ground contact (Alston, 1982). It is a useful fuel and charcoal, but mainly planted as an ornamental and for windbreak and boundary plantings. It is not the best long-term windbreak, as its canopy becomes open and wide spreading as the tree matures. It is also affected by Seychelles scale pest. Key references are Keppel and Ghazanfar (2011), Thomson and Thaman (2016) and Smith (1981).

3.2 *Gymnostoma papuanum*

Gymnostoma papuanum naturally occurs in New Guinea and the Solomon Islands from near sea level to 2,000 m elevation. In the Solomon Islands it is mainly found on ultramafic soils in south Santa Isabel and south-east Choiseul (Whitmore, 1966, as *Casuarina papuana*). It is a pioneering, long-lived, dioecious large tree up to 50 m tall and > 50 cm dbh. *G. papuanum* is adapted to diverse edaphic environments including acidic, infertile, ultrabasic soils. In nature the species forms monospecific stands on the margins of forest and anthropogenic grasslands.

Fig. 6. *Gymnostoma vitiense* (low bushy trees) and *Casuarina equisetifolia* (sparse overstory and growing off-site due to very high rainfall) being used as windbreak and sandalwood host, Laulau, Naitasiri Province, Viti Levu, Fiji (Photo: L. Thomson).

Table 1. Summary information on Pacific Islands Casuarinaceae species.

Name	Natural distribution	Uses[1]	State of knowledge[2]
***Casuarina* species**			
C. collina J. Poiss. ex Pancher & Sebert.	New Caledonia	MR, O, SP, W, F, T, SH	****
C. equisetifolia L.	Cook Is, Fiji, French Polynesia, New Guinea, Marshall Is, Mariana Is, New Caledonia, Solomon Islands, Tonga, Tuvalu, Vanuatu,	CP, O, SP, W, F, T, TM, SH	****
C. elassodonta L.A.S. Johnson & K.L. Wilson mss in herb	Vanuatu	W, SH	*
C. grandis L.A.S. Johnson	Papua New Guinea	F, T	**
C. oligodon L.A.S. Johnson	Papua New Guinea	W, F, T	***
C. orophila L.A.S. Johnson	Papua New Guinea	SP	**
C. paracletica L.A.S. Johnson & K.L. Wilson mss in herb	Vanuatu	W, F, T, SP	*
C. teres Schltr	New Caledonia	SP	**
***Gymnostoma* species**			
G. chamaecyparis (J. Poiss.) L.A.S. Johnson	New Caledonia	MR, O, M	***
G. deplancheanum (Miq.) L.A.S. Johnson	New Caledonia	MR, O	***
G. glaucescens (Schltr.) L.A.S. Johnson	New Caledonia		*
G. gracillimum L.A.S. Johnson & K.L. Wilson sp. nov. ined.	New Guinea		*
G. intermedium (J. Poiss.) L.A.S. Johnson	New Caledonia	O, SP	**
G. leucodon (J. Poiss.) L.A.S. Johnson	New Caledonia	O	**
G. mesostrobilum L.A.S. Johnson & K.L. Wilson sp. nov. ined.	New Guinea		*
G. nodiflorum (Thunb.) L.A.S. Johnson	New Caledonia	O	**
G. papuanum (S.Moore) L.A.S. Johnson	PNG, Solomon Is	MR, T, F, O	***
G. poissonianum (Schltr.) L.A.S. Johnson	New Caledonia	MR, SP	***
G. spicigerum L.A.S. Johnson & K.L. Wilson sp. nov. ined	New Guinea		*
G. vitiense L.A.S. Johnson	Fiji	F, T, SP, O	***
G. webbianum (Miq.) L.A.S. Johnson	New Caledonia	O, SP	**
Gymnostoma sp. nov. (Beaufort Bay, Guadalcanal)	Solomon Islands		*

[1] Uses include current and potential uses. CP=coastal protection, F= fuelwood, MR= mine rehabilitation, M= medicine, O=ornamental, SH=sandalwood host, SP=soil protection, TM= Traditional medicine, T=timber, W=windbreak

[2] Key to State of Knowledge: **** Well known, ***Moderately well known, **Little known with limited or no research undertaken, *Very poorly known, ±undescribed

Fig. 7. *Gymnostoma papuanum* being used for mine site rehabilitation. Four-year old saplings in rehabilitation trial of mine site, Santa Isobel, Solomon Islands. Brownish/red clay loam with pH 4.95, very low exchangeable bases Ca, Mg and K (low N, available P) and elevated Mn and Ni levels, typical of laterite soils originating from ultramafic parent materials (Photo: S. Tutua).

In traditional agroforestry systems in the PNG highlands, the tree is intercropped with food crops – both as shade trees or in rotation with crops. *Gymnostoma papuanum* has extremely high potential for rehabilitation of degraded sites, including mined sites. It is also a highly attractive ornamental and useful for fuelwood, charcoal and for production of wooden artefacts.

4 Green growth opportunities

A key identified green growth opportunity is to utilize selected, faster-growing *Casuarina* and *Gymnostoma* species (*C. collina, C. equisetifolia, C. grandis, C. oligodon, G. papuanum* and *G. vitiense*) in the rehabilitation of degraded and infertile sites in the Pacific Islands, while at the same time producing round timbers for building, woodchips for export to pulp mill sand biomass/fuelwood/charcoal.

There is also high potential to introduce and trial promising *Casuarina* hybrids, especially *C. equisetifolia* x *C. junghuhniana* that have developed in southern India and Thailand; this research needs to be coupled with adequate precautions to protect the purity of native genetic resources.

5 Research and development needs

There is a need to research and better document Pacific Islands Casuarinaceae, including an updated taxonomy of the genera/species in the region along with phylogeny/evolutionary studies; assessment of simple vegetative propagation techniques, breeding systems and conservation status, and an investigation of their potential in agroforestry systems and in order to contribute to green economies and rehabilitation of degraded sites. This applies especially to two Pacific Islands riparian *Casuarina* species, *C. grandis* and *C. sp. nov.*, (Santo), which are tropical analogues of *C. cunninghamiana*, as well as *C. oligodon* and *C. collina*, and faster-growing and promising *Gymnostoma* species, especially *G. papuanum* and *G. vitiense*. It is recommended that Pacific Islands Forestry Departments seek donor support to undertake R&D on their Casuarinaceae species, with the technical assistance of SPC, CSIRO and other R&D organizations in the region.

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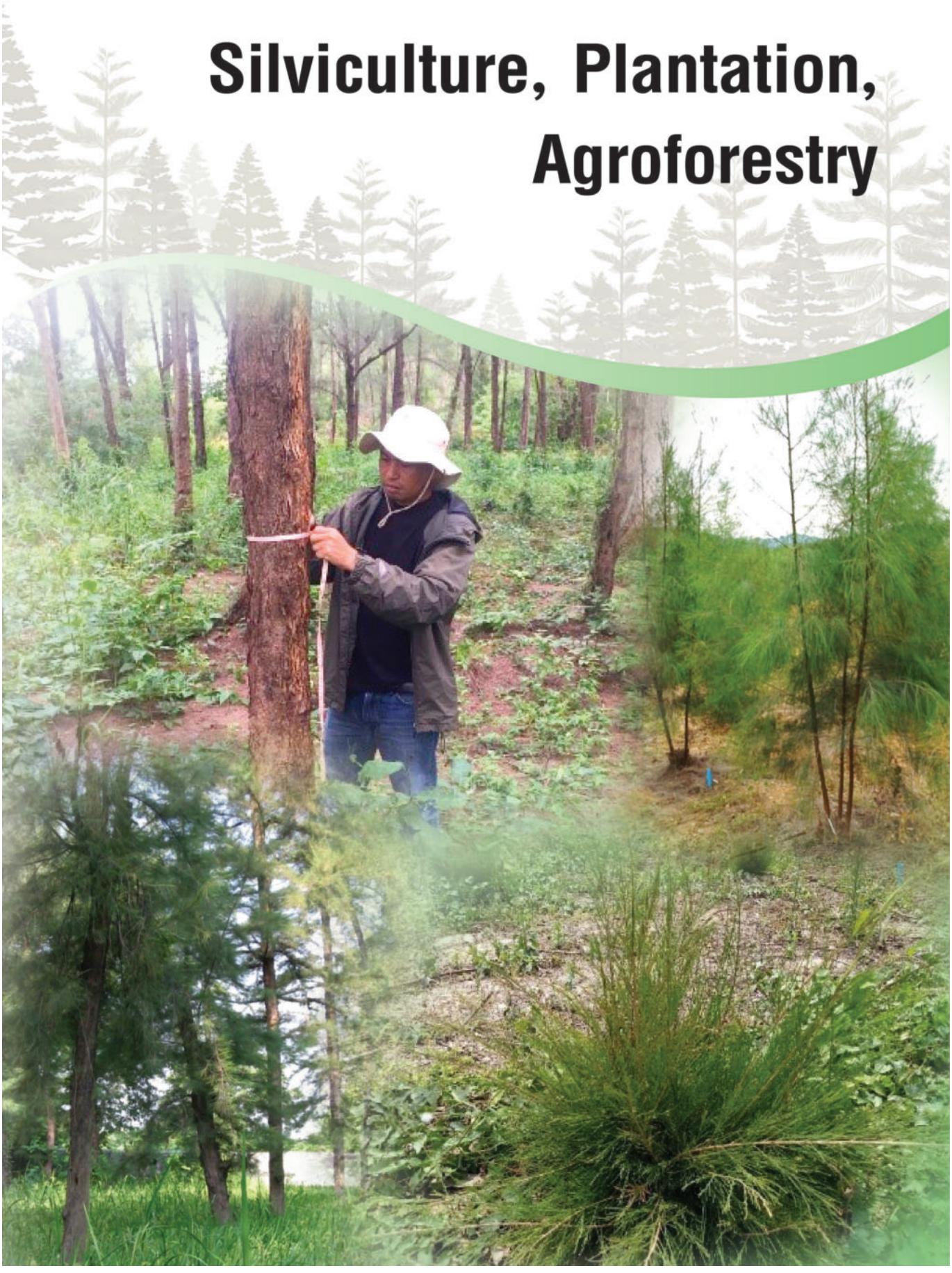
Silviculture, Plantation, Agroforestry



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Effect of thinning on growth and yield of naturally regenerated *Casuarina equisetifolia* on tsunami-affected sites in Thailand

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Abstract

The 2004 tsunami in the Andaman Sea was a major natural disaster that severely impacted on human life, communities, livelihoods, and coastal ecosystems of southern Thailand. *Casuarina equisetifolia* littoral forests were also impacted. The objectives of this study were to investigate the effect of thinning on growth and yield of *C. equisetifolia* that had naturally regenerated on tsunami-affected land at the Andaman Coastal Research Station for Development, Ranong province, Thailand. A completely randomized design with three replications and plots of 30 m x 30 m was used. Three different thinning regimes were applied to an 8-year-old stand, 177 (T1), 266 (T2), and 435 (T3) retained trees ha^{-1} were compared with the control (584 trees ha^{-1}). Diameter at breast height (DBH) and total height (H) were measured, with an estimation made of aboveground biomass (AGB), and stem volume (V). DBH, H, AGB, and V were subjected to analysis of variance. The results showed that DBH and H of 14-year-old *C. equisetifolia* were significantly different among treatments ($p<0.05$). Trees in T1 had the highest in DBH and H. On the other hand, the control trees had the lowest in DBH and H. Total aboveground biomass and total stem volume were not significantly different ($p>0.05$) between treatments, while volume per tree was the largest in T1 followed by T2, T3, and control, respectively. These findings indicated that heavy thinning could increase growth and yield of *C. equisetifolia* individual trees. Hence, heavy thinning of *C. equisetifolia*, with about 2/3 of trees removed and final stocking of 150-200 trees ha^{-1}, is strongly recommended to promote growth of larger trees, leading to an establishment of seed production area and better protecting the coastal zone from erosion.

Keywords: *Casuarina equisetifolia*, tsunami, thinning, growth, yield

1 Introduction

The 2004 tsunami in the Andaman Sea in southern Thailand, the Indian Ocean and many countries in Asia and Africa caused immense damage. Approximately 230,000 people lost their lives and major damage was caused to homes, schools, temples, infrastructure, business and coastal ecosystems. Six provinces in

southern Thailand were hit by the 2004 tsunami including Phuket, Phangnga, Krabi, Ranong, Trang, and Satun (Asian Disaster Preparedness Center, 2006). Near-coastal forest was destroyed and damaged in southern Thailand. Large trees were uprooted and small trees were destroyed. Moreover, the root system of residual trees was also damaged with major soil erosion. Tree species prominent in the coastal and beach forest areas include *Casuarina equisetifolia, Pongamia pinnata,* and *Hibiscus tiliaceus.* Especially, *C. equisetifolia* was damaged by the giant waves of the tsunami (Kashio, 2005).

C. equisetifolia has a wide natural distribution along the coastal areas of Thailand, Malaysia, Indonesia, Philippines to subtropical Australia, Melanesia, and Micronesia (National Reseach Council, 1984). It grows well in tropical and sub-tropical humid lowlands with annual rainfall approximately 700-2,000 mm. *C. equisetifolia* is fast-growing tree and planted widely in plantation for wood production in many countries. It has rapid growth and high above-ground biomass compared to *Leucaena leucocephala* and *Eucalyptus robusta* (Parrotta, 1990). Wood of *C. equisetifolia* was mainly used for fuelwood, woodchip, raw material for pulp, construction, and poles (National Reseach Council, 1984). In addition, this species is a nitrogen fixing tree, suitable for soil improvement in degraded lands (National Reseach Council, 1984; Parrotta, 1990; Warrier et al., 2013).

C. equisetifolia is a pioneer tree in coastal sites. Thaiutsa and Tanpibal (1988) reported that *C. equisetifolia* displayed good natural regeneration in a post-tin mining area in Phang Nga province with density of 8,000 seedlings ha^{-1}. High density of trees contributed to low growth rate and productivity (Wongprom et al., 2013), and strong competition occured in dense tree stand leading to high mortality (Thaiutsa, 1990). Thinning is often used in plantation management to promote tree growth, health and productivity. *Casuarina equisetifolia* on a coastal site in the Andaman Coastal Research Station for Development had high density regenerating following the 2004 tsunami. Thinning trials were undertaken on 8-year-old *C. equisetifolia* for the purpose of informing forest management and landscape restoration. Thus, the objectives of this study were to estimate growth and productivity of 14-year-old *C. equisetifolia,* six years after application of the three thinning regimes.

2 Materials and methods

2.1 Study site

The study site was located on tsunami-affected area in 2004 at the Andaman Coastal Research Station for Development, Ranong province, southern Thailand. Mean annual rainfall and number of rainy days per annum were 4,119 mm and 194 days, respectively. Mean maximum and minimum temperature were 32.0 and 23.2°C, respectively (Amatayakul and Chomtha, 2017). Dominant tree species in these coastal forests include *Casuarina equisetifolia, Pongamia pinnata, Terminalia catappa,* and *Hibiscus tiliaceus.*

2.2 Experimental plot and data collection

A completely randomized design with three replications and plots of 30 m x 30 m was used. Low thinning method was practiced in an 8-year-old *C. equisetifolia* stand naturally regenerated after the tsunami leading to high stocking of seedlings and saplings of *C. equisetifolia.* Three different thinning regimes, with 177 (T1), 266 (T2), and 435 (T3) retained trees (trees ha^{-1}) were compared with the control (584 trees ha^{-1}). Small and irregular trees in T1, T2, and T3 were removed. DBH of 14-year-old *C. equisetifolia* was measured using diameter tape and H was estimated with relationship between DBH and H. Ten representative trees were measured. The height equation was shown as follow:

$$Y = 5.1712\ (DBH)^{\wedge 0.5793} \quad R^2 = 0.9262$$

where Y is total height of tree (m) and DBH is diameter at breast height (cm).

Ten representative trees in different DBH classes were cut for establishing an allometric equation for biomass in relation to DBH. The aboveground biomass was determined by using the stratified clip technique (Monsi and Saeki, 1953). Each tree was separated as logs: 0-0.3 m, 0.3-1.3 m, 1.3-2.3 m, etc. every 1.0 m. Total height and diameter of logs were measured. However, volume of top log was estimated by 1/3 x basal area x length. Fresh weight of stems, branches and leaf were weighed in the field and representative samples from each tree were taken for dry weight determination. All samples were oven-dried at 80°C for 48 h to constant weight. The volume of each log was calculated using the Smalian's formula. Aboveground biomass of stem, branch and leaf was estimated by allometric equation:

$$Y = a\,(DBH^2H)^b$$

where a and b are specific constants, DBH is diameter at breast height (cm), H is total height (m), and Y is aboveground biomass of tree component (kg tree^{-1}) or stem volume (m3 tree^{-1}).

2.3 Data analysis

DBH, H, aboveground biomass (AGB), and stem volume (V) among treasments were analyzed and tested using one way analysis of variance. The means were compared using the Tukey test at a 95% confidence level.

3 Results

3.1 Growth performance

Growth of 14-year-old *C. equisetifolia* after thinning showed significant differences among treatments ($p<0.05$) for DBH and H. DBH and H of trees in the T1, T2, and T3 plots were greater than the control plot, especially T1 was the highest for both DBH and H (Table 1). Self-thinning in unthinned plot was the highest with a value of 48%. High tree density in the control plot had a negative impact on growth rate.

3.2 Above-ground biomass

The above-ground biomass was estimated using allometric equations generated from ten representative trees (n=10). Relationship between DBH and H was used ($Y = a \times (DBH^2H)^b$) with their high coefficient of determination. The allometric equations of stem, branches, and needle biomass are shown in Table 2. The above-ground biomass of each component in the 14-year-old *C. equisetifolia* did not significantly differ among treatments ($p>0.05$). Stem biomass was a main biomass with 85.9-89.0% of total above-ground biomass, followed by branches and leaf needles, respectively (Table 3). Total above-ground biomass

Table 1. Diameter at breast height (DBH), total height (H), growth increment and self-thinning of 14-year-old *C. equisetifolia* after thinning.

Treatment	Density (trees ha^{-1})	DBH (cm)	Total height (m)	Self-thinning (%)
T1	177	30.7 a	37.5 a	7.93
T2	266	28.3 ab	35.5 ab	22.0
T3	435	26.3 b	34.6 b	39.8
Control	584	22.3 c	27.7 c	48.0
F-value	-	18.32*	7.74*	-

NB. * = significant different at $p<0.05$ and different letters in the same column indicated significant differences at $p<0.05$.

Table 2. Allometric equations ($Y = a \times (DBH^2H)^b$) developed to estimate above-ground biomass (dry weight : kg tree^{-1}) and stem volume (m^3 tree^{-1}) of various components for 14-year-old *C. equisetifolia.*

Components	Regression parameters			
	a	b	R^2	n
Stem	0.0910	0.8361	0.9741	10
Branches	0.0006	1.0906	0.9547	10
Leaf needle	0.0003	1.0813	0.9541	10
Stem volume	0.0001	0.8858	0.9941	10

NB. a and b are regression coefficients, R^2 is correlation cocffcient, n is number of observations, DBH is diameter at breast height (cm) and H is height (m).

Table 3. Above-ground biomass of stem (W_S), branches (W_B), needle (W_L), total above-ground biomass (AGB_T), stem volume per tree (V_S), and total stem volume (V_T) of 14-year-old *C. equisetifolia* stand after thinning.

Treatment	W_S (MT ha^{-1})	W_B (MT ha^{-1})	W_L (MT ha^{-1})	AGB_T (MT ha^{-1})	V_S (m^3 tree^{-1})	V_T (m^3 ha^{-1})
T1	96.6	9.37	4.22	110	1.01 a	180
T2	103.7	10.2	4.52	118	0.96 ab	192
T3	102.6	12.5	4.37	120	0.78 bc	162
Control	89.3	7.48	3.59	100	0.57 c	161
F-value	0.929 ns	0.976 ns	1.266 ns	0.942 ns	10.380*	2.885 ns

Remarks: * = significant different at $p<0.05$, ns is not significant different at $p>0.05$, and different letters in the same column indicated significant difference at $p<0.05$.

was between 100-120 MT ha^{-1}. Biomass production of thinned plots was similar to unthinned plots. Trees exhibited very good growth after thinning, especially heavy thinning.

3.3 Stem volume

Total stem volume of *C. equisetifolia* after thinning was between 161 and 192 m^3 ha^{-1} and not significantly different between treastments ($p>0.05$). However, stem volume per tree was significantly different between treatments ($p<0.05$). T1 was the highest in stem volume per tree followed by T2, T3, and control. Total stem volume of T1, T2, and T3 plots was 1.77, 1.68 and 1.37 times, respectively greater than control plot. The intensity of thinning resulted in increasing stem volume per tree.

4 Discussion

Our results from growth and yield evaluations showed that thinning significantly affected the growth and yield of *C. equisetifolia*. *C. equisetifolia* exhibited good growth after thinning with DBH of 26.3-30.7 cm. It grows well, similar to *Acacia mangium* at the same age, on abandoned tin mining area in southern Thailand (Wongprom et al., 2018). At the same age, *C. equisetifolia* in this area grows faster than other fast-growing tree species planted in Thailand (Table 4). *C. equisetifolia* typically grows rapidly in coastal sites. High rainfall at this site, approximately 4,100 mm year^{-1} and appropriate site conditions are favourable for growth. However, *C. equisetifolia* is a pioneer tree developing a large canopy in open spacings. High density of *C. equisetifolia* led to high mortarity, because of strong competition and shading from neighboring trees. In this study the unthinned plot (control) displayed high natural mortarity rate due to self-thinning. Thaiutsa (1990) found that density of *C. equisetifolia* plantations decrease with age leading to low productivity. Thinning was reccommented for enhancing vigour and health of retained trees.

Thinning intensity significantly influences above-ground biomass and stem volume of retained trees (Hawley, 1947). Heavy thinning (T1) gave the highest value for biomass and volume increments of retained trees. Tree increment increased with thinning intensity levels (Wongprom et al., 2013). Total above-ground biomass of *C. equisetifolia* in this site was similar to *Acacia mangium*, which was the second thinning on abandoned mining area with value of 108-147 MT ha^{-1} (Wongprom et al., 2018). However, total above-ground biomass in this study was lower than that of 12-year-old *C. equisetifolia* on post mining in southern Thailand with value of 255 t ha^{-1} (Thaiutsa, 1990), because number of retained trees was higher than in the *A. mangium* study. Growth and yield of *C. equisetifolia* depends on various factors, including tree density, genetic variation, site conditions, and silvicultural practices (Thaiutsa, 1990; Thaiutsa et al., 2001; Pinyopusarerk et al., 2004; Ravi et al., 2012; Wang et al., 2013; Kumar et al., 2017). In addition, Thaiutsa (1990) reported that biomass productivity of

Table 4. Growth performance of *C. equisetifolia* and other fast-growing tree species at aged 14 years in Thailand.

Species	Density (trees ha^{-1})	DBH (cm)	H (m)	Rainfall (mm)	Remarks
Casuarina equisetifolia	177	30.7	37.5	4,119	Current study
	266	28.3	35.5		
	435	26.3	34.6		
	control	22.3	27.7		
Acacia mangium	150	34.5	30.8	3,669	Wongprom et al., 2018
	200	30.9	30.4		
	300	28.4	30.8		
Acacia mangium	487	21.1	22.4	1,312	Peawsa-ad and Viriyabuncha, 2002
Eucalyptus camaldulensis	1,187	13.2	20.1	1,316	Viriyabuncha et al., 2009
Acacia crassicarpa	2,361	16.2	15.0	1,050	Meunpong, 2012
Azadirachta indica	2,361	11.2	9.1		

C. equisetifolia decreased with age, while tree mortality increases with age. Thus, age of *C. equisetifolia* and tree density need to be taken into account when considering plantation management options and objectives.

Large diameter stems of *C. equisetifolia* have multiple uses, i.e., house construction, timber, and pole. Number of trees in large DBH class were mostly recorded in the thinned plots (Fig. 1), especially the most heavily thinned treatment; in contrast the greatest number of trees in small DBH classese was recorded in the unthinned plots. Thinning promotes both larger stems and increased wood value, such as saw-logs (Beadle et al., 2013). This result can be applied to the management of *C. equisetifolia* plantations.

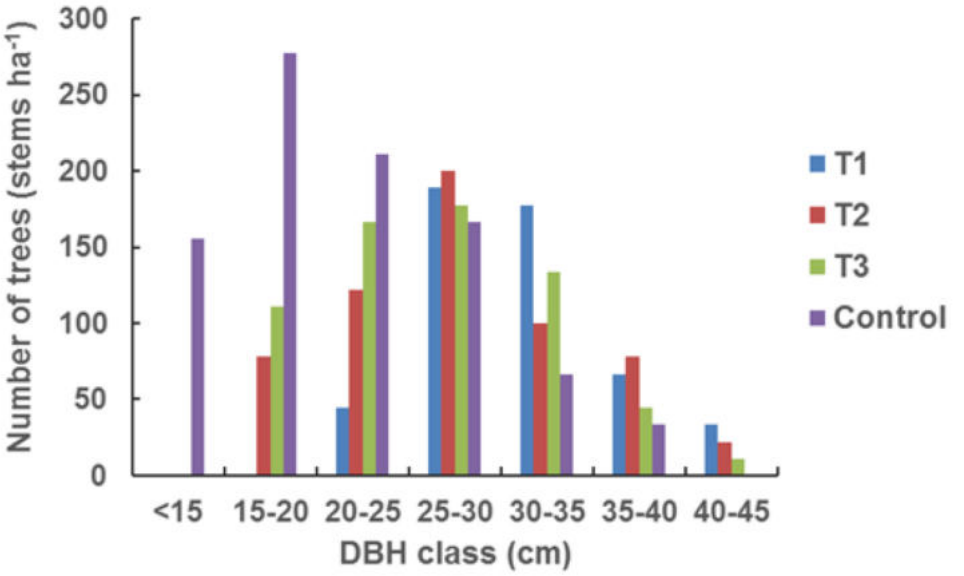

Fig. 1. Number of trees in each diameter class of 14-year-old *C. equisetifolia* after thinning.

C. equisetifolia is a pioneer tree with typically good natural regeneration in coastal areas. Our study has shown that thinning increases wood productivity (more larger trees useful for wood production) and ecological services. However, different levels of thinning intensity could be considered depending on the objectives. For wood utilization, Thaiutsa (1990) reccommented that light thinning with 25% of *C. equisetifolia* stand and moderate thinning with 25-50% of *C. equisetifolia* stands be done for woodchips and poles, respectively. On our research site, heavy thinning is recommended where timber production is the main objective. Native *C. equisetifolia* on this site exhibited high survival, growth and productivity compared to exotic *C. equisetifolia* provenances (Thaiutsa et al., 2001). Accordingly, it could be managed as both a seed production area and coastal erosion protection.

The thinned trees were used by the Andaman Coastal Research Station for Development and local community, such as for pole, fuelwood, and house construction as well as fish and squid traps, which was an important role in livelihoods of coastal communities. *C. equisetifolia* is highly recommended for planting in coastal sites for multiple purposes including as fuelwood, windbreak, and erosion control (National Reseach Council, 1984). Thinning should be undertaken for utilizing wood while at the same time enhancing the vigor and health of the retained trees. Moreover, these trees can maintain ecosystems, reduce storm damage, and protect coastal sites from erosion.

5 Conclusion

Thinning is a highly valuable management tool for natural regenerated *C. equisetifolia* stands. It can promote growth and yield of the individual retained trees. DBH, above-ground biomass, and tree volume of thinned trees increased substantially in comparison to unthinned trees. However, above-ground biomass and stem volume among treatments were not different. T1 with density of 177 retained trees ha^{-1} is recommended for *C. equisetifolia* stands in Thailand. A large number of thinned trees in T1 were able to be used for fuelwood, pole, and timber: if left unthinned many of these stems would have been wasted due to both high self thinning and low growth. Therefore, thinning should be practiced for increasing tree growth and productivity of remaining trees. These results were usful for *C. equisetifolia* plantation and coastal forest management. Moreover, thinning can be used to establish a seed production area of *C. equisetifolia* stand and at the same time reduce coastal erosion.

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Reclamation of limestone mine spoil through casuarina plantation

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Abstract

Mine dumps and spoils create various environmental problems if not reclaimed. Mine site reclamation and ecological restoration is an urgent need for environmental management and increasing biodiversity of mine spoil. Identification of plants that are adapted for growth on polluted sites can be used for restoration of degraded soil. This study was conducted in Associated Cement Company (ACC) open cast limestone mine site in Coimbatore, South India. This mine manufactures cement leading to the dumping of huge amounts of infertile overburden material on adjacent non-mined land as well as another waste product called floatation reject, a quartz sand which is separated as an impurity in the manufacture of cement. An experiment was conducted to compare the growth of five tree species viz. *Acacia auriculiformis, A. nilotica, Casuarina equisetifolia, Eucalyptus tereticornis* and *Leucaena leucocephala*. Total tree height, girth at breast height (GBH) and biomass were measured at 10 years after planting.

The fastest growing species was *C. equisetifolia* reaching 10.9 m tall, 20.2 cm girth at breast height with a total biomass of 20.8 kg tree^{-1}. Biomass allocation of the components of three multipurpose trees shows that there is an increase in the above ground biomass of all the tree components such as stem, branch, twig and foliage was higher in *C. equisetifolia* except bark followed by *E. tereticornis*. The total biomass produced in the order was *C. equisetifolia*>*E. tereticornis*>*A. nilotica*>*L. leucocephala*>*A. auriculiformis*.

Keywords: *Casuarina equisetifolia, Leucaena leucocephala, Acacia nilotica, Acacia auriculiformis, Eucalyptus tereticornis,* growth, biomass

1 Introduction

Mining is an important and imperative land use; however mine dump and spoils create various environmental problems if not reclaimed in a thorough and timely manner. In Madukkarai (Coimbatore District, Tamil Nadu State, India) extensive lime stone mining has been undertaken on an area of 30 km^2. After collecting the limestone from the mining site, the residual rock is dumped within the mining leased area

as heap ranging from 10-15 m high. Every year the dumping area is increasing at the rate of 50 ha. In addition to the mine spoil dumps, there is another material called mine reject is also dumped near the mining area. Management of top soil is important for reclamation plan to reduce the N losses and to increase soil nutrients and microbes. Re-vegetation constitutes the most widely accepted and useful way to reduce erosion and protect soils against further degradation during reclamation. Mine restoration efforts have focused on leguminous or N-fixing species as well as grasses, herbs, and trees. Plants tolerant of high metal levels can be effective for acidic and heavy metals bearing soils. Reclamation of abandoned mine land is a complex and challenging process. Once the reclamation plan has been implemented and vegetation has established, the assessment of the reclaimed site is desirable to evaluate the success of reclamation. Different physical, chemical and biological remediation technologies have been developed during previous decades and technology selection in remediation process is site specific (Mulligan et al., 2001). However, physical and chemical technologies are unsuitable for large areas such as mining sites (Khan., 2005). Bioremediation strategies using plants and microbes are more suitable and efficient for improving the quality of degraded landscapes (Rosenvald et al., 2011) and soil fertility, as a parameter of soil quality, is one of the major factors for plant growth and improvement (Sarma, 2005).

In the process of cement manufacture, the rejected quartz-rich material was dumped in an area adjoining the factory which is an inert material more powdery and less dense than sand. Since the material is dumped 3 m above the road level it flies in the wind causing serious problems and a hazard to adjacent traffic.

Mined site reclamation and ecological restoration is urgently needed to stop local pollution hazards and restore the biodiversity of mine spoil. Identification of plants with adaptation mechanisms for growth on polluted sites can aid in the restoration of degraded soil. This floral diversity data will be useful for developing appropriate strategies for introduction of suitable tree species as soil binders and nitrogen fixers for improving the soil fertility. Hence, it is aimed to assess the deterioration of chemical, physical properties of mine dump area for their management to get productive mine soil by introducing suitable tree species.

2 Methodology

2.1 Study site

The study was conducted on Associated Cement Company (ACC) land in Coimbatore, a open cast limestone mine for manufacture of cement. This mining results in the dumping of huge amounts of infertile overburden material on adjacent un-mined land as well as another material called floatation reject which is quartz sand separated as impurity in the manufacture of cement. The study area situated in Madukkarai, at an elevation of 650 – 700 m, with a sub-humid to semi-arid climate, mean annual rainfall of 600 – 750 mm, and temperature ranges from 21 °C during winter to 39 °C during summer.

2.2 Remedial measure undertaken

The prime problem was to arrest the flying wind-blown quartz material. The first step was to flatten the heaps, then wet the material close to the road and pour over slurry of cow dung to stabilize the dust from blowing around. At the same time casuarinas seedlings of 1 m in height were planted about 25 cm apart in a grid pattern to cover the roadside to prevent wind effect. The seedlings were watered profusely during evening to stop the seedlings from drying-out and stabilize the quartz dust.

Seedlings of five fast-growing tree species (*Acacia auriculiformis, A. nilotica, Casuarina equisetifolia, Eucalyptus tereticornis* and *Leucaena leucocephala*) were obtained from the Institute of Forest Genetics and Tree Breeding in Coimbatore. Six-month old seedlings were planted in 30 cm x 30 cm x 30 cm pits at a spacing of 2 x 2 m with a randomized block design. These seedlings were treated with microbial inoculants (Arbuscular mycorrhiza fungi, *Frankia, Rhizobium, Azospirillum, phosphobacteria*), coir pith, urea, single super phosphate and muriatic of potash. In addition, a cover crop of *Indigofera sumatrana* and *Tephrosia purpurea* was planted. Soil cultivation and regular watering was done in the initial stage.

2.3 Biometric observations

Tree height and girth at breast height (GBH) were measured at 10 years after planting. Biomass was estimated at the end of ten years, using a random sampling method. Selected trees were clear felled and carefully uprooted to ensure that as much as

possible of the root system was harvested. Biomass components such as foliage, branch, stem, bark and root were separated and fresh weight was measured directly in the field. Samples were taken for biomass estimation.

3 Results

3.1 Growth

The growth of different species is presented in Table 1. *C. equisetifolia* recorded the maximum height (10.9 m) followed by *E. tereticornis* and *L. leucocephala*. The shortest trees were *Acacia nilotica*. Similarly, girth at breast height of *C. equisetifolia* recorded the maximum (19.8 cm) followed by *E. tereticornis* and *L. leucocephala*, with plants of *A. auriculiformis* having the smallest girth.

3.2 Biomass production

Biomass data presented in Table1 shows the biomass allocation of the different components of various multipurpose trees. Trees of *C. equisetifolia* had the highest above ground biomass for all the tree components except bark, followed by *E. tereticornis* and *A. nilotica*. The biomass production was generally low for trees of *L. leucocephala* and similar to *A. auriculiformis*. Extensive root system of *Casuarina* root recorded higher below ground biomass when compared to other species followed by *E. tereticornis* which was similar to *A. nilotica*. Estimated total biomass was in the order: *C. equisetifolia*>*E. tereticornis*>*A. nilotica*>*L. leucocephala*>*A. auriculiformis*.

4 Discussion

In farm forestry plantings on the east coast district of Tamil Nadu, India the height of *C. equisetifolia* ranged from 9.9 m to 11.9 m and GBH ranged from 16.8 cm to 23.2 cm at two years after planting. When inoculated with bio-fertilizers, *C. equisetifolia* recorded an average height of 11.0 m and 21.3 cm GBH and basal diameter of 26.8 cm after three growing seasons (Uma et al., 2011; Uma et al., 2014). However, for casuarinas grown in adverse soil conditions the survival percentage is lower, with increased branching and with extensive root system associated with nitrogen availability through *Frankia*. *Frankia* invades root hairs of *Casuarina*, developing within cortical cells in the lobes of resultant

Table 1. Growth and biomass (kg) of 10-year-old multipurpose trees planted at mine reclamation area. Means followed by a common letter(s) in the same column are not significantly different at the 5% level by DMRT.

Species	Height (m)	GBH (cm)	Stem (kg)	Bark (kg)	Branch & twig (kg)	Foliage (kg)	Total above-ground biomass (kg)	Roots (kg)	Total biomass (kg)
Casuarina equisetifolia	10.9e	19.8d	10.8d	1.21b	2.89c	3.28c	18.1d	2.70c	20.8d
Acacia nilotica	4.75a	18.6bc	4.32a	1.46b	1.24ab	1.16a	8.17b	1.84b	10.0b
Eucalyptus tereticornis	9.07d	19.2c	7.86c	1.31b	1.60b	1.71b	12.5c	1.99b	14.5c
Leucaena leucocephala	7.59c	16.2b	4.85b	0.74a	0.95a	1.64a	8.18b	1.57a	9.74a
Acacia auriculiformis	6.59b	13.2a	3.65a	0.64a	1.55b	1.84b	7.68a	1.77a	9.443a
LSD (0.05)	0.37	2.25	1.57	0.24	0.41	0.44	2.28	0.48	2.62

nodules. *Frankia* is able to incorporate atmospheric nitrogen into amino acids, which are the building blocks of proteins. *Frankia* exchange fixed nitrogen for carbohydrates from the plant. As the plants drop organic matter, or when the plants die, the nitrogen from their tissues is made available to other plants and organisms. This process of accumulating atmospheric nitrogen in plants and recycling it through organic matter is the major source of nitrogen in tropical ecosystems.

Nitrogen fixing tree species

Approaches to soil management, including problems of soil degradation and low soil fertility, have undergone major changes in recent times. Trees play an important role in improving the soil fertility due to extensive root system, extending below the root zone of crops and herbaceous plants. Establishment of N_2 fixing plants is a crucial phase in successful afforestation of disturbed lands like mine spoils; available nitrogen levels are a major limiting factor and regular addition of nitrogen may be required to maintain healthy growth and persistence of vegetation (Yang et al., 2003; Song et al., 2004; Valente et al., 2012). Selection of appropriate plant species can be made for different mine spoil environments by considering factors such as (a) growth rate and colonising capacity, (b) nitrogen-fixation capacity and ability to increase soil organic matter and fertility, (c) fruit yielding plants attracting birds, butterflies and other forms or wildlife and also encourage soil fauna, and (d) plants having social and economic values for local population. Both N_2-fixing legume and non-legume tree species play critical roles in reclamation of areas degraded by soil erosion, construction and mining activities. We recommend an alternative approach to introduce legumes and other nitrogen-fixing species. Nitrogen fixing species have a dramatic effect on soil fertility through production of readily decomposable nutrient rich litter and turnover of fine roots and nodules. *Casuarina equisetifolia* is a fast growing multipurpose actinorhizal tree and commonly used for wasteland development (Kondas, 1983; Ponnampalam et al., 1999), and is suitable for farm forestry, agro-forestry plantation, soil reclamation due to its association with symbiotic nitrogen fixing microorganism of *Frankia* and arbuscular mycorrhizae which is used for soil reclamation and widely adopted by farmers of southern part of Tamil Nadu India (Rajendran et al., 2000; Rajendran et al., 2003; Rajendran and Devaraj, 2004; Uma et al., 2011; Rajendran and Mohan, 2014). Results of species trial conducted in Quartz sand dump at Madukkarai, Coimbatore district of Tamil Nadu indicate that *C. equisetifolia* root cuttings exhibited better growth (3.34 m height) and total biomass of 5.8 kg tree^{-1} at three years. Litter production and heavy root infection and maximum number of AM fungal spores were greater than *Casuarina junghuhniana* seedlings and it can be used for afforestation and reclamation of Quartz sand dump (Ponnampalam et al., 1999). *Casuarina equisetifolia* recorded the highest suitability index of 364 for the afforestation of quartz sand dumps (Ponnambalam et al., 1999; Párraga-Aguado et al., 2016). In order to restore forest ecosystem especially in mined areas suitability of tree species has to be assessed for its suitability estimation based on suitability index which is summation of biomass index score (BIS) plus biomass score (BS) plus rooting potential (RP) plus survival score (SS). Therefore suitability index = sum total (BIS+BS+RP+SS). These tree species not only fix the atmospheric nitrogen but also produce a high amount of litter which can be used to improve soil fertility and moisture conservation. They can also grow well in semi-arid zones in the southern part of Tamil Nadu, India. Mineralization of N-rich litter from these species allow substantial transfer to companion species and subsequent cycling, thus enabling the development of a self-sustaining ecosystem (Zhang et al., 2001).

5 Conclusion

Reclamation of mine spoiled area is a difficult task which requires sound research related to the soil/mine spoils on the site, selection of suitable tree species for reclamation and binder plant species for soil improvement. Moreover, it is important to standardize suitable methods of soil moisture conservation using locally available organic waste, beneficial microbes particularly helper bacteria and fungi (AM fungi) for the sustainable nutrient management and afforestation. Application of beneficial microbes during afforestation in mine spoils appears to be a cost effective and environment friendly approach. The reclaimed and rehabilitated sites are fast restoring, increasing the

bio-diversity by attracting host of soil microbes and invertebrates, insects and birds. Based on the results of the present study, we suggest that reclamation of mine spoil dump through establishment of most suitable tree species will elevate biological diversity. Nitrogen fixing species, notably *C. equisetifolia*, are especially important for improving the physical, chemical and biological properties of the soil and soil water conservation. Revegetation of mine site overburden has greatly enhanced the aesthetic and ecological values of the reclaimed areas.

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Enhancing climate resilience in coastal areas of Bangladesh using plantations of *Casuarina equisetifolia* L.

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Abstract

Coastal regions of Bangladesh, covering 32% of the country's land area, are highly vulnerable to climate-induced disasters. Creation of greenbelts through the establishment of mangrove and non-mangrove tree plantations along the coastal belt can play crucial role in reducing vulnerabilities and hazards of extreme weather events. Bangladesh Forest Department under several initiatives conducted massive tree plantations in the coastal areas of the country for enhancing resilience to climate change and extremes. Plantations were conducted with a number of mangrove and non-mangrove species including *Sonneratia apetala, Excoecaria agallocha* and *Avicennia officinalis* in the mangrove and *Casuarina equisetifolia, Acacia auriculiformis, Neolamarckia cadamba* and *Swietenia mahagoni* in the non-mangrove plantations. This study assessed the growth performance and potential of *Casuarina equisetifolia,* a prominent species in the new plantations, for creating a coastal green belt in order to combat climate-change induced hazards. The study was conducted in 2017, in four forest divisions extending to six administrative districts of Bangladesh, through random quadrat survey of pure *C. equisetifolia* plantations as well as in mixed plantations with other species. The quadrats were 20 m × 20 m sized in the block plantations whereas in the strip plantations they were 20 m × 2 m sized. Plantations of three different ages (0.5, 1.5 and 2.5 years) were surveyed which revealed very promising growth of *C. equisetifolia* in terms of diameter, height, mean annual increment and survival percentage. The survival percentage varied from 83-97% at 2.5-years old and 85-98% at 0.5 years old plantations across the forest divisions which are much higher than observed in other mangrove plantations. The trend of height growth is satisfactory as the study indicated that mean annual increment (MAI) of height for 2.5-year aged *C. equisetifolia* plantations ranged from 1.2 m in Chittagong Coastal Forest Division (block plantation) to 3.2 m in Feni Social Forest Division (strip plantations). A consistent trend of DBH growth was also observed across the forest division and plantations types which were maximum in 4.5 cm in Noakhali Coastal Forest Division. Among the four forest divisions, Noakhali and Patuakhali Coastal Forest Divisions showed comparatively better

height and diameter growth in the three different aged plantations. A comparison with the growth parameters of other species conducted in the same or adjacent area showed better performance of *C. equisetifolia* which indicates its higher potentiality for creating coastal green belt as well as for enhanced resistance to the extreme climate events. The study concluded that *C. equisetifolia* is a promising species in the coastal region of Bangladesh for establishing a more resilient forested environment against climate-induced extreme weather events.

Keywords: coastal plantation, climate-resilient, cyclone, vulnerability, sandy beaches, windbreakers

1 Introduction

Coastal areas of Bangladesh, stretching along 711 km, comprise 20% of the total land area of the country with a population of 38 million or 27% of the total population (BBS, 2018). Such a huge population is living in a vulnerable condition due to the highly natural disaster prone nature of this zone. Every year a number of cyclones and storms impact the coast of Bangladesh some of which are highly destructive. Establishment of coastal green belts along with the creation of a resilient ecosystem is one of the most important issues for fighting climate change induced calamities and reducing the damage associated with those calamities. Bangladesh Forest Department has undertaken a number of measures to enhance resilience and adaptability of coastal people to climate change impacts. Climate Resilience Participatory Afforestation and Reforestation project is one of the largest initiatives where a substantial number of native and exotic tree species have been planted to build a coastal greenbelt and thus resilience of the coastal ecosystem to climate induced hazards (Bhuyian et al., 2019). Shelterbelts have been widely developed in coastal areas with different trees species. Coastal shelterbelts are well recognized for their roles in mitigating climate change and reducing the impacts of climate induced or sea-side natural hazards (Bai, 2010; Tanaka et al., 2011; Czerepowicz et al., 2012), soil stabilization (Prasetya, 2007; Bai, 2010), reducing damage of tsunami along the shore line (Fritz and Blount, 2007; De Zoysa, 2008; Miah et al., 2013). *Casuarina equisetifolia* is one of the fast growing species that is widely planted in the shelterbelt (Takle et al., 2007). In recent plantations of Bangladesh, along with *C. equisetifolia, Acacia auriculiformis, Swietenia mahagoni, Artocarpus chama, Syzygium* spp., *Swintonia floribunda* etc. are the frequent species that planted in the core zone and buffer zone plantation whereas *Sonneratia apetala, Excoecaria agallocha, Avicennia officinalis* are the mangrove species that planted in the saline conditions (Eusuf and Associates, 2016). In addition, *C. equisetifolia* is being planted in the coastal areas of Bangladesh since 1966 for protecting coastal people from cyclone and tidal surge (Revilla et al., 1998; Miah et al., 2013).

C. equisetifolia, locally known as *jhau,* is able to establish on barren sand. Its leaves are reduced to scales and the green twigs or cladodes are the main photosynthetic tissues (Orwa et al., 2009). The tree is able to fix atmospheric nitrogen with the help of symbiotic bacteria living in close contact with the root system (Rawat et al., 2010). The latter and litterfall create favorable soil conditions for other beach forest species which subsequently replace the *Casuarina,* since it is not able to establish under closed canopy conditions (Göltenboth et al., 2006). It is very fast-growing (1.5 to 3 m year^{-1}), salt tolerant and readily colonizes rocky coasts, dunes, sandbars, islets and islands: it may also invade moist sites far inland, forming dense stands where grazing animals are absent. Moreover, this species can act as a wind break in the coastal region (CPWD, 2013). It can retard the spread of salt spray towards the agricultural lands (Edward et al., 2006). There are some studies also that reported negative effects of *C. equisetifolia* shelterbelt

(Jadhav and Gaynar, 1995; Namboothri et al., 2008).

There are few studies that reported the impacts of *C. equisetifolia* as a coastal shelterbelt species on social and economic conditions in Sri Lanka (De Zoysa, 2008), carbon stock in *C. equisetifolia* plantations in Timor Leste (Lasco and Cardinoza, 2007), socio-economic and environmental impact of *C. equisetifolia* shelterbelt in Bangladesh (Miah et al., 2013). Although there is a vast plantation area in Bangladesh, there is a dearth of scientific information regarding the comparative growth performance of this species in these plantations. Perceptions of growth performance may help the climate change practitioners to choose species and decide about the extent of shelterbelt plantations with this species. The present study was undertaken with the aim of assessing and evaluating the comparative growth and potentials of *Casuarina equisetifolia* for enhancing resilience of coastal Bangladesh to climate change.

2 Methodology

2.1 Study area

The study was conducted in the four forest divisions of Bangladesh Forest Department in which there are substantial areas planted with *C. equisetifolia* along with other species (Eusuf and Associates, 2016). The four divisions are- Chittagong Coastal Forest Division, Noakhali Coastal Forest Division, Patuakhali Coastal Forest Division and Feni Social Forest Division. These divisions extend from the Southeast of Bangladesh towards the Southwest through the southern coastal shoreline of Bangladesh. The entire area is almost flat having been formed by gradual sediment deposition from upstream. Most of the areas of Chittagong Coastal Forest Division are represented by stable and muddy flat land. The soil is mostly alkaline and sometimes with neutral pH (Haque et al., 2000). Uddin and Hossain (2013) reported almost neutral to slightly alkaline soil in the stable coastal plantations of Noakhali Coastal Forest Division where soil salinity varied from 7.1 to 9.0 dSm^{-1}. The texture is silty loam to sandy loam soil. Rainfall varies seasonally with a maximum (93% of the mean annual rainfall 3,670 mm) from May to October (Khatun et al., 2016). The annual average temperature is 33 °C and a minimum of 16 °C. The average rainfall is 4,285 mm (BBS, 2012). This coastal area frequently faces sea borne natural disasters, notably cyclones and tsunamis. In some areas, flooding and saline water intrusion may be severe especially during the monsoon. Sediments deposition causes accretion of new lands in the nearby areas of the Bay of Bengal and river. Forests in these areas are characterized by mangrove species. BFD conducted plantations with *Sonneratia apetala, Avicennia officinalis* and other species in many of the newly accreted land parcels subjected to regular inundation. In some areas *Excoecaria agallocha* along with other species grows naturally.

2.2 Study design

The study was conducted through stratified sampling method. The quadrats were 20 m × 20 m in the block plantations whereas in the strip plantations they were 20 m × 2 m. Plantations of three different ages i.e. 0.5, 1.5 and 2.5 years were surveyed. Data has been collected from three different types of plantations: mound plantations, non-mangrove plantation and strip plantation. In those plantation *jhau* was planted along with *A. auriculiformis, Terminlia arjuna, Albizia procera, Acacia mangium, Acacia nilotica, Acacia farnesiana* and *Samanea saman,* among which first four were very common and present in almost all the plantations. Growth of these species was also considered an indicator of the comparative growth of *C. equisetifolia.* In each of the plantations, sample plots were located at least 100 m inside of the boundary to avoid the edge effect.

2.3 Data collection and analysis

Growth data particularly the number of individuals per plot, total height and diameter at breast height (DBH) was measured and recorded for all the seedlings occurring in the sample plot. In case of six months (0.5 yr) old plantations, many of the seedlings were too small to measure. Locations, planation type and age of the plantations were recorded. Collected data was then input into a spreadsheet and used for estimating the survival percentage, average height, average DBH, mean annual increment of height and DBH. The mean annual increment of the seedlings in terms of height and DBH were calculated for 1.5- and 2.5-year ages to represent the growth rate. Comparisons were made with similar common plantation species. In most of the plantations *Acacia auriculiformis, A. farnesiana,*

A. mangium, A. nilotica, Albizia procera, Samanea saman, Terminalia arjuna were frequently planted with *C. equisetifolia,* and facilitating direct comparisons. Moreover, a number of species including *Phyllanthus emblica, Terminalia bellirica, Chukrasia tabularis, Syzygium fruticosum* and *Engelhardtia spicata* were found occasionally in the plantations which were treated as 'other species' in growth comparisons with *C. equisetifolia.*

3 Results

3.1 Survival rate of seedlings in the plantations

Jhau (*C. equisetifolia*) showed high survival in the plantations of the four forest divisions. However, plantations at Feni Social Forest Division exhibited highest survival of the seedlings in all the 0.5-year (98%), 1.5 years (97%) and 2.5 years (97%) aged plantations in comparison to three other forest divisions (Table 1).

There were four types of plantations i.e. *jhau,* mound, non-mangrove and strip. In the *jhau* plantations, only *C. equisetifolia* is planted in the sandy sea shore, in mound a number of both short and long rotation species were planted in soil mounds in mixed pattern. Non-mangrove plantations were also composed of mixed species but these are positioned on stable coastal shores on more elevated ground. The strip plantations are also composed of mixed species planted in both sides of the roads and embankments. In all cases, *jhau* (*C. equisetifolia*) is one of the major species. However, the study indicated that up to 2.5 years the survival percentage of is very high in the *Jhau,* non-mangrove and strip plantations (Table 2). The survival percentage is lowest in mound which may be because of the unsuitability of the site to some of the planted species and influence of tidal surge.

3.2 Growth performance of seedlings in the plantations

Height and DBH are the key indicators of growth of the seedlings of *C. equisetifolia* and associated species. The Figure 1 and Figure 2 present the average height and DBH for some of the most common species in each forest division, plantation type at the three studied ages. At 1.5 years of age *C. equisetifolia* is represented by highest mean height (3.2 m) in Noakhali Coastal Forest Division among all the species as well as all the forest divisions. At 1.5 years the average height of *C. equisetifolia* is also highest in Feni Social Forest Division. Average DBH of *C. equisetifolia* is also maximum (3.78 cm) in those two forest divisions at

Table 1. Survival percentage of the plants in different plantations at three different ages. Data are presented as mean ±SE.

Forest Division	0.5 year	1.5 years	2.5 years
Chittagong Coastal Forest Division	85 ± 5.82	83 ± 5.37	83 ± 5.52
Feni Social Forest Division	98 ± 1.33	97 ± 1.00	97 ± 1.79
Noakhali Coastal Forest Division	86 ± 3.34	85 ± 1.65	83 ± 4.47
Patuakhali Coastal Forest Division	93 ± 3.30	88 ± 3.04	83 ± 4.57

Table 2. Survival percentage of the seedlings in different plantations at three different ages. Data are presented as mean ±SE.

Plantation type	0.5 year	1.5 years	2.5 years
Jhau	98 ± 0.67	98 ± 1.20	93 ± 3.21
Mound	68 ± 2.58	64 ± 0.50	60 ± 0.87
Non-mangrove	98 ± 0.90	96 ± 2.08	95 ± 0.75
Strip	97 ± 1.01	91 ± 2.18	96 ± 1.38

the same age. In Chittagong Coastal Forest Division, *A. auriculiformis* attained the maximum height (1.53 m) and in Patuakhali Coastal Forest Division *A. catechu* exhibited the fastest height growth. In terms of DBH, *A. procera* and *A. catechu* dominated all the species in both Chittagong Coastal and Patuakhali Coastal Forest Divisions. However, in those cases where *C. equisetifolia* is not dominant, its mean height and DBH are close to the maximum height and DBH. In all cases the height of *C. equisetifolia* at 1.5 years age varied between 1.1 and 3.2m with DBH from 2.7 to 3.78 cm (Fig. 1).

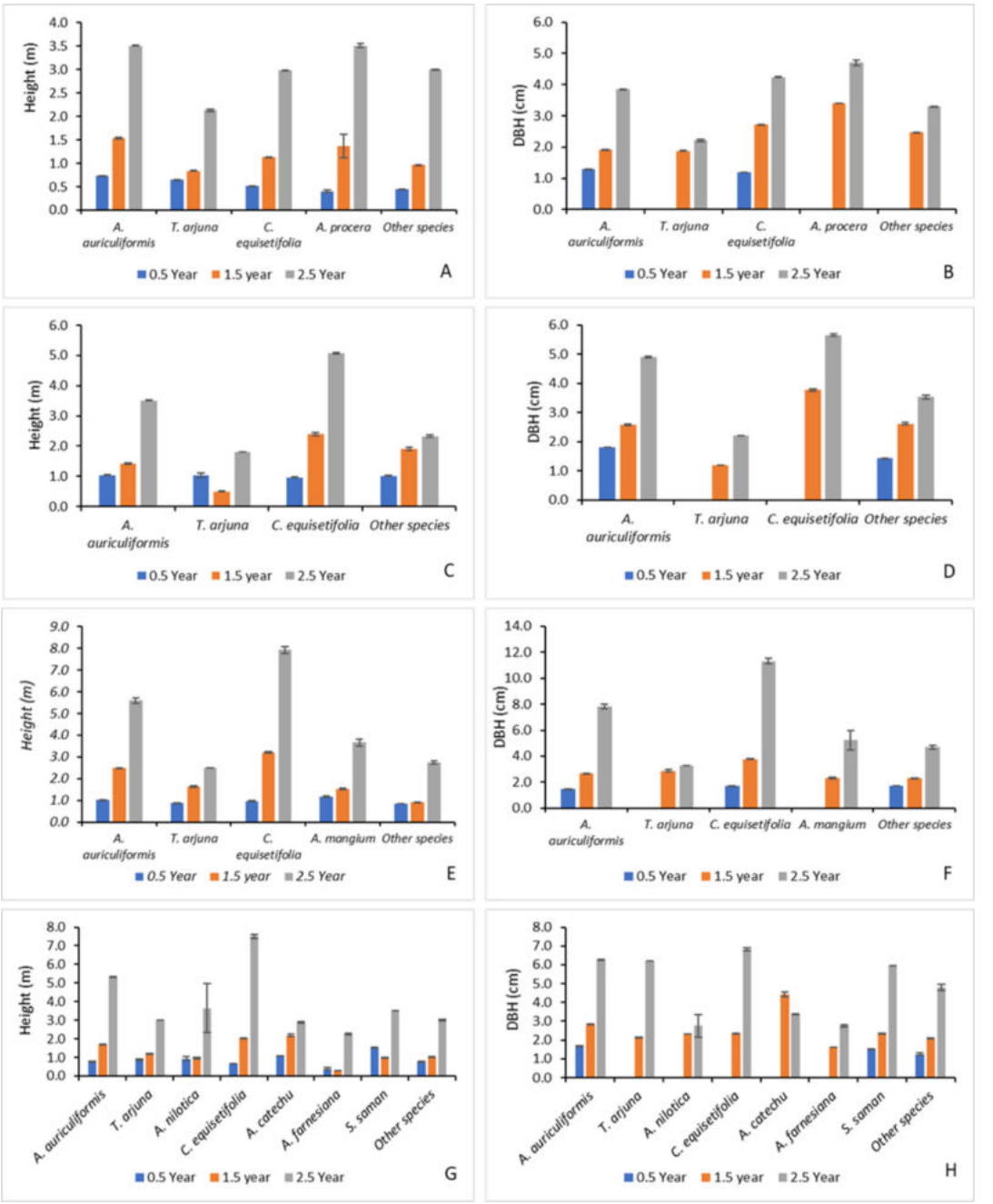

Fig. 1. Growth performance of the seedlings of different species at different age in four forest divisions. Note: A and B = Height and DBH in Chittagong Coastal Forest Division, C and D = Height and DBH in Feni Social Forest Division, E and F = Height and DBH in Noakhali Coastal Forest Division.

Casuarina equisetifolia became dominant in all the forest divisions at 2.5 years aged plantations except in Chittagong Coastal Forest Division as it attained maximum height in those three divisions ranging from 5.1 m to 7.9 m. The mean height of this species is maximum (7.9 m) in Noakhali Coastal Forest Divisions. In 2.5-year-old plantations in Chittagong Coastal Forest Division, *A. auriculiformis* and *A. procera* attained the maximum average height (3.5 m) nevertheless the height (3.0 m) of *C. equisetifolia* is not far behind those species. The DBH growth of *C. equisetifolia* is even more promising as it is maximum in all the three forest divisions. In the three forest divisions, the mean DBH of *C. equisetifolia* varied from 5.7 cm in Feni Social Forest Division to 11.3 cm in Noakhali Forest Division. In the case of Chittagong Coastal Forest Division *A. procera* attained the maximum DBH (4.7 cm) which is followed by the *C. equisetifolia* (4.2 cm DBH) (Fig. 1). Considering the height and DBH growth of *C. equisetifolia* in all the regions, it appears that its growth is comparatively better in coastal areas of Noakhali and Patuakhali covering the eastern parts of Bangladesh in comparison to that of Chittagong in the south-east.

Growth performance of *C. equisetifolia* was also studied in three types of plantations as shown for the survival rate of the seedling. The average height of *C. equisetifolia* at 1.5 years varied from 2.4 m in non-mangrove plantations to 3.3 m in strip plantations (Figure 2). In comparison with other species, *C. equisetifolia* attained 2nd highest height in all three sorts of plantations. The maximum height attaining species at 1.5 years is *A. procera* (3.40 m) in mound plantation, *A. auriculiformis* (3.0 m) in non-mangrove plantations and *A. catechu* (3.6 m) in strip plantations. Interestingly, the DBH of *C. equisetifolia* is maximum in all three types of plantations which varied from 2.2 cm non-mangrove to 2.4 cm mound plantation. Similarly, at aged 2.5 years, *C. equisetifolia* exhibited maximum height and DBH in both mound and strip plantations where height varied from 6.5 m to 7.6 m and DBH varied from 5.7 cm to 6.6 cm. In case of 2.5-year-old non-mangrove plantations *C. equisetifolia* seedlings showed maximum mean height (8.0 m) but maximum mean DBH (8.7 cm) was revealed by *A. auriculiformis* which was followed by *C. equisetifolia* (7.6 cm).

3.3 Growth rate of seedlings in the plantations

Mean Annual Increment (MAI) of height and DBH in 1.5- and 2.5-year-old plantations present the growth rate of *C. equisetifolia* seedlings in comparison with other common species included in the plantations. The MAI of the studied species at different forest divisions indicated that growth rate of *C. equisetifolia* up to 2.5 years in terms of MAI for DBH and height is maximum

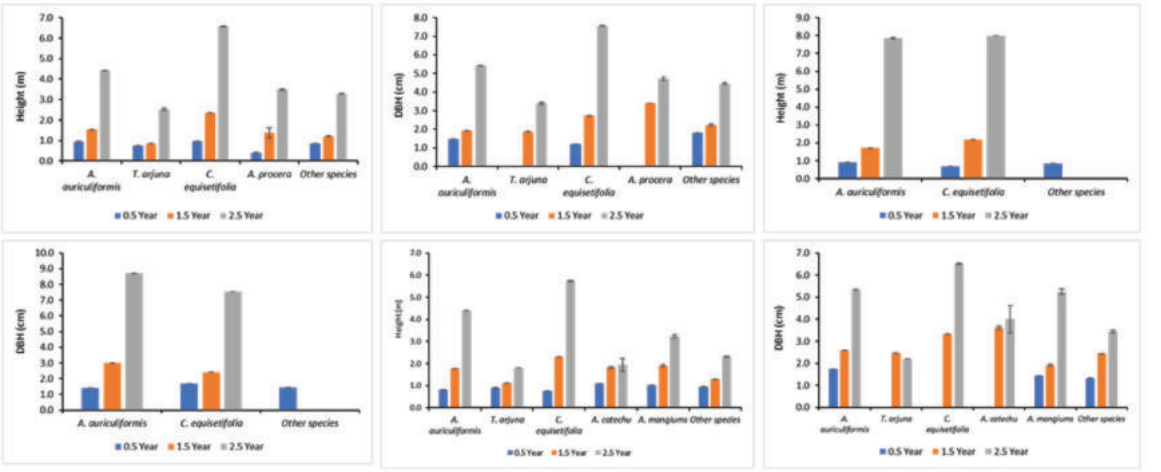

Fig. 2. Growth performance of *C. equisetifolia* seedlings in comparison with other species at three ages in different sorts of plantations.
Note: A and B = Height and DBH in Mound plantation, C and D = Height and DBH in Non-mangrove plantation, E and F = Height and DBH in Strip plantation.

in all forest divisions except in Chittagong Coastal Forest Division (Table 3). The MAI for DBH varied from 1.69 cm in Chittagong Coastal Forest Division to 4.53 cm in Noakhali Coastal Forest Division. On the other hand, MAI of height ranged from 1.2 m in Chittagong Coastal Forest Division to 3.2 m in Noakhali Coastal Forest Division. It is relevant to mention here that at 2.5 years of age the maximum MAI for DBH (1.88 cm) was shown by *A. procera* in Chittagong Coastal Forest Division whereas the maximum MAI of height (1.4 m) was exhibited by *A. auriculiformis* in the same forest division. A similar pattern of MAI growth was observed for *C. equisetifolia* at 1.5 years of age.

In mound and strip plantations the MAI of *C. equisetifolia* indicated a promising growth rate. The MAI of height and DBH are highest in Mound in comparison with all other species which ranged from 1.81 cm (at 1.5 years) to 3.02 cm (at 2.5 years) for DBH and 1.57 m (at 1.5 years) to 2.63 m (at 2.5 years) for height (Table 4). However, *C. equisetifolia* exhibited highest MAI of height in all three plantation types (i.e. mound, non-mangrove and strip) in comparison with other species where it is maximum (3.2 m) at 2.5 year age in case of non-mangrove. Besides, the maximum

Table 3. Growth rate of *C. equisetifolia* in comparison with other species at the different aged plantations of four forest divisions.

Forest division	Tree species	Mean annual increment for DBH at two different ages		Mean annual increment of height at two different ages	
		1.5 years	2.5 years	1.5 years	2.5 years
Chittagong Coastal Forest Division	*A. auriculiformis*	1.27 ± 0.007	1.54 ± 0.002	1.02 ± 0.007	1.4 ± 0.001
	T. arjuna	1.25 ± 0.007	0.88 ± 0.013	0.56 ± 0.006	0.85 ± 0.009
	C. equisetifolia	1.81 ± 0.002	1.69 ± 0.003	0.76 ± 0.001	1.2 ± 0.002
	A. procera	2.27 ± 0.03	1.88 ± 0.035	0.91 ± 0.164	1.4 ± 0.017
	Other species	1.65 ± 0.002	1.32 ± 0.002	0.65 ± 0.001	1.2 ± 0.002
Feni Social Forestry Division	*A. auriculiformis*	1.72 ± 0.016	1.96 ± 0.01	0.95 ± 0.01	1.4 ± 0.007
	T. arjuna		0.88 ± 0.001	0.34 ± 0.006	0.72 ± 0.001
	C. equisetifolia	2.52 ± 0.024	2.26 ± 0.014	1.6 ± 0.033	2.03 ± 0.01
	Other species	1.75 ± 0.023	1.41 ± 0.028	1.27 ± 0.036	0.93 ± 0.016
Noakhali Coastal Forest Division	*A. auriculiformis*	1.77 ± 0.019	3.14 ± 0.072	1.65 ± 0.015	2.24 ± 0.052
	T. arjuna	1.92 ± 0.049		1.08 ± 0.026	
	C. equisetifolia	2.52 ± 0.021	4.53 ± 0.082	2.14 ± 0.018	3.17 ± 0.062
	A. mangium	1.28 ± 0.035	2.09 ± 0.305	1.27 ± 0.027	1.47 ± 0.067
	Other species	1.54 ± 0.01	1.88 ± 0.055	1.01 ± 0.009	1.09 ± 0.026
Patuakhali Coastal Forest Division	*A. auriculiformis*	1.91 ± 0.002	2.51 ± 0.002	1.14 ± 0.001	2.14 ± 0.002
	T. arjuna	1.41 ± 0.015	2.48 ± 0.013	0.81 ± 0.01	1.2 ± 0.001
	A. nilotica	1.53 ± 0.02	1.1 ± 0.24	0.64 ± 0.033	1.46 ± 0.523
	C. equisetifolia	1.58 ± 0.002	2.73 ± 0.031	1.37 ± 0.002	3 ± 0.041
	A. catechu	2.95 ± 0.078	1.35 ± 0.016	1.46 ± 0.041	1.15 ± 0.019
	A. farnesiana		1.1 ± 0.017	0.2 ± 0.001	0.9 ± 0.013
	S. saman	1.4 ± 0.012	1.92 ± 0.012	0.69 ± 0.013	1.2 ± 0.001
	Other species	1.55 ± 0.008	2.38 ± 0.067	0.66 ± 0.014	1.4 ± 0.014

Table 4. Growth rate of *C. equisetifolia* in comparison with other species in different plantation types.

Plantation type	Species	Mean annual increment for DBH at two different ages		Mean annual increment of height at two different ages	
		1.5 years	2.5 years	1.5 years	2.5 years
Mound	*A. auriculiformis*	1.27 ± 0.007	2.17 ± 0.003	1.02 ± 0.007	1.77 ± 0.003
	T. arjuna	1.25 ± 0.007	1.36 ± 0.027	0.56 ± 0.006	1.01 ± 0.02
	C. equisetifolia	1.81 ± 0.004	3.02 ± 0.007	1.57 ± 0.002	2.63 ± 0.008
	A. procera	2.27 ± NA	1.88 ± 0.033	0.91 ± 0.164	1.39 ± 0.016
	Other species	1.48 ± 0.033	1.78 ± 0.014	0.8 ± 0.022	1.31 ± 0.006
Non-mangrove	*A. auriculiformis*	1.99 ± 0.003	3.48 ± 0.01	1.14 ± 0.002	3.14 ± 0.009
	C. equisetifolia	1.61 ± 0.003	3.02 ± 0.03	1.45 ± 0.002	3.19 ± 0.04
Strip	*A. auriculiformis*	1.73 ± 0.004	2.13 ± 0.005	1.19 ± 0.003	1.75 ± 0.004
	T. arjuna	1.64 ± 0.009	0.88 ± 0.02	0.74 ± 0.006	0.72 ± 0.01
	C. equisetifolia	2.22 ± 0.009	2.6 ± 0.012	1.53 ± 0.009	2.29 ± 0.008
	A. catechu	2.41 ± 0.049	1.6 ± 0.249	1.21 ± 0.022	0.78 ± 0.118
	A. mangium	1.28 ± 0.035	2.1 ± 0.05	1.27 ± 0.027	1.29 ± 0.034
	Other species	1.62 ± 0.003	1.38 ± 0.023	0.86 ± 0.004	0.92 ± 0.012

MAI of DBH is shown by *A. auriculiformis* (3.48 cm) at 2.5 years age in non-mangrove plantations. The results indicate that *C. equisetifolia* has a comparatively faster growth rate in both mound and strip plantations. In the case of non-mangrove plantations, its height and diameter growth rate are promising but *A. auriculiformis* shows faster diameter growth rate.

3.4 Casuarina as climate resilient species

Climate change is a major threat to the human lives and resources of the communities living in the coastal region of Bangladesh (World Bank, 2000). The devastating cyclones that occurred in Bangladesh in 1991, 2007 (Sidr), 2008 (Nargis), 2009 (Aila) and 2019 (Bulbul) killed thousands of people and caused major damage to housing and infrastructure. The plantations of mangrove and non-mangrove tree species in the coastal belt of Bangladesh are of immense importance to reduce the vulnerabilities to climate-induced hazards like cyclone and storm surges, as well as tsunamis (Islam and Rahman, 2015).

Coastal areas of Bangladesh is increasing as new char lands are continuously accreted with about 0.2 million tons of sediments flowing downstream through the rivers of Bangladesh. Historically, Bangladesh Forest Department (BFD) has established coastal plantations mainly with mangrove species i.e. *Sonneratia apetala, Avicennia officinalis, Excoecaria agallocha, Cerios decandra* and *Bruguiera sexangular* and non-mangrove species i.e. *C. equisetifolia* (Uddin et al., 2014). The mangrove plantations have good growth performance and coastal conservation potential but only in the areas that receive regular tidal inundations. There are vast stable and comparatively raised coastal lands where mangrove species are not suitable (Siddiqi and Khan, 2004). BFD and Bangladesh Forest Research Institute (BFRI) is trying to establish *C. equisetifolia* along with some other suitable mesophytic species. The present study indicated very good growth performance of *C. equisetifolia* in across different regions and different types of plantations. According to Moula (2010) the mesophytic species plantation in the coastal areas is likely to offer sustained yield and render a permanent forest cover in the coastal belt.

Miah et al. (2013) mentioned *C. equisetifolia* as a potential species for mitigating climate change and adaptation in coastal areas of Bangladesh through

sequestration of carbon in its woody tissues and thereby reducing greenhouse gases. *C. equisetifolia* is a major component of the coastal green belt formed by the coastal afforestation. The green coverage plays a key role in mitigating the catastrophic effects of cyclones and storm surges on the coastal communities. Moreover, the species provides timber, conserves and stabilizes newly accreted lands and accelerates accretion of new lands (Iftekhar and Takama, 2008; Siddiqi, 2001). Hasan (1987) reported that afforestation in the newly accreted land helps the raising land above the tide level along with retaining soil sediments. Uddin and Hossain (2013) also mentioned the important role of coastal plantations of *C. equisetifolia* and other species in minimizing the vulnerability to natural disasters in coastal localities.

Saenger and Siddiqi (1993) reported that the coastal plantations of Bangladesh are contributing for coastal protection and stabilization, wood production and land reclamation, socio-economic development. In coastal areas of Bangladesh, in addition to the dynamic geomorphology, browsing by deer and damage by Lesser Bandicoot rats (*Bandicota bengalensis*) are two important threats to the plantations. In this situation, *C. equisetifolia* is a most suitable species as it is neither browsed by deer nor damaged by Lesser Bandicoot rats (Siddiqi and Khan, 2004). Since *C. equisetifolia* showed promising performance in the strip plantations, and accordingly afforestation with this species along the embankments and shelterbelts is easily achieved (Islam and Rahman, 2015): it is as cheaper option and ecologically beneficial than any other measures for protection from cyclones and storm surges. Recent experiences from tsunami and major storms from South Asia and different parts of the world showed that forests can reduce the damage by absorbing the wave energy and preventing debris movement (Latief and Hadi, 2007). So, the dense vegetation can protect human lives, housing and infrastructure, crops and livestock from climate-change induced extreme weather events. The root system of *C. equisetifolia* can also protect soils from erosion and build sand dunes in the sandy beaches. Islam et al. (2014) mentioned that the species can tolerate moderate to high salinity. Since, salinity intrusion is increasing at coastal areas of Bangladesh particularly in dry season, so this species can be a good choice for plantations on well-drained, saline soils.

4 Discussion

There are a number of studies of the growth performance of seedlings of different mangrove and non-mangrove species. Uddin et al (2014) studied the growth performance and regeneration potential of some mangrove afforestation species at Mirersarai forest range but not the non-mangrove species. Uddin and Hossain (2013) reported that *C. equisetifolia* attained 17 cm mean DBH and 32.5 m mean height at the age of 27 years while *Samanea saman* attained the maximum mean DBH (54 cm) and maximum mean height (35.5 m) at the same age in strip plantation. Moula (2010) studied the survival and growth of a number of mesophytic species in the coastal areas of Char Kashem of Patuakhali. He reported that *C. equisetifolia* has a high survival rate (96.6 ± 3.1 percentage) at 7 years old which is similar to survival of the best species *S. saman* (98.0 % survivability). Moreover, the study indicated promising height (13.2 ± 0.76 m) and DBH (12.2 ± 1.2 cm) growth of *C. equisetifolia* where the height was highest among the 7 mesophytic species and mean DBH was the same as *S. saman* (13.2 ± 1.8 cm) showing maximum DBH. Another study by Serajuddoula et al. (1995) showed *C. equisetifolia* and *A nilotica* to be the best performing species followed by *S. saman* and *A. procera* in a 4-year old plantation at Rangabali of Patuakhali. In another study, Siddiqi and Khan (2004) showed 65% survival of *C. equisetifolia* after 6 years. In addition, the mean height and diameter were reported to be 6.54 + 0.21 m and 6.60 + 0.34 cm, respectively, whereas the MAI of height and diameter were 0.93 m and 1.10 cm, respectively. In comparison to this study, we found greater annual increment of height and DBH. It is may be due to the environmental influence of the dynamic coastal region of Bangladesh.

Casuarina equisetifolia plantations with appropriate human management can be utilized for attaining sustainable green coverage. Sustainable management of the plantations can enhance the climate resilience of communities living along the coast of Bangladesh. The survival rate and growth performance of *C. equisetifolia* was also reported from two sites (i.e. Rangabali and Char Kukri-Mukri) of Patuakhali by Islam et al. (2014). They showed *C. equisetifolia* to be one of the top performing species at both sites in terms of growth but with lower survival rate at Char Kurki-Mukri.

The survival rate after 17 years of plantation was 69% in Rangabali whereas it was 25% at Char Kukri-Mukri, the latter low survival being due to illegal felling of the trees from the plantation. However, the mean height and DBH was 15.26 ± 0.38 m and 19.45 ± 0.33 m, respectively, with growth rates (MAI of height 0.90 m and MAI of DBH 1.15 cm) similar to that found in the present study.

5 Conclusion

C. equisetifolia along with a number of mesophytic species are suitable for planting in the raised and stable coastal lands of Bangladesh. In the previous studies the performances of the species was reported mostly for experimental plantations. The present study examined the growth performance and suitability of the *C. equisetifolia* along with few mesophytic species from a massive government plantation established in the coastal districts of Bangladesh in different forms i.e. mound, strip, non-mangrove. The study indicated that the survival and growth of *C. equisetifolia* is very promising. Survival percentage, mean height, mean DBH, annual increment of these growth parameters are among those of the best performing tree species in almost all studied regions (i.e. Chittagong, Noakhali and Patuakhali Coastal and Feni Social Forest Divisions) and plantation types. Moreover, for enhancing climate change resilience the species has several encouraging aspects i.e. building coastal green belt, providing protection against cyclones and tidal surges, helping land accretion, raising accreted lands, providing timber and fuelwood, resistant to damage from wildlife, and a strong protective barrier to storms and high winds. Therefore, *C. equisetifolia* along with the other promising species in the present study i.e. *A. catechu, A. farnesiana, A. nilotica, A. procera, S. saman* and *T. arjuna* can be planted in the newly accreted raised lands, roadsides, embankments and marginal lands to create shelterbelts. These dense shelterbelts can reduce the impact of all-weather events resulting from climate change.

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Varietal comparison of wheat and paddy under *Casuarina equisetifolia* based agroforestry system on sodic wasteland of India

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Abstract

To optimize the efficiency of agroforestry systems (AFS), the present study was conducted to evaluate the performance of wheat and paddy varieties under 5-year-old *Casuarina equisetifolia* plantation at different distances from the tree line (1, 2, 3 and 4 m). The system comprised three varieties of wheat (NW-1067, NW-1014 and HD-2643) and three varieties of paddy rice (Sarjoo-52, Narendra Usar-2 and Narendra Usar-3). Growth and yield attributing parameters of wheat and paddy rice varieties were significantly reduced at different distances from *Casuarina* plantations in comparison to sole agricultural crop (devoid of trees). While effects on crop produce were limited for all wheat and paddy rice varieties with the increasing distance from tree line, there were considerable yield reductions near to trees (at 1 m). The maximum reduction in grain yield over control was recorded in wheat variety HD-2643 (37.4%) and in paddy variety Narendra Usar-3 (29.1%) when the crop was grown at 1 m away from the tree line, which indicated that wheat variety HD-2643 and paddy variety Narendra Usar-3 were very sensitive to shade. However, two other wheat (NW-1067 and NW-1014) and paddy varieties (Sarjoo-52 and Narendra Usar-2) showed only moderate decrease in grain yield at all the distances from *Casuarina* based AFS. Among wheat and paddy varieties, NW-1067 and Narendra Usar-2 were observed to be more shade tolerant varieties and showed significantly higher grain and straw yield at all the distances from tree line under this study. However, the yield of three wheat and paddy varieties was substantially reduced in the *Casuarina*-based AFS over control. We also quantified soil properties such as soil pH, electrical conductivity (EC), soil organic carbon (SOC) and available N, P and K. After 5 years of plantation, the organic carbon in soil decreased with an increase in distance from tree line and it was maximal near the tree line (1 m) after the harvesting of wheat crop.

Keywords: agroforestry, intercropping, casuarina, different distances, productivity

1 Introduction

Agroforestry (AF) is a system of land use, in which woody perennials are deliberately integrated into the same land management unit as annual agricultural crops and/or animals either sequentially or simultaneously with the aim of obtaining greater output on a sustained basis (Albrecht and Kandji, 2003; Montagnini and Nair, 2004; Nair et al., 2009). Under agroforestry systems (AFS), forest tree species/woody vegetation continuously added leaf litter into the soil through litter fall and decomposition which may lead to highersoil organic carbon (SOC)than for mono-cropping systems (Lenka et al., 2012; Abbaset al., 2017). As a result, AF have been recommended as an environmentally friendly practice, now receiving wider attention not only in terms of agricultural sustainability but also as a land use system to mitigate climate change (Oelbermann et al., 2004; Takimoto et al., 2009; Oliveira and Carvalhaes, 2016; FAO, 2017). Realizing the importance of agroforestry, the Government of India has pronounced and implemented a National Agroforestry Policy (2014). Agri-silvicultural systems are one type of AFS which is gaining ground in most developing countries including India, and which ensures a better and more intensive utilization of the land (Kumar et al., 2017).

Casuarinais planted for fuel wood as well as timber and to lower the salinity of infertile sodic land. Apart from being a fuel/timber wood species, *Casuarina* trees are efficient N-fixers and through a symbiosis with *Frankia* improve the N and C status of the soils through litter input. It is tolerant of high soil pH (8.5-9.5) and accordingly has been introduced into agroforestry systems on problematic sodic soils (Parihar and Rana, 1999) in UP. Salt-tolerant tree species can be used to reclaim salt-affected lands, at the same time increasing the size of carbon sink in the plant-soil system and improving soil microbial activity. *Casuarina* wood may also be used in pulp and rayon production. It is fast growing, multipurpose and actinorhizal tree species (Kumar et al., 2017). Thus, wheat and rice paddy as part of *Casuarina equisetifolia* based agroforestry systems provide the basic requirements of food, fodder, fuel, pulp and green manure. However, limited comprehensive scientific information is available on the effect of crops and woody trees on each other when grown in such associations. Therefore, the present study was undertaken to study the performance of different varieties of wheat and paddy under *C. equisetifolia* based agroforestry system on sodic soil wastelands.

2 Materials and methods

A field experiment was conducted at Main Experiment Station (Forestry), Kumarganj, Faizabad, Uttar Pradesh during the years 2009-10 to study tree growth and crop productivity under certain agri-silviculture systems on sodic soil. The main experimental site is located at Narendra Nagar lies 26°27′ N latitude and 82°12′ E Longitude, 113 m elevation and 42 km from Faizabad, on Faizabad – Raibarelly Road, and lies between the Gomti and Saryu Rivers. The mean monthly maximum temperature ranged from 17.9 °C (January) to 41.4 °C (April) and mean monthly minimum temperature from 7.4 °C (December- January) to 27.2 °C (June) during the year 2009-2010. Total annual rainfall during the year was 773 mm. The meteorological observations recorded during the experimental period from November 2009 to October 2010. The soil of experimental site is partially improved sodic soil having a pH of 8.5. It has intermediate levels of available nutrients and soil organic carbon (%). The three varieties of wheat (NW-1067, NW-1014, and HD-2643) and three varieties of paddy rice (Sarjoo-52, Narendra usar-2, Narendra usar-3) were sown under five-year-old *C. equisetifolia* plantation on 15th November 2009. All cultural practices were adopted as per recommended for cultivation of wheat and paddy. The present study was laid out in a factorial randomized block design with three replications.

3 Results and discussion

3.1 Chemical properties of soil

The chemical properties of soil such as the soil organic carbon and available nitrogen, phosphorus and potassium in the *C. equisetifolia* based AFS and in control plot are depicted in Fig. 1.

Organic carbon and available N, P and K

It is evident from Fig. 1 that *Casuarina* based agroforestry systems showed a significant positive effect on soil organic carbon. The results revealed that the organic carbon content of the soil decreased with

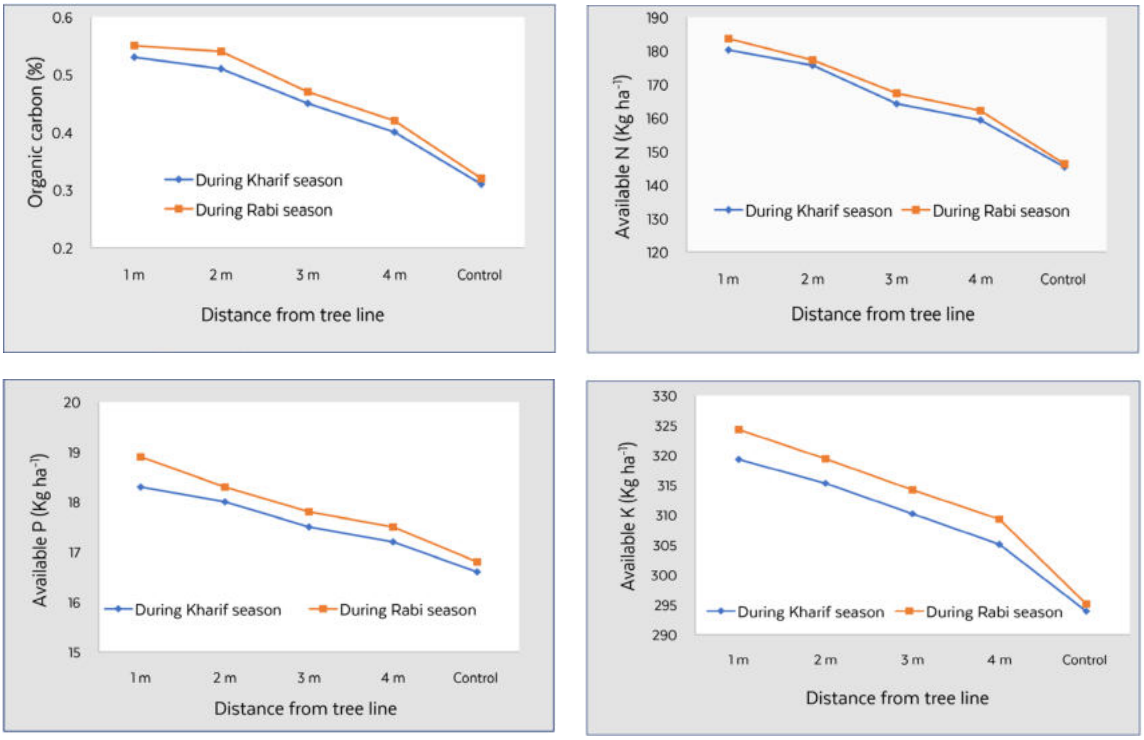

Fig. 1. Effect of *Casuarina equisetifolia* based agroforestry system on soil organic carbon (%) and available N, P, K (kg ha^{-1}) during *Kharif* and *Rabi* season.

the increase in distance from casuarina tree line. In *Casuarina* based agroforestry system, organic carbon was found significantly higher at 1m distance from tree line (0.58%) as compared to 4 m distance (0.48 %) from casuarina tree line after the harvesting of wheat crop. These findings are similar to those of Bambrick et al. (2010) where there was an increase in soil organic carbon (SOC) of 6.2 tonne OC ha^{-1} in the 0–20 cm soil layer after 21 years of intercropping with poplar in comparison to arable cropping without trees. Thevathasan and Gordon (2004) also found a 35% relative increase in SOC (0–15 cm soil layer), within 2 m distance from poplar trees (8 years old plantation) on an alley cropping field. However, minimum organic carbon content was found 4 m away from casuarina tree line as compared to other distances after harvesting of *Kharif* crops under study. Litterfall input typically decreases exponentially with distance from the tree row (Oelbermann et al., 2004). Sole cropping exhibited significantly lower organic carbon (0.31 and 0.32%) before sowing of wheat and paddy crops.

The amount of available soil N at different distances between two crop growing seasons under casuarina based agroforestry system was higher than sole crop during the year of observations (Fig. 1). In *Casuarina*-based AFS, the available N was significantly higher (187.2, 180.4 and 173.2 kg ha-1) up to a distance of 3 m from tree line as compared to sole crop after harvesting of wheat. The availability of N was significantly lower in *Casuarina* based AFS during both the crop growing seasons at a distance of 3 m to 4 m from tree line but this was significantly higher than control. A similar trend was also observed in available P and K in both the crop growing seasons under *Casuarina*-based AFS (Fig. 1). The availability of P and K content was also significantly higher under *Casuarina*-based AFS compared with sole crops (devoid of trees). These lines are similar to the findings of Chauhan et al. (2012) who found that the total soil nitrogen and available P and K (kg ha^{-1}) was higher in the top layer of soil and decreased with depth; however, the differences in available P and K contents with distance from the tree line were non-significant but slightly better than the control plot. The overall improvement in fertility status of soil could be ascribed to the regular addition of *Casuarina* cladode litter and root decay

in situ, higher microbial activity, more favourable soil condition viz., soil moisture and temperature under trees (Patel et al., 2010).

3.2 Crop growth and yield attributes

3.2.1 Growth and yield parameters of three wheat varieties under C. equisetifolia *based agri-silvicultural system*

There were no significant differences in the plant height of wheat varieties under casuarina and in open (sole wheat) (Fig. 2). The maximum plant height of wheat was recorded in sole crop as compared to *Casuarina*-based AFS.However, the minimum plant height was found when the crop was growing close (1 m distance) from tree base. The growth of wheat crops under *Casuarina*-based AFS was lower than in plots without trees. The results are in conformity with Rana et al. (2005) who reported that significant variations in growth character of different wheat varieties under agroforestry systems. The maximum test weight (1000 – grain weight) was recorded for wheat variety NW 1067, when the crop was grown in open conditions (control). However, wheat grown in the alleys of casuarina at 1, 2, 3 and 4 m distance from tree base produced minimum test weight (1000 – grain weight g.) compared to the control plot. The test weight was significantly influenced by variety but not distance from the tree base, however the interaction between variety and distance were found significant (Fig. 3). Similar results have also been reported by Artru (2017) which was partly confirmed by the supplementary observations where the thousand grain weight (TGW) of wheat was examined at different distances from tree line.

The data presented in Fig. 4 showed that the grain and straw yield of wheat crop under *Casuarina*-based AFS decreased significantly as compared to open field (crop without casuarina trees). However,the interaction between wheat variety and distance were found to be significant. The maximum grain yield (2.42 t ha^{-1}) was recorded in wheat variety NW-1067 which was closely followed by HD-2643 (2.35 t ha^{-1}) in open area (devoid of trees).However grain yield of wheat varieties exhibited significant differences in *Casuarina*-based AFS at different distances from tree line. The reduction in the grain and straw yield in wheat varieties under *Casuarina*-based AFS was likely due to both reduced solar radiation on crop canopy and lower availability of moisture and nutrients. Yield reduction depends on the light intensity. On an average, the reduction in grain yield of different wheat varieties under *Casuarina*-based AFS varied from 12% (4 m distance) to 51% (1 m distance) over control (sole wheat). The rate of decrease in straw yield was comparatively

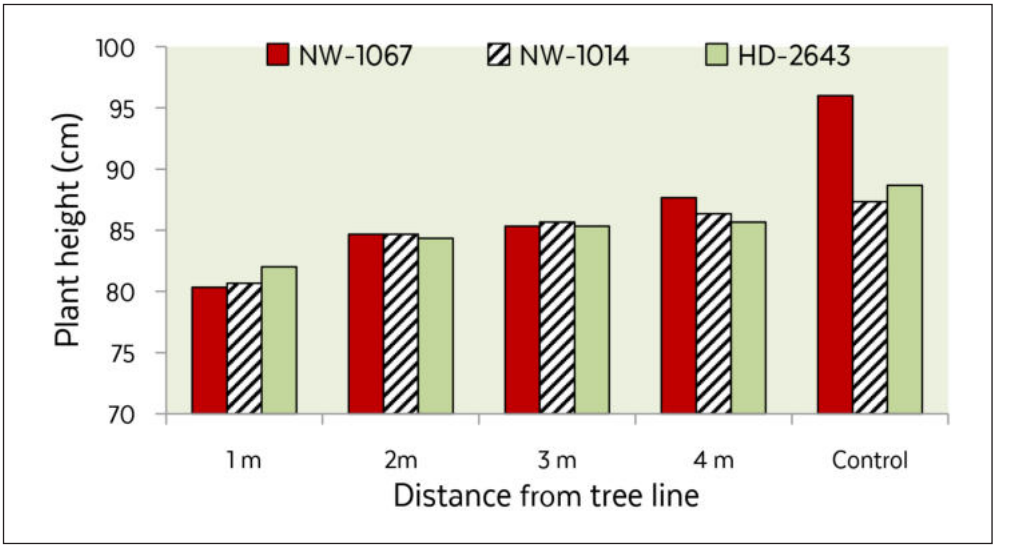

Fig. 2. Plant height (cm) of wheat varieties at different distances from trees in *Casuarina equisetifolia* agroforestry system.

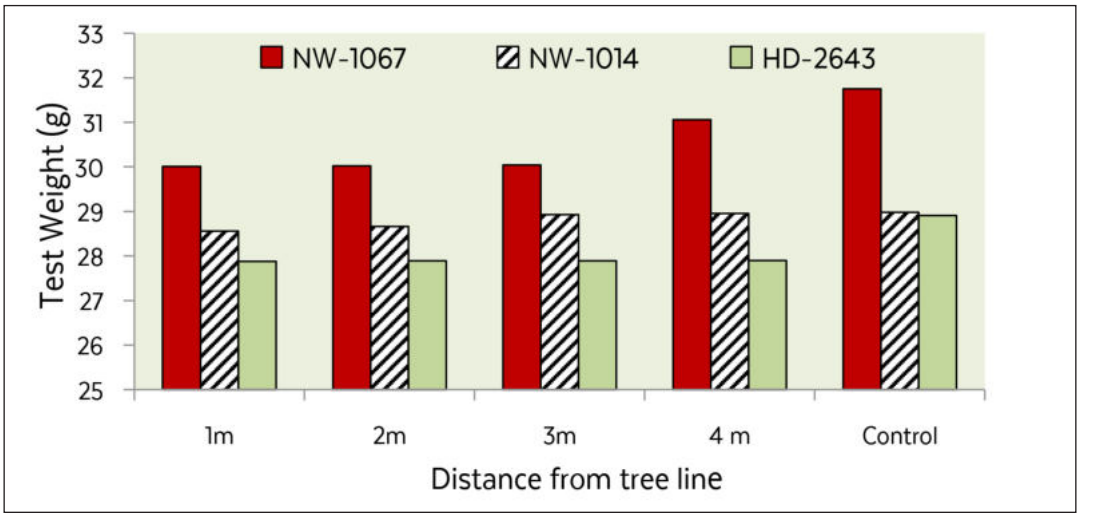

Fig. 3. Test weight (g) of wheat varieties at different distances of *Casuarina equisetifolia* agroforestry system.

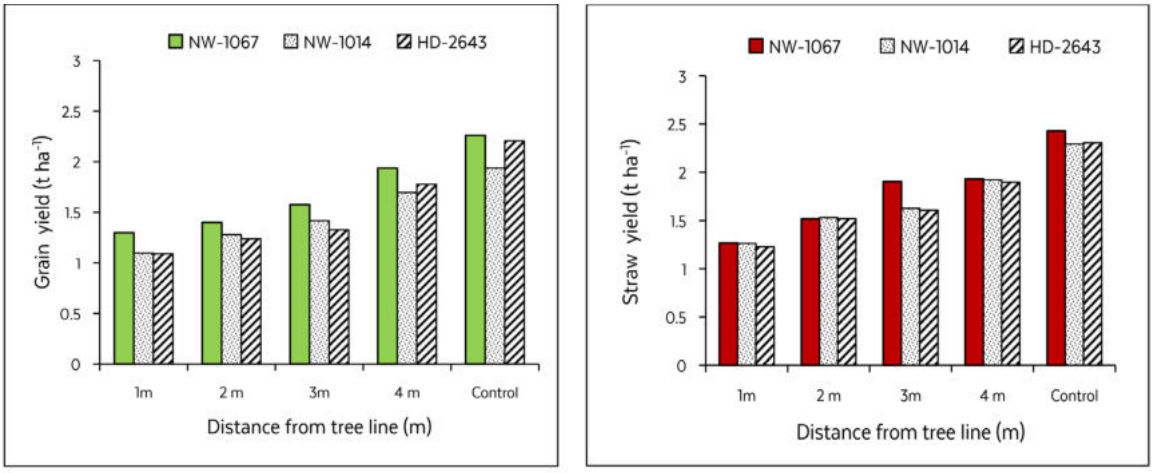

Fig. 4. Grain and straw yield (t ha^{-1}) of wheat varieties at different distances of *Casuarina equisetifolia* based agroforestry system.

lower than grain yield at different distances from the casuarina tree line.The findings are in general conformity with the results reported in the previous studies in whichwheat was intercropped with a walnut-based agroforestry system (Guadalupe Arenas-Corraliza et al. 2018; He et al. 2012).

3.2.2 Growth and yield parameters of three paddy rice varieties under Casuarina*-based AFS*

Data presented in Fig. 5 shows that the growth characters of the three paddy varieties were significantly affected by perennial tree *C. equisetifolia* when the crop was grown at 1m away from tree base. Among all the paddy rice varieties, the maximum plant height was recorded in Narender Usar-2 under *Casuarina*-based AFS as well as sole crop (without tree). The growth of paddy varieties under *Casuarina*-based AFS was lower than open area cultivation. Fig. 6 indicates that the maximum test weight (26 g) was also recorded for Narendra user-2 variety in open area. It may be due to the fact that direct light intensity favours increase the solar radiation in case of open area as compared to *Casuarina*-based AFS. Khybri et al. (1992) noted that when compared with a monoculture, yield was reduced by 38, 33, 25, and 8% up to 1, 2, 3, and 5 m from trees, respectively, in a rice–wheat system.

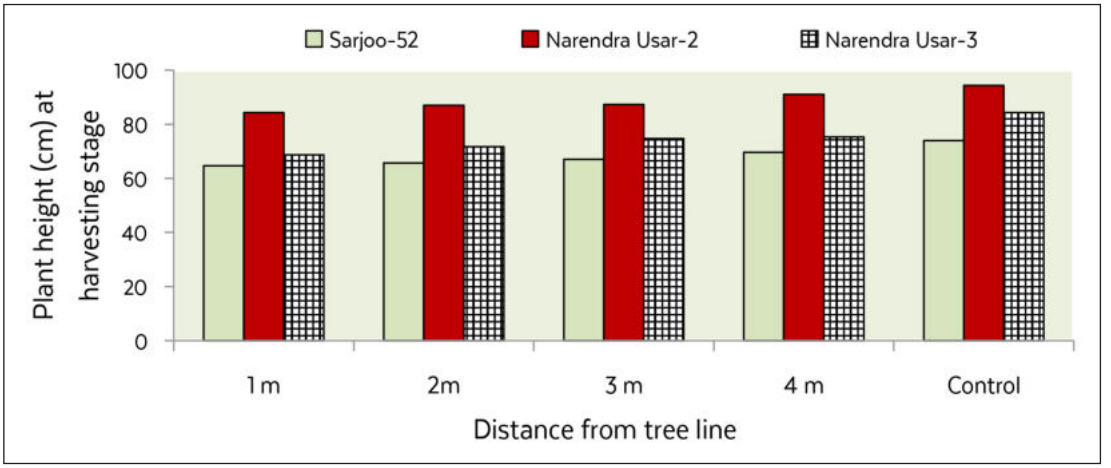

Fig. 5. Plant height (cm) of paddy varieties at different distances of *Casuarina*-based AFS.

The result depicted in Fig. 7 indicates that variety and distance from the tree line significantly affected the yield of paddy rice crop. Among all the paddy varieties the maximum grain yield (2.22 t ha^{-1}) was recorded in variety Narendra usar-2 in open area (without tree). Whereas the maximum straw yield (2.41 t ha^{-1}) was also observed in same variety Narendra Usar-2 which was closely followed by the Narendra Usar-3 (2.32 t ha-1) when the crop was grown in an open area (without tree). However, all the paddy rice varieties exhibited lower grain and straw yield under *Casuarina*-based AFS at different distances from the tree line during the study period. The reduction in the rice grain yield under tree canopies may be due to the fact that shade negatively affected grain yield. Yield reduction depends on the light intensity. Ourfindings are in conformity with the results of Bao et al. (2012),who found that average wheat yield increased by 30% within the sheltered zone (0–255 m) of a 14 m tall poplar shelterbelt in comparison to an unsheltered area (open area). Zhang et al (2013) showed that root competition led to decreases in yield and biomass in wheat under agroforestry system.

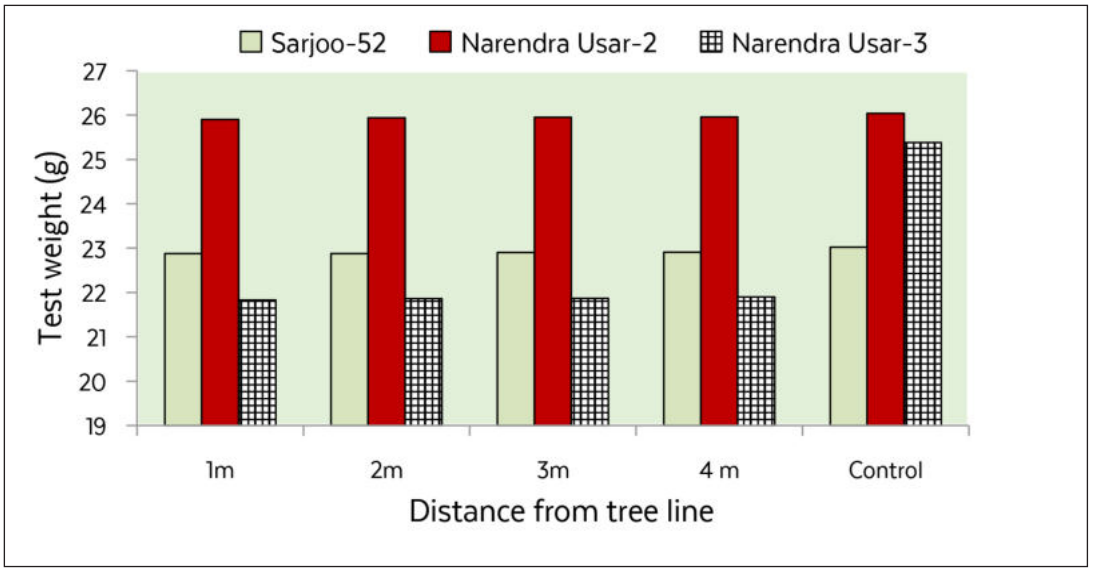

Fig. 6. Test weight (g) of paddy varieties at different distances of *Casuarina*-based AFS.

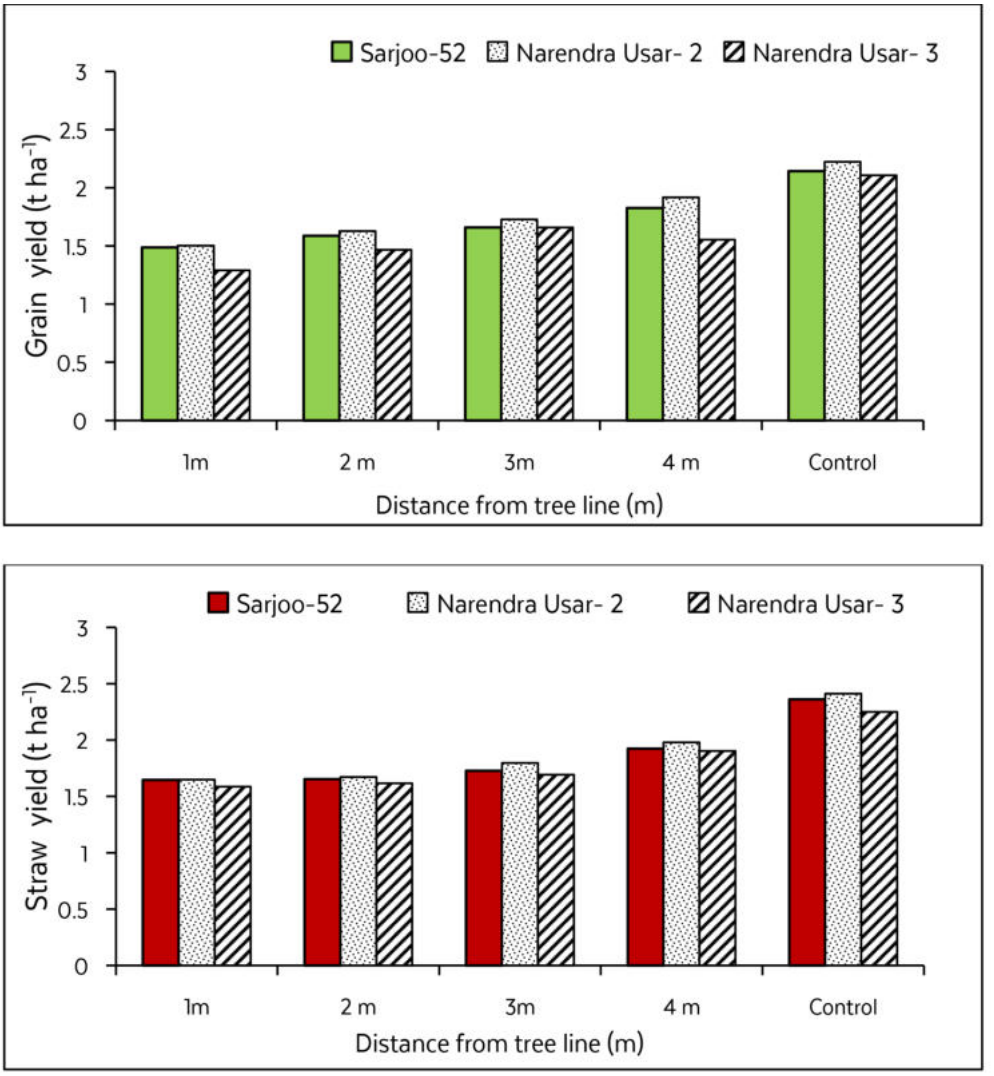

Fig. 7. Grain and straw yield (t ha^{-1}) of paddy varieties at different distances of *Casuarina equisetifolia* based agroforestry system.

4 Conclusion

Among the three varieties each of wheat and paddy, NW-1067 (wheat crop) and Narendra Usar-2 (paddy crop) gave better yields under *Casuarina*-based AFS. Wheat and paddy crop yield in regard to varietal performance within *Casuarina*-based AFS showed notably positive relation with soil fertility status which ultimately enhanced the yield of wheat and paddy varieties.Our results indicate that *Casuarina*-based AFS can play a significant role in adaptation strategies providing food, improving soil and sequestering atmospheric CO_2 in the present changing climate.

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Casuarina coastal shelterbelt – a protection forest for countering natural calamities

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Abstract

Casuarinas were first introduced in shelterbelt plantings to protect the coasts primarily from drifting of sand dunes, subsequently finding a place as a cash crop in more inland areas. Casuarina shelterbelts, of a width of 50-100 meters, may acts as a vital barrier and offer resistance to tsunami waves. Therefore, in post-tsunami construction the new coastal stone walls are being built behind the casuarina 'bio-wall', which does not interfere with the turtle movement for nesting. Coastal dwellers have long understood the values of conservation of coastal trees and shelterbelt. The study looks at the role of casuarinas in the protection of coasts and communities with reference to recurring cyclones to better plan for future calamities, including tsunamis.

Keywords: coastal, shelterbelt, casuarina, mangroves, cyclone, tsunami

1 Introduction

The Coromandel Coast witnesses frequent natural disasters such as the tsunami in December 2004 followed by increasingly frequent Tropical Cyclones including TC Thane in December 2011, TC Vardah in December 2016 and TC Gaja in November 2018. These calamitous natural phenomena not only lead to loss of human lives and livestock, but also severely damage agriculture and with increasing cyclone frequency, before recovery is made another cyclone may strike adversely affecting the livelihoods of people living near the coast. In earlier times the coastal area was bestowed with tropical dry evergreen forests, which could help offset and mitigate the vagaries of cyclone to greater extent but due to encroachment and development these forests were removed exposing the coast to the vagaries of tropical cyclones and consequently more damage. In order to protect and shelter the coast, man-made casuarina coastal plantations are being developed. Since tropical cyclones and tsunamis originate from the sea and move towards the land the strong winds and waves generated are reduced and dissipated as they pass through dense coastal casuarina plantations.

Why casuarinas as coastal shelter plantation?

The coast is already denuded without any plantation, there are few species including *Lannea coromandelica* (Indian ash tree), *Prosopis juliflora* (mesquite) and *Cocos nucifera* (coconut) in that area, but these trees are of limited protective value. The *Lannea* tree is very light and soft, mesquite is very thorny and bushy it cannot grow straight and it may be counter protective as people may get caught in the bush when fleeing a

tsunami, while coconuts usually grow 8 m apart and may allow destructive waves to wash through (although a tsunami-protective function for coconuts has been observed in the Pacific Islands). The ideal tree species will be fast-growing and long lived with close planting. Some species such as *Acacia auriculiformis* can grow very fast, at similar rates to casuarinas, but cannot withstand the salinity and adverse climate, and accordingly casuarinas are preferred. The advantages of casuarinas include:

i) Fast growing
ii) Non grazable - even if nibbled it regrows rapidly
iii) Hardy, and not requiring most post-establishment care
iv) Most popular farm forestry tree in coastal lands
v) Salt tolerant in aerobic edaphic environments
vi) Wind resistant with limited crown breakage, except under extreme wind speeds
vii) Adapted to infertile soils
viii) Not a high-water demander and reasonably drought-resistant
ix) Disease resistant with no damage observed although Trichosporium attack is seen on farmland
x) Withstand salt-laden sea spray
xi) Can tolerate high day time temperatures, including when sand gets very hot
xii) Has a root system that can proliferate in loose sands and bind them
xiii) They have a tap root that assist with anchoring in loose sands.

Plantation of casuarinas

The planting of casuarinas in the coastal areas requires proper verification of the existing plantation and the after=effects on turtle movement as the beaches are known for turtle nesting and breeding. It was noticed in Odisha coast where casuarina plantations were established close to the high tide line that there was an adverse impact on sea turtle nesting. Accordingly, care was taken to plant *Casuarina* seedlings 500 m beyond the high tide line so that even if there is change in the tide line level due to tsunami or cyclonic storm there will not be any adverse impacts on turtle nesting. Karaikal, one of the districts of Puducherry union territory, is prone to annual cyclones and therefore a fast-growing tree species needs to be utilized with plantations established within a short time, and the plantation well established prior to being hit by cyclonic winds.

Plantation monitoring and evaluation

Monitoring and evaluation of plantations is essential owing to the successive calamities striking the coastal areas. The plantation is approaching 15 years and well established, but large gaps have developed compared to the initial spacing of 1m x 1m due to self-thinning. Though the trees put on good girth increment and were generally sturdy, the gaps in the plantation are a matter of concern whereby the death of trees or their illegal removal requires the replacement of trees by the Forest Department to maintain stand density. Since the *Casuarina* tree canopy is dense and not generally allowing any species to regenerate it was decided to infill and underplant with the littoral *Calophyllum inophyllum*, a species that is shade-tolerant in seedling and sapling phases. Once *C. inophyllum* is established the problem of openness underneath *C. equisetifolia* plantations is minimized.

Effects of cyclones on the survival of plantation and protective role

The location of Karaikal means that it is impacted by a tropical cyclone almost every year. TC Thane cyclone struck in December 2011 followed by TC Vardah in December 2016 and TC Gaja in November 2018. Though the average cyclonic wind was only about 120 km h^{-1}, the casuarina plantation protected the coastal environment and lives and properties of the communities. The casuarina plantations suffered little damage aside from removal of needles and small branches and the trees remained intact, and re-grew very quickly, emphasizing the value of casuarina compared to other tree species.

Mangroves in back waters

Mangroves are considered as land builders, and a front line defense against natural calamities in the coastal areas, protecting lives and properties, as was evident during the 2004 tsunami, with many men, women and children taking shelter in the mangrove

areas and escaping from the wrath of this tsunami in Pitchavaram mangrove area of Tamil Nadu, India. In Karaikal the present area under mangrove was barren and the tsunami waves rushed through the back water to the town taking a heavy toll on human lives. In response mangrove plantations were planted in 2006, and are now well established and grown over the barren area offering excellent protection against natural calamities.

Problems of protection of shelterbelt and mangroves

Shelterbelt:

The shelterbelts were established with the active involvement of fisherwomen of the local community. Since they were without any work after the tsunami they had no alternative employment and accordingly they came for planting and maintenance of casuarinas and raising of mangroves in nurseries and planting. They were also paid from project funds, and once the project concluded there was no money to keep paying them. Though the people realized the importance of shelterbelt and mangroves, they resorted to cutting for firewood and anchoring boats, due to absence of other livelihood options. There is no law to take action against those cutting the casuarina trees, as the Forest Act, is not applicable to casuarinas.

Mangroves:

Mangroves have well grown and completely covered the entire back water area as a thick mat of vegetation. Unlike the casuarinas the mangroves are not much damaged although there were occasional incidents of cutting, and the people have been cautioned that action would be taken against offenders under the Wildlife Protection Act.

Conclusion

Though casuarinas were introduced as shelterbelt to protect the coasts primarily from drifting of sand dunes, the importance and value of shelterbelt was only realised after tsunami. A systematic approach of coastal shelterbelt was taken up to cover the coasts against vagary of natural calamities especially frequent cyclones. The damage to live and properties was found in the field that there is openness and direct exposure to open sea. There is no barrier in between the sea and residential complex. But due to development these areas were denuded forests and thereby exposed to open sea. It was also observed that wherever the vegetation predominantly of casuarina, the damage was minimal and the plantation acts as a first line defense as a natural barrier and protected the dwellings behind without damage or if damage occurred at minimal in spite of heavy waves. The plantation of casuarina and mangrove were undertaken in the year 2005-06 and now they have well established and remained intact. This is primarily due to the awareness among the people about the protective role of the important man-made biome not mere law.

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Seasonal dynamics and spatial distribution pattern of seed rain in a monocultural *Casuarina equisetifolia* coastal protection forest

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Abstract

In order to study whether the quality and quantity of *Casuarina equisetifolia* L. seeds is a barrier to its natural regeneration, 30 seed rain collection nets (SRCN) were set up in the *C. equisetifolia* coastal protection forest in Haikou city, Hainan island, and germination experiments of seed rain were carried out in the laboratory, to study the seasonal dynamics, spatial and temporal distribution pattern and germination characteristics of seed rain. Key findings were: (1) the dispersal period of *C. equisetifolia* seed rain began mid-July and ended in mid-April of the following year, i.e. the duration of seed rain dispersal was up to nine months and the dispersal period was synchronized for most seed trees. The seasonal dynamic of seed rain was greater, with obvious mast and lean years, in which, the total average density of seed rain in mast year was up to 1765 seeds m^{-2}, or only 713 seeds m^{-2} in the lean year, but the germination rate of seed rain was low, from 11 to 18% in different years. (2) the seed rain dispersal pattern was unimodal with a peak in December. The quantity of seed rain in the half month of peak reached 43 to 44 % of total seed density in different years with 80% of the seed rain occurring in the dry season from November to April. (3) the seed rain exhibited an aggregated distribution with seed rain density positively correlated with the number of cones on *C. equisetifolia* mother trees surrounding the SRCN, indicating that *C. equisetifolia* seeds had very limited dispersal in the forest, even though the seed is small, light and winged. (4) the quantity and quality of seeds are not barriers to the observed lack of natural regeneration of *C. equisetifolia*.

Keywords: *Casuarina equisetifolia*, seed rain, seasonal dynamic, distribution pattern, Hainan Island

1 Introduction

Casuarina equisetifolia L. is an evergreen tree native to South-east Asia, Australia and the Pacific islands. It was first introduced to Quanzhou, Fujian province, China, in 1919, and planted in a large area in the southeast

coast, especially Leizhou Peninsula and Hainan Island, after 1950s (Li et al., 2002). The area of *C. equisetifolia* coastal protection forest reached 56,000 ha in Hainan island in the early 2000s (Tan et al., 2003). *C. equisetifolia* has desirable characteristics, such as rapid growth, strong wind resistance, resistance to sand burial, salinity and high pH, and has become the main pioneer tree species for wind protection and sand fixation in near coastal locations in southern provinces of China (Lin, 2000). However, there are also problems such as single tree species, simple stand structure (Han, 2006), and difficulty in natural regeneration (Zhang et al., 2000).

Seed rain refers to the process that mature seeds are shed by mother trees and reach the ground in specific time and space by their own gravity and external forces (Lü et al., 2012), which is an important link affecting the natural regeneration of plants (Levine and Murrell, 2003). The quantity, quality, dispersal period and distribution pattern of seed rain have important effects on seed dispersal, germination and seedling settlement (Clark et al., 1998), and finally affect the quality of natural regeneration. The size of seed rain density is not only related to the age of the mother trees (Zhang et al., 2009; Yin and Liu, 2005) and the diameter at breast height (*DBH*) of the mother trees (Wu, 1997), but also related to their own reproductive characteristics, environmental conditions and external transmission factors (Hu et al., 2005). Seed rain characteristics of different species in different regions often vary, which is the result of the adaptation of species or populations to the environment (Hu et al., 2005).

Research on *C. equisetifolia* has more often focused on growth characteristics, stress resistance, allelopathy, soil nutrient and microorganism, but there have been few studies of *C. equisetifolia* seed rain. Yang, et al. (2019) investigated the seed rain in CCPF of Hainan island for one year and got the preliminary results, but this study was too limited to investigate seasonal variation of seed rain. Based on Yang bin's investigation, seed rain investigation was carried out for another year, as reported in this paper, to reveal seasonal dynamics, spatial distribution pattern and germination dynamic of *C. equisetifolia* seed rain in Hainan island. It is informative to study the seed rain characteristics of *C. equisetifolia* in different regions to better understand the adaptability of this species to local areas and its regeneration and recovery potential.

2 Research area environment and methods

2.1 Study area

The study area is located in casuarina coastal protection forest (CCPF), Haikou city, in the north of Hainan Island. The region has a tropical monsoon climate, with an annual average temperature of 24.8 °C, an annual average precipitation of 1,646 mm, an average humidity of 82%, and a sunshine duration of 1878 h per annum (Che et al., 2014). The soil is sandy, with a pH of 6.1. The soil organic matter content was low (0.02%), the ammonium nitrogen content was 16.6 mg kg^{-1}, the available phosphorus content was 11.6 mg kg^{-1}, and the available potassium content was 10.7 mg kg^{-1}. Three sample plots were laid out in the study area. Density, growth and sex of casuarina trees in the sample plots are given in Table 1. In addition, the survey found that *C. equisetifolia* is mainly female fruit-bearing plant, but there are also a few monoecious trees bearing fruit.

2.2 Research methods

2.2.1 Seed rain collection

(1) Arrangement method of seed rain collection nets (SRCN): The three sample plots were set up in the middle of July, with the distance of at least 200 m between them in the research area of CCPF. In each sample plot, 10 SRCN of 1 m × 1 m were arranged, thus the total SRCN was 30. The spacing of SRCN is 5 m × 10 m. SRCN are nylon net, which were placed directly above the litter of *C. equisetifolia*, and the four corners were fixed with disposable bamboo chopsticks.

(2) Collection time for seed rain: Undertaken from 2017 to 2019. The duration of seed rain collection was about nine months from the beginning of August, until April of the following year, during which seed rain was collected 18 times from 2017 to 2018, and 16 times from 2018 to 2019. The seed rain was collected twice per month, usually in the beginning and the middle of the month, in case of typhoon, rainy days or other unexpected circumstances, it was collected in advance or delayed by 1-3 days.

(3) Statistics of seed rain quantity: the seed rain was collected from each SRCN and put it into zip-lock bag, marked with the number of the SRCN. The seeds in the zip-lock bags were taken back to a laboratory, and the

Table 1. Basic condition of the sample plots in the study area.

Sample plot	Longitude (E)	Latitude (N)	Density (plants ha^{-1})	Mean *DBH* (cm)	Max *DBH* (cm)	Min *DBH* (cm)	Mean tree height (m)	No. of male tees	No. of female tree
1	20°02′36″	110°26′30″	1750	7.27	22.2	1.80	9.33	61	44
2	20°02′23″	110°26′46″	1133	8.57	15.0	2.30	11.67	51	18
3	20°02′43″	110°26′31″	1167	7.09	16.0	1.50	7.00	35	35
Mean			1350	7.64	17.7	1.87	9.33	49	32

quantity of seeds in each zip-lock bag was recorded after removing the impurities and the seeds which appeared to have an incomplete embryo.

2.2.2 Germination of seed rains

In order to examine the dynamic of seed rain quality, a seed germination test was conducted simultaneously with each seed rain collection. The between-paper method was used for seed germination in petri dishes. Germination test was conducted in 3 replicates each consisting 100 seeds. All 30 bags of seeds after counting were mixed together and divided into 4 equal parts. After that 25 seeds were randomly selected from each part, giving a total of 100 seeds as representative sample for each collection. The quality of seed rain in each collection was reflected by average germination rate. The germination test was carried out under laboratory conditions without other treatment. Water was replenished regularly and the number of germinants was counted daily, generally lasting 20 days.

2.2.3 Cone quantity survey of C. equisetifolia

In order to understand the distribution pattern of seed rain, the quantity of cones around SRCN was investigated. The cone survey was centered on each SRCN to investigate the quantity of female or monoecious trees within 5 m and the quantity of cones per plant. If the quantity of cones was small, they were directly counted by observation. If the quantity of cones was large, the number of cones on one quarter of the crown was counted, and then multiplied by 4.

2.3 Data processing and analysis

2.3.1 Calculation of seed rain density

The average density of seed rain (M) = $\sum_{i=1}^{i=30} N / 30$ (seeds m^{-2}), where N is the quantity of seeds in each SRCN.

Relative density of seed rain (M1) = seed rain density per time/total seasonal density of seed rain ×100%.

Coefficient of variation (C_v) = ($S/\overline{X}$)×100%, where S is the standard deviation and $\overline{X}$ is the mean.

2.3.2 Spatial analysis of seed rain

The spatial distribution patterns of seed rain were determined by four indexes:

variance/mean ratio (C_x), negative binomial index (K),

aggregated degree index (I) and Cassie. R.M. index (C_A). The calculation and determination methods are shown in Table 2 (Lan et al., 2003).

2.3.3 Data analysis

Microsoft Excel 2019 and SPSS 19.0 were used to analyze the data.

Table 2. Test method of spatial distribution patterns.

Variance/mean ratio (C_x)	Negative binomial index (K)	Aggregation index (I)	Cassie. R.M. index (C_A)
$C_x = S^2 / \bar{X}$	$K = \bar{X}^2 / (S^2 - \bar{X})$	$I = (S^2 / \bar{X}) - 1$	$C_A = (S^2 - \bar{X}) / \bar{X}^2$
Uniform distribution if $C_x < 1$	Non aggregated distribution if $K < 0$	Uniform distribution if $I < 0$	Uniform distribution if $C_A < 0$
Aggregated distribution if $C_x > 1$	Aggregated distribution if $K > 0$	Aggregated distribution if $I > 0$	Aggregated distribution if $C_A > 0$
Random distribution if $C_x = 1$		Random distribution if $I = 0$	Random distribution if $C_A = 0$

Note: $\bar{X}$ is the mean value, $\bar{X} = \sum X/N$; S^2 is the variance, $S^2 = \sum(X - \bar{X})^2/(N-1)$; N is the quantity of SRCN in each sample plot.

3 Results

3.1 Density of *C. equisetifolia* seed rain

There were considerable differences in seed rain density between years and among sample plots (Table 3). Total average density of seed rain during 2017-2018 was higher with 1765 seeds m^{-2} while 2018-2019 was a lean year for seed rain with an average density of 713 seeds m^{-2}. In addition, the differences in the seed rain density between SRCN were highly significant ($P < 0.01$), in which the seed rain density for sample plot 1 was the highest and that for sample plot 3 was the lowest. Over the two years, the coefficients of variation of seed rain density in sample plot 2 was the largest, at 1.67 and 1.078, respectively, while that in sample plot 3 was smaller, 0.97 and 0.59, respectively.

3.2 Temporal distribution pattern of *C. equisetifolia* seed rain

3.2.1 Temporal dynamics of* C. equisetifolia *seed rain density in different years

In both years, the dispersal period of seed rains began in the middle of July and ended around mid-April in following year, with a continuous dispersal period of nine months during which the seed rain density increased slowly over time, reaching a peak in early December-January, and then decreased in an irregular manner and slowly over time (Fig. 1). The change trend of seed rain density in both years was basically the same, but the change curve of seed rain density in 2017-2018 was significantly higher than that in 2018-2019, indicating that the mast and lean years of the seed rain was obvious. In addition, the dispersal pattern of seed rain was obviously unimodal, with the peak period in the lean year being one month later than that in the mast year, and the peak value in the lean year was also significantly lower than that in the mast year.

3.2.2 Temporal dynamics of seed rain density in different seed rain collection nets (SRCN)

The peak of seed rains for SRCN mainly occurred in December, among which the peak of seed rains for 28 SRCN occurred in the first half of December in 2017-2018, accounting for 93%, and the peak of seed rains for 26 SRCN occurred in the second half of December in 2018-2019, accounting for 87% (Fig. 2). Fig. 3 shows that six nets with relatively high seed rain density (>33%) all appeared in early December, 81% of 37 nets with relatively medium density (16-33 %) appeared in

Table 3. Density of seed rain in different sample plots over two years.

Year	No. of Sample plots	No. of Seed rain collections	Mean of seed rain density (seeds m^{-2})	Max density in single net (seeds m^{-2})	Min density in single net (seeds m^{-2})	Coefficient of variation
	1	18	2 659 ±2 810	8 800	432	1.06
2017-2018	2	18	1 772 ±2 965	10 100	179	1.67
	3	18	863 ±835	2 500	78	0.97
Mean*		18	1 765 ±2 439	10 100	78	1.38
	1	16	1 007 ±592	1 846	325	0.59
2018-2019	2	16	694 ±747	2 751	216	1.08
	3	16	437 ±259	888	135	0.59
Mean*		16	713 ±599	2 751	135	0.84

NB. * Mean value is derived from a total of 30 seed rain collection nets.

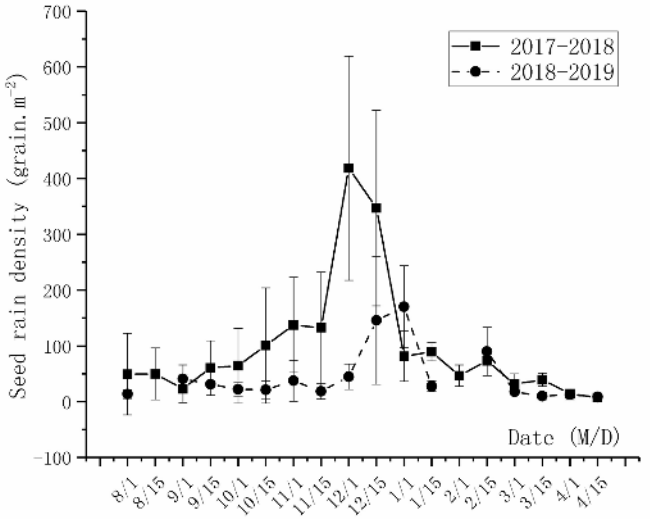

Fig. 1. Temporal dynamics of seed rain density.

early December, and only 4.8% of 497 nets with relatively low density (<16.%) appeared in early December in 2017-2018. All seven nets with relatively high seed density were in late December, and 46 nets with relatively medium seed rain density were in late December, accounting for 69.6%, and only 4.9% of 427 nets with relatively low density recorded in late December in 2018-2019 (Fig 4).

3.2.3 Temporal dynamics of the vigour of C. equisetifolia *seed collected as seed rain*

The germination rate of seed rain in two years fluctuated with time, and it was higher in the early dispersal period, and then gradually decreased. The germination rate of seed rain increased again during the peak period of seed rain in early December to early January, and then decreased in an

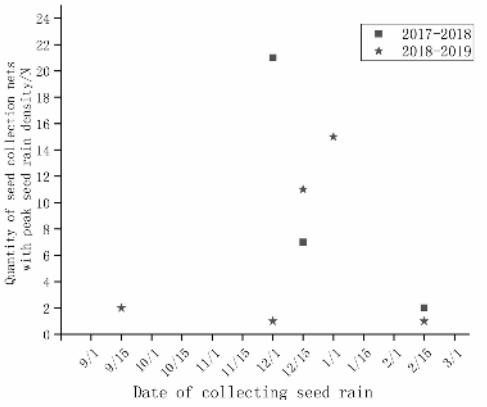

Fig. 2. Distribution of SRCN quantity with peak seed rain density over different collection date.

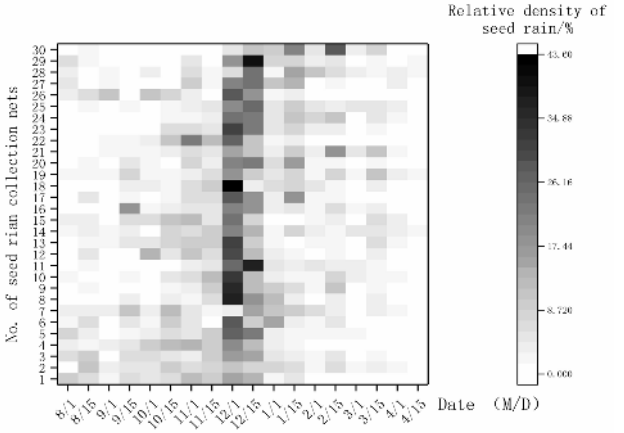

Fig. 3. Hot spots of relative seed rain density for each SRCN in 2017-2018.

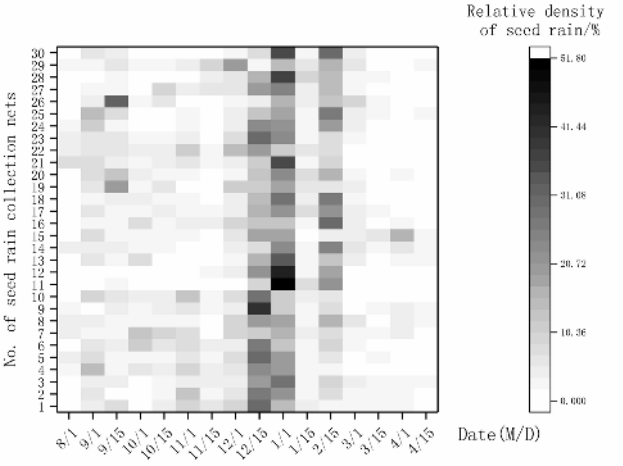

Fig. 4. Hot spots of relative seed rain density for each SRCN in 2018-2019.

irregular manner The average germination rate of seed rain in the mast year (2017-2018) was low (11%), while that in the lean year (2018-2019) was relatively high, with 18%. In addition, the maximum germination rate of seed rain and times of high germination rate were not the same in both years, but the lean year was still better than mast year for those two items, in which the times with high seed rain germination rate (≥22%) in the mast year was four times, the maximum germination rate was 30%, however, the times with high seed rain germination rate was six times and the maximum germination rate was 50% in lean year. Meanwhile, there were eight times of low germination rate (< 6%) in mast year, but only three times in lean year. The germination rates of seed rain in different years and periods were significantly different (Duncan, $P < 0.05$), as shown in Fig. 5.

3.3 Spatial distribution pattern of *C. equisetifolia* seed rain

Seed rain density can only provide information about the quantity of seed rain distribution, while the distribution pattern of seed rain can explain how seeds are arranged in space. Seed rain distribution patterns are mainly divided into three patterns of uniform, aggregated and random distributions. The calculation results of four discriminant methods (Table 4) showed that variance/mean ratio for different years, various sample plots and total sample plots was greater than 1 ($C_X > 1$), negative binomial index $K > 0$, aggregation index $I > 0$, Cassie. R. M. index $C_A > 0$, therefore, the spatial distribution patterns of the seed rain were all aggregated distribution, with very high aggregated degree (C_X and $I >> 1$), and discriminant results of four methods were consistent.

In order to further distinguish the spatial distribution pattern of seed rain, linear regression analysis was used to analyze the relationship between the seed rain densities in different SRCN and the quantity of cones in the vicinity of the SRCN. The results showed that the seed rain density and the quantity of cones were positively correlated ($r = 0.53$; $P = 0.003 < 0.01$), which indicates that the seed rain in the SRCN mainly comes from the surrounding mother trees, therefore, the more cones in mother trees surrounding the SRCN the higher the seed rain density in the SRCN. The results of linear regression analysis further confirm that the distribution pattern of seed rain has an aggregated distribution, and

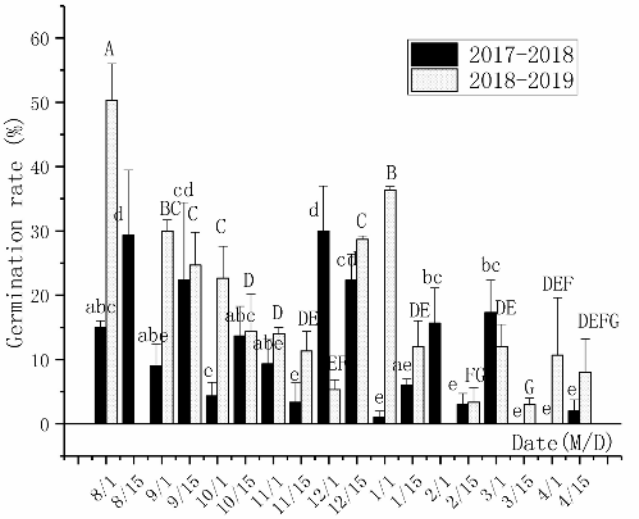

Fig. 5 Temporal dynamics of seed rain germination rate.
(Difference is significant with different lowercase letters for 2017-2018 and uppercase letters for 2018-2019)

Table 4 Spatial distribution patterns of seed rain in different years.

Year	No. of plots	C_x	Distribution pattern	K	Distribution pattern	I	Distribution pattern	C_A	Distribution pattern
2017-2018	1	2969.17	Aggregated	0.90	Aggregated	2968.17	Aggregated	1.12	Aggregated
	2	4959.81	Aggregated	0.36	Aggregated	4958.81	Aggregated	2.80	Aggregated
	3	807.74	Aggregated	1.07	Aggregated	806.74	Aggregated	0.93	Aggregated
Total		3056.75	Aggregated	0.58	Aggregated	3055.75	Aggregated	1.73	Aggregated
2018-2019	1	348.30	Aggregated	2.90	Aggregated	347.30	Aggregated	0.35	Aggregated
	2	802.88	Aggregated	0.87	Aggregated	347.30	Aggregated	1.15	Aggregated
	3	807.74	Aggregated	2.87	Aggregated	152.39	Aggregated	0.35	Aggregated
Total		503.35	Aggregated	1.42	Aggregated	502.35	Aggregated	0.70	Aggregated

seed rains in SRCN mainly fell from mother trees in the immediate vicinity. *C. equisetifolia* seeds are small, light and winged, therefore, they should be well-suited to long-distance dispersal, from the perspective of the structure and morphology of the seeds. However, the results of aggregated distribution suggested that the dispersal distance of *C. equisetifolia* seed rain is very limited in the forests.

4 Discussion and conclusion

(1) High density, great seasonal variation of *C. equisetifolia* seed rain

The quantity of seed rain is an important index to measure the seed yield and regeneration potential of the plant. Due to the different reproductive strategies of different plants, their seed yields often vary greatly, which leads to different seed rain densities among different species (Urbanska and Fattorini, 2000). The observation results of seed rain for two consecutive years showed that the seasonal variation of seed rain density for *C. equisetifolia* was great, with obvious mast and lean years. The average annual seed rain density over both years was 1239 seeds m^{-2}, and the density in the mast year was more than double that in the lean year, among which the density in the mast year is 1764.63 seeds m^{-2} and that in the lean year was 712.60 seeds m^{-2}. This is consistent with the seasonal variation of seed rain for *Larix olgensis* in the Liaodong mountains (Liaoning province, China). For example, the seed rain density in the mast year was 937 seeds m^{-2}, while the that in the lean year is only 64 seeds m^{-2} (Yue et al., 2010), and the density in the mast year is more than twice as high as that in the lean year. However, the differences in seed rain density for some species are less marked, for example, *Taxus chinensis* var. *mairei* in Fujian Province (China) did not show mast and lean years (Yue et al., 2010). In addition, the seed rain density of *C. equisetifolia* is higher, even the seed rain density in the lean year is higher than that of many tree species, such as *Castanopsis delavayi* (2.3 ~ 2.8 seeds m^{-2}) in southwest Sichuan mountains, China (Fei et al., 2006), *Picea asperata* (86 seeds m^{-2}) in Sweden (Hofgaard, 1993), *Haloxylon ammodendron* (189 seeds m^{-2}) in the Jungar Basin, north-western China (Lv et al., 2012) and *Taxus chinensis* var. *mairei* (252 seeds m^{-2}) in Fujian Province (Yue et al., 2010). However, it is lower than that of *Betula platyphylla* at different forest ages in the southern part of the greater Hinggan mountains (Mongolia), which was 8478 ~ 35273 seeds m^{-2} (Feng et al., 2019). Due to the difference of age structure, density, ratio of male and female plants in CCPF on Hainan Island, the seed rain density will have significant variation from place to place. In the research area the *Casuarina* stands are of overall poor quality, due to low density of stands and smaller *DBH*, therefore, the seed rain density for higher quality stands is expected to be higher.

(2) Dispersal period long, synchronization and Unimodal pattern

The dispersal period of *C. equisetifolia* seed rains was long over a total of nine months starting in mid-July and ending to mid-April in the following year, which is much longer than *Pinus tabuliformis* in Ziwuling, Loess plateau (China) and *Castanopsis delavayi* in the mountain of southwest Sichuan (China), with two months of seed rain dispersal period (Fei et al., 2006), and *Taxus chinensis* in Fujian Province (China), with three months of seed rain dispersal period (Yue et al., 2010). In addition, the seed rain density displayed a clear temporal dynamic during the whole seed rain dispersal period, and the dispersal pattern of seed rain was unimodal with an distinct peak period. The peak period in both years occurred from early December to early January, and the seed rain density during peak period reached 43-44% of the total seed rain density. The temporal dynamic of *C. equisetifolia* seed rain is similar to that of *Cunninghamia lanceolata* (Feng et al., 2019), Pinus tabulaeformis (Zhang et al., 2009) and other tree species, that is: the density is low in the early stage of dispersal, then increases to peak in the middle stage, and then gradually decreases. The peak density of seed rain occurred in December in 97% of SRCN indicating that the dispersal period of different *Casuarina* trees was synchronized. That is, the longer dispersal period of *C. equisetifolia* forests was not caused by the unsynchronized dispersal period of different trees, but only a few trees lagged behind.

(3) Seed rain germination rate low and fluctuated with time

The average germination rate of *C. equisetifolia* seed rain was low, and the seasonal variation was greater, ranging from 11% to 18%, with the germination rate in the mast year being higher than that in the lean year. Seed rain has a high germination rate in the early and peak stages of seed rain dispersal, but the highest germination rate of seed rain varies greatly in different years, ranging from 30% to 50%, which is far lower than that of *Haloxylon ammodendron* in Junggar basin (80%) (Lv et al., 2012), and from seed of a planted 65-year old *Picea asperata* stand in the sub-mountain of west Sichuan (71%) (Yin et al., 2011). However, the germination rate of *C. equisetifolia* seeds, collected directly from the tree at the same period with the seed rain was 58%, far higher than that of *C. equisetifolia* seed rain. The reason is that earliest seeds of the seed rain had already lost viability and decreased seed vigor under the ecological environment of high temperatures, high humidity and microbial infection, because the collection interval of seed rain is longer, typically 15 days. During the whole dispersal period, in general, the germination rate of seeds increases with the increase of seed maturity, and the germination rate reaches maximum as seed rain reaches the peak, and then decreases. For example, the germination rate of *Haloxylon ammodendron* seed rain in Junggar basin was relatively low in the earlier stages of dispersal, then gradually increased, and slightly decreased at the end of dispersal (Lü et al., 2012). However, the vigor of *C. equisetifolia* seed rain does not follow this pattern, fluctuating with no obvious trends. The reason is not the maturity of seeds, but the heterogeneity of aging conditions, under the original habitat conditions, because there is no significant difference in the germination rate of seed rain in the dispersal beginning and at the peak. In addition, the rainy season on Hainan is from May to October, during which the quantity of seed rain is only 18 to 20%, and more than 80% of the seed rain is dispersed in the dry season. Dispersal in the dry season and rapid loss of viability of seed, falling as seed rain, are all unfavorable for *C. equisetifolia* seeds to germinate and establish naturally.

(4) The seed rain of C. *equisetifolia* being aggregated distribution

Spatial heterogeneity often exists in seed rain dispersion, and its spatial pattern will affect population regeneration pattern, and then affect population distribution (Ran et al., 2000). According to results of four spatial distribution discriminant methods, the seed rain of *C. equisetifolia* had an aggregated distribution, and the seed rain density was positively correlated with the number of cones on mother trees surrounding the SRCN ($r = 0.530$). Seed rain is usually distributed around the mother tree, and dispersal quantity of seed rain gradually decreases with the increase of the distance from the mother tree (Hu et al., 2005). The dispersal distance of seed rain is affected by many factors such as seed weight and morphology, topography, height of seed release and wind strength (Han and Wang, 2002). *C. equisetifolia* seeds are small, light and winged, which is well-suited for long-distance dispersal. However, the research results showed that

the seed rain presented a strong aggregated distribution, indicating that the dispersal capacity of *C. equisetifolia* seeds in the forest was limited due to the physical hindrance of the tree canopy and on wind speed through the canopy.

(5) Quantity and quality of seeds not being the barrier to the regeneration

Although the germination rate of seed rain was low, the quantity of seed rain with germination potential (germination rate × density) was higher, with about 128 seeds m^{-2} (lean year) to 200 seeds m^{-2} (mast year), because of high density of seed rain, therefore, the quantity and quality of *C. equisetifolia* seed rain is not considered a major factor hindering its natural regeneration on Hainan Island.

Acknowledgements

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Allelopathy of *Casuarina equisetifolia* on Hainan Island, China

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Abstract

Allelopathy is a chemical ecological phenomenon which regulates the growth of neighboring organisms and the establishment of population through the release of secondary metabolites, it is an important reason for the difficulty of self-renewal of *Casuarina equisetifolia* forest in Hainan of China. This paper presents the study on the allelopathy and autoallelopathy of *Casuarina equisetifolia* in Hainan from the identification of allelochemicals, the diversity of endophytes and their allelopathic potential.

Keywords: autoallelopathy, allelopathy, diversity of endophytes, *Casuarina equisetifolia*

1 Introduction

Casuarina equisetifolia is the main species used in coastal shelterbelt plantings in Hainan Province, its planting area is over 60,000 ha, and plays an important role in restoring, improving and protecting coastal ecosystems. It also plays a prominent role in mitigating winds and preventing sand erosion and improving of soil (Yang et al., 2015). However, at present, *C. equisetifolia* forest has difficulties in natural regeneration and is affected by serious diseases and insect pests, which results in a reduced site protection efficiency. One of the main reasons for this phenomenon is the allelopathy of *C. equisetifolia* (Deng et al., 1996; Lin et al., 2005). Allelopathy is a chemical ecological phenomenon which regulates the growth of neighboring organisms and the establishment of population through the release of secondary metabolites. In recent years, our work on allelopathy of *C. equisetifolia* has focused on: (1) Identification of the components of the aqueous extract from *C. equisetifolia*; (2) Allelopathic effects of *C. equisetifolia* on native tree species; (3) Diversity of soil microorganisms and endophytes of *C. equisetifolia* and their allelopathic potential.

2 Identification of components in the aqueous extract of *C. equisetifolia*

Allelopathy is a phenomenon by which the secondary metabolites produced by plant roots or litter accumulate in the soil and inhibit the growth of surrounding plants. In order to study the secondary metabolites in *C. equisetifolia* root, litter and rhizospheric soil, we identified the extracts from litter, root and rhizosphere soil of *C. equisetifolia* using GC-MS chromatograms in stands of different ages.

GC-MS analysis identified 36 and 38 chemical compounds present in the root and litter extracts, respectively (Fig. 1). However, only 14 chemicals were found in the soil extracts. The amount of these constituents increased with stand age. The 2,4,7,9-Tetramethyl-5-decyn-4,7-diol; 7,9-di-tert-butyl-1-oxaspiro (Hejl et al., 1993; Hata et al., 2012); deca-6,9-diene-2,8-dione and 2,4-bis (1,1-dimethylethyl)-phenol) were found in all

three extracts, while 4,5-dihydro-2-methylimidazole-4-one, L-Arabinitol, Pyridine, 2,4-dimethyl-Benzo[h] quinoline, DL-Threitol, 2H-Pyran-3(4H)-one-dihydro and mannopyranoside were only present in the root and litters extracts. This suggests that these substances are synthesized by plants and are released into the surrounding environment through roots and from litter. Earlier studies have reported that 2, 4-bis (1, 1-dimethylethyl) phenol was most inhibitory to rice, lettuce and barnyard grass (Qin et al., 2011) and it was also the main allelochemical of barnyard grass. This allelochemical is also present in Rehmannal glutinosa that affects the Sesamum indicum (Wang et al., 2009). Deng et al. (1996) isolated the 5- glycosides from *C. equisetifolia* and found that three of them inhibited the growth of *C. equisetifolia* seedlings, especially root growth, suggesting auto inhibition. However there have been no other reports in support of these observations.

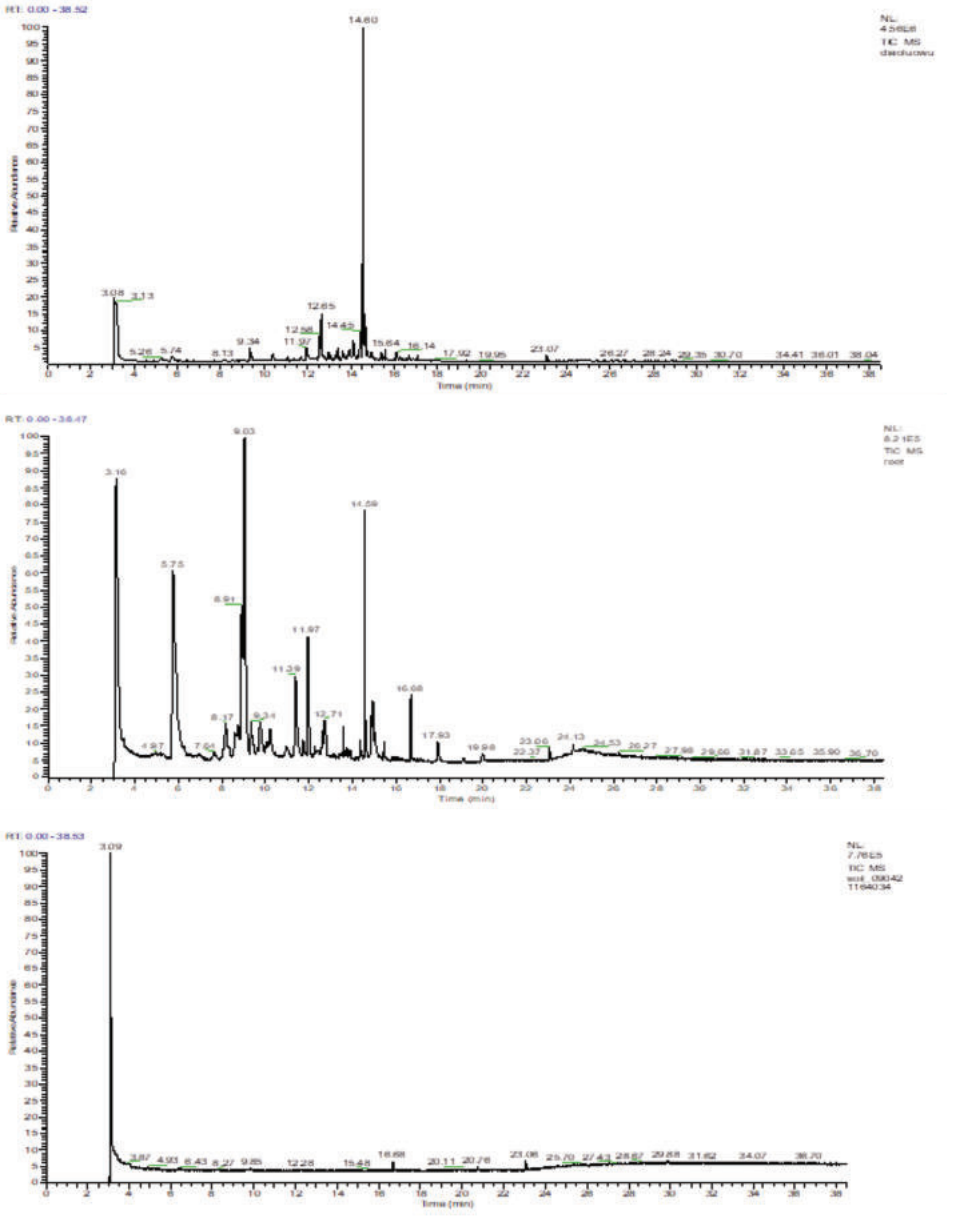

Fig. 1. GC-MS chromatograms of *C. equisetifolia* mature forest aqueous extracts. a. Litter extract; b. Root extract; c. Rhizospheric soil extract.

3 Allelopathic effects of *C. equisetifolia* on native tree species

Mixed planting of *C. equisetifolia* with other native species is undertaken to improve the stability of these forests as well as to protect the Hainan coastline. In the current study we planted three native tree species viz. *Calophyllum inophyllum, Thespesia lampas* and *Vatica mangachapoi,* in casuarina forest of different stand ages.

These results showed that *C. equisetifolia* leachates markedly decreased the germination of *V. mangachapoi, T. lampas* and *C. inophyllum* (Fig. 2). With the increase in stand age, the allelopathic effects of *C. equisetifolia* became more evident. *C. equisetifolia* leachates decreased the seed germination of the three native species. In young forest, *C. equisetifolia* is characterized by small tree size, low total biomass and hence, limited release of allelopathic chemicals into the soil. With the increasing stand age, *C. equisetifolia* released more allelochemicals into the soil and these accumulated more in the biomass. The accumulated allelochemicals inhibited the seed germination of plants in their immediate environment. Yang (2006) investigated the biodiversity of understory vegetation in Manchurian-walnut plantations of different ages. With increasing stand age of *Juglans mandshurica,* the metabolic pattern in changed and increased juglone was released. Different plants have variable abilities to adapt to the presence of juglone and the stand age difference, led to distinct plant diversity (Rietveld, 1983; Neave and Dawson, 1989; Hejl et al.,1993; Yang, 2006). Based on this premise and our observations, we conclude that the young stand age of *C. equisetifolia* is the most suitable period to transplant other tree species to avoid alleopathic inhibition of their survival and growth.

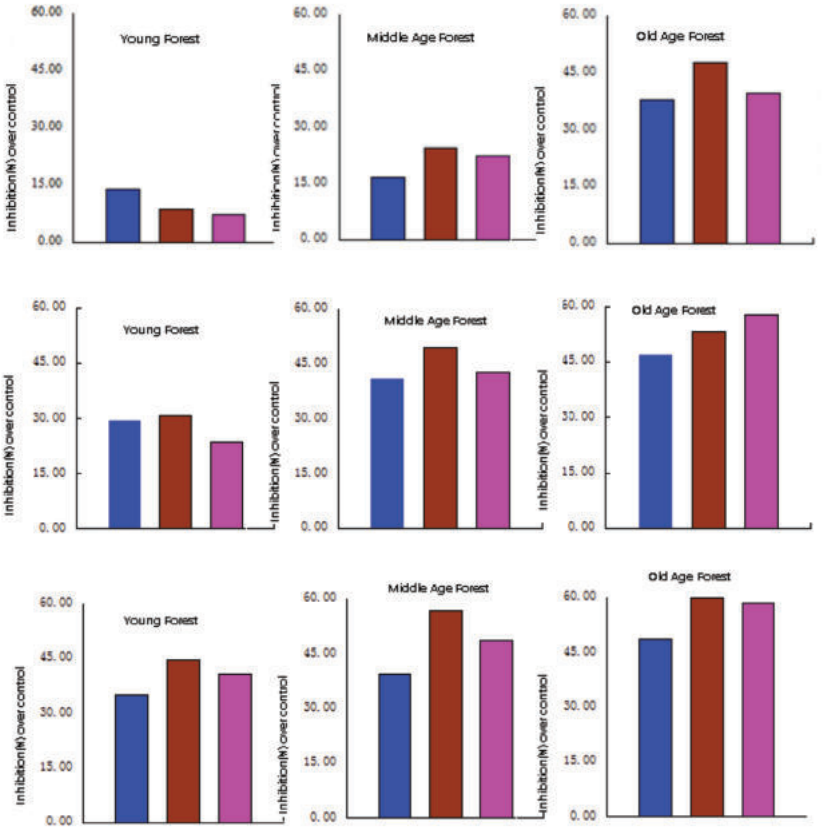

Fig. 2. Inhibitory effects of aqueous extracts of *C. equisetifolia* roots, soil and litter on the germination of seeds of three native tree species. (: roots, : soil, : litter)

4 Diversity of soil microorganisms and endophytes in C. *equisetifolia* woodlands

We choose the 18S rRNA universal gene primers of fungi, using high-throughput sequencing: the results showed that the rhizospheric fungal communities of young and middle-aged forests were more diverse than those of mature forests (Fig. 3). The endophytic fungal community was less diverse in young forests than in the older forest age classes. The evenness indices revealed that rhizospheric fungal community evenness in young- and middle-age forest was higher than in mature forest: endophytic fungi community evenness was highest in mature forests, with significant differences between them.

The results of the fungal community's composition at different taxonomic levels showed that the rhizospheric and endophytic fungi communities consisted of 31 different phyla (Fig. 4). The two most prevalent phyla represented at least 90% of the profile. *Ascomycota* and *Basidiomycota* were the most dominant fungal phyla both in rhizosphere and endosphere. The rhizospheric and endophytic fungi communities among the different aged *C. equisetifolia* were composed of 70 orders. Rhizospheric and endophytic fungi communities were mainly dominated by *Eurotiales* and unclassified orders of *Sordariomycetes* and *Agaricomycetes*

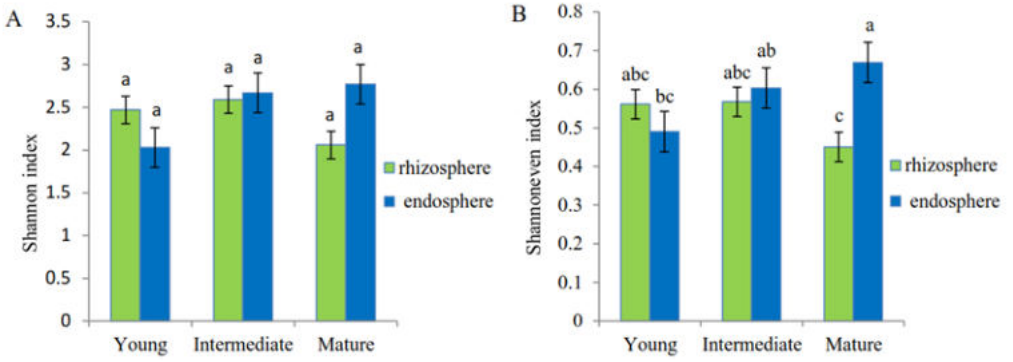

Fig. 3. Diversity and evenness index of rhizospheric and endophytic fungi community of *C. equisetifolia* at different ages. A, Diversity index; B, Evenness index.

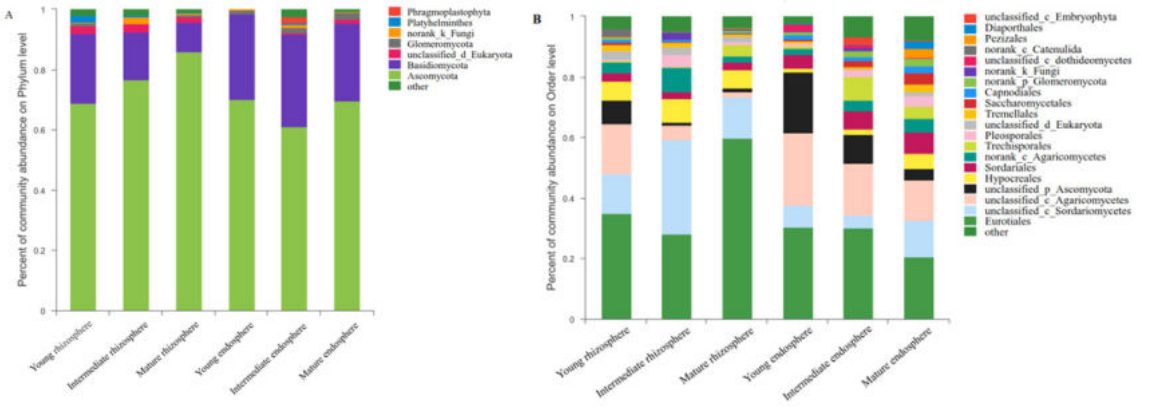

Fig. 4. Rhizospheric and endophytic fungi community composition at different taxonomic levels. A, Phylum level; B, Order level.

PCoA analysis (Fig. 5) indicated differences between rhizospheric and endophytic fungal communities of *C. equisetifolia*. Based on the different communities, all samples were clustered into two regions, one corresponded to rhizospheric samples and another represented endophytic samples.

The results showed a clear division between the rhizosphere and endosphere compartments. Compared to the rhizosphere, endophytic communities were more variable between sampling locations. The results suggested that changes in the microbiome were mainly due to different rhizo-compartments (rhizosphere and endosphere) and sampling locations, but not the development stages of the forest.

Forest stand age changes microbial community structures and environmental characteristics. The disrupting effect of stand age on microbial communi ties may primarily be mediated by aboveground plant and soil physicochemical characteristics. Therefore, we investigated whether microbial community structure

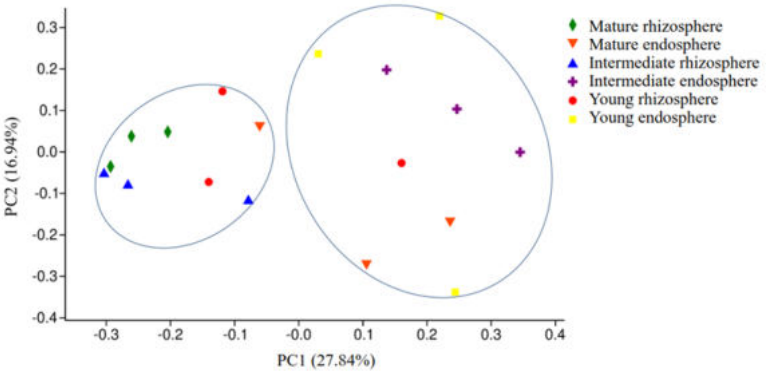

Fig. 5. Principal coordinate analysis for rhizospheric and endophytic fungi communities of different aged forests of *C. equisetifolia* (OUT level).

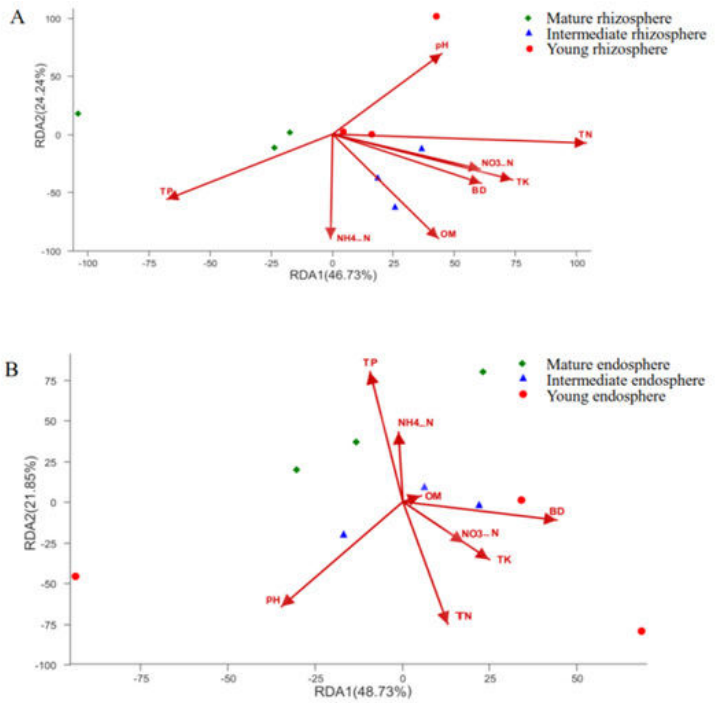

Fig. 6. Redundancy Analysis (RDA) of rhizospheric (A) and endophytic (B) fungal communities.

and environmental characteristics were related.

Through a Redundancy Analysis (RDA) (Fig. 6), we investigated the relationships between environmental factors and fungal communities. The results showed that organic matter (P = 0.049) and total nitrogen (P = 0.039) significantly affected the rhizospheric fungi community structure, while total phosphorus significantly affected (p = 0.049) the endophytic fungi community structure.

5 Allelopathic potential of *C. equisetifolia*'s endophytes

Four fungal species were isolated from rhizosphere and root endosphere. It can be seen that the fermentation broths of the four fungi produce different allelopathic effects on the seeds of *T. lampas* and *C. inophyllum*. The allelopathy effect index of *T. lampas* and *C. inophyllum* was the strongest when treated with *A. aculeatus* fermentation broth, which was -0.26 and -0.17, respectively (Fig. 7). This result showed that the allelopathy potential of different strains is different.

Also, with the same fungal fermentation broth, the allelopathic effect indices on *T. lampas* were stronger than on *C. inophyllum*. This suggested that *C. inophyllum* was less susceptible to allelopathy, which is consistent with earlier findings (Li et al., 2016a). Among the fungal fermentation broths, the allelopathic effect index of the same seed was strongest with *A. aculeatus* fermentation broth and this was significantly different from the effects of *P. melinii, N. fisccheri* and *P. solitum* fermentation broths. This finding showed that the allelopathic potential of *A. aculeatus* was strongest.

6 Chemical constituents from litters of *C. equisetifolia*

Previous studies on the litter of *C. equisetifolia* have shown that the litter's decomposition is slow and the nutrient return ability is poor, and the removal of forest litter has no significant effect on soil nutrient levels, but it can enhance forest pest resistance (Hu et al., 1992; Huang et al., 2000; Tan et al., 2003; Uma et al., 2014; Xue et al., 2016). Our study showed the secondary metabolites in litters have physiological activity.

Twenty one compounds were isolated from the ethyl acetate (EtOAc) extract of the litters of *C. equisetifolia*, and their structures were elucidated using UV, IR, MS, 1D and 2D NMR (Fig. 8). Compounds 1-4 were new compounds. Compounds 5-7, 9-10 and 13-17 were obtained from *C. equisetifolia* for the first time.

Using an *in vitro* by MTT method, compounds 9 and 11 showed cytotoxicity against human chronic myelogenous leukemia cells line (K562), human hepatoma cell line (BEL-7402) and human gastric cancer cell line (SGC-7901), while compound 9 displayed cytotoxicity against a human cervical cancer cell line (Hela). By Ellman's method, compounds 5-7, 9 and 11-12 exhibited moderate acetylcholinesterase (AChE) inhibitory activity. By PNPG method, compounds 5-6,

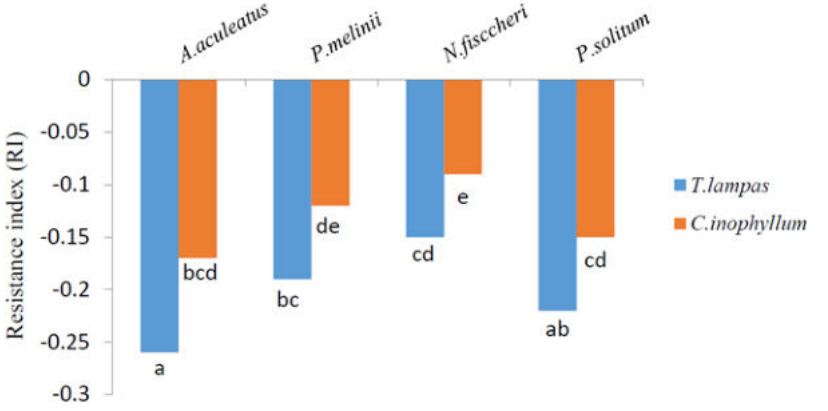

Fig. 7. The allelopathy index (Resistance index) of endophytic fungi fermentation broth on seeds germination. Values marked with different letters are significantly different at P < 0.05.

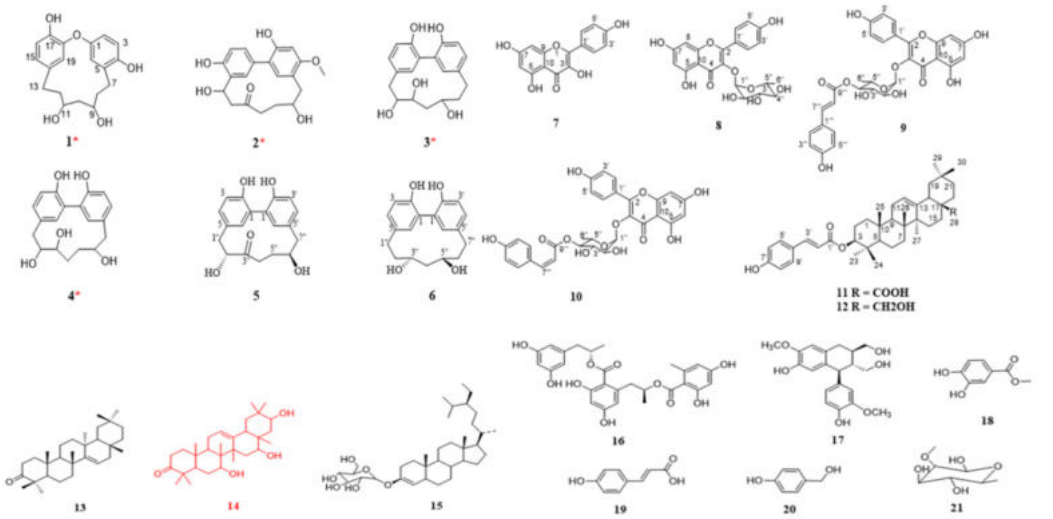

Fig. 8. Compounds from the ethyl acetate (EtOAc) extract of the litters of *C. equisetifolia,*

8 and 11 exhibited significant α-glucosidase inhibitory activity.

The results of these physiological activities indicate that the litters of *C. equisetifolia* have the ability to produce various bioactive substances, which have drug research value, worthy of further study and possibly exploitation.

Using the same method, we analyzed the diversity of rhizosphere and endospheric bacterial microbiome in *C. equisetifolia* woodlands, and the diversity of endophytic and epiphytic fungi and bacterium of the litter in differently aged Casuarina forests (Li et al., 2016b; Wang et al., 2017). By comparison, some common compounds were found in the soil, root, litter, rhizospheric and endophytic bacterium (fungi). This result suggested that endophytes and soil microorganisms were involved in the synthesis of the allelochemicals. But how these endophytes and soil microorganisms participate in the synthesis of allelochemical is unclear.

Our future research will explore the mechanism of allelochemical synthesis under the interaction of endophyte and *C. equisetifolia*. We will select endophytes and soil microorganisms which can produce the common allelochemicals, then establish the interaction system composed of plants and core microbial community that simulates the natural environment, discuss the molecular regulation mechanism of the synthesis of allelochemicals underplant-microbial interaction by conjoint analysis of transcriptomics and metabolomics.

Acknowledgements

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Coppice management in *Casuarina junghuhniana* – preliminary results

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Abstract

Planting fast-growing tree species in short rotation for production of biomass feedstocks used in electricity generation is receiving increasing interest in Thailand. Widely planted *Eucalyptus* species are commonly used because they grow fast and coppice strongly making harvest of coppice regrowth feasible. In recent years, *Casuarina junghuhniana* has proved to be a fast-growing multipurpose tree. The wood is dense with high calorific value suitable for bioenergy production. The species also coppices readily, a characteristic required for short-rotation bioenergy crops. However, there has been no information regarding coppice management to optimise wood production of the regrowth for this casuarina species. A study is currently underway in order to understand factors influencing coppicing ability, such as time of cutting, and time and frequency of pruning. Three 3-year-old stands were cut near ground level and subjected to the following treatments: (1) pruning once at 6 months; (2) pruning once at 12 months; (3) pruning at 6 months and then repeat at 12 months; and (4) control, coppice regrowth left unmanaged. In the pruning treatments, the three most vigorous shoots were retained on each stump. Preliminary results showed that season of tree cutting was important as the trees which were cut in the dry season had poor sprouting (<40%) and the coppice shoots died within a few months. Tree cutting in the rainy season yielded strong coppice sprouts (>90%), however one stand suffered dieback soon after and the cause is not yet known. The remaining stand is making good progress and coppice regrowth now over 12 months old is developing strongly. Mean diameter of the stump surface varied from 8.7 cm to 9.0 cm but there was no effect of diameter on the coppicing ability. Mean height of the three most vigorous shoots was not significantly different among treatments at 12 months, however coppice shoots of the control treatment (mean 3.62 m) were shorter than the other treatments (mean 3.74–3.96 m). Monitoring of the growth characteristics of the coppice regrowth is continuing.

Keywords: *Casuarina junghuhniana*, coppicing ability, coppice growth, pruning, Thailand

1 Introduction

In Thailand, production of woody biomass for bioenergy is a priority of the government and a subject of keen interest for the private sector. Fast growing tree species especially eucalypts have already been planted on short rotations of 3 years for this purpose. Rapid early growth, ability to coppice and high calorific value are key tree species attributes for wood energy plantations.

Casuarina junghuhniana was first introduced into Thailand for provenance trials in 1997-1998 (Pinyopusarerk et al., 2005). The species has shown wide adaptability and promising growth for plantation development. The species is also known to coppice readily making it suitable as a candidate for wood energy plantations. However, there has been no information available on coppice management for wood production in *C. junghuhniana*. The objective of this study was to determine how to best manage the coppice shoots to maximise wood production in *C. junghuhniana*.

2 Materials and methods

2.1 Study sites

Three mixed-clone *C. junghuhniana* stands aged 3 years in different regions of Thailand were used to study coppice management. The sites differed in climate and soil conditions (Table 1).

2.2 Experimental design

The study involved four pruning treatments as follows:

(1) Pruning coppice shoots once at 6 months after tree felling

(2) Pruning coppice shoots once at 12 months after tree felling

(3) Pruning coppice shoots at 6 months after tree felling and repeat at 12 months

(4) Control, no pruning after tree felling

At each pruning, the three tallest shoots were retained and numerically tagged (1–3).

The treatments were laid out at random in 4 replicates and 8 coppice stumps plot^{-1}.

2.3 Assessment and analysis

Soon after laying out the experimental plots, all trees in the stands were clear felled. Trees were cut low to the ground, retaining a stump height of about 10 cm. Diameter of stump surface in the experimental plots was measured in cross direction (east-west and north-south) and the average calculated. Three months after cutting, the number of stumps developing sprouts was recorded. Height of the coppice shoots was measured at 6 and 12 months after tree felling.

Data were subjected to analysis of variance in Genstat version 16 (VSN International, Hemel Hempstead, UK), and least significant difference ($P<0.05$) calculated.

Table 1. Locations of *C. junghuhniana* coppice study sites in Thailand.

Site	Altitude (m)	MAR (mm)	No. dry months	MAT (°C)	Soil
Fang (Far North)	507	1660	5	18.8 (16.4-22.6)	Clay, pH 5.6-5.9
Lad Krating (East)	36	1875	3	27.0 (22.3-33.0)	Sandy loam, pH 4.1
Kanchanaburi (West)	63	1030	4	28.7 (23.3-34.0)	Sandy loam, pH 6.3-6.8

NB. MAR = mean annual rainfall; MAT = mean annual temperature with mean minimum and mean maximum in brackets. Monthly rainfall less than 40 mm is regarded as dry month.

3 Results

3.1 Development of coppice shoots

Coppice sprouts appeared within two weeks after tree felling. Most of the coppice sprouts grew from adventitious buds which developed from callus tissues formed between the bark and wood at the cut surface. In general, each cut stump had up to ten shoots.

At Fang site in the far north, trees were cut down in the beginning of the dry season in November 2017. Thirty nine percent of the stumps had coppiced but sprouts died in the following summer (March–April).

At Lad Krating site on the east, the stand was cut down in the wet season in July 2018, and 91% of the stumps coppiced. Coppice shoots were growing well then stump surface showed sign of fungus development. Nearly all coppice shoots died a few months later.

At Kanchanaburi site in the west, the stand was cut down in the wet season in July 2018 and overall coppice rate was 98%. Coppice shoots have continued to grow well and provided the basis for the results reported here.

3.2 Coppice growth and development

Mean stump diameter ranged from 8.7 cm to 9.0 cm among the treatments and there was no association between size and the coppice ability of the stumps.

Mean height of coppice shoots at six months varied significantly from 1.48 m to 1.93 m (Table 2). However, the differences were not significant at 12 months.

4 Discussion and conclusion

This study confirmed the strong coppicing ability of *C. junghuhniana* as previously reported in India (Nicodemus et al., 2003). However, the low rate of sprouting and severe mortality of coppice shoots at Fang indicated that the season of cutting needs to be considered. It appears best to avoid coppicing in the dry season.

At Lad Krating site in the eastern region with high annual rainfall, nearly all coppice shoots died likely due to fungal disease developing on the stump surface. Therefore, it is suggested that when coppicing was carried out at high rainfall site, the stump surface should be treated with fungicide. There was no fungal growth at Kanchanaburi although trees were also cut in the wet season. Kanchanaburi site has a mean annual rainfall of 1030 mm which is much less than for Lad Krating.

The results so far are not sufficient to determine the effects of pruning on coppice growth in *C. junghuhniana.* Nevertheless, coppice shoots of the control, unpruned treatment were shorter than those of the pruned treatments. Whether or not this was caused by a stronger competition among coppice shoots in the unpruned treatment needs further investigation.

Table 2. Mean stump diameter and mean height of coppice shoots of *C. junghuhniana* at Kanchanaburi, Thailand.

Treatment	Stump diameter (cm)	Height 6 months (m)	Height 12 months (m)
1 Pruned once at 6 months	8.9	1.48	3.96
2 Pruned once at 12 months	9.0	1.93	3.86
3 Pruned at 6 months and repeat at 12 months	8.7	1.71	3.74
4 Control, no pruning of coppice shoots	8.9	1.71	3.62
Overall mean	8.9	1.71	3.80
LSD (P<0.05)	NS	0.21	NS

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Genetics, Breeding, Tree improvement

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Increasing productivity of casuarina plantations in India through genetically improved seeds and clones

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Abstract

Casuarina is among the most widely cultivated tree species in India mainly by farmers of smallholdings. It is generally cultivated in short rotations of three to five years but in fertile soils harvest can start as soon as two years after planting. The principal uses of casuarina are pulpwood for papermaking and poles for temporary house construction and agricultural tools. Farmers benefit from the nitrogen-fixing ability of casuarina by rotating with rice cultivation to sustain soil fertility and yield from both agricultural and tree crops. The industrial need for casuarina wood has maintained a stable market and profitability for casuarina wood for the last two decades sustaining farmers' interest in its cultivation. Short rotations of high density plantations require a large amount of genetically improved planting material. Any improvement in the productivity of plantations results in significant gains for the farmers and industry. A long-term breeding programme started in 1996 by the Institute of Forest Genetics and Tree Breeding with technical guidance from the CSIRO's Australian Tree Seed Centre has completed two generations of breeding of *Casuarina equisetifolia* and *C. junghuhniana*. Breeding populations initially planted as progeny trials with range-wide provenance-family seedlots were later converted into seedling seed orchards. Outstanding individuals in terms of growth, stem form and wood properties were clonally propagated and deployed in multilocation clonal tests. Best clonal accessions were used as clones for large scale planting and the clonal trials were converted into clonal seed orchards. Elite clones of the two species possessing specific end-use characters were involved in control pollination programme to produce interspecific hybrids with new combinations of traits. Making the improved planting material accessible to thousands of smallholding farmers has been a challenge: only through widespread deployment will the programme's genetic gains be fully realised and converted into economic gain. Decentralized seed orchards were established with community and industry in major casuarina planting districts. Non-exclusive licenses have been issued to paper industries and nursery operators to commercially multiply the clones and supply to farmers in different

regions, which helped in reaching a large number of farmers with the genetically improved planting stock and to increase the area under their cultivation. The cumulative area of plantations raised with genetically improved seeds and clones is 50,000 ha and 18,000 ha respectively. The average increase of improved seeds from second generation seed orchards over local source is 20% and that of clones is 40%. Every one per cent gain corresponds to 1.2 tonnes of additional pulpwood per ha valued USD 50 at the time of harvest. The new varieties have strengthened the farming economy and also benefited the paper industries by securing their pulpwood raw material need with more uniform wood properties. The output from the Indian breeding programme can also be made use of by countries with similar edaphic and environmental conditions after undertaking a limited field testing.

Keywords: breeding, clone, farmers, genetic gain, industry, seed orchard

1 Introduction

Casuarina is an extensively cultivated tree crop in Peninsular India. The current estate of plantations is around half a million hectares mostly raised by smallholding farmers. The major species under cultivation is *Casuarina equisetifolia* in coastal areas and *C. junghuhniana* in the interior regions. The main uses of casuarina wood is papermaking and poles for construction and scaffolding. Casuarina meets around 25% of pulpwood raw material need of Indian paper industry. It is also a preferred fuelwood owing to its high calorific value. Shelterbelts, windbreaks, amelioration of salt-affected and mined areas are some of the environmental services derived from Casuarina. All these benefits and its nitrogen-fixing ability in association with the actinomycete, *Frankia* make casuarina truly a multipurpose and farmer-friendly tree in India.

Casuarina is under cultivation for more than a century in India enabling the farmers to evolve a standard practice of cultivation. Some of the unique aspects of its cultivation is high density plantations (around 10,000 trees ha^{-1}), short duration of four to five years, planting bare-rooted seedlings, intercropping during the first six months by availing space between saplings and in the recent years trickle irrigation and targeted fertilizer application. Despite all these innovations, the productivity of plantations remained low mainly due to the lack of genetic improvement of planting material. Till recently almost the entire plantation was raised through seeds collected from the existing plantations which hardly underwent any genetic improvement. Over a period of time, the growth among trees became highly variable, 10% of trees contributing 23% of utilizable wood biomass, leading to reduction in overall wood production (ICFRE, 1994).

Casuarina cultivation rapidly increased during the decade starting 1990 due to major forest and economic policy changes effected in India. The new forest policy adopted in 1988 mandated the wood-based industries to entirely source their wood raw material from trees grown outside the natural forests. Almost at the same time due to liberalization of economic policies, most of the paper industries have expanded their production capacity up to 50% necessitating increased demand for pulpwood. Farm forestry programmes were launched by the industries to encourage farmers to grow pulpwood trees by providing seedlings and technical support for cultivation, harvest and sale of the wood. This partnership between tree farmers and industries lead to the realization of increasing productivity through undertaking a long-term selection and breeding programme. A systematic breeding

programme was launched in the year 1996 and is being continuously implemented in the country since then. This paper is a review of India's casuarina breeding programme detailing the strategy, implementation and the genetic and the economic gains realized by farmers (growers) and industries (end users).

2 India's casuarina breeding programme

The breeding programme of casuarina for India was prepared by Khongsak Pinyopusarerk of CSIRO Australian Tree Seed Centre in 1996 and funded by UNDP/FAO Regional Project on improving productivity of man-made forests through application of technological advances in tree breeding and propagation (RAS/91/004; FORTIP) (Pinyopusarerk, 1996). It was prepared by the author after undertaking extensive field visits in South India and interacting with farmers, managers of forest department and paper industries, faculty of Universities and Research Institutions. Since it was the first instance in the country that a breeding programme was to be implemented as per a written plan, the expertise and resources available for the programme was also considered in the process. The Institute of Forest Genetics and Tree Breeding, located at Coimbatore in Tamil Nadu State was identified as the coordinating organization to implement the programme in collaboration with other organizations interested in casuarina improvement.

2.1 Breeding objective

Pulpwood and poles are the most important uses of casuarina in India and fuelwood is generally derived as a by-product. The breeding objective is to maximise the yield and quality of pulpwood and poles in casuarina plantations. To achieve this objective adaptation (survival and health), fast growth, straight stems were set as the selection criteria for the breeding programme.

2.2 Overview of breeding strategy

The breeding strategy was based on open-pollinated mating in a two-tier breeding population. Since the knowledge on reproductive biology of casuarina was limited at the start of the programme, open-pollination approach was considered the best to make early gains. However, as the understanding on floral biology and breeding system started accumulating, control pollination was also employed during the second generation to produce inter-provenance and inter-specific hybrids. The breeding populations were established as large progeny trials planted with nearly 300 open-pollinated families representing 39 natural provenances and land races in Africa, Asia and Australia. A list of seedlots used for establishing breeding populations is provided in Table 1. This population with a broad genetic base is referred to as the 'Main Population' expected to provide maintain adequate genetic diversity both for long-term genetic improvement and gene conservation.

2.3 Breeding populations

The 'Main Populations' were subjected to two thinning for removing the inferior individuals and seedlots and converting them into seedling seed orchards. The progeny tests were generally planted with small sized plots consisting 3 – 4 trees of a family in each replication. The first thinning undertaken at the age of three years was mostly a within-plot thinning which removed the most inferior two trees in each plot. The second thinning was taken up at the age of 5 -6 years in which only the most dominant tree was retained in each plot of better-than-average seedlots. In addition, all trees of the poorest 10% of the families were removed. An important consideration while undertaking the second thinning was to maintain an overall ratio of 1:1 for female and male trees. Seed collection after the second thinning and when more than two thirds of the trees in the orchard flowered marked the completion of one generation of breeding. The breeding cycle in each generation was originally intended to be six years but while implementing it took around 10 years due to late flowering of some of the newly introduced natural provenances. The breeding programme was advanced to the second generation by establishing a progeny trial with around 200 open-pollinated families collected from the best trees of first-generation breeding populations and about 50 families collected from other unrelated populations.

In each generation, a 'Core Population' was developed as a subset of the 'Main Population'. After the first thinning, 50 trees (25 each female and male) with outstanding growth and stem form from the best 25 families were selected. They were vegetatively

Table 1. Details of *Casuarina equisetifolia* provenances deployed in first generation breeding populations.

Prov. No.	CSIRO No.	Provenance	Country	Latitude (° ')	Longitude (° ')	Altitude (m)
Natural provenances – Australia Pacific						
1	15958	Wangetti Beach, Cairns, Qld.	Australia	16 41 S	145 34 E	30
2	18378	Prince of Wales Island, Qld.	Australia	10 20 S	142 10 E	10
3	18008	Darwin, NT	Australia	12 25 S	130 50 E	20
4	16166	Danger Point, NT	Australia	11 07 S	132 20 E	10
5	18271	Vanua Levu	Fiji	16 58 S	178 49 E	24
6	18272	Viti Levu	Fiji	17 45 S	168 18 E	30
7	18402	Kolombangara	Solomon Is.	8 07 S	157 08 E	2
8	18153	Ela Beach	PNG	9 05 S	147 17 E	10
9	18121	Mariana Island	Guam	13 20 N	144 04 E	2
Natural provenances – SE Asia						
10	18374	Kedah	Malaysia	1 36 N	104 16 E	N/A
11	18244	Sarawak	Malaysia	1 44 N	110 30 E	30
12	18348	Kuantan Penang	Malaysia	3 48 N	103 20 E	N/A
13	18299	Had Samira, Songkhla	Thailand	7 09 N	100 37 E	2
14	18298	Had Chao Mai, Trang	Thailand	7 33 N	100 37 E	2
15	18296	Ban Bang Sak, Phang Nga	Thailand	8 46 N	98 16 E	5
16	18297	Ban Kam Phuam, Ranong	Thailand	9 21 N	98 27 E	2
17	18154	Tangalan, Akalan	Philippines	11 55 N	122 30 E	30
Land races - Africa						
18	18355	Cotonou	Benin	6 24 N	2 31 E	8
19	18141	Robinson Island	Kenya	2 58 N	40 10 E	2
20	18135	Malindi	Kenya	3 15 N	40 09 E	7
21	18142	Kilifi	Kenya	3 38 N	39 51 E	20
22	18134	Kenyatta Beach	Kenya	4 00 N	39 00 E	10
23	18144	Baobob	Kenya	4 00 N	39 06 E	25
24	18122	Montazah	Egypt	31 16 N	30 05 E	13
25	18126	Agamy	Egypt	31 13 N	29 45 E	9
Land races - Asia						
26	18152	Ninh Thuan	Vietnam	11 33 N	108 59 E	2
27	18128	Non Nuoc	Vietnam	16 06 N	106 20 E	2
28	18119	South Arcot, Tamil Nadu	India	11 42 N	79 44 E	40
29	18014	Balukhanda, Orissa	India	19 50 N	85 53 E	10
30	18013	Peempur, Orissa	India	20 12 N	86 38 E	7
31	18015	Chandipur, Orissa	India	21 30 N	86 54 E	2
32	...	Cuddalore, Tamil Nadu	India	11 42 N	79 50 E	10
33	...	Tamil Nadu	India	11 30 N	79 35 E	10
34	...	Andaman Islands	India	11 20 N	93 15 E	N/A
35	...	Balukhanda, Orissa	India	19 25 N	86 05 E	10
36	...	West Bengal	India	22 46 N	89 50 E	15
37	18268	Hainan	China	19 58 N	110 59 E	10
38	18586	Da Dun Hai, Beihai	China	21 35 N	109 00 E	2
39	18267	Guangzhou	China	23 00 N	113 03 E	4

Table 2. Chronological list of past casuarina breeding programme activities conducted in India from 1996 to 2018. CE = *C. equisetifolia,* CJ = *C. junghuhniana,* CC = *C. cristata,* CCU = *C. cunninghamiana,* CH = *Casuarina* hybrid.

Year	Activity
1996-98	Establishment of three breeding populations ('Main Populations') as progeny trials in Andhra Pradesh and Tamil Nadu – CE Establishment of international provenance trials - CJ
1999	Selection and vegetative propagation of 50 phenotypically superior trees (25 male + 25 female) to establish 'Core Population' – CE
2000	Establishment of three clonal tests with 'Core Population' – CE
2001	First thinning in 'Main Populations' and conversion into Seedling Seed Orchards (SSO) – CE Thinning in provenance trials for conversion into ESSO– CJ
2003	Seed collection from SSO and ESSO for commercial supply to tree planters – CE and CJ
2005	Thinning in clonal tests and conversion into Clonal Seed Orchard (CSO) – CE
2006	Seed collection from CSO and commercial supply to tree planters - CE
2007	Control crossing involving outstanding clones selected trough clonal testing – CE and CJ Second thinning in SSO/ESSO and seed collection from best 5% of trees to establish second generation breeding population – CE and CJ
2008	Establishment of three second generation breeding populations – CE and CJ Establishment of hybrid progeny trials – CH Establishment of clonal trials - CJ
2010	Release of four outstanding clones for deployment in commercial plantations - CE Establishment of community seed orchards to increase accessibility of genetically improved seed – CE and CJ
2010-11	Selection of outstanding hybrid individuals from progeny trials and establishment of hybrid clonal tests – CH
2014	Release of 10 outstanding clones for deployment in commercial plantations – CE (3); CJ (7) Selection and vegetative propagation of 50 phenotypically superior trees (25 male + 25 female) to establish second generation 'Core Population' – CE and CJ
2015	Establishment of progeny tests with infusion populations received from Thailand and Indonesia - CJ Large scale clonal propagation of clones released for commercial cultivation. Licensing of clones to paper industries and private nurseries for commercial supply -CJ
2016	First thinning in second generation breeding populations and conversion into SSO – CE and CJ
2017	Release of five outstanding hybrid clones for deployment in commercial plantations - CH Establishment of second generation clonal tests - CJ Control pollination involving three species: CE, CJ and CCU
2018	Genetic gain trials of interspecific hybrid clones - CH

propagated through rooting of sprig cuttings and used to establish clonal tests. These tests were established with a large number of replications (8 -10) and small plot-size (2-3 trees clone^{-1} replication^{-1}).

At the age of three years, only one tree was retained in each plot and a few most inferior clones were altogether removed for converting the trial into a clonal seed orchard. The best 10% of the clones were assembled in a clone bank as hedges for producing cuttings for rooting and establishing commercial plantations. The 'Main Population' and the 'Core Population' of the breeding programme took care of the needs of both seed and cutting planting material needs of farmers and industries in India.

2.4 Interspecific hybridization

The strategy described above was adopted for two species of *Casuarina* viz. *C. equisetifolia* and *C. junghuhniana* almost simultaneously. A chronology of breeding programme events for the two species is provided in Table 2. After one generation of breeding, the distinct characters of the two species became clear and were accordingly deployed depending on the site and/or end use. Overall *C. junghuhniana* was at least 30% faster growing and substantially more resistant to drought and diseases than *C. equisetifolia*. While *C. equisetifolia* is a non-coppicer, *C. junghuhniana* possessed coppicing ability which provided two benefits. There is a possibility of harvesting coppice crop without the need to replant after each harvest and production of clonal plants with juvenile vigour through rooting of coppice shoots to raise clonal plantations. But the stem of *C. junghuhniana* had more bends compared to that of *C. equisetifolia*. The bark was thick and fissured in *C. junghuhniana* whereas *C. equisetifolia* had a thin and smooth bark for which *C. equisetifolia* was more preferred as a pole crop. Taking advantage of the similar chromosome number and the dioecious nature of both the species, interspecific hybrids were produced by crossing the outstanding clones across the species. The most outstanding clones selected from Core Populations of each species were hybridized through control pollination. The full-sib families were screened in progeny tests conducted at contrasting locations to select the individuals with vigorous growth and superior stem form from the best families (Nicodemus et al., 2011). They were vegetatively propagated by rooting of coppice shoots and deployed in multilocation clonal tests. Those clones with a desirable package of characters like fast growth, drought resistance, straight stems and high pulp content were released for commercial planting programmes.

Table 3. Geographic variation in growth of *Casuarina equisetifolia* in the first generation breeding populations.

Region	Pondicherry (coastal)		Sadivayal (inland)	
	Height (m)	**DBH (cm)**	**Height (m)**	**DBH (cm)**
Natural – Australia and Pacific	7.8 (6.1-9.2)	6.0 (4.9-7.6)	6.9 (6.6-7.2)	4.2 (4.1-4.3)
Natural – SE Asia	10.0 (9.1-10.7)	7.6 (7.2-8.4)	6.4 (5.7-6.8)	4.1 (3.7-4.5)
Landraces - Asia	8.6 (8.2-9.2)	7.0 (6.5-7.3)	6.3 (5.9-7.0)	3.7 (3.6-4.3)
Landraces - Africa	8.4 (7.6-9.1)	6.3 (5.3-7.0)	6.3 (4.6-7.3)	3.8 (2.3-4.5)
Mean	8.5	6.6	6.4	3.9
S.E.D.	0.1898	0.1544	0.1318	0.1069

Values in parenthesis indicate range for individual seedlot means under the region.

3 Results and output from breeding programme

By the time first thinning was undertaken in the Main Populations of *C. equisetifolia,* it was clearly evident that many native provenances and a few landraces were faster growing than local seed sources and also possessed better qualitative characters like stem form, wood density and resistance to blister bark disease. In general, provenances from South East Asia were the best in all test locations in terms of growth. Native provenances of Pacific Islands and land race seedlots from Africa were fast growing in some locations over the others while those from Australia were generally slow growing (Table 3). In the case of *C. junghuhniana,* six provenances from low elevations of Indonesian Islands, Timor and Wetar were faster growing than native provenances from higher elevations and landraces from Kenya and Tanzania. The mean performance of the six provenances is comparable to the best performing provenance of *C. equisetifolia.* The most outstanding provenance of *C. junghuhniana* was around 40% better than that of *C. equisetifolia* (Nicodemus et al., 2005; 2015).

The second-generation breeding populations ('Main Populations') also showed superior growth of progeny of different first-generation seed orchards over the locally used seedlots (Table 4). Convinced by the superior growth of the many accessions in the breeding populations cum seed orchards, supply of seeds collected from them commenced in the year 2003. In order to demonstrate the better qualities of the orchard seeds, genetic gain tests were established in farmers' land by planting seed orchard progeny, a native provenance seedlot and the local unimproved seedlot in large sized plots (49 trees per replication) to simulate plantation-like environment. In all test locations orchard seeds outperformed the local seed from 15 to 50% (Nicodemus et al., 2011; Varghese et al., 2011). Data from the genetic gain tests also help in efficient matching between orchard and planting site. When the first few harvests were made after 3 -4 years, the increased wood production from the genetically improved seed encouraged farmers to adopt them at a large scale which connected the breeding programme and the commercial plantation programme.

Table 4. Growth and stem straightness of different first generation seed orchards deployed in second generation breeding populations of *Casuarina equisetifolia* at the age of five years.

Location and type of first-generation orchard	Pugalur (irrigated)				Tirupati (rainfed)			
	Height (m)	DBH (cm)	Volume index	Stem st'ness	Height (m)	DBH (cm)	Volume index	Stem st'ness
Karunya - SSO	20.08	10.16	2633	3.52	10.55	5.97	462.4	3.67
Pondy - SSO	19.8	9.48	2340	3.41	10.17	5.74	408.3	3.57
Nellore - SSO	18.02	9.22	2269	3.39	10.49	5.78	434.7	3.61
Rajahmundry - SSO	18.51	9.10	1851	3.49	9.13	5.11	292.1	3.51
Rajahmundry - CSO1	18.57	9.35	2138	3.45	10.42	5.90	446.7	3.59
Rajahmundry - CSO2	17.95	8.82	1913	3.38	10.11	5.79	421.7	3.56
Local seedlot	17.42	8.06	1667	3.19	9.51	5.23	314.0	3.12
Mean	18.81	9.37	2210	3.44	10.25	5.77	422.5	3.59
P	<0.001	<0.001	<0.001	0.101	<0.001	<0.001	<0.001	<0.001
LSD-Min	2.567	1.706	960.3	NS	1.146	0.662	137.8	0.291
LSD-Max	0.894	0.594	334.4	NS	0.399	0.23	48.01	0.101

Clonal forestry is not widely practiced for casuarina in India till 2010 and more than 90% of the plantations were raised through seeds. Outstanding clones shortlisted from the Core Populations were released in three batches: *C. equisetifolia* clones in 2010, *C. junghuhniana* in 2014 and interspecific hybrids in 2017. Since *C. equisetifolia* does not coppice, stem cuttings collected from the hedges of ortets were used to produce planting material. These plants grew slowly during first two years and then showed faster rate of growth (Warrier et al., 2010). As the popular rotation age of casuarina being 3 to 4 years, farmers were not keen to plant these clones. However their genetic potential was utilized through seed production in clonal seed orchards. Clones of *C. junghuhniana* with their faster growth than *C. equisetifolia* coupled with vigorous coppicing nature perfectly suited for clonal forestry (Nicodemus et al., 2003). Clones showed around 40% gain in terms of growth, stem straightness and pulp yield compared to the seeds (Table 5). The uniformity in growth and stem form in clonal plantations quickly attracted farmers' attention and the area under clonal planting expanded rapidly.

Table 5. Growth and pulping characters of casuarina clones at 4 years age.

Parameter	CJ-9	Commercial clone
Height (m)	14.8	12.6
DBH (cm)	10.3	6.9
Volume (m^3)	0.044	0.017
Bulk density (kg m^{-3})	247	270
Basic density (kg m^{-3})	556	484
Chemical addition (%)	17	17
Screened pulp yield (%)	51.71	50.62
Kappa Number	17.1	25

As mentioned earlier, *C. junghuhniana* clones had a thick and fissured bark. Although pleased with their faster growth, farmers and industries preferred new clones with a thin and smooth bark for different reasons. Generally, casuarina wood fetches a higher price when sold as poles than as pulpwood. A bark with a smooth outer surface is preferred for poles used in construction. Paper industry preferred a thin bark since casuarina wood is pulped with bark and low bark content will increase the pulp yield and reduce chemical consumption. Interspecific hybrids that combined the *C. equisetifolia* characters of thin and smooth bark with the fast growth and coppicing ability of *C. junghuhniana* met the needs of farmers and industries. Expectedly they were faster growing than all parental clones (Kannan et al., 2016). They also showed hybrid vigour for adventitious rooting which helped in shortening the nursery cycle from 14 weeks to 10 weeks. In a short span of five years, the share of clones has crossed 50% of annual planting area. Early growth results (age: one year) from genetic gain trials in four contrasting soil types showed consistent superiority of hybrid clones over seed orchard seeds ranging from 20 to 30% for height and 7.7 to 36.8% for diameter (Table 6).

4 Changes in nursery and cultivation practices

While achieving the objective of the breeding programme, i.e. increasing production and quality of wood in plantations, many associated benefits were also gained particularly changes in cultivation practices. Overall, the advent of high yielding varieties has transformed casuarina cultivation from a low-input activity to a high-input one. Farmers realized that it is necessary to change the old practices for realizing the full potential of the new varieties. The most important change is reduction in the stocking of plantations especially those raised with clones. From 10,000 trees ha^{-1} planted at a spacing of 1 m x 1 m, the stocking has come down to around 4,500 trees ha^{-1} at a spacing of roughly 1.5 m x 1.5 m. The space available between the trees during the first six months after planting is utilized for raising intercrops like peanuts and pulses. Lesser number of trees facilitates more attention given to them like fertilizer application, pruning of side branches and early detection and controlling of insect and disease incidence. The awareness on nitrogen-fixing ability of casuarina has increased among farmers who now apply less nitrogenous fertilizer and instead use biofertilizers like VAM and biocontrol agents like *Trichoderma* and *Psuedomonas*. Such optimization of resources needed for planting and tending of plantations offsets at least

Table 6. Early growth of interspecific hybrid clones and seedlots of casuarina in genetic gain trials at the age of 12 months.

Clone name	Height (m)				DBH (cm)			
	GPPM	RMCH	RGPT	SKGU	GPPM	RMCH	RGPT	SKGU
CH1	4.57	3.98	3.15	3.57	3.75	4.01	3.29	1.92
CH2	4.80	4.53	3.60	3.91	4.23	4.32	3.97	2.38
CH3	4.49	4.02	3.62	3.70	3.98	3.68	3.92	1.91
CH4	4.02	3.97	3.09	3.42	3.31	3.67	3.24	1.86
CH5	4.86	4.88	3.67	3.80	4.36	4.52	4.06	2.09
CJ01	3.18	2.60	2.50	1.99	2.87	3.01	2.86	0.96
Seedling-Orchard	3.78	3.74	3.04	3.09	3.81	3.95	3.77	1.74
Seedling-Local	3.66	3.31	3.19	2.52	3.83	3.67	3.72	1.36
Mean	4.17	3.88	3.23	3.25	3.77	3.85	3.61	1.78
LSD (P>0.01)	0.688	0.773	0.448	0.483	0.452	0.388	0.618	0.321
Superiority of best clone over best seedlot (%)	28.44	30.52	20.48	26.38	14.44	14.43	7.69	36.78

Trial location and soil type: GPPM - Gopalpuram (inland -red clayey); RMCH - Rampachodavaram (upland - red loam); RGPT- Rangampet (inland - clayey); SKGU - Shankaraguptam (coastal - sandy)

partly the higher cost of genetically improved planting material particularly clones and brings overall increased income to the planters.

5 Large scale production and commercialization of improved planting material

Reaching the output of the breeding programme to a large number of tree growers and thereby increasing the extent of its adoption and economic benefits is a formidable challenge in India. Farmers raise almost the entire casuarina plantations in India. The average landholding in India is less than two hectares and most of these farmers of smallholding lack the initial resources for procuring the improved planting material. Paper industries do not have planting area of their own but work with farmers through various farm forestry programmes. The amount of seed and clones needed to take care of annual planting is huge; the seed demand alone is estimated to be two tonnes year^{-1}. Considering these facts, the following actions were taken to increase the accessibility and affordability of improved planting material to a large number of tree growers in partnership and government and industrial organizations.

5.1 Community seed orchards

This is an effort to take the seed orchards where their seeds are needed. Seed orchards were raised by communities that are involved in casuarina planting and raising traditional nurseries through technical and financial support provided by a group of government departments and research institutions. The experience of Australian Tree Seed Centre in similar areas in different countries was also made available to this initiative through funding from the Public Sector Linkages Project of the AusAID. Seed orchards were raised in village or government land located close to the traditional nurseries that produce a major share of planting material in south India. The clones required for establishing the orchard were provided from the casuarina breeding programme and the nursery

operators were trained to maintain and collect seeds from the orchards. All the cost of establishing and maintaining till seed production was borne by the organizations involved and thereafter the orchards were managed by the nursery operators themselves. Seeds collected from these orchards were used to raise seedlings in the nursery hubs which ensured that a large number of farmers accessing the genetically improved seedlings at an affordable cost.

5.2 Industrial seed orchards

When the supply of seeds produced from orchards begun, many paper industries placed orders for purchasing large quantities of seed far beyond the production capacity of the orchards. Industries were then encouraged to grow their own orchards using the superior family and clonal accessions developed in the breeding programme. Many industries established orchards in their own nursery area so that the maintenance and timely seed collection could be easily undertaken. Technically these clonal seed orchards are copies of the breeding programme's 'Core Populations' producing genetically improved seed possessing adequate genetic diversity.

5.3 Licensing of clones for commercial propagation

Large scale clonal multiplication requires investments on infrastructure and recurring expenses on human power and maintenance works. Only if the clones developed in the breeding programme are propagated at the commercial scale will their planting be carried out in large areas and make an impact on the farm income of the growers and pulpwood raw material sufficiency of industries. Paper industries which use casuarina as a major pulpwood and a few nursery operators were given non-exclusive license to propagate the clones at commercial scale and sell to farmers and other tree growers in their respective areas. These licensees have introduced many innovations to reduce the production cost and to transport the plants to long distances in large numbers. During the period 2015-19 the cumulative area of plantations raised with genetically improved seeds and clones is around 50,000 ha and 18,000 ha respectively. The average increase of improved seeds from second generation orchards over local seed source is around 20% and that of clones is 40%. Every one per cent gain corre sponds to 1.2 tonnes of additional pulpwood per ha valued USD 50 at the time of harvest. This return from the breeding programme justifies all the investments made in the breeding programme from its inception. The new varieties have also strengthened the farming economy and benefited the paper industries by securing their pulpwood raw material need with more uniform wood properties. They have set benchmarks for developing new varieties from the advance generation breeding. The output from the Indian programme can also be made use of by countries with similar edaphic and environmental conditions after undertaking a limited field testing.

6 Constraints

New introductions from the natural populations flowered later than the normal reproductive maturity leading to overshooting of breeding cycle in the first generation. Casuarina trees particularly that of *C. equisetifolia* require intensive management with irrigation to prevent mortality and to ensure good flowering and fruit setting. Trees of this species are also susceptible to the blister bark disease when retained beyond five years and the infected / dead trees have to removed from orchards and prophylactic actions carried out. While advancing the breeding programme to the second generation, obtaining unrelated germplasm as infusion population was a difficulty. Since all ongoing breeding activities in different countries involve the same set of seedlots provided by the Australian Tree Seed Centre, they are all related in varying degrees. Unless fresh seed collections made in new areas of natural distribution are included in the third-generation breeding, sustaining the genetic gain and diversity in future generations will be difficult.

Although casuarinas are in general prolific seed producers at individual tree level, their reproductive characters pose difficulties for commercial seed production. Being mostly dioecious species, only half the entries in a seed producing population are females that produce seed which brings down the seed production per unit area of orchard. The fruit is a samara (winged fruit) but for all practical purposes it is treated as seed and the infructescence is generally called as 'cone' or 'fruit'. The seed to fruit weight is very low in species like *C. equisetifolia*, normally 40 kg of fruits yield one kg of seeds. Although the seed rate is high at 600,000 kg^{-1}, collecting large volumes of

fruits from orchards at distant locations and extraction of seeds is a laborious activity. As a result, even from a well managed and productive orchard the annual seed collection is around 10 kg ha^{-1} which can be used to raise 100 ha of plantations. Considering the large extent of annual planting area (>100,000 ha) and the amount of seed required, it is difficult to meet them from seed orchards alone.

Clonal propagation significantly compensated the limitations faced in seed production due to biological constraints. Juvenile coppice shoot cuttings showed high percentage of rooting (>90%) and were free from influence of seasons in a year. In particular, interspecific hybrid clones had a faster cutting production and rooting cycles than the parent species leading to higher production per unit time and nursery space. But despite releasing around ten clones to maintain variation in the plantations and to ensure clone-site matching, farmers and industries tend to be limiting their choice to the most productive clone. Expectedly disease problems started appearing in clonal plantations, particularly the collar rot disease caused by *Diplodia natalensis* is major problem in hybrid clones planted in clayey and water-logged areas.

7 Advance generation breeding

Before moving to the third generation of breeding, a review of the breeding undertaken so far for over two decades was conducted. After careful consideration of experiences and genetic gain obtained from the first phase of breeding together with the preferences of farmers and paper industries, a revised programme was prepared to further increase the plantation productivity while maintaining a broad genetic base (Pinyopusarerk et al., 2019). The revised programme is expected to be implemented during the next two decades starting from 2019. The objective of the revised breeding programme is to maximise the growth, stem form and pulp yield from the casuarina plantations to meet the principal end uses in India viz., poles and pulpwood. Given the experience gained during the implementation of first phase of the breeding programme and the new knowledge generated with respect to genetic diversity, flowering biology and control pollination (Yu et al., 2020; Zhang, 2016; 2020), the breeding strategy for the second phase will rely on both open pollination breeding populations and control pollinated accessions together with the use of vegetative propagation to meet the demand for genetically diverse seeds and high-yielding hybrid clones. The open pollination based breeding will be conducted through establishing 'Main Populations' and Core Populations for *C. equisetifolia* and *C. junghuhniana* separately as in the previous generations. Outstanding clones selected in the Core Populations of each species will be deployed as parents in a control pollination programme to produce interspecific hybrids. Hybrid progeny in multilocation progeny tests and best individuals will be vegetatively propagated and subjected to clonal testing for releasing high-yielding clones in each generation. This strategy will ensure meeting the requirements of genetically improved seeds of each species, hybrid seed and clones for deployment based on location, end use and affordability of the grower.

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Studies on control pollination, pollen-pistil interaction, cross compatibility in four *Casuarina* species

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Abstract

The members of the genus *Casuarina* species share a common chromosome number (2n=18) providing an opportunity for hybridization among species. Experiments on controlled pollination, pollen-pistil interactions and cross compatibility were carried out involving four species of *Casuarina* viz., *C. cristata, C. cunninghamiana, C. equisetifolia* and *C. junghuhniana*. Crossing between *C. equisetifolia* and *C. cristata* resulted in the highest percentage of fruit set (38.3%). *C. junghuhniana* as a female parent recorded average fruit set for inter-specific combinations with *C. cristata* (15.9%) and *C. equisetifolia* (11.2%). The initial fruit set for different cross combinations ranged from 14.4 to 76.6% but reduced to 2 to 57% when the fruits matured five months after pollination. Viable seed set ranging from 75 to 94% and seed germination from 3.6 to 91% was observed for the control pollinated families. At the time of pollination, the ovules were found undeveloped in the ovary, and required about 45-50 days for fertilization. Fluorescent microscopic studies showed pollen tubes travelling in a zigzag line through the stigma and style. They were branched in the upper region of the style, and their further growth was found to be arrested until the ovules fully develop. The mean number of pollen tubes (8.0) was reduced significantly at different intervals, maximum one or two pollen tubes reached the style and only one finally reached the ovules. The reduction of number of pollen tubes in the lower region of the style shows that the self-incompatibility systems operating in the stigma and style. Since casuarina cultivation is increasingly using interspecific hybrid clones in India and China, the results from the present study will help in developing new hybrid combinations specific to planting environment and end use requirements.

Keywords: controlled pollination, cross compatibility, genetic resources, pollen-pistil, seed set

1 Introduction

Casuarina species are wind-pollinated and can hybridize across species resulting in the movement of genes between species (Wilson and Johnson, 1989; El-Lakany, 1990; Boland et al., 1996; Ho et al., 2002).

They have a similar chromosome number (2n=18) and hybridization occurs whenever they are brought together. Natural hybrids have been reported in both natural and introduced environments (Bardan et al., 1976; Wang et al., 1984; Wilson and Johnson, 1989). Successful intra- and inter-specific hybrids of *Casuarina* have been developed in India and China (Nicodemus et al., 2011; Zhang et al., 2014). Low level of pollination success was reported for crosses between *C. equisetifolia* and *C. junghuhniana* and no information is available on control pollination involving other species of *Casuarina*. The present study was undertaken with the objectives of conducting control pollination experiments involving *C. cristata, C. cunninghamiana, C. equisetifolia* and *C. junghuhniana* and producing hybrids between them, and to understand the pollen-pistil interactions, cross compatibility between different species.

2 Material and methods

2.1 Tree selection and pollen collection

The controlled pollination studies were carried out in two locations: a species-provenance trial established in 2002 at Karunya, Coimbatore, India (latitude 11º00′ N, longitude 76º 58′ E, altitude 300 masl); and a potted seed orchard at the Institute of Forest Genetics and Tree Breeding (IFGTB) in Forest Campus, Coimbatore (latitude 11º 01′ N, longitude 96º 93′ E, altitude 410 masl). The selected parent trees were established with rooted cuttings of *C. equisetifolia* and *C. Junghuhniana* from 4-year-old second generation progeny trials located at Karur and Salem in Tamil Nadu. In the case of *C. cunninghamiana* and *C. cristata*, trees were selected in a species provenance trial located at Karunya, Tamil Nadu, India. Details of selected species and clones are given in Table 1. Male inflorescences were harvested from the selected trees at least one hour before anthesis (around 06.00 hours) in clean Petri dishes and brought to a laboratory of the IFGTB. The dishes were incubated at 35 ºC for one hour and the pollen that was shed in the dishes was harvested and passed through 25µm sieve to remove anther lobes and other debris, and collected in Eppendorf tubes and stored at 4 ºC.

2.2 Controlled pollination

Secondary branches bearing female inflorescences were covered with pollination bags three days before anthesis when flower buds started appearing pinkish in colour. Needles in the branch were trimmed to facilitate pollen application and to avoid rubbing of needles to the flowers. A coiled wire was tied around the branch as scaffolding to prevent the pollination bag touching the flowers and to facilitate aeration inside the bag. The pollination bag was placed around the wire-scaffoling and tagged. Each branch had 10 to 15 inflorescences and pollen was applied to the fully expanded stigma using a fine hairbrush between 6.00 and 7.00 hrs. One secondary branch in each parent was tagged and observed for fruitset under open-pollinated conditions. Different parental cross combinations used in control pollination are given in Table 2. Control pollination could not be conducted in *C. cunninghamiana* due to lack of flowering during the period of experiment. While no emergence of female inflorescences was observed, male inflorescences developed up to a certain stage but did not matur into functional flowers.

2.3 Pollen-pistil interactions

Open and cross pollinated female inflorescences of different combination of parents were collected at 3, 5, 7, 10, 15, 20, 25, 30, 35, 40, 45 and 50 days after pollination and fixed in FAA (Formalin: glacial acetic acid: 50% ethanol; 5:5:90) for 24 h. Each inflorescence was washed three to four times in distilled water and stored in 70% ethanol at 4 °C for microscopic investigation of pollen tube growth in pistils. The pistils were decolourised by overnight treatment with a diluted solution of sodium hypochlorite at room temperature. After gently washing with distilled water for three to four times, pistils were treated with 1N NaOH at 60 °C for 1 to 2 h. Pistils were washed three - four times with distilled water and then stained with 0.5% decolourized water soluble aniline blue in 0.1N K_3PO_4 for 3 to 12 h following the procedure described by Martin (1958). The pistils were mounted in staining medium and a drop of diluted glycerol on to slides on which two glass covers were placed and pressed gently to loosen the pistil tissue for easy observation of pollen tubes. The processed slides were observed under Nikon epifluorescence microscope (OPTIPHOT-2, Nikon Optical Inc., Japan). The images were captured with Leica DFC 295 camera connected to a computer.

Table 1. Details of trees of *Casuarina* species used for studying controlles pollination, pollen- pistil interaction, cross compatibility.

Clone ID	Location	Sex	Height (m)	DBH (cm)	Stem St'ness
C.equisetifolia					
CE18	Second generation progeny trial, Karur, Tamil Nadu	Female	13	9.5	4
CE40		Male	12.5	7.9	4
CE166		Female	12	9.8	4
CE164		Female	13	9.3	4
CE183		Male	12	6.5	4
Mean of selected trees			12.5	8.6	4
Overall mean			9.8	6.3	3.5
C. junghuhniana					
CJ23	Second generation progeny trial, Salem, Tamil Nadu and Clonal bank	Female	12.5	6.5	3
CJ9		Male	13.9	10.7	4
CJ7		Male	10.7	8.6	4
CJ38		Male	13.5	9.5	3
CJ55		Female	11	9.0	4
CJ85		Female	12.5	7.9	4
CJ89		Male	12.5	7.2	4
Mean of selected trees			12.3	8.5	3.7
Overall mean			9.3	6.9	3.1
C. cunninghamiana (Rollingstone provenance; CSIRO number 13519)					
CU16/1	Species-provenance trial, Karunya, Tanil Nadu	Male	21	17.4	4
CU16/2		Female	20	16.4	4
Mean of selected trees			20.5	16.9	4
C. cristata (Dalby Moonie provenance; CSIRO number 17757)					
CR18/1	Species provenance trial, Karunya, Tamil Nadu	Male	12	14.8	4
CR18/2		Female	14	15.0	4
Mean of selected trees			13	14.9	4
Overall mean			15.2	11.0	3

2.4 Hybrid seed set and germination

Matured fruits of different hybrid combinations were collected six months after pollination for studying seed set and seed germination potential. The number of normal and aborted seeds were counted under dissection microscope (Nikon Smz-U, Japan) attached

Table 2. Details of parental cross combinations of *Casuarina* species involved in control pollination study.

Species	Clone ID of parents	
	Female	Male
C. equisetifolia		
	CE166	CJ38
	CE166	CJ89
	CE18	CJ38
	CE18	CJ89
	CE164	CR18/1
	CE166	CR18/1
C. junghuhniana		
	CJ85	CR18/1
	CJ85	CE183
	CJ85	CJ38
	CJ55	CJ38
	CJ55	CJ89
	CJ23	CR18/1
	CJ23	CE183
	CJ23	CJ89
C. cristata		
	CR18/2	CE183
	CR18/2	CJ89
	CR18/2	CR18/1

with fiber optic light source. The small-sized, shrivelled and unfilled seeds were recorded as aborted seeds. Seed germination studies were carried out under laboratory conditions at 25 ± 3 °C using 4 replications of 25 seeds from each accession. The seeds were germinated in glass Petri dishes with two layers of filter paper moistened with distilled water twice a day. A seed was considered to have germinated when the radical had emerged about 1 cm long. Germination was recorded from the fourth day and final count was made after 20 days. Data of control pollination success rate, fruit (cone) set and seed set, normal and aborted seed, seed germination and number of pollen tubes in the stigma region were subjected to analysis of variance (ANOVA) using the software GenStat (15th Edition) and following the methods of Williams and Matheson (1994).

3 Results

3.1 Controlled pollination success

The extent of fruit set showed highly significant differences ($P<0.0001$) among various cross combinations involving three *Casuarina* species viz. *C. equisetifolia, C. junghuhniana* and *C. cristata*. It ranged from 14.4% to 76.6% at 45 days after pollination but reduced to 2 to 57% five months after pollination (Table 3). Among the 17 cross combinations performed, three crosses involving *C. cristata* resulted in no fruit set. There was a reduction in the overall initial fruit set (31.3%) as they matured five months after pollination (19.7%). The highest final fruit set of 56.9% was obtained from the cross CJ23 x CJ89. In general, *C. equisetifolia* as a female parent recorded high percentage of fruit

set ranging from 12.2% to 38.3% five months after pollination, followed by *C. junghuhniana* (2 to 56.9%) and *C. cristata* (2.8%). Fruit set was higher in open pollinated inflorescences than controlled pollinated in *C. junghuhniana* (46.4%) and *C. cristata* (12.56%), but *C. equisetifolia* recorded lower fruit set (20.8%) under open-pollination than most of the controlled crosses after five months. Among the interspecific crosses, CE166 crossed with *C. cristata* yielded the highest fruit set (38.3%), followed by CE166 x CJ38 (35.9%), CE166 x CJ89 (34.2%), CE18 x CJ38 (23.1%) and CE18 x CJ89 (21.2%). Another accession of *C. equisetifolia* (CE164) crossed with *C. cristata* (CR18) recorded the lowest percentage of fruit set (12.2%). *C. junghuhniana* as a female parent recorded average fruit set for interspecific combinations of *C. junghuhniana* x *C. cristata* (15.9%) and *C. junghuhniana* x *C. equisetifolia* (11.2%). Inter-specific cross combination of *C. cristata* as female parent with *C. equisetifolia* and *C. junghuhniana* did not result in fruit set.

Table 3. Fruit set in cross and open-pollinated flowers of *Casuarina* species.

Parents		Fruit set (%)	
Female	**Male**	**45 days**	**5 months**
CE166	CJ38	63.40^{b}	35.96^{d}
CE166	CJ89	39.53^{d}	34.26^{d}
CE166	CR18/1	54.40^{c}	38.33^{c}
CE18	CJ38	29.93^{ef}	23.16^{ef}
CE18	CJ89	76.66^{a}	21.20^{fg}
CE164	CR18/1	34.50^{e}	12.20^{i}
CE166	OP	22.13^{h}	20.8^{g}
CJ85	CE183	14.43^{i}	11.23^{i}
CJ85	CJ38	23.63^{gh}	11.06^{i}
CJ85	CR18/1	23.06^{gh}	15.96^{h}
CJ55	CJ38	28.03^{gf}	25.36^{e}
CJ55	CJ89	34.70^{de}	17.36^{h}
CJ55	OP	52.03^{c}	46.40^{b}
CJ23	CR18/1	0.00^{j}	0.00^{k}
CJ23	CJ38	33.20^{e}	2.00^{j}
CJ23	CJ89	59.86^{b}	56.96^{a}
CR18/2	CJ38	0.00^{j}	0.00^{k}
CR18/2	CJ89	0.00^{j}	0.00^{k}
CR18/2	CR18/1	23.20^{gh}	2.80^{j}
CR18	OP	14.43^{i}	12.56^{i}
	Mean	31.36	19.77
	P	<0.0001	<0.0001
	LSD	4.9674	2.2085

Note: CE- *C. equisetifolia*; CJ- *C. junghuhniana*; CR- *C. cristata* and OP- Open pollinated

Mean data with same superscript letter were not significantly different by Duncan's multiple range test (P<0.05).

3.2 Pollen tube growth in pistil

All the species of *Casuarina* examined showed similar pattern of pollen germination and pollen tube penetration in the pistil (Appendix 1). The pollen grains started germinating on the stigma one or two days after anthesis. The mean number of pollen tubes germinated on the stigma eight days after pollination is presented in Table 4. The analysis of variance did not show any significant difference in the number of pollen tubes between the open pollinated and cross pollinated flowers. The number of pollen tubes found in a flower ranged from 3 to 5 among the accessions. On the third day, the pollen tubes passed through the transversely arranged stigma cells. Pollen tube branching was seen in *C. equisetifolia* and *C. cristata* on the fifth day. One branch of the pollen tube grew towards the stigma (Appendix 2a) and its growth was arrested on the seventh day when it reached the top of the stigma. The other branch of the pollen tube showed vigorous growth (Appendix 2b). At this stage curling of stigma and change in its colour from pink to dark brown happened, pollen tube branching was commonly observed in the stigma region. On the eighth day, mostly one or two pollen tubes reach the lower region of the stigma (Appendix 2c). At this stage, the style starts developing into the seed wing. On the 10th day the pollen tube reaches the stylar region. By this time the stigma was completely dried up and the inflorescenc appeared as a young cone. The pollen tube remained like spiral line in the style region in *C. junghuhniana* (Appendix 2d) and zigzag lines in *C. cristata* and *C. equisetifolia* (Appendix 2e). When the pollen tube reached the stigma region its growth was arrested till the ovules mature approximately 10 days later. On the 20th day pollen tube growth was resumed and entered the mid-stylar region on the 25th day. The pollen tubes were found to grow straight towards the ovary and reached the upper surface of the ovary on the 35th day in *C. equisetifolia* and *C. junghuhniana* and 40th day in *C. cristata* (Appendix 2f). In all the pistils examined, only one tube reached the ovary. By this time the fruit was enclosed with bract and bractoles.After the pollen reached the ovary the pollen tube branching was observed on the 40th day in *C. equisetifolia* and *C. junghuhniana* and 45th day in *C. cristata* (Appendix 2g). Pollen tubes remained in the ovary till the ovules developed. The pollen tubes reached the ovule and fertilized the egg cell on the 45th day in *C. equisetifolia* and *C. junghuhniana* and 50th day in *C. Cristata.*

The cross between *C. cristata* and *C. junghuhniana* showed incompatibility through barriers like lack of pollen germination on the stigma. Sometime late germination of pollen grains on the stigmatic surface occured 3 days after hand pollination. In a few crosses between *C. equsetifolia* and *C. junghuhniana,* pollen tubes passed through the stigma region 15 days after pollination and the pollen tubes got arrested in the mid stylar region and showed crooked tubes 35 days after pollination. In the intra-specific crosses of *C. cristata,* pollen tubes passed through the stigma and stylar regions and finally reached the ovary after the 45th day but resulted in low fruit set (Table 4).

3.3 Hybrid seed set and germination

The analysis of variance showed highly significant variation (<0.0001) in seed set and germination potential of different crosses (Table 5). Seed set was generally high (>75%) for all crosses. Among the seven inter-specific cross combinations, *C. equisetifolia* as maternal parent crossed with *C. junghuhniana* as male parent showed the highest seed set (93.8%) and C. cristata as a male parent had the lowest seed set (75.1%) and also had the highest rate of aborted seeds. However another interspecific cross, CJ85 x CR18 recorded high seed set (84.2%). Aborted seeds either failed to germinate or died within 3 days of germination.

Seeds of different crosses showed high level of variation in germination ranging from 3.6 to 88.7% (mean 45.2%, Table 5). Among the various combinations, *C. junghuhniana* (CJ85) crossed with *C. cristata* (CR18) had the highest germination (88.7%). Even though the crosses between *C. equisetifolia* and *C. cristata* had the high seed set, the germination was low (22.8 and 23.5%). Germination was also poor (3.6%) for the cross between *C. equisetifolia* (166) and *C. junghuhniana* (38) among the different cross combinations.

4 Discussion

4.1 Control pollination success

The extent of fruit set, seed production and germination obtained with different interspecfic cross combinations confirms the possibility of developing hybrids with

Table 4. Mean number of pollen tube in different cross combinations of *Casuarina* species.

Parent		Mean number of pollen tubes in stigma	SD
Female	Male		
CJ85	CR18/1	5.7	2.1
CJ85	CJ38	4.0	3.6
CJ85	CE183	5.7	1.2
CE166	CJ38	4.3	0.6
CE166	CJ38	5.6	1.5
CE18	CJ38	4.0	1.7
CE166 OP		3.0	0.6
CJ OP		5.6	2.5
CR18 OP		3.3	0.6
	Mean	4.6	1.6

Table 5. Seed set and seed germination of interspecific crosses of *Casuarina* species.

Cross combination		Seed Set (%)		Germination (%)
Female	Male	Normal	Aborted	
CE166	CJ38	93.81^{a}	3.32^{f}	23.08^{d}
CE166	CJ89	88.58^{c}	23.1^{b}	19.16^{g}
CE166	CR18/1	75.086^{e}	25.26^{a}	23.5^{d}
CE18	CJ38	91.56^{b}	8.15^{e}	3.67^{e}
CE18	CJ89	90.11^{c}	8.98^{e}	10.69^{f}
CE164	CR18	76.71^{e}	22.22^{b}	22.87^{d}
CJ85	CR183	88.58^{c}	11.12^{d}	91.32^{a}
CJ85	CJ38	84.68^{d}	14.06^{c}	81.19^{c}
CJ85	CR18/1	84.24^{d}	14.14^{c}	88.73^{b}
CJ55	CJ38	84.54^{d}	14.25^{c}	88.03^{b}
	Mean	84.48	14.76	45.22
	P	<0.0001	<0.0001	<0.0001
	LSD	1.668	0.9553	1.2227

Note: Mean data with same superscript letter were not significantly different by Duncan's multiple range test (P<0.05).

desirable traits by involving *C. equisetifolia, C. junghuhniana* and *C. cristata.* Each of these species has unique adaptability and end use characters and hybridization through control pollination is the best way to make different combinations of these characters to suit the planting environment and end use requirements. The availability of genetic resources across species is also an insurance to face challenges

in the future triggered by events like climate change and outbreaks of pest attack. The difference in fruit set involving different species and clones may be influenced by efficiency of the hand pollination techniques adopted and the choice of genotypes involved as female and male parents. In the previous studies involving *C. equisetifolia* and *C. junghuhniana,* fruit set obtained through control pollination was lower than open pollination (Nicodemus et al., 2011a). It was observed that the pollination success was affected by hand pollination, which caused damage to the flowers while applying pollen on the stigma, and by high temperature and relative humidity inside the pollination bags. In the present study, cloth bags were used, instead of paper bags, to facilitate aeration inside the bags and placing of a wire coil inside the pollination bag prevented physical damage to the inflorescences. As a result the fruit set was higher than what was reported for *C. equisetifolia* and *C. junghuhniana* crosses (less then 1%) (Nicodemus et al., 2011a). Zhang et al., (2014) reported a novel approach of using pollination chambers to increase fruit set in control pollination programmes. They adopted a factorial mating design inside closed chambers which functioned like pollination bags and increased fruit set and seed germination from 7% to 89.9% and 8.15 to 51.8% respectively compared to conventional controlled pollination method involving bags.

Selection of suitable species for deploying as maternal and paternal parents is essential to overcome the reproductive barriers that may prohibit creating desirable cross combinations. Using *C. cristata* as male parent for crossing with *C. equisetifolia* and *C. junghuhniana* resulted in high percentage of fruit set and seed germination. But when *C. cristata* was used as female parent with other species as paternal parents, no fruit set was obtained. The structural differences in the female flowers among different species could be a reason for failure in fertilization and fruit set. The size of the *C. cristata* flower was the largest among the four species studied. The structural barrier is reported to be unilateral and is due to the pollen tubes of small-flowered species being unable to grow the full length of the style of large-flowered species (Potts, 1988; Gore et al., 1990). It appesrs that such structural barrier prevented fertilization in crosses between *C. equisetifolia* with large-sized flower as the female parent and *C. junghuhniana* with small-sized flower as male parent and the absence of this barrier resulted in fruit and seed set for the reciprocal crosses. A similar pattern of crossability barriers was found in *Eucalyptus* species. Gore et al. (1990) found that *Eucalyptus globulus* markedly differed from *E. nitens* in flower size and the F1 hybrid can only be produced using the smaller flowered *E. nitens* as the maternal parent since the style of *E. globulus* being too long for the short pollen tube of *E. nitens* to reach the ovary. In the present study, production of hybrid progeny involving *C. cristata* can be achieved only by using it as the male parent whereas *C. equisetifolia* and *C. junghuhniana* can be used as both female and male parents.

The fruit maturation percentage and seed germination were low in both control and open pollination five months after of pollination. This reduction shows that different species of *Casuarina* might have various post-fertilization barriers occurring during the fruit maturity period as reported for different *Eucalyptus* species (Ellis and Sedgley, 1992).

4.2 Pollen-pistil interactions

The pollen tube germination and fertilization pattern in different cross combinations of *Casuarina* species showed similar growth pattern. Many pollen grains germinated in the stigmatic surface and grew downwards to reach the upper region of the style. Pollen tube branching was observed in *C. equisetifolia* and *C. cristata*. The cross combination with *C. junghuhniana* did not show pollen tube branching in the stigma region. Pollen tubes lie in a zigzag line in *C. equisetifolia* and spiral pollen tubes in *C. junghuhniana* in the upper region of the style. This indicated that the pollen tubes were arrested in the stylar region till the ovules developed as described by Swamy (1948), Barlow (1958) and Sogo et al. (2004). In all three *Casuarina* species in this study, the duration between pollination and fertilization is about 45-50 days and that pollen tube growth continues without any break for the entire period.

The reduction in the number of pollen tubes from the upper region to the lower region of the style suggests that selection between pollen tubes is occurring in the stylar region. This may be resulted from competition for space to penetrate the stigma and enter the style, or competition between the fastest growing tubes to reach the locules and the ovaries (Ellis and Sedgley, 1992; O' Brien, 1996). Our results obtained with the

three different species and cross combinations showed that number of pollen tubes and rejections of pollen tubes were similar in controlled and open-pollinated flowers. This indicates that rejection of pollen tube in the stigma and style by self-incompatibility.

5 Conclusion

Hybridization among three species of *Casuarina* viz. *C. equisetifolia, C. junghuhniana* and *C. cristata* is feasible thorough control pollination. Species that have large-sized flowers like *C. cristata* better function as male parents than as maternal parents. Studies on induction of flowering in shy-flowering species like *C. cunninghamiana* is necessary to facilitate control pollination involving such species. The cross compatibility among different species of *Casuarina* provides scope to produce site- and end-use specific hybrids to meet the commercial and livelihood needs.

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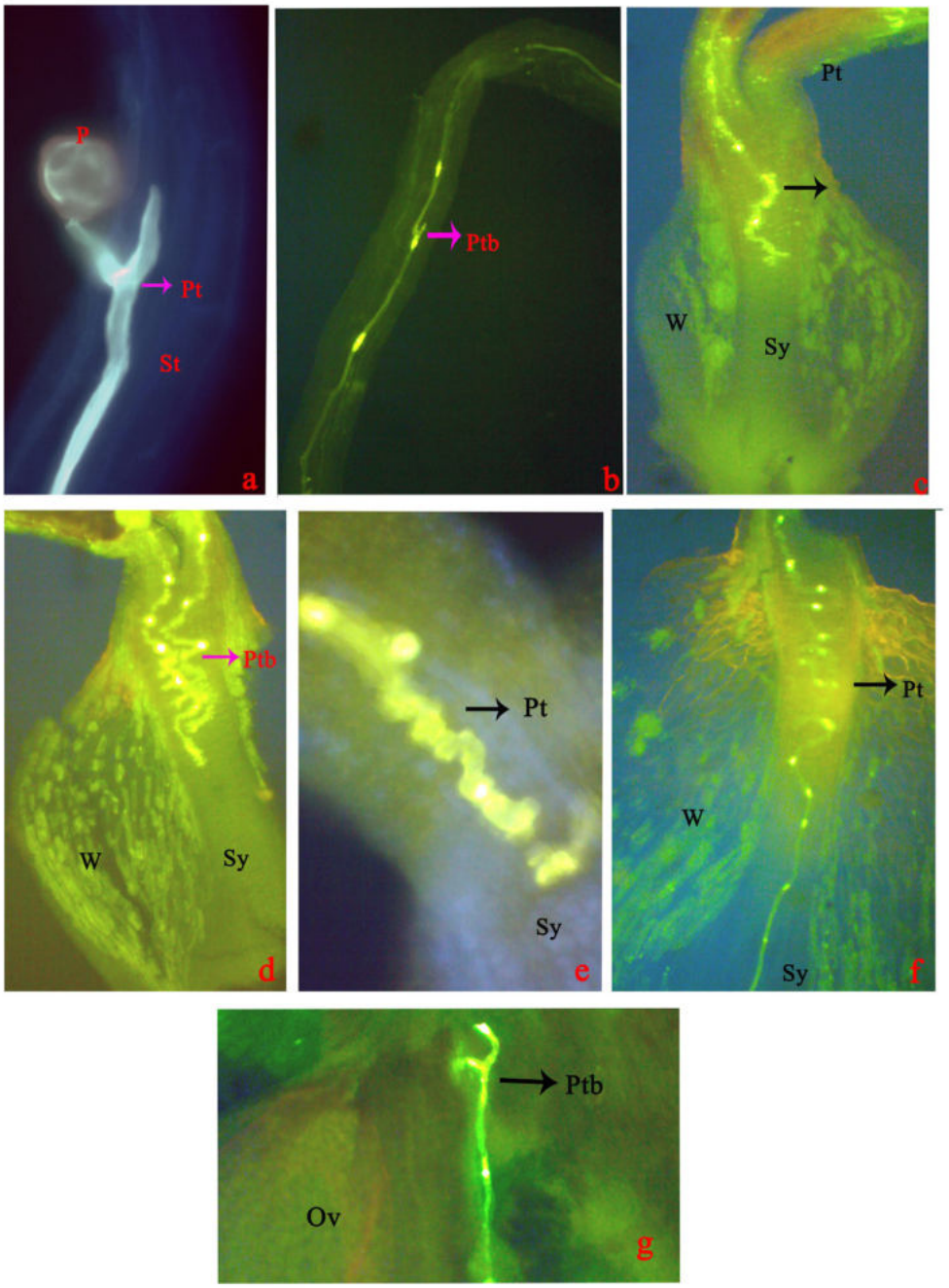
Appendix 1. Pollen tube growth and fertilization in *Casuarina* species at different time intervals pollination.

Cross combination	3rd day	5th day	10th day	20th day	35th day	40th day	45th day
CE x CJ CE x CE	Pollen germination	Short pollen tube branch towards tip of stigma	Pollen tube reach the stigma retained in a zigzag line	Pollen tube growng straight to lower style	Pollen tbe entering the ovary	Pollen tube branches and llies in the ovule	Fertilization
CE x CR	Pollen germination	No pollen tube branch	Pollen tube reach the stigma retained in a zigzag line	Pollen tube growing straight to lower style	Pollen tbe entering the ovary	Pollen tube branches and llies in the ovule	Fertilization
CJ x CE	Pollen germination	No pollen tube branch	Pollen tube reach the stigma retained in a spiral line	Pollen tube growing straight to lower style	Pollen tube entering the ovary	Pollen tube branch and lying on the ovule	Fertilization
CJ x CR CJ x CJ	Pollen germination	No pollen tube branch	Pollen tube reach the stigma retained in a spiral line	Pollen tube growing straight to lower style	Pollen tube entering the ovary	Pollen tube branch and lying on the ovule	Fertilization
CR x CE	Pollen germination	Pollen tube coiled ad growth was arrested	--	--	--	--	--
CR x CR	Pollen germination	Pollen greminated above the stigma	No further pollen tube growth in the stigma	--	--	--	--

Note: CE - *C. equisetifolia,* CJ - *C. junghuhniana,* CR - *C. cristata*

Appendix 2. Flourescent microscope images of pollen germination, pollen tube growth in stigma and fertilization in *Casuarina* species at different time intervals after controlled pollination.



(a) Pollen tube branching in stigma of CE x CCR after 5th day, (b) Pollen tube branching in stigma of CE x CCR after 8th day, (c) Pollen tube in upper style region in CJ x CCR after 8th day, (d) Coiled pollen tube in upper style region in CJ x CCR after 10th day, (e) Zig-zag pollen tube in upper style region in CCR x CE, (f) Pollen tube growing towards ovary in CE x CCR after 35th day and (g) Pollen tube branching on the ovary in CE x CJ after 40th day (Pt - pollen tube, Ptb- pollen tube branch, St- stigma, Sy- style, W- wing and Ov- ovary).

Population structure and diversity of *Casuarina equisetifolia* – results from 25 years of research

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Abstract

Casuarina equisetifolia is a highly cosmopolitan species with a natural range throughout Australasia, the Pacific and southeast Asia. It has been successfully introduced into parts of Asia beyond its natural range, including to India and China, where it is a major plantation species. Significant introductions have also been made in Africa. A series of international provenance trials comprising range-wide provenance seedlots was established and evaluated in the 1990s. These trials strongly suggest significant subpopulation differentiation in the species. More recently, morphological and microsatellite-based nuclear DNA studies have further elucidated population structure and provided evidence for the origins of land races.

Provenance trial data showed a performance differential between southeast Asian provenances (and southeast Asian land races), which were generally more vigorous than those from Australia, the Pacific and the African land races.

Morphological markers, including both growth and physiological traits such as cladode characters, also indicated two main clusters of subpopulations that coincide with those suggested by the field trials. The first cluster comprises provenances from Australia, Pacific Island nations including Papua New Guinea, Solomon Islands, Fiji and Tonga as well as the Philippines. The second cluster includes southeast Asian provenances from Vietnam, Thailand and Malaysia. Asian land races appear to have affinity with the southeast Asian cluster while African land races appear likely to be derived from the Australia-Pacific region.

Molecular maker-based evidence gathered from putatively selectively-neutral markers also suggests two main clusters of subpopulations. However, the composition of these clusters varies somewhat from those suggested by the morphological marker study. While Asian and Pacific clusters are still evident, different Australian seed sources are aligned to different clusters. We postulate that differences between morphological and molecular marker-based clusters may be attributed to the fact that morphological markers are likely to be under selection, whereas the SSR markers are presumed to be selectively neutral.

We discuss the implications of population structure on domestication and breeding of *C. equisetifolia* in the context of breeding population management, germplasm exchange among programmes and infusion of new selections.

Keywords: molecular markers; population differentiation; land race origins

Introduction

Casuarina equisetifolia is a cosmopolitan species with a very wide global distribution throughout the Asia-Pacific region (Fig. 1). The species comprises two subspecies, *C. equisetifolia* ssp. *equisetifolia* and *C. equisetifolia* ssp. *incana*. The latter species is shorter in stature and is not as widely planted as *C. equisetifolia* ssp. *equisetifolia* (references to *C. equisetifolia* hereafter refer to *C. equisetifolia* ssp. *equisetifolia*). There is evidence that the species has been cultivated for over a thousand years (Haberle, 2007; Fall, 2010). Within the last fifty years, it has been extensively planted for industrial purposes and coastal protection and is the subject of tree breeding and domestication programs in India (Rawat et al. 2011; Kumar et al. 2016) and China (Zhong et al., 2019).

International provenance trials

A milestone in the domestication of a wild species is gaining an understanding of the genetic variation at the subpopulation or provenance level. As *C. equisetifolia* is widely distributed, it would be expected that this might be significant. A major landmark was the establishment of an international series of provenance trials by the Australian Tree Seed Centre and international collaborators between 1992 and 1994. The ATSC has collected widely throughout the Asia-Pacific region and procured land race seedlots from this region as well as Africa, where the species is well established, and even Cuba (Table 1). This trial series tested 67 provenance and land race seedlots from 22 countries across 40 trial sites in 20 countries. The results of this study (Pinyopusarerk and Williams, 2000; Pinyopusarek et

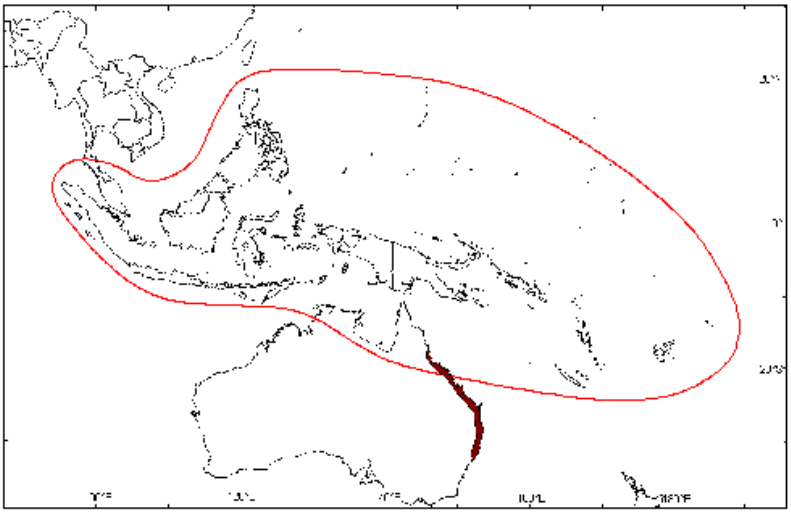

Fig. 1. Distribution of *Casuarina equisetifolia* is within the marked line. Closely related species, *C. equisetifolia* ssp. *incana*, is distributed on the Australian east coast at lower latitudes than *C. equisetifolia* (shaded area) as well as in Vanuatu and New Caledonia.

Table 1. Summary of ATSC historic holdings of *C. equisetifolia*.

Wild	No. seedlots	Planted	No. seedlots
Australia	27	**Africa**	21
NSW*	5	Benin	1
NT	8	Egypt	5
QLD	14	Kenya	10
		Mauritius	4
Asia	30	Seychelles	1
Indonesia	3		
Malaysia	12	**America**	3
Philippines	5	Cuba	2
Thailand	10	Puerto Rico	1
Pacific	28	**Asia**	22
Fiji	3	China	7
Guam	11	India	6
New Caledonia	1	Sri Lanka	4
PNG	7	Vietnam	5
Samoa	2		
Solomon Is	2		
Tonga	1		
Vanuatu	1		
Total wild	85	Total planted	46

Note: * *C. equisetifolia* ssp. *incana*

al., 2004) confirmed that there is indeed significant provenance variation in terms of growth and other traits, with provenances from southeast Asia and land races from Asia generally being the best performers across a range of target planting sites. Provenances from Australia and the Pacific generally performed less well, though some families were outstanding: these should not be ignored as a potential source of germplasm for future breeding.

Morphological markers

Provenance-level differences in tree morphology were observed in the early stages of domestication of *C. equisetifolia*. A seedling based study was established by the Research Institute for Tropical Forestry across two nursery sites in southern China to quantify the differences and provide further evidence for subpopulation differentiation in the species (Hu et al., 2016). This study measured 10 traits, including cladode traits, namely cladode node count, colour, length, number of teeth and branch density, as well as growth traits such as collar diameter and height. In total, 28 wild and land race seedlots were included. The study showed significant variation among seedlots. Some traits, including teeth, node count density and length were stable across the two sites (i.e. the provenance rankings did not change), indicating an absence of genotype-by-environment interaction (GxE). Conversely, provenance ranks for cladode colour,

thickness and plant height and diameter changed across the sites, indicating significant GxE. Despite this, the early growth performance of seedlings at the provenance level was moderately-well correlated with the results of the earlier field trials: Asian wild and land race seed sources tended to be more vigorous than seed sources from Australia, the Pacific and the African land races. A key finding of this study was that, based on principle component analysis of the 10 traits, African land race seed sources had close affinity to seed sources from Australia, Pacific islands (Fiji, Tonga, Vanuatu, Solomon Islands) and the Philippines which together formed a major cluster of similar provenances (Fig. 2). Land races from China, India, Sri Lanka and Vietnam clustered together with southeast Asian wild provenances including Malaysia and Thailand, forming a second group. These two groups of wild and land race provenances make sense in the context of the information from the earlier provenance trials and provide solid evidence for the origins of both Asian and African land races.

Enzyme markers

No studies on *C. equisetifolia* population structure using isoenzyme markers have been published. However, in 2000, CSIRO (Khongsak Pinyopusarerk, Charlie Bell and Gavin Moran) carried out a study using 23 loci on 33 provenances and land races of *C. equisetifolia* and *C.* ssp. *incana,* most of which had been tested in the international provenance trials. We retrieved the raw data from the ATSC data repository to estimate genetic distances among provenances, perform analysis of molecular variance (AMOVA) and undertake a principle coordinates analysis (PCOA) using GenAlex (Peakall and Smouse, 2006). The results are shown in Fig. 3.

The study indicated major differentiation among provenances with an estimated F_{ST}=65%. The PCOA diagram shows clustering of *C.* ssp. *incana* with Queensland, Australia provenances of *C. equisetifolia* in the upper right quadrant. Northern Territory and Prince of Wales Island (which is in the Torres Strait

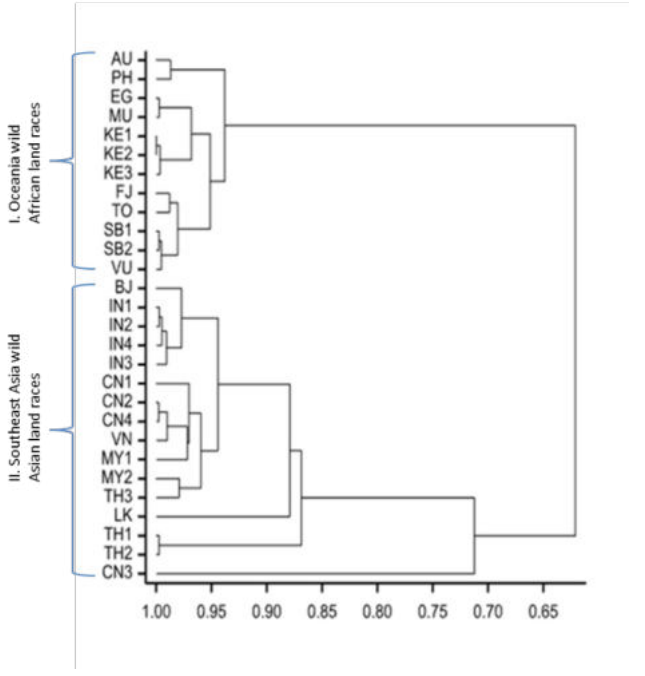

Fig. 2. Dendrogram based on stable traits from a morphological study by Hu et al (2016). Two clusters are evident: (I) wild Oceania and the wild Philippines provenances plus most African introduced material, and (II) all other wild Asian material, all introduced Asian material and the introduced provenance from Benin. Wild provenances include sources from *AU* Australia, *FJ* Fiji, *PH* Philippines, *SB* Solomon Islands, *MY* Malaysia, *TO* Tonga, *TH* Thailand, *VU* Vanuatu. Land race seedlots included *BJ* Benin, *CN* China, *EG* Egypt, *IN* India, *KE* Kenya, *LK* Sri Lanka, *MU* Mauritius, *VN* Vietnam.

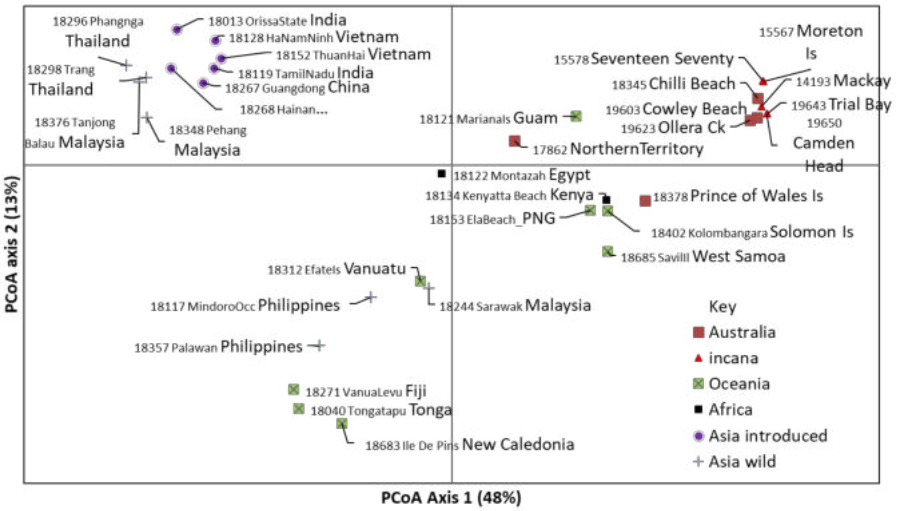

Fig. 3. Principle coordinates analysis based on genetic distances estimated with 21 isozyme loci among wild and land race seedlots of *C. equisetifolia* throughout the Asia-Pacific region and Africa. Provenances are prefixed with their ATSC seedlot number which can be cross-referenced to seedlots used in the international provenance trials reported by Pinyopusarerk et al. (2004).

between Australia and PNG) provenances cluster with Guam, PNG and West Samoan natural provenances and land races from Egypt and Kenya. The lower left quadrant contains Vanuatu, Philippines and one Malaysian provenance plus Fiji, Tonga and New Caledonia. Another cluster (upper left quadrant) comprises wild provenances from Thailand and Malaysia and land race populations from China, India and Vietnam. This clustering is broadly in accord with the associations suggested by the provenance trials and the morphological study.

DNA marker studies

The first molecular DNA studies of *C. equisetifolia* used dominant markers including RAPD (Ho et al., 2002a; Ho et al., 2002b). The first of these studies was based on the 12 wild provenances of *C. equisetifolia* established in Taiwan as part of the international trial series in 1992. The study indicated significant population differentiation (F_{ST} = 39%). The study population split into two main clusters, the first comprising only the Cairns provenance, which the authors contended might be *C. equisetifolia* ssp. *incana,* and a second cluster that further bifurcated into two more groups (Fig. 4). This classification differs significantly from the previously discussed ones in that Asian wild provenances, such as Thailand, are clustered with some from Oceania (Darwin, Australia; Viti Levu and Vanua Levu, Fiji). Among other differences, Philippines provenances are present in both clusters.

The RAPD study was followed by another dominant marker study using AFLP (Huang et al., 2009). This study was based on 7 wild and 11 cultivated seedlots established as part of the international trial series in 1992. Again, considerable subpopulation differentiation was indicated (F_{ST} = 47%). However, like the RAPD study, clustering of wild provenances did not follow the general pattern indicated by the morphological and isozyme studies (Fig. 5). Though this study did estimate genetic parameters for the land race seedlots, it unfortunately did not attempt to cluster these with the wild accessions.

Two studies, published within the last twelve months, have used codominant microsatellite (SSR) markers to further elucidate population differentiation in *C. equisetifolia*. The first study (Yu et al., 2019) compared samples from the very extensive southern Chinese clonal plantation population with 13 wild accessions from Asia and Oceania, with an objective of determining the origins of the clones. The plantation population was definitively shown to have a very narrow genetic base, though its ancestral origins were less clear: the authors concluded a southeast Asian

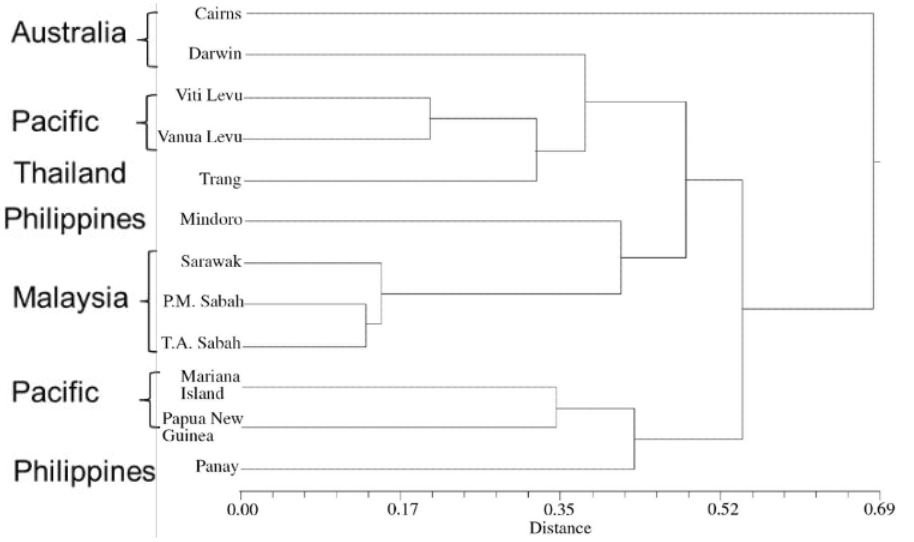

Fig. 4. Phenogram based on RAPD markers after Ho et al. (2002a).

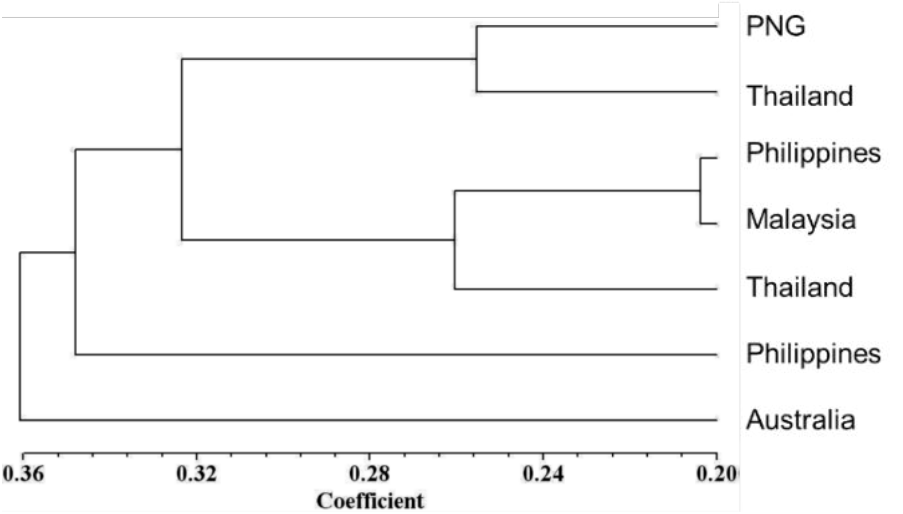

Fig. 5. Phenogram based on AFLP molecular markers (after Huang et al., 2009).

origin is most likely, but no strong evidence for a more-precise origin was found.

The most recent study, again using SSR markers (Zhang et al., 2020) examined population differentiation among 14 wild provenances and 13 land races of the species. Estimated population differentiation was high (F_{ST}=30%) and two main clusters of subpopulations were suggested. Like the AFLP- and RAPD-based studies, Australian, Oceanian, African and Asian seed sources were split between the two clusters (Fig. 6). This is at odds with earlier indications from morphological and isozyme studies that show that Australian and Oceania provenances have a close affinity and that African land races are likely derived from these, while Asian plantations in China, India and elsewhere are derived from Asian wild sources in Thailand and Malaysia.

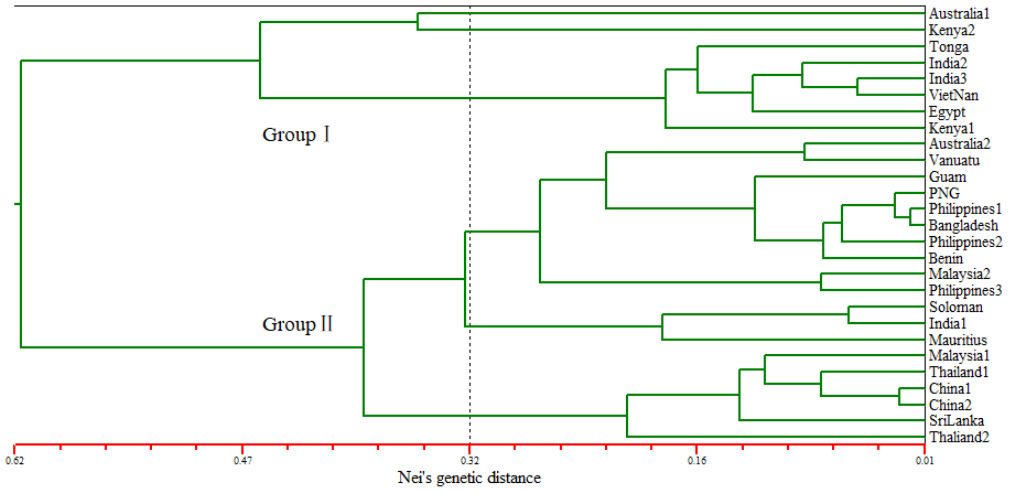

Fig. 6. Clustering of 27 natural and introduced subpopulations of *C. equisetifolia* using 13 SSR loci (after Zhang et al., 2020).

What does it all mean?

Taken together, the results of studies discussed in the preceding sections do not present a clear picture of population differentiation in *C. equisetifolia*. The provenance trials, morphological markers and isozymes give indications that are broadly in accord. These studies indicate a separate "Oceania cluster" of Australia and Pacific material (which generally, but not always, performs relatively poorly in plantations) which appear likely to be the source of planted stands in Africa. The wild Philippines accessions appear to have some affinity to the Oceania cluster. The second cluster comprises Asian wild provenances from Malaysia and Thailand which appears to be the origin of planted stands in India, China and other Asian locations. This "Asian cluster" was the source of some of the best-performing material in the international provenance trials and was generally more vigorous in the early growth assessments of the morphological marker study. The suggested origins of the planted populations are highly plausible given the likely human-mediated flow of germplasm throughout Asia and into Africa.

The DNA marker studies (RAPD, AFLP and SSR markers) present results that do not accord with the picture built up from the previous three sources. These studies show a pattern of inter-relatedness that is less easy to interpret in terms of geographic distribution of wild populations. Though some clustering (for example the branch containing Malaysia, Thailand, China and Sri Lankan populations - Group II, Fig. 6) does accord with the indications of the non-molecular studies, the cluster containing Tonga, India and Vietnam sources is puzzling.

There are various possible explanations for the discord between the two groups of studies (i.e. "non-DNA" and DNA marker studies). One is that the non-DNA studies are examining traits that are likely to be controlled by genomic regions that are under selection, while the DNA markers used are putatively neutral (Zhang et al., 2020). This seems the most plausible explanation, though isozymes are also often regarded as being neutral, or at least that a high proportion of loci could be expected to be neutral. Another contributing factor that should also be considered is weak confidence in dendrograms caused by small sample sizes in some studies (the RAPD and AFLP studies sampled 12 and 13 plants per seed source, respectively, while the isozyme and SSR studies examined 40 and 30 plants respectively). Though the studies did not all include the same genetic materials, they all had some overlap of common ATSC seed sources which aids direct comparison among them.

One common finding clearly evident among all

studies was the large amount of population differentiation in this species. This is to be expected in a species with such a wide geographic distribution, especially from those studies that included accessions from the *C. equisetifolia* ssp. *incana* intergrade zone in northern Queensland.

Concluding remarks

Casuarina equisetifolia has emerged as one of the world's key plantation species, with extensive plantings in China and India. It has a very wide distribution with substantial subpopulation differentiation. Though consistent growth performance and morphological patterns are evident from outgrown material, examination of DNA-based variation is less-readily interpretable in terms of population structure. In the future, it is possible that next-generation genomics including SNP marker studies and genome assembly may help to answer some of the remaining questions about the population genetics of this species.

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Cultivar identification, genetic diversity and relatedness analyses of *Casuarina equisetifolia* clones in South China based on EST-SSR markers

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Abstract

Casuarina equisetifolia is planted extensively in clonally-established coastal shelterbelts in South China. Analyses of samples from 109 separate plantations in three littoral provinces of South China, namely Hainan, Guangdong and Fujian, using microsatellite molecular markers, revealed that 22 separate clones are being used in coastal shelterbelts of *C. equisetifolia*, and most of them presented close genetic distances and low genetic diversity. Mutual introduction and repeatedly naming and mislabeling of clones in different provinces or regions were found. We recommend developing more disease-resistant planting material and planting a more-diverse mix of unrelated clones at local and regional scales for coastal shelterbelts of South China.

Keywords: *Casuarina equisetifolia,* Microsatellite, Clone identification, Genetic diversity and relatedness

1 Introduction

Casuarina which belongs to Casuarinaceae, occurring naturally from Australia to the Pacific islands and in Southeast Asia (Turnbull, 1990), is a nitrogen-fixing tree genus of considerable importance in many tropical and subtropical regions of the world. It has been estimated that over 2 million hectares of *Casuarina* plantation, predominantly *Casuarina equisetifolia* subsp. *equisetifolia* (hereafter referred to as *C. equisetifolia*), has been planted for wood production, coastal shelterbelts, land rehabilitation and ornamental purposes around the world (Zhong et al., 2011). Large-scale clonal plantations of *C. equisetifolia* in coastal areas of South China have been established since the 1990s (Pinyopusarerk et al., 2004). In the past three decades, some superior clones of *C. equisetifolia* have been developed for coastal shelterbelts in three main coastal provinces of South China: Fujian, Guangdong and Hainan, but their diversity is unclear. It is usual to introduce new clones for plantation establishment from other provinces or regions, but we strongly suspect that some introduced clones, though bearing different names, may be genetically similar or identical to those already found in southern China because of their morphological similarities. It is therefore necessary to find out which and how many clones have been renamed in introduced in South China. This is very important for appropriate use of clones in coastal shelterbelts, including monitoring of genetic diversity, and intellectual property protection of breeders.

Visual identification of *C. equisetifolia* clones using morphological characteristics is likely to be inaccurate due to the strong influence of environment and genotype-by-environment interaction on clonal phenotypes. The development of simple sequence repeat (SSR) markers in *C. equisetifolia* will provide a more-reliable alternative for clone identification ("fingerprinting") of *C. equisetifolia*. A reliable SSR marker panel has been developed and applied for parentage analysis (Zhang et al., 2016), genetic diversity assessment (Xu et al., 2018) and population structure analysis (Kullan et al., 2016) of *C. equisetifolia*, but clone identification using SSR markers is still limited.

The objectives of this study were: 1) to identify and find out how many clones of *C. equisetifolia* are being used in coastal shelterbelts of South China, and whether different names have been applied to commercial clones in different regions, and 2) to analyze and assess the genetic diversity and relationship among these clones.

2 Materials and methods

2.1 Clonal samples collection

Clonal samples were collected from coastal shelterbelts of *C. equisetifolia* in three provinces (Fujian, Guangdong and Hainan) of South China. Two or three large-area clonal plantations were selected for sample collection in every littoral county of the three provinces. In total, 109 samples were collected from 42 counties within 3 provinces (Fig. 1). The number of samples collected from Fujian, Guangdong and Hainan were 31 (sampling No. 1-31), 50 (sampling No. 32-81) and 28 (sampling No. 82-109), respectively. Young branchlets of clonal individuals of each plantation mentioned above were collected and dried in a plastic preservation box filled with silica gel, then were stored in ultra-low temperature freezer for further DNA extraction. During sampling, we asked local forestry authorities for the clone names of samples if available. At the same time, we collected

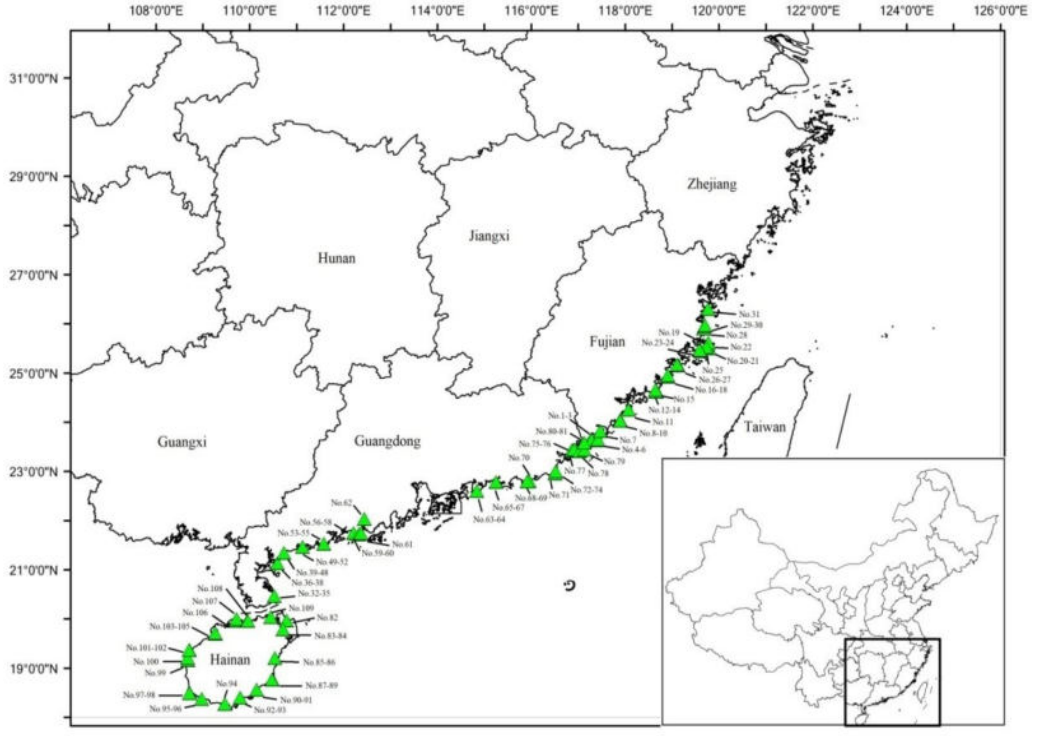

Fig. 1. Collection location of 109 clonal samples in Fujian, Guangdong and Hainan provinces. Numbers in boxed indicated the sampling numbers, sample No. 1-31, 32-81 and 82-109 were collected in Fujian, Guangdong and Hainan provinces respectively.

all the local known clones which were used for large-scale planting as reference clones for clone identification. Finally 9 well-known reference clones were collected from the three provinces, namely clones "hui-1" , "Pingtan-2", "501" and "601" were collected in Fujian province, clones "A-13" and "A-8" in Guangdong province, and clones "Bao-9", "Dong-2" and "Zen-4" in Hainan province.

2.2 DNA extraction and EST-SSR markers screening

Genomic DNA of the 109 clonal samples and 9 reference clones was extracted from young branchlets using a modified CTAB method. Twelve SSR markers were selected by means of screening and optimization of 58 markers from published papers (Zhang et al., 2016; Kullan et al., 2016). The marker primer sequences and repeat motif of each primer pairs are shown in Table 1.

2.3 PCR amplification and genotyping

Polymerase chain reaction (PCR) system, 10 μL in volume, was composed of 10 ng DNA template, 1.2 × buffer (Mg^{2+}plus), 10 mmol dNTP, 0.5 μM forward primer, 0.5 μM reverse primer, 10 pmol dUTP (Fermentas International Inc.), 1 U *Taq* DNA polymerase (Fermentas International Inc.). A Touchdown PCR programme was employed to thermo cycler (Applied Biosystems, Foster City, CA, USA). The touchdown amplification protocol was as follow: denaturation at 94°C for 5 min; followed

Table 1. Microsatellite loci used for PCR amplification in clonal samples of *C. equisetifolia.*

Primer	Repeat motif	Primer Sequence(5'-3')	Expected product	Annealing Tm (°C)
EST-C13	(CATCTT)3	F: ATGGGACATTTTGGTGAT R: CTTTGCTTTAGGCGTTTT	282	50
EST-M03	(AG)11	F:GATCGTCAGGGGAAGAGTGA R:CATCGGTTTCAGCGAGTACC	290	60
EST-M26	(AGA)15	F:GCCGTCTCTGAGAGTTTTGG R:TAGAGACTGATCCTCGGCGT	263	54
EST-M27	(GAA)13	F:TTTTTAGATGGGTTTCCCCC R:GAAATGAGCACTTCCTTGCC	269	54
EST-M32	(TC)12	F:AGCATGAAAGCAGGACCACT R:AAGCCTGGCTGGCTCTGA	179	60
EST-M46	(TA)9	F:TGTTTCCATTTCTTATTTGCTTCA R:TCAAATCTCCCAAACCCATC	196	60
EST-M36	(GAG)10	F:CGGAGGAGCTGTTGGTACTC R:TACTTGCAGGTCGCTTTCCT	248	60
EST-M37	(GAA)16	F:TGCTTCAGCCTCACTCTTGA R:CACCAACCCAATCAACTCAAC	175	58
EST-M38	(AG)20	F:TAACGGTCGACATGATTGGA R:AAAATCATCGTCCAATCTCAGAA	161	60
EST-M39	(AG)18	F:CGTAAACGTGCGTGTGTTTT R:TTGATTTTCGATTTCTCCGC	160	60
EST-M40	(AT)11	F:CAACATAATTGAATGCCCGA R:GTCTGGCCCTGGATGTAAGA	163	60
EST-M41	(CT)17	F:CCTCCCCCTGTGGTTTTTAT R:GAGATCTGAGGGTGCGAGAG	170	58

with 20 cycles touchdown program of 30 s denaturation at 95°C, annealing from 60 to 50°C for 30 s with a decrease of 0.5°C per cycle, and 30 s extension at 72°C; then 26 cycles of normal PCR with 30 s denaturation at 94°C, 30 s annealing at 60°C, and 30 s extension at 72°C, ended with a final extension at 72°C for 10 min. Capillary electrophoresis detection of amplified fragments of each EST-SSR locus was performed on an ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA) when PCR products were confirmed through agarose gel electrophoresis.

2.4 Data analyses

Genotyping data was collected using software GeneMapper 4.0, and each allele and its fluorescence intensity were verified by visual inspection. Identify Analysis function of software CERVUS 3.0 was used to undertake clone identification of 109 samples based on 9 reference clones. The genetic diversity parameters of the 109 clonal samples based on 12 EST-SSR markers, including number of alleles per locus (N_o), number of effective alleles per locus (N_e), Shannon information index (*I*), observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphism information content (*PIC*), and probability of identity (*PI*) were calculated using GenAlEx 6.5 (Peakall and Smouse, 2006). The genetic relationship among the clones was determined by calculating Nei's genetic distance and dendrogram was constructed with UPGMA using software NTSYS-pc 2.2 (Rohlf, 2009). Genetic distance and relatedness among the identified clones were characterized by principal coordinate analysis (PCoA) based on Nei's genetic distance using GenAlEx 6.5 (Peakall and Smouse, 2006).

3 Results

3.1 Genetic genotyping and clone identification

Based on identifying matching of 12 EST-SSR loci genotypes of 109 clonal samples and 9 reference clones using Identify Analysis function of software CERVUS 3.0, all 109 samples were categorized as one of 22 clones, if they shared exactly same genotypes across all 12 loci. As shown in Table 2, 9 out of the 22 clones were identical in 12-locus genotypes with the 9 reference clones, but the remaining 13 clones were unknown and named as UC-1 to UC-13 (UC means unknown clone). According to genotyping and matching results of 109 clonal samples, there are 11 clones, 19 clones and 12 clones are being used in Fujian, Guangdong and Hainan provinces respectively. The duplicates of clone A-8, Pingtan-2, Bao-9, Dong-2, 601 and UC-1 were sampled in all the 3 provinces, indicated that many clones had been mutually introduced and used in different provinces or regions. Moreover, some clones bearing local names actually are introduced clones from other provinces or regions. For example, 4 clones sampled in Wuchuang city, Guangdong province (local names are No.7, No. 16, No.20 and No.21 respectively) and 1 clone sampled (local name is Zhen-4) in Daodong Forest Farm, Hainan province actually are duplicates of clone 601 because of their entirely identical genotypes across all the 12 EST-SSR loci (data not shown).

3.2 Genetic diversity parameters

Detailed genetic parameters of 109 clonal samples regarding the 12 EST-SSR loci are shown in Table 3. The 12 EST-SSR markers yielded 49 alleles and a range of 3 to 6 alleles (N_o), with an average of 4.10 alleles per locus in 109 clonal samples. Observed heterozygosity (H_o) and expected heterozygosity (H_e) of the 109 samples in each locus ranged from 0.23 – 1.00 and 0.36 – 0.76 respectively; the Shannon index (*I*) of 109 clonal samples ranged from 0.55 to 1.48, with an average of 1.17; the polymorphism information content (*PIC*) of 109 clonal samples varied from 0.35 to 0.72, with a mean of 0.58; Nine of 12 markers (75%) contain high levels of *PIC* in 109 clonal samples according to the suggested criterion of high (*PIC* > 0.5), moderate (0.25 < *PIC* < 0.5), and low (*PIC* < 0.25) (Botstein et al., 1980).

The UPGMA cluster analysis based on Nei's genetic distances (1978) revealed two main clusters (Fig. 2). The first group consisted of 10 clones, and the second group comprised 12 clones. Some clones were extremely closely related, such as two pairs clones, UC-1 and A-8, Bao-9 and UC-12, both had only 0.036 at Nei's genetic distance. Furthermore, the Nei's genetic distances of most clones were less than 0.2, and maximum Nei's genetic distance between the two main groups was 0.59, revealing the fact that most of the 22 clones had low genetic diversity.

3.3 Genetic structure

Principal coordinate (PCoA) analysis based on covariance matrix of Nei's genetic distances was carried

Table 2. Clone identification of 109 clonal samples of *C. equisetifolia* through matching genotypes with 9 reference clones across 12 EST-SSR loci.

Clone	Sampling No. with identical genotypes across 12 loci	Sample
Reference clones		
Bao-9	13,39,82,85,87,91,94,95,97,98,100,104,108	13
A-8	3,7,8,10,11,22,27,61,70,81,89,96,99,101,103,107	16
501	4,38,68,69,71	5
Dong-2	24,46,47,83	4
Duanza-1	18,42,43	3
A-13	45,50,52	3
601	30,36,40,41,44,48,84	7
Pingtan-2	2,17,19,20,23,25,28,29,31,72,106	11
Hui-1	5,6,9,12,14,15,16,102	8
Previously unidentified clones		
UC-1	21,62,86	3
UC-2	26,93	2
UC-3	33,88	2
UC-4	34,37,67	3
UC-5	35,65,105	3
UC-6	32,54,56,74	4
UC-7	49,75,109	3
UC-8	51,76,77	3
UC-9	55,57,58,73	4
UC-10	63,66	2
UC-11	1,59,60,64,78,80	6
UC-12	90,92	2
UC-13	53,79	2

out using the GenAlEx 6.5. The first principle coordinate accounted for 44.1% of the total genetic variation and the second principle coordinate accounted for 14.7%. Genetic structure analysis of the 22 clones used in coastal shelterbelts of South China indicated that most of the clones have close genetic distances, but they were clustered as 3 groups (Fig. 3) by PCoA. For example, clone 601 was clustered as the first group by PCoA due to its genetic distance divergence from other clones; clones UC-8 and Pingtan-2 were clustered as the second group; the remaining 19 clones were clustered together as the third group.

4 Discussion

This study demonstrated that EST-SSRs were effective in identifying *C. equisetifolia* clones. The 12 SSR loci selected were high discrimination markers except for locus C-13 which had moderate level of PIC (0.25 < PIC < 0.5) based on the criterion of Botstein et al. (1980). Cumulative PI of the 12 loci at discriminating the 109 samples reached 2.88×10^{-10}, which means that the probability of matching any two different clones at all 12 loci by chance in the 109 samples is 2.88×10^{-10}. The extremely low error probability of identification in this

Table 3. Genetic diversity parameters at 12 EST-SSR loci for 109 clonal samples of *C. equisetifolia*.

Primer	Genetic diversity parameters of 109 clone samples						
	Na	*Ne*	*Ho*	*He*	*I*	*PIC*	*PI*
C-13	2	1.57	0.23	0.36	0.55	0.35	0.39
M-03	3	2.31	0.84	0.57	0.95	0.50	0.27
M-26	5	3.80	0.72	0.74	1.44	0.69	0.10
M-27	6	3.41	1.00	0.71	1.44	0.66	0.09
M-32	3	2.94	0.94	0.66	1.09	0.59	0.19
M-46	5	3.47	1.00	0.71	1.39	0.67	0.11
M-36	3	2.44	1.00	0.59	0.97	0.50	0.21
M-37	6	4.10	1.00	0.76	1.53	0.72	0.08
M-38	4	2.24	0.78	0.55	0.97	0.50	0.24
M-39	3	2.50	0.94	0.60	1.00	0.53	0.23
M-40	5	3.91	1.00	0.74	1.48	0.70	0.09
M-41	4	2.89	1.00	0.65	1.21	0.60	0.16
Mean/ cumulative	4.08	2.97	0.86	0.64	1.17	0.58	2.88 X 10^{-10}

N_a = Number of alleles per locus, N_e = Number of effective alleles, H_o = Observed heterozygosity, H_e = Expected heterozygosity, *I* = Shannon information index, *PIC* = Polymorphic information content, *PI* = Probability of Identity.

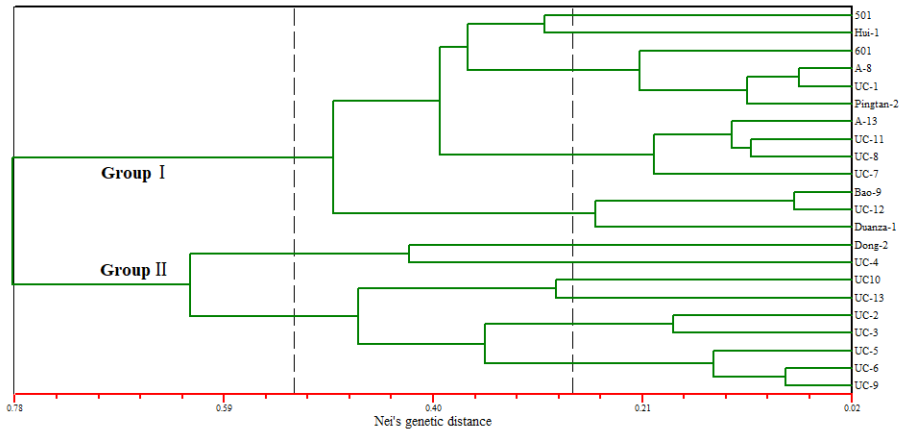

Fig. 2. Dendrogram generated by Unweighted Pair Group Method showing the 22 clones of *C. equisetifolia* were clustered as two main group, based on Nei's genetic distance derived from 12 EST-SSR markers.

study has given us great confidence in the ability of the 12 SSR markers to identify any clones that having been mislabeled or repeatedly named.

Based on genotyping results of 109 clonal samples and 9 reference clones of *C. equisetifolia,* 70 samples were determined as duplicates of 9 reference clones, the remaining 39 samples were identified as 13 previously unknown clones. We failed to obtain the relevant information on name, origin and breeder etc. of the 13 clones from local forestry authorities in the process of sample collection. It is probable that these clones had been selected and vegetatively propagated

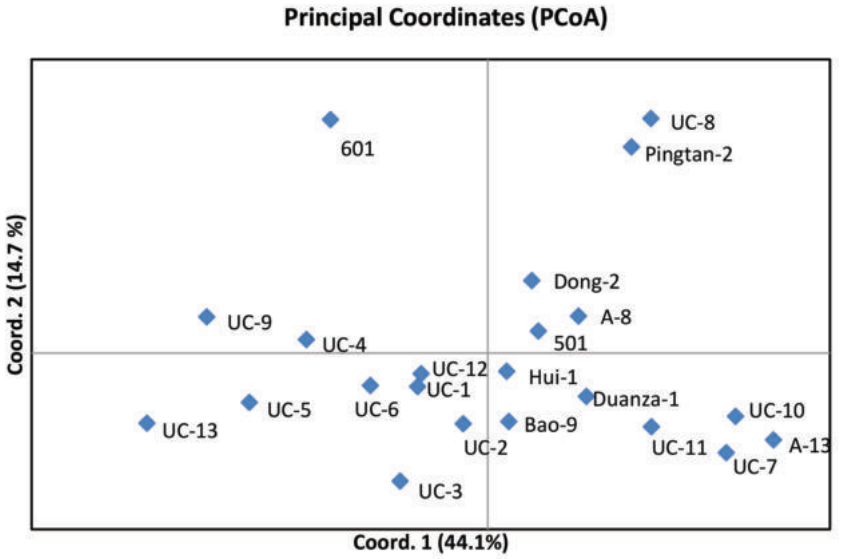

Fig. 3. Genetic relationship between the 22 clones of *C. equisetifolia* revealed by principal coordinates analysis (PCoA) based on mutually Nei's genetic distances. Twenty-two clones were clustered as three main groups.

from some superior individuals of *C. equisetifolia* by local forest farms or private nurseries (Xu et al., 2018). Deployment of these clones for large-scale planting without scientific and systemic genetic test poses a potential risk to coastal shelterbelts of *C. equisetifolia* in South China, as low genetic diversity exposes the plantations to the threat of disease incursion. Appropriate guidance and supervision should be taken by administrative sectors of forestry for selection and deployment of *C. equisetifolia* clones in South China.

In the process of clone identification, some local names were confirmed to be synonymous (i.e. describing the same clone), indicating that mislabeling or re-naming of *C. equisetifolia* clones is not unusual in South China. A similar situation was also found in another clone collection of *C. equisetifolia* (Xu et al., 2018), an apple collection (Hokanson et al., 1998) and grape species (Lamboy and Alpha, 1998). The re-naming or mislabeling of widely-used clones would undermine the legitimate rights and interests of breeders, and produce adverse effects on ecological protection functions of coastal shelterbelts of *C. equisetifolia.* For intellectual property protection and appropriate utilization purpose, fingerprints of some widely used clones should be constructed using SSR markers (Fossati et al., 2005). In this study a minimum of 7 informative SSR markers can entirely discriminate all the 22 clones, with cumulative PI of 4.71×10^{-6} (data not shown), that would reduce the cost of clone identification.

Genetic diversity assessment is an essential component in germplasm characterization and conservation and for future breeding program construction (Friedt et al., 2007). This study gave a valuable insight into the genetic variation of *C. equisetifolia* clones in shelterbelt plantations of South China. Low genetic diversity of the 22 clones can be confirmed by their close Nei's genetic distances (most of the clones were less than 0.20 and the maximum was 0.59) revealed intuitively in the UPGMA cluster analysis (Fig. 2), and similar results were verified by the principle coordinates cluster (Fig. 3). In our opinion, the relatively high H_o and H_e (mean value 0.86 and 0.64 respectively) detected in 22 clone accessions can be explained by the high heterogeneous nature of *Casuarina,* which is caused by its strongly outcrossing characteristic (Zhang et al., 2016). Similar results were found in *C. junghuhniana* and *C. cunninghamiana* (Kullan et al., 2016) and other outcrossing plant species such as *Tectona grandis* (Huang et al., 2016) and some perennials (Nybom, 2004).

It is well known that clonal plantations generally

are threatened by biotic and abiotic stressors due to their low adaptive genetic diversity. Roberds et al. (1997) concluded that 30 to 40 related clones in plantations can provide protection against catastrophic failure, but Wu (2018) suggested that a "safe" number of clones is between 5-30 related clones, with an optimum level of diversity at around 18 clones. Although there are about 22 clones of *C. equisetifolia* being used in clonal plantations of South China, individual plantations typically comprise only one clone. Moreover, they are closely related and have low genetic diversity, making them at risk of catastrophic failure. In recent decades, clonal shelterbelts of *C. equisetifolia* in South China have been facing a serious threat from bacterial wilt, often leading to rapid and widespread mortality, caused by *Ralstonia solanacearum* (Zhong et al., 2014). There is an urgent need to develop a diverse set of new clones, with superior disease resistance, for coastal shelterbelt construction in South China.

5 Conclusion

This study showed that *Casuarina* shelterbelts in South China are mainly planted to only 22 clones of *C. equisetifolia*. Most of these clones appear to be closely related, with close genetic distances and overall low genetic diversity. Mutual introduction, re-naming and mislabeling of clones in different provinces or regions was found. Developing a diverse set of new clones, with superior disease resistance, for coastal shelterbelt construction in South China is warranted.

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Genomic selection for economic traits in *Casuarina junghuhniana* natural population

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Abstract

Genomic selection (GS) is a tool to predict genetic value of untested genotypes based on genome wide markers. The current study is aimed at developing genomic selection model for economic traits such as wood yield (WY), pulp yield (PY) and blister bark disease (BBD) tolerance. A total of 240 individuals, representing 55 half-sib families were evaluated across four different agro-climatic locations. Genotyping, carried out using the Diversity Arrays Technology (DArT-seq) identified ~58461 SNPs for WY, 88400 SNPs for PY and 45185 SNPs for BBD *in-silco*. After applying stringent filtering criteria (MAF >0.05 and missing genotype <10%), GBS approach yielded 10334,11392,9082 SNP markers for WY, PY and BBD respectively. An association mapping using generalised linear model (GLM) at false discovery rate (FDR) adjusted to P<0.05 resulted 8,722 SNPs for WY and 9,862 SNPs for PY and 6,872 SNPs for BBD. Highly associated markers were used for the development of genomic selection model. Further, RR-BLUP (Ridge regression-best linear unbiased prediction) model was used to estimate the marker effect. The *Casuarina junghuhniana* accessions were divided into two sets: training population (80%) and validation population (20%). Genomic selection model was developed based on the marker effects using training data set (Genotype and Phenotype) and the accuracy of the GS model was validated using validation data set (based on genotype only). The GS model was able to predict the phenotype for WY (accuracy of 0.65), PY (0.55) and BBD (0.76). Application of genomic selection in untested population to predict genetic value of individual genotype based on genomic estimated breeding values (GEBV) for early selection is discussed.

Keywords: : genomic selection, single nucleotide polymorphism, genomic estimated breeding values, wood yield, pulp yield, blister bark disease

1 Introduction

Casuarinas are grown extensively in tropics, subtropics and Mediterranean countries due to their adaptation to different environment, fast growth and atmospheric nitrogen fixation. Casuarinaceae family has more than

96 species (Turnbull, 1990). *Casuarina junghuhniana, C. glauca, C. cunninghamiana* and *C. equisetifolia* are widely cultivated in various parts of the world, of which *C. junghuhniana* and *C. equisetifolia* are extensively grown. In India, there are approximately 800,000 ha of casuarina plantations (Pinyopusarerk and Williams, 2000), of which 500,000 ha are in coastal states such as Andhra Pradesh, Odisha, Tamil Nadu and the Union Territory of Puducherry (Nicodemus, 2009). *C. equisetifolia* and *C. junghuhniana* are suited for various end-uses such as fuel wood, windbreaker, scaffolding, paper and pulp industries. Raw material to pulp and paper industry in India is supplied through existing clones derived from *C. equisetifolia, C. junghuhniana,* and the F_1 hybrid derivative of Cj x Ce (Thai hybrid). Casuarina is still not very popular among farmers due to lack of coppicing ability and poor adaptability in inland dry zone. These issues can be addressed through informed tree breeding approaches, inadequate due to limited availability of genomic tools such as genome wide associated SNPs for various traits. Early selection of superior genotypes through associated markers with desirable trait(s) would help to deploy high yielding varieties. These tools would also help in supplying genetically improved seeds and clones to increase the economic value, which involves recurrent selection to increase frequency of favourable alleles for a number of traits in the population. Genetic gain is achieved through repeated cycles of selection, inter-mating and genetic testing of the superior planting material. Tree breeding programs depend on long term breeding objectives, species biology, deployment plan and source of planting material (seed/clone) (Namkoong et al., 1988; White et al., 2007). In addition, tree breeding is expensive due to late expression of economical traits and long-time breeding cycle, hence use of Marker assisted selection (MAS) is particularly gaining importance in tree breeding. Implementation of MAS is difficult in tree species due to a large number of QTLs controlling each trait and each QTL with small and unpredictably variable effect. Recent advancement in Genomic selection (GS) or Genome wide selection (GWS) estimates all marker effects simultaneously, retaining all of them as predictors of performance, thus precluding the prior search for significant marker-trait associations, but focusing exclusively on selection efficiency (Meuwis sen et al., 2001; Goddard and Hayes, 2009). Wilt or blister bark disease is the most destructive disease of casuarina, caused by a hyphomycete fungus, *Subramanianospora vesiculosa* Narayanan, Sharma & Minter (=*Trichosporium vesiculosum* Butler) (Narayanan et al., 2003); it was first reported in India in the early 1900s. Later, epidemic outbreaks were reported in India, Mauritius and Sri Lanka (Narayanan et al., 1996). In recent years the disease has been reported to cause large-scale mortalities in India as well as in China, Kenya, Thailand and Vietnam (Narayanan et al., 2003) causing yield loss to the tune of 10-15% in coastal plantations (Mohan and Sharma, 1993). Although there are several *adhoc* recommendations for management of these diseases, use of resistant host material for eco-friendly (reduced use of pesticides) management of the disease for the sustainable productivity of casuarina is of high priority. Hence, disease management strategies in casuarina may be adopted with the emphasis on host plant resistance. In view of the above, Current investigation was aimed at two objectives, (a) identifying molecular markers associated with traits and to develop genomic selection model for growth and pulp yield, wood yield; and (b) screening and classification of susceptible and tolerant for blister bark pathogen *Trichosporium vesiculosum* causing blister bark disease in casuarina and development of genomic selection model

2 Material and methods

2.1. Plant material

The plant materials used in the study were collected from Indonesian Islands. A total of 240 individuals of *C. junghuhniana* representing 55 half-sib families was established at Mettupalayam, southern India (11°14′43.6″ N, 76°52′17.1″ E) as mother stock plants for vegetative propagation. After propagation, all the individuals were planted across four different locations (Visakhapatnam, Ongole, Cuddalore and Mayiladuthurai) for evaluation using randomised complete block design (RCBD) with five replications. Plants were planted in Visakhapatnam and Cuddalore during 2013-2014, whereas at Ongole and Mayiladuthurai plantation was taken place during 2014-2015 (Table 1).

Table.1. Details of the site location.

Parameters	Visakhapatnam	Cuddalore	Ongole	Mayiladuthurai
Latitude (N)	17°41′	11° 44′ 47″	15° 31′	11° 6′
Longitude (E)	83°1′	79° 45′ 52″	84° 4′	79° 40′
Altitude (m asl)	30	9	39	10
Soil type	Sandy soil	Red soil	Red soil	Clay soil
Mean annual precipitation (mm)	1050	1252	825	1197
Mean annual temperature (°C)	27.6	28.2	28.5	28.5

2.2 Phenotyping

2.2.1 Stability analysis of growth traits

The stability of wood yield of each genotype was calculated by regressing the mean yield of individual genotypes on environmental index and calculating the deviations from regressing the mean yield of individual genotypes on environmental index and calculating the deviations from regression as suggested by Eberhart and Russell (1966). Regression coefficient (*bi*) was considered as an indication of the response of the genotype to varying environment, while the environment and genotype x environment interactions were partitioned into three components viz., environment (linear), genotype x environment (linear) and deviation from regression (pooled deviation over the genotypes). The stability analysis was done using the linear regression model suggested by Eberhart and Russell (1966).

2.2.2 Wood quality traits

Wood basic density was defined as oven-dry wood mass per unit volume of green wood, and was measured using the water displacement method (Kube and Raymond, 2002). The 5-mm increment cores from pith to bark were extracted at a height of 1.37 m in the south-north orientation from each genotype, immediately stored in aluminium tubes with both ends sealed. Wood basic density was determined using the water displacement method, with two weights for every sample: weight of water displaced by immersion of core samples (w1) and oven dry weight (w2) (Nguyen et al., 2008). Basic Density was then calculated as:

Basic Density = Dry weight/Volume (g cm^{-3}).

Pulp yield estimation: NIR measurements were made with a Bruker Multi Purpose Analyzer (MPA) (Bruker Optics, Ettlingen, Germany) using the integrating sphere accessory. Wood meal (powdered core samples) was placed in quartz vials for analysis. The average value of three measurements was used for each sample. Spectral processing and calibrations were performed using the Bruker QUANT software package within OPUS version 6 (Bruker Optik, 2006). Calibration models were developed using partial linear regression analysis (PLR). Prior to calibration the spectra were processed using a straight line subtraction for lignin and no spectral processing was used for KPY calibration. NIRS calibration models developed for casuarina (Ramadevi et al., 2009) was used to estimate KPY of casuarina genotypes. Wood quality traits such as pulp yield and wood density was measured in Visakhapatnam plantation.

2.2.3 Disease screening for blister bark disease

The pathogen was isolated from the affected tissues using the standard tissue isolation method on potato dextrose agar medium and preserved under refrigerated conditions. Sub-culturing and reconfirmation was carried out for the pathogen. Identified pathogens were mass multiplied on Potato dextrose broth (Karthikeyan et al., 2011) giant culture mixture containing sand: sorghum at 1:1 ratio. Two-month-old casuarina ramets (80 clones) were planted in poly bags containing sterile sand soil mixture from trays along with susceptible clones. For disease expression, 5 ml of pathogen spores

Table 2. Disease severity index scale.

Score	Type of symptoms	Disease reaction
0	No symptoms,	Resistant
1	Healthy stems and shrunken needles	Moderately resistant
2	Partially drying and brittle needle leaves	Susceptible
3	Completely dead needle leaves and necrotic roots	Highly susceptible

(10^6 spores ml^{-1}) was inoculated to 2-month-old ramets by making an injury at the collar region with the help of a small needle and injured part was covered with parafilm to provide humidity. Infected plants were inoculated with *T. vesiculosum* (4 ramets for each clone) and maintained in polyhouse with 25-40 °C temperature and 75% RH for disease expression for the period of 4-6 months. Casuarina clones were classified as per the disease severity index scale (Table 2).

2.3 Genotyping

Genotyping was carried out using the Genotyping by Sequencing approach (DArT-seq), which was performed by Diversity Arrays Technology Pty Ltd (DArT P/L).

2.3.1 Structure analysis

To infer population structure, the germplasm was analysed with a set of 2204 SNP markers that were common between two species using model-based program STRUCTURE. Five independent runs of K = 1 to 20 were performed at 50,000 Markov chain Monte Carlo (MCMC) repetitions and 500,000 burn-in period using prior population information with no admixture model assuming correlated allele frequencies. In addition, ΔK values based on the rate of change in the *log* probability of data between successive K-values was also estimated.

2.3.2 Association mapping and genomic selection analysis

For genomic selection, first we conducted an association mapping analysis to select a subset of markers that were showing some association with pulp yield, wood yield and blister bark disease. SNPs markers associated with growth were determined using generalised linear model (GLM) using population structure as covariate at false discovery rate (FDR) adjusted P<0.05. The RR-BLUP (Ridge regression-best linear unbiased prediction) prediction model was used to estimate the marker effect, which assumes equal variance of all markers and shrinks all the marker effects to zero and was implemented using the "rrBLUP" library in R – statistical package.

During the process of model development *C. junghuhniana* accessions were divided into two sets: training population (80%) and validation population (20%). Genomic selection model was developed based on the marker effects estimated using training data set (Genotype and Phenotype) and the accuracy of the GS model was validated using validation data set (based on genotype only).

2.3.3 Estimation of effective population size (N_e)

Effective population size (N_e) was estimated using below formula (Grattapaglia et al., 2011). Number of markers were chosen based on prediction accuracy, linkage disequilibrium and recombination rate.

$$N_e = (4N_m N_f)/(N_m + N_f),$$

where N_m is number of males and N_f is number of females.

3 Results

3.1 Stability analysis of growth traits

For wood yield traits genotypes derived from families (Fig.1) with phenotypic index (*pi*) greater than zero, stability index (*bi*) close to 1 and no significant deviation from regression, hence these genotypes are classified as stable over the locations (Table 3).

3.2 Wood quality traits

3.2.1 Pulp yield

Pulp yield was estimated for 337 genotypes from Visakhapatnam trial using NIRS. This population has variation for PY, values are ranging from 45.6 to 49.2%, maximum (49.2%) PY was in family CJ1 (Fig. 2) with population mean of pulp yield recorded was 47.2%.

3.2.2 Wood density

Wood density is by far the most intensively studied wood quality trait in tree breeding program. Wood basic density was estimated from 279 genotypes of 55 families collected from Vishakhapatnam plantation. Wood density ranged from 0.51 g cm^{-3} to 0.69 g cm^{-3}, population has trait variation for wood density (Fig. 3). Average wood density of the population was 0.5985.

3.3 Screening for blister bark disease

Totally 88 genotypes out of 218 genotypes showed zero symptoms after 60 days of inoculation, these have been considered as resistant clones for blister bark disease. Needles of 73 genotypes showed shrunken and stem healthy and scored as 1 indicating

Table 3. List of stable genotypes.

S. No.	Family name	No. of genotypes
1	CJ10, CJ11, CJ19, CJ33, CJ37, CJ3, CJ45, CJ46, CJ47, CJ49, CJ51, CJ52, CJ53	13
2	CJ6, CJ7, CJ8, CJ13	4
3	CJ1	1

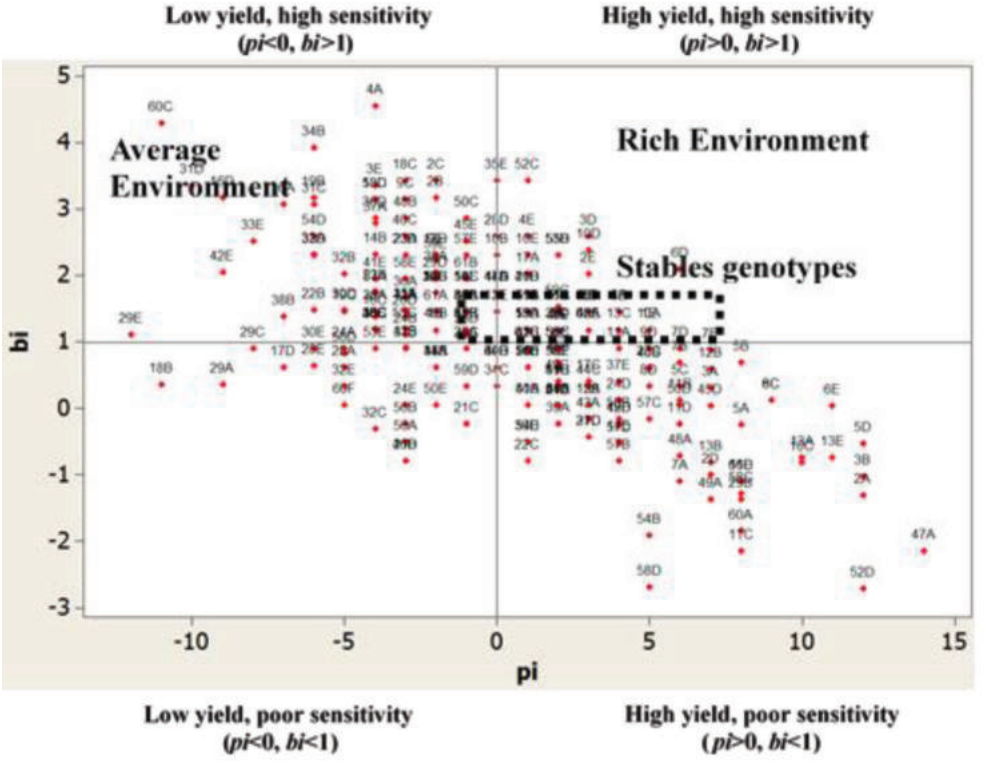

Fig.1. Stability analysis of selected genotypes. The genotypes highlighted are stable genotypes.

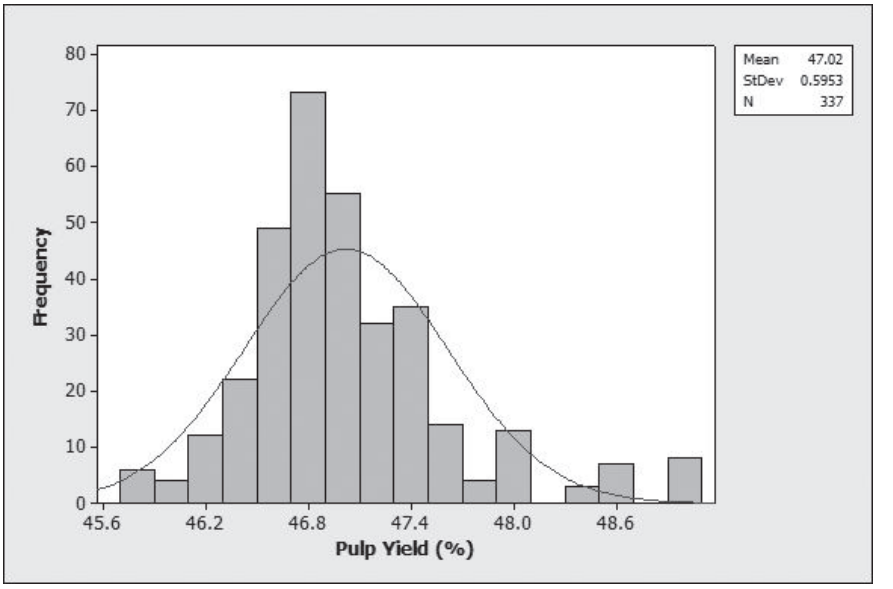

Fig. 2. Histogram for pulp yield.

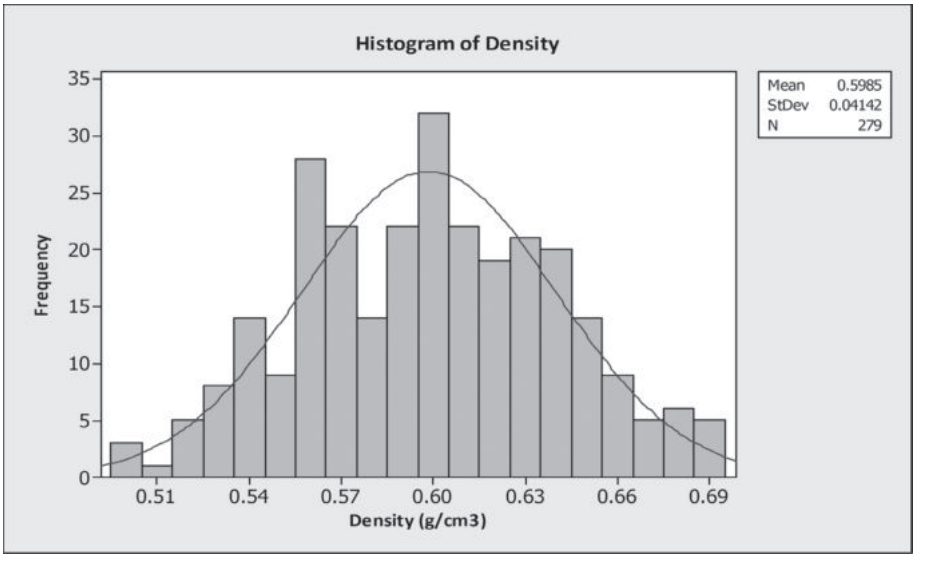

Fig. 3. Histogram for wood density.

moderately resistant for blister bark disease. Needles of 37 genotypes showed partially drying and brittle, with a score of 2 and were classified as susceptible for blister bark disease. A total of 20 genotypes showed complete death and necrotic roots and these were classified as highly susceptible to blister bark disease. The resistant genotypes showed higher phenol contents (19-21 mg g^{-1}) whereas susceptible genotypes showed lower phenol contents (14.5-16 mg g^{-1}) (Fig. 4).

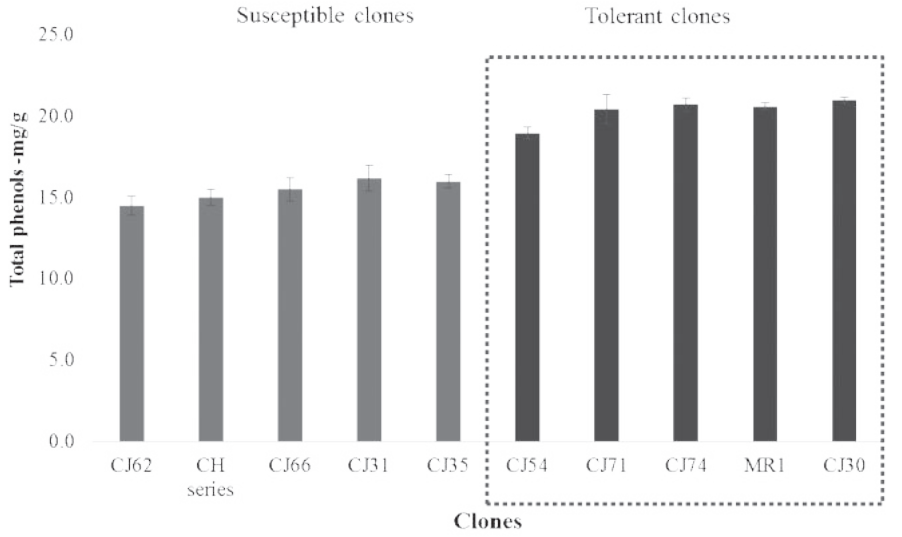

Fig. 4. Total phenol estimation in susceptible and tolerant clones for blister bark disease.

3.4 Genotyping

3.4.1 Association mapping and genomic selection analysis

An association mapping analysis was carried out to select a subset of markers that were showing association with wood yield, pulp yield, wood density and blister bark disease in 10334 SNPs identified as mentioned.

Genome-wide association study identified a total of 8722 SNPs for wood yield, 9862 SNPs for pulp yield, 9800 SNPs for wood density and 6872 SNPs for blister bark disease without FDR correction (Table 4). Multiple testing correction was not carried out for these makers, because multiple testing correction may drastically reduce the number of markers associated with traits, which may affect the GS model development (GS requires genome-wide markers for the model development).

Genomic selection model was developed based on the marker effects estimated using training data set (Genotype & Phenotype) and the accuracy of the GS model was validated using validation data set (based on genotype only). Accuracy of the GS model developed based on RR-BLUP model able to predict the phenotype with an accuracy of 0.65, 0.55, 0.65 and 0.76 for wood yield, pulp yield, and wood density and blister bark disease (Fig. 5).

3.4.2 Validation of genomic selection material in 2nd generation material

Clonal testing area (CTA) derived from training population was converted into clonal seed orchard (CSO), from which seeds were collected to raise seedlings. These seedlings were genotyped and the breeding values were estimated. Simultaneously, these seedlings were planted in various locations and the developed genomic model was used to predict

Table 4. Prediction accuracy for wood yield, wood properties and blister bark disease.

Details	WY	PY	Density	BBD
Total markers	32000	32000	32000	32000
Markers associated with trait	8722	9862	9800	6872
Prediction accuracy	0.65	0.55	0.65	0.76

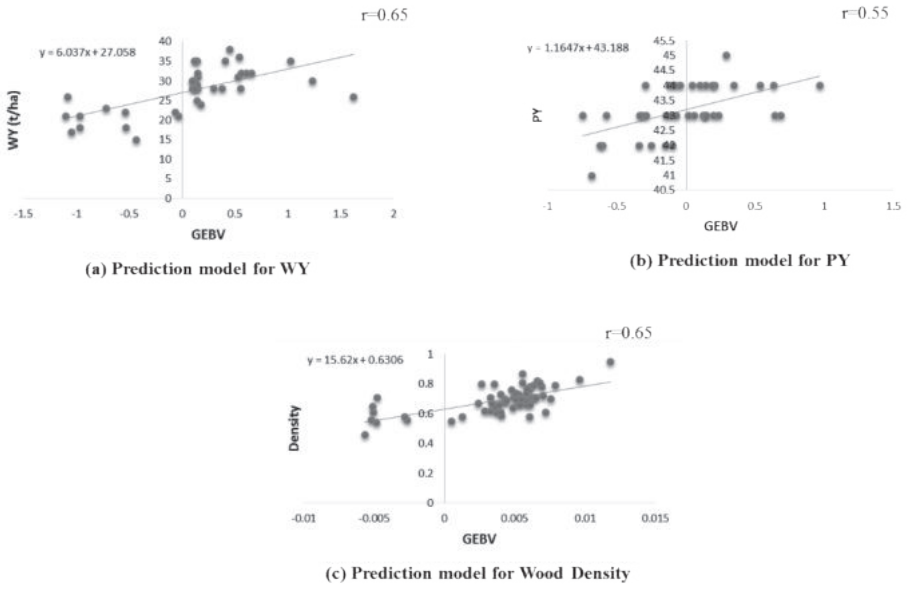

Fig. 5. Prediction models for wood yield (a) pulp yield (b) and wood density (c).

phenotype. We found 11 families which were superior for PY, WY and density. Predicted data were correlated with the phenotypic data and found 80-90% in agreement, which gave us confidence on genomic selection early selection of superior genotypes (Fig. 6).

4 Discussion

The scope of any tree breeding program is highly dependent on variation. The earlier germplasm col lections showed skewed distribution for trait diversity and genetic diversity indicating narrow genetic variation (data not presented). Introduction of new germplasm has helped to achieve higher trait and genetic diversity, which was further used in genomic selection studies. Major hurdles in MAS strategies in tree breeding are long generation breeding cycle, the costs involved in establishing and maintaining large progeny trials, and the low heritability of most targeted traits (Grattapaglia et al., 1992; Williams and Neale, 1992; White et al., 2007). Despite several issues, QTL's were identified for a number of traits in most of the plantation

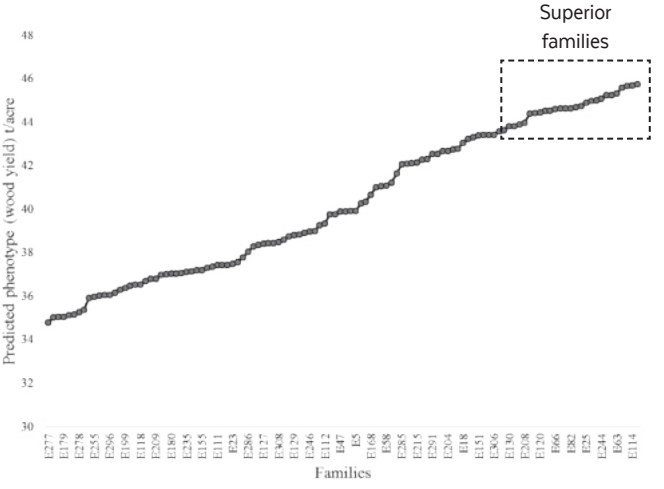

Fig. 6. Wood yield prediction based on Genomic selection model.

crops. Initially quantitative traits were dissected by building linkage maps through RFLP, RAPD, AFLP, and microsatellite based markers. In all these studies due to the small sample size, major QTL's had overestimated the phenotypic effects (Sewelland Neale, 2000; Kirst et al., 2004; Grattapaglia et al., 2009). Studies with larger progeny size and more precise phenotyping have increasingly shown a different scenario regarding the number and distribution of QTL effects (Brown et al., 2003; Dillen et al., 2008; Rae et al., 2008). Due to larger number of QTL controlling each trait, and each QTL with a small and unpredictably variable effect, the likelihood of implementing cost-effective MAS for several traits simultaneously has been practically difficult in tree improvement programs. Limitations of QTL-based MAS can be addressed by association genetics (Neale and Savolainen, 2004). This method is most appropriate in tree breeding due to large random mating unstructured populations with high nucleotide diversity and low-range LD. Population-wide marker–trait associations would help in dissection of complex traits. Major traits in tree breeding programs are complex, SNP's in candidate genes associated with wood property, disease resistance, and drought tolerance are reported (Gonzalez-Martinez et al., 2007; Gonzalez-Martinez et al., 2008; Eckert et al. 2009).

Four parameters are majorly contributing accuracy of genomic selection, (a) Linkage disequilibrium between markers and QTL's, (b) Training population size, (c) Heritability of the traits, and (d) Distribution of marker effects. Diverse genetic material is a prerequisite for any tree improvement programs. Existing casuarina germplasm pool showed skewness for trait diversity and genetic diversity. This study strongly suggests for enhancing the diversity by introducing new germplasm. Population structure based on SNP markers of introduced germplasm revealed two groups specific to CJ and CC, which suggested to develop GS model specific to each species for higher accuracy. Casuarina being outcrossed species, high level of recombination rate is observed, low LD is observed in CC and CJ. Genomic selection accuracy depends on population size and number of markers. In casuarina, high accuracy could be realised with 10-20 markers cM^{-1} with effective population size (*Ne*) of 66, which necessitates approximately 10,000-15,000 markers to reasonably cover entire genome of casuarina for unbiased estimates, assuming that the trait factors are distributed across the genome.

Scaffolds of CE genome was mapped using English walnut (*Juglans regia*). Around 84% of sequences of CJ was mapped onto CE indicating complete capture of CJ genome used for genomic selection study. Approximately 1062 SNP's are found in genic region and 517 SNP's non genic region (Fig.7).

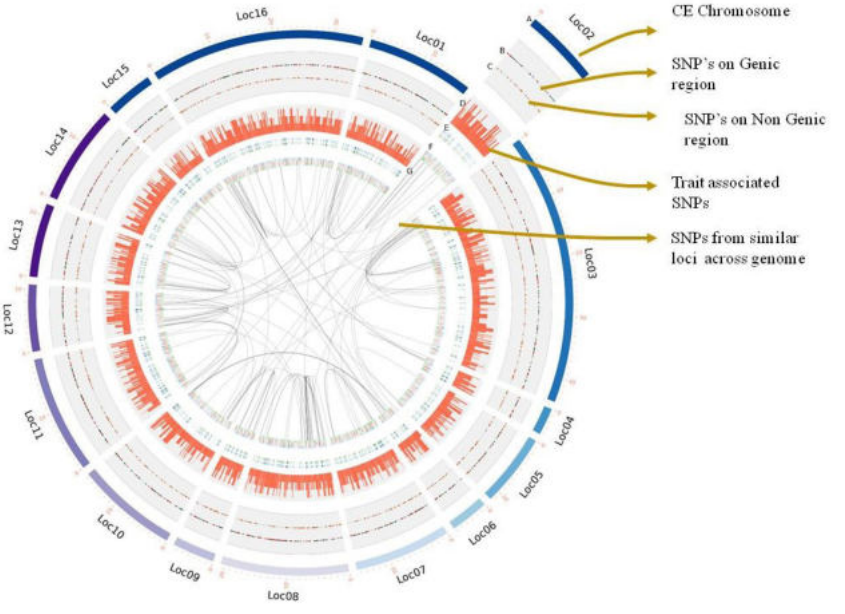

Fig. 7. Landscape of CJ SNPs in CE genome.

This study clearly shows that medium distributed markers, well distributed over genome can be used in GS for predicting breeding values with significant prediction accuracy, while similar prediction accuracy was observed in pine and white spruce (Resende et al., 2012; Zapata et al., 2012; Beaulieu et al., 2014; Isik et al., 2016). The accuracy of the GS model obtained with the present casuarina natural population was high for blister bark disease, wood yield and density, reaching values of 0.65 to 0.76. Accuracy estimates from casuarina GS studies are comparable with other pedigree-based counterparts. Results obtained were on par with other tree species such as white spruce, Eucalyptus for a population of half sib families (Beaulieu et al., 2014). The study also revealed that GS can be efficiently utilised in casuarina breeding population with structured population of half sib families resulting in higher gains per year than conventional selection. Moreover, applying GS scheme at an early stage is possible for selecting better genotype. The present study showed accuracy estimates of GS is on par with estimates previously obtained for similar traits in half sib families of other tree species. Simulation results of Gattapaglia and Resende (2011) suggested that to reach prediction of 0.60 to 0.75 prediction accuracy for genomic selection relying on 10-20 markers cM^{-1}, with an effective population size close to 66 is essential. Overall, the accuracy estimates showed minor differences among traits. Model accuracy for pulp yield was lower than that for wood density, similar results are reported in pine and white spruce (Beaulieu et al., 2014; Isik et al., 2016). This is due to higher heritability of wood density compared to pulp yield as often noted in conifers (Lenz et al., 2013; Beaulieu et al., 2014; Gamal et al., 2015). Heritability of a trait is an important consideration for prediction accuracy of complex traits (Hayes et al., 2007). Through genomic selection, we have successfully demonstrated traits of low heritability can be improved using informed selection. Upon deployment of mentioned methods, we have estimated an improvement of 10.2% WY, 4.5% PY and 6.2% increase in wood density per year over normal 16-year breeding cycle in the existing germplasm.

5 Conclusion

This study showed genetic gains in complex traits such as wood yield, pulp yield, wood density and blister bark tolerance can be achieved in casuarina by implanting marker-based selections at early stage as against traditional selections. Based on present results, the genomic selection would be useful to select superior individuals with high accuracy in a full sib family. This technique would also help in controlled crosses that were used to build the GS models to generate much larger full sib families and thus, apply higher selection intensities and obtain larger genetic gains. Individuals identified through genomic selection at the nursery stage could be selected and propagated for high genetic gain per unit time with reduced breeding cycle.

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Three-year performance of *Casuarina equisetifolia* in a provenance trial in South Sumatra

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Abstract

Seed collection of *Casuarina equisetifolia* was carried out in six locations in Belitung Island, Sumatra (Lilalang, Serdang, Burung Mandi, Sungai Linggang, Mentigi, Menduong). The seeds were used to establish a limited provenance trial at a site belonging to a forestry company, PT. Musi Hutan Persada (MHP) in South Sumatra. The company has grown *Eucalyptus pellita* plantation for pulpwood production. The need to find alternative species for pulp plantation and also suitable species for energy plantation motivated the establishment of the *Casuarina* provenance trial.

The trial site was located at an altitude of 108 m, with a mean annual rainfall of 2,700 mm. The soil is classified as red-yellow podsolic (Ultisol) with high clay content (52%). At three years of age, differences between provenances were significant for survival (p<0.04), tree height (p<0.005) and stem diameter at breast height (p<0.0001). The survival rate of all provenances was very good, better than 85% except at Mentigi (65%). Tree height ranged from 7.6 m to 9.5 m and stem diameter from 6.6 cm to 9.2 cm. Provenances with higher survival rate tended to have better growth. No apparent pest and disease problems were detected in any provenances tested. All provenances had excellent axis persistence, with no difference between provenances (p=0.23); differences in stem straightness between provenances were, however, significant (p=0.002). Differences in branch density between provenances were also significant (p=0.02). At three years of age all provenances started flowering even though the percentage of trees producing flowers was very low, ranging from 0.5% to 5.4%.

Keywords: *Casuarina equisetifolia*, provenance variation, growth, stem form, branching habit

1 Introduction

PT. Musi Hutan Persada (MHP), a forestry company located in South Sumatra, Indonesia has developed pulpwood plantations (110,000 ha) since 1990 for feeding a bleached kraft pulp mill with an annual capacity of 4,500 tonnes of pulp. Recently the company has also been interested in growing biomass energy

plantations, with some of its areas to be allocated for this purpose. In addition, the energy plantations may be developed in community lands surrounding MHP's areas in partnership with local people. The local people are expected to be interested in growing the biomass plantations as they have a short-rotation time, provided the income is reasonably competitive relative to traditional commodities such as rubber or palm oil.

MHP has considered that *Casuarina equisetifolia* has a good potential to be grown on its estate and is regarded as the "best-bet" species for initial testing., Other species including *Calliandra callothyrsus, Gliricidia sepium,* and *Leucaena leucocephala* have also been considered to have some potential However, the growth and productivity of these species including *C. equisetifolia* on MHP's estate are as yet untested. A provenance test of *C. equisetifolia* was considered the important first step to identify the best provenance that can be further developed for biomass energy plantation at the company site. This paper reports the results of the provenance test at age 3 years.

2 Materials and methods

2.1 Establishment

Seeds of *C. equisetifolia* were collected along the east and south coast of Belitung Island (Sumatra) at seven locations (provenances) in August 2015. The seeds of each provenance were sown in germination boxes and the transplanted into side-slit container (90 cc) previously filled with cocopeat growing medium. Each cubic metre of the potting medium was mixed with 4 kg slow released NPK (10:26:11) and 3 kg triple superphosphate (TSP) fertilizer. Transplanting was done when the germinant had two pairs of leaves, about 3 weeks after sowing.

The provenance test was established on a site of ex-third rotation of *Acacia mangium*. The site is flat, located at 3°68′99″ S and 103°99′86″ E and at an altitude of about 108 m. The mean annual rainfall at the site measured at the nearby weather station averaged 2,700 mm over the last 25 years. Rainy season normally occurs from October to April, while dry season occurs from May to September. The soil of the site belongs to red-yellow podsolic soil or Ultisol (Soil Survey Staff 1992). The details of soil properties of the trial site are given in Table 1.

In April 2016 five-month old seedlings were hand planted in planting hole of 20 cm x 20 cm x 20 cm previously prepared at spacing distance of 3 m x 3 m. Triple superphosphate (TSP) fertilizer at a rate of 100 g tree^{-1} was applied in a hole of 10 cm depth, about 15 cm from the seedling stem given at planting time. The trial was arranged in a randomized complete block design, 64 trees plot^{-1} replicated three times. Weeding was controlled regularly by a combination of hand weeding and herbicide (a mixture of glyphosate and methyl-metsulfuron) application, particularly before trees reached one-year of age.

Table 1. Soil properties of the *Casuarina equisetifolia* provenance test site in South Sumatra.

Soil property	Value	Remark
Texture		
Clay (%)	52.4	
Silt (%)	24.2	
Sand (%)	23.4	
pH	4.76	Acid
Organic C (%)	4.85	High
Total N (%)	0.23	Medium
Extractable P (mg kg^{-1})	3.68	Low
Exchangeable K (mg kg^{-1})	43.68	Low
Exchangeable Ca (mg kg^{-1})	1130.40	Low
Exchangeable Mg (mg kg^{-1})	105.12	Low

2.2 Assessment

The provenance test was assessed at age 3 years for growth traits, namely height (Ht) and diameter at breast height (Dbh). Plot mean survival was also calculated. In addition, the stem form (axis persistence and stem straightness) and branching habit (density, thickness and angle) were also assessed in every tree using the procedures of Pinyopusarerk et al. (2004). Details of assessment are presented in Table 2.

Table 2. Characteristics assessed in the provenance test of *Casuarina equisetifolia* in South Sumatra.

Characteristic	Unit	Remark
Growth		
Height (Ht)	m	Total height of the tallest stem
Diameter(Dbh)	cm	Measured at 1.3 m above ground
Stem form		
Axis persistence (Axpst)	1-6	1= double or multiple stem form ground level 2= axis loses persistence in the first quarter of the tree 3= axis loses persistence in the second quarter of the tree 4= axis loses persistence in the third quarter of the tree 5= axis loses persistence in the fourth quarter of the tree 6= complete persistence
Straightness (Strst)	1-6	1= not vertical with more than two bends 2= roughly vertical with more than two bends 3= not vertical with one to two bends 4= roughly vertical with one to two bends 5= roughly vertical and straight 6= completely vertical and straight
Branching habit		
Density (Denpb)	1-4	1= very high, regularly branching with internode length around 15 cm 2= high, irregular branching with internode length mainly around 15 cm 3= low, irregular branching with internode length mainly around 30 cm 4= very low, sparse branching with internode length generally > 30 cm
Thickness (Thkpb)	1-4	1= very heavy, more than three branches, diameter > one-third of adjacent stem 2= heavy, one to branches, diameter > one-third of adjacent stem 3= light, branch, diameter up to one-third of adjacent stem 4= very light, branch, diameter < one-third of adjacent stem and uniform
Angle (Angpb)	1-2	1= branch angle <60° (upright) 2= branch angle >60° (horizontal)
Flowering (Flwre)	1-2	1= no flowering 2= flowering (regardless of sex)

2.3 Data analysis

Each trait, except branch angle and flowering was analyzed using analysis of variance to determine differences between provenances. Significant differences between provenances were identified by Least Significant Difference (LSD) test at p=0.05. All analyses were performed using R statistical software version 3.6.1.

3 Results and discussion

Results of the assessment of the provenance test at age 3 years are presented in Table 3. The survival of all provenances was good, (> 91%), except Mentigi which had markedly lower survival (66%). No pest and disease infestation was observed in any provenance. Height growth differed significantly between provenances (p=0.005). Similarly, diameter growth was significantly different between provenances (p<0.0001). Tree height ranged from 7.6 m to 9.5 m, and stem diameter from 6.6 cm to 9.2 cm. Provenances with higher survival rate tended to have better growth. Lilalang and Serdang provenances had the highest rate of growth.

The growth of *C. equisetifolia* provenance test in South Sumatra was relatively faster than that of a trial in Bangladesh, established on brown-forest soil at spacing of 1.5 x 5 m and measured at 4 years of age which had mean height and diameter of 4.8 m and 4.0 cm respectively (Hossain et al., 1995). It was also faster compared with that of a provenance test of *C. equisetifolia* in southern India grown on clay loam soil (Sandivayal site) and loamy soil (Neyveli site) at spacing of 1 x 1 m and assessed at 4.5 years of age. At Sandivayal the height and stem diameter growth ranged from 3.97 m to 4.97 m and from 2.96 cm to 3.29 cm, respectively. Meanwhile at Neyveli the height and stem diameter were varied from 5.80 m to 7.53 m and from 3.13 cm to 5.02 cm, respectively (Nicodemus et al., 1996).

All provenances had excellent axis persistence and did not differ significantly between provenances (p=0.23). However, differences in stem straightness between provenances were significant (p=0.002), Serdang having the highest score (4.7). Branching density also differed among provenances (p=0.02); Serdang, and Metigi had the densest branches, while Burung Mandi and Linggang had sparsest branches. Serdang and Mentingi tended to have more-horizontal branches compared with the other provenances. By 3 years of age, all provenances had commenced

Table 3. Growth, stem form and branching habit of *Casuarina equisetifolia* in the provenance test in South Sumatra at age 3 years.

Provenance	Characteristic								
	Ht (m)	Dbh (cm)	Survival (%)	Apxst	Strst	Denpb	Thkpb	Angpb	Flwre (%)
Lilalang	9.5	9.2	94.0	5.8	4.1	2.8	4.0	1.1	1.3
Serdang	8.5	8.3	96.1	5.6	4.7	1.9	4.0	1.6	1.3
Burung Mandi	8.4	7.4	91.8	5.4	4.1	3.8	4.0	1.2	1.1
Linggang	7.6	7.3	84.7	5.7	4.4	3.6	4.0	1.2	1.0
Mentigi	6.9	6.6	65.6	5.8	4.5	2.3	3.9	1.6	1.3
Menduong	6.8	6.9	85.2	5.8	4.3	3.5	4.0	1.1	1.2
P-value	0.0005	<0.0001	0.04	0.23	0.002	0.02	0.31	0.07	0.01
LSD (p=0.05)	0.94	0.56	19.16	0.34	0.26	1.15	0.03	0.49	0.15
CV (%)	6.53	4.06	12.21	3.33	3.24	21.11	0.40	20.92	7.19

Notes: Ht=total height, Dbh= diameter at 1.3 m, Apxt= axis persistence, Strst= stem straightness, Denpb= density of permanent branch, Thkpb= thickness of permanent branch, Angpb= angle of permanent branch, Flwre= flowering.

flowering, was albeit at a very low intensity, ranging from 0.5% to 5.4% of trees.

Despite the provenance test being established using only seven provenances from Belitung Island, the trial showed that the selection of superior provenances will likely result in plantations with better survival, growth and form. In the future, seed collection of *C. equisetifolia* from a wider range of provenances will be very worthwhile, as the species has very wide natural distribution and great genetic variation among provenances (Pinyopusarerk et al., 2004). The wide ranging collection of provenances can then be used to establish a breeding program of *C. equisetifolia* incorporating provenance tests and establishment of a breeding population for further selection and genetic improvement.

Acknowledgements

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Growth performance of *Casuarina junghuhniana* provenances in two sites in Kenya

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Abstract

Two provenance trials of *Casuarina junghuhniana* were established in April 1999 in Uplands forest and Kiandongoro forest stations in Kenya on opposite sides of Aberdares Ranges as part of the ACIAR International Provenance Trial. The trials compared growth performance of 21 seed sources from the natural range of the species in Indonesia, and planted stands from Kenya and Tanzania. Fifteen provenances were common to the two sites while three additional different provenances were included at each site as the number of their seedlings was low. Assessments of the trials were carried out in April 2003 (for Uplands) and October 2005 (for Kiandongoro) for: height and diameter growth; axis persistence; stem straightness; density, thickness and angle of permanent branches; and length, thickness and stiffness of branchlets; results presented are for the growth parameters only. In 2015, health status of both trials was assessed including incidence and severity of pests and diseases. The growth characteristics differed significantly among the provenances and the land races. The average survival of the 18 provenances in Uplands was 53.4% while in Kiandongoro it was 78.3%. This is despite the fact that Kiandongoro trial was assessed at 6.5 years while the Uplands trial was assessed at 4 years. Provenance 18848 from Mt. Abang, Bali, Indonesia had the highest growth performance with 80%, 15 cm and 18.3 m (survival, diameter and height respectively). It was followed by 18844 from Mt. Tapak, Bali with 78.5 %, 13.0 cm and 12.8 m respectively. Other provenances worth considering for the Kenyan highlands include 18951 (Mt. Arjuno, East Java, Indonesia); 18846 (Mt. Pengalongan, Bali Indonesia) and 19239 (a land race from Muguga Kenya). The least adapted of the provenances was 18853 from Kwai Mission in Tanzania. However, the best performing provenances showed considerable within-provenance variation. There is therefore a need to establish stands of these suitable provenances and carry out selection of outstanding individual trees to optimize plantation productivity in future. The health status data revealed no major pests at either site but there was a significant difference in incidence of leaf blights between the two sites. The Kiandongoro site was severely affected by a leaf blight caused by a *Fusarium* species which

seemed to negatively affect growth there.

Keywords: *Casuarina junghuhniana,* provenance trials, highlands, growth, stem form, branching habit

1 Introduction

Casuarina junghuhniana is native to Indonesia where it grows naturally on the slopes of volcanoes and is a pioneer species of deforested land (Orwa et al., 2009). The species grows into tall forest trees of 15-25 m height and 30-50 cm diameter but can grow up to 35 m in height and 1 m in diameter. This species grows rapidly with a strong apical dominance. It was introduced in Kenya in 1956 and naturalized over time (Potgieter, 2014). Where it is endemic, annual average rainfall varies between 700 and 1500 mm and up to 2500 mm in Java (Pinyopusarerk et al., 2005). The mean minimum and maximum temperatures are 13°C and 28°C respectively.

There has been increasing enthusiasm to plant *Casuarina* in Kenya as an agroforestry tree species but unfortunately this has seen the mixing of *C. junghuhniana* and *C. equisetifolia* – the latter a lowland species. No trials of *C. junghuhniana* had been established in the country until the 1990s when CSIRO Australian Tree Seed Centre organised a coordinated seed collection from the natural occurrences in Indonesia and the planted land races in Australia, Kenya and Tanzania. There is therefore a need to assess the performance of *C. junghuhniana* in countries such as Kenya, Tanzania, Senegal, Uganda, China, India, Taiwan and Thailand where large-scale plantings have been established in areas with climates similar to its native range. Two provenance trials were established in Kenya among others established in other countries in Africa and Asia. This paper presents results from the two trials planted in Kenya (Uplands at the age of 4 years and Kiandongoro at 6.5 years). Though the trials are still standing to date, they have been greatly interfered with over time through illegal cutting for firewood and poles. However, photos of the standing trees have been obtained to guide in selection of provenance to promote for future plantings.

2 Materials and methods

Seedlots of 22 provenances of *Casuarina junghuhniana* and one of *Casuarina oligodon* in 5 g packages were received from the Australian Tree Seed Centre and sown in Muguga, Kenya. The *C. oligodon* did not germinate, while the other provenances had varying germination rates. After germination and development of the first leaves, the seedlings were pricked out into polythene tubes. When they had attained a height of approximately 30 cm, the seedlings were divided into two batches for two sites. These sites were: Uplands Forest Station (1° 3S′; 36° 39′E and 2354masl) in Kiambu County; and Kiandongoro Forest Station (0° 27′S; 36° 50′E and 2,300 masl) in Nyeri County, both on Aberdares Ranges. Due to varying germination among the diverse provenances, not all were replicated in the two sites, while others were also not replicated in the four blocks in a given site. Deliberate attempts were made to have all the seedlots established even where the particular seedlot could not fill the plot of 25 seedlings. In such situations, the available seedlings were planted first in the inner 3 by 3 planting spots of the plot. The outer row was then planted with seedlings of other seedlots that had excess seedlings and clearly marked in the plot layout. The details of the seedlots are shown in Table 1.

The Uplands Forest Station trial had 18 seedlots which include four local seedlots and one seedlot from Tanzania, and Kiandongoro Forest Station trial had 17 seedlots including five local seedlots. A randomized complete block design was used at each site, with 25 trees plot^{-1} planted at 2 m by 2 m and replicated in four (4) block replicates. Only nine seedlots had enough seedlings to be replicated in all four blocks at both sites.

Tree heights (ht) and their diameters (dbh) were measured between 1 and 4 years in Uplands and between 1 and 6.5 years in Kiandongoro. Plot mean survival was also calculated. Some qualitative traits

Table 1. Details of seed sources in the provenance trials of *Casuarina junghuhniana* established at Uplands and Kiandongoro Forest Stations in April 1999.

Prov No.	Soruce	Latitude (° ′ N)	Longitude (° ′ E)	Altitude (masl)	Site							
					Kiandongoro				Uplands			
					I	II	III	IV	I	II	III	IV
18951	Mt Arjuno, East Java, Indonesia	07 45	112 35	1350	√	√	√	√	√	√	√	√
17878	Noelmina River, Timor, Indonesia	09 59	124 06	170	√	√	√	√	√	√	√	√
18846	Mt Pengalongan, Bali Indonesia	08 50	115 15	1500	√	√	√	√	√	√	√	√
19491	Buat Soe, Timor Indonesia	09 51	124 16	800	√	√	√	√	√	√	√	√
19237	Meru, Kenya	00 07	037 37	1750	√	√	√	√	√	√	√	√
18844	Mt Tapak, Bali Indonesia	08 45	115 15	1500	√	√	√	√	√	√	√	√
18954	Mt Bromo, East Java Indonesia	07 55	112 55	2500	√	√	√	√	√	√	√	√
18948	Mt Kawi, East Java Indonesia	07 55	112 5	2000	√	√	√	√	√	√	√	√
17877	25km SW Soe Timor, Indonesia	09 54	124 14	550	√	√	√	√	√	√	√	√
19239	KARI, Muguga Kenya	01 16	036 36	2060	√	√	√	-	√	√	-	-
19238	KEFRI Hqs, Muguga Kenya	01 13	036 39	2080	√	√	-	-	√	√	-	-
18847	East Batu Kawu, Bali Indonesia	08 40	115 05	1500	√	√	-	-	√	√	√	√
19240	Muka Mukuu, Kenya	01 05	36 39	1460	√	√	-	-	√	√	-	-
18952	Mt Willis, East Java Indonesia	07 50	111 47	1500	√	-	-	-	√	√	-	-
18848	At Abang, Bali Indonesia	08 55	115 25	1500	√	-	-	-	-	-	-	-
19241	Thika, Kenya	01 02	37 12	1440	√	-	-	-	-	-	-	-
17844	Old Uhak, NE Wetar Indonesia	07 36	126 30	5	-	-	-	-	-	-	-	-
19490	Camplong, Timor Indonesia	10 05	123 57	600	-	-	-	-	√	-	-	-
18853	Kwai Mission Tanga Tanzania	04 19	038 14	1600	-	-	-	-	√	-	-	-
19489	Kapan, Kupang Timor Indonesia	10 13	123 38	600	-	-	-	-	√	-	-	-
18845	Mt Pohen Bali Indonesia	08 40	115 05	2000	-	-	-	-	√	-	-	-

on tree stem and branch features were observed and scored. These included: stem axis persistence, stem straightness, permanent branches density, permanent branches thickness, permanent branches angle and permanent branches length (Pinyopusarerk et al., 2004). The two trials were assessed for health status including incidence and severity of pests and diseases in 2015 at the age of 16 years.

3 Results

3.1 Diameter growth

In Kiandongoro, diameter growth among the provenances differed significantly. The provenances with the highest diameter growth were 18948, 18848, 18844, 18951, 18952, 18846 and 19239 all attaining at least 14 cm mean dbh at 6.5 years (Fig. 1). The poorest performing provenances were 19241 and 19491 with mean dbh less than 10 cm. In Uplands at 4 years, the best performing provenances were 18846, 18847, 18844, 18951, 18954, 18952 and 19239 with at least 10 cm mean diameter. Poor growth was registered among 18853, 17878, 17877, 19237, 19489 and 19491 provenances with mean diameters less than 8 cm.

At 20 years, five of the provenances had suffered high mortality with very few trees remaining. As a result, they were not included in the analysis. Higher diameter growth was recorded in Uplands where trees were sparsely distributed among all provenaces than in Kiandongoro where there was minimal disturbances of the plots. In Uplands, seven of the provenaces had attained a mean diameter of 30 cm. These were 19239, 18954, 19238, 18948, 19240, 18846 and 18844 (Fig. 2). The least growth was recorded among provenaces 17878 and 19491 at approximately 25 cm mean diameter.

In Kiandongoro, where reduced growth was registered, mean dbh ranged from 15.9 cm to 27.4 cm. Among the provenances, only two (18846 and 18948) had attained a mean dbh of 26 cm (Fig. 2).

3.2 Height growth

In Kiandongoro, the mean height ranged from 8.2m to 18.4m at six and half years. The best provenances were 18848, 18948, 18844 and 18846 with mean heights of around 17m (Fig. 3). The least performers were 19489 and 19241 with mean heights less than 9m. In Uplands mean heights at 4 years ranged from 4.5 m to 8.5 m. The best performing provenances were 18846, 18844, 18847, 18847 and 18948 with mean heights above 8m (Fig. 3). The worst performance was recorded for provenances 19491, 17877, 17878 and 19489 with mean heights less than 5 m.

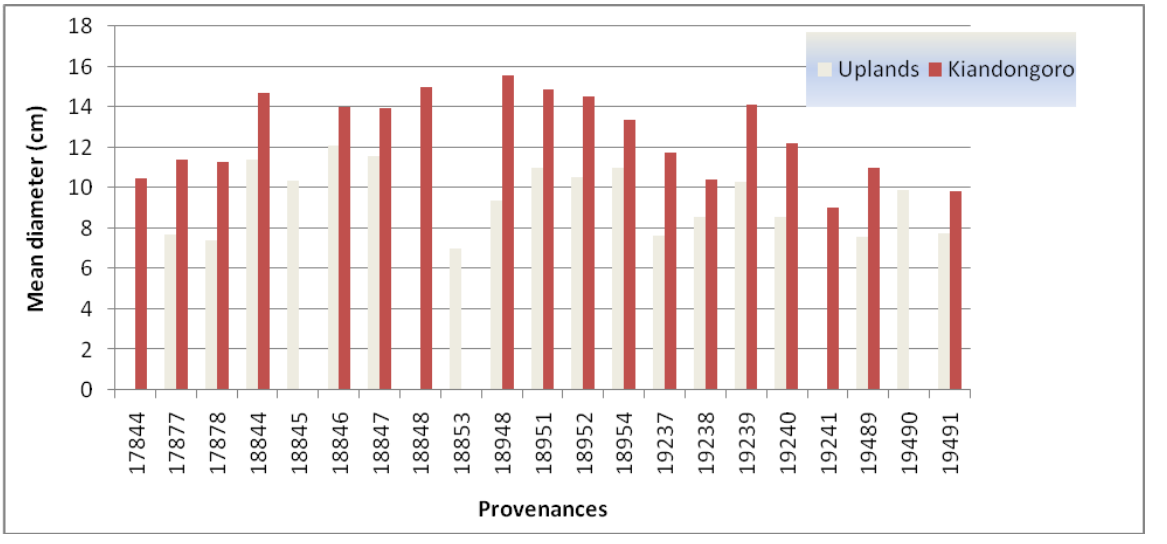

Fig. 1. Mean diameter growth of provenances at 4 years in Uplands and 6.5 years in Kiandongoro, Kenya.

In Kiandongoro at 20 years, the height ranged from 5.9 m to 22.2 m. The height growth pattern among the provenances generally remained stable relative to observations at 4 and 6.5 years with 3 out of 4 provenances still among the best performers (18846, 18948 and 18844). In Uplands, mean height ranged from 9.9 m to 20.7 m (Fig. 4). However, provenances that performed well in Kiandongoro also recorded above average height growth. For example, provenances 18948 (18.5m) and 18844 (17.1 m).

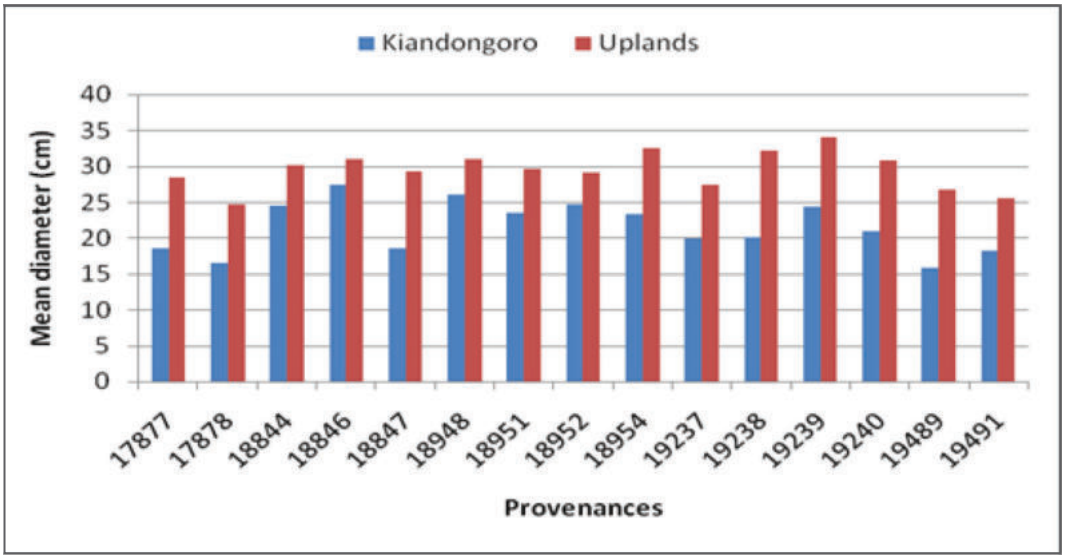

Fig. 2. Mean diameter growth of provenances at 20 years in Uplands and Kiandongoro, Kenya.

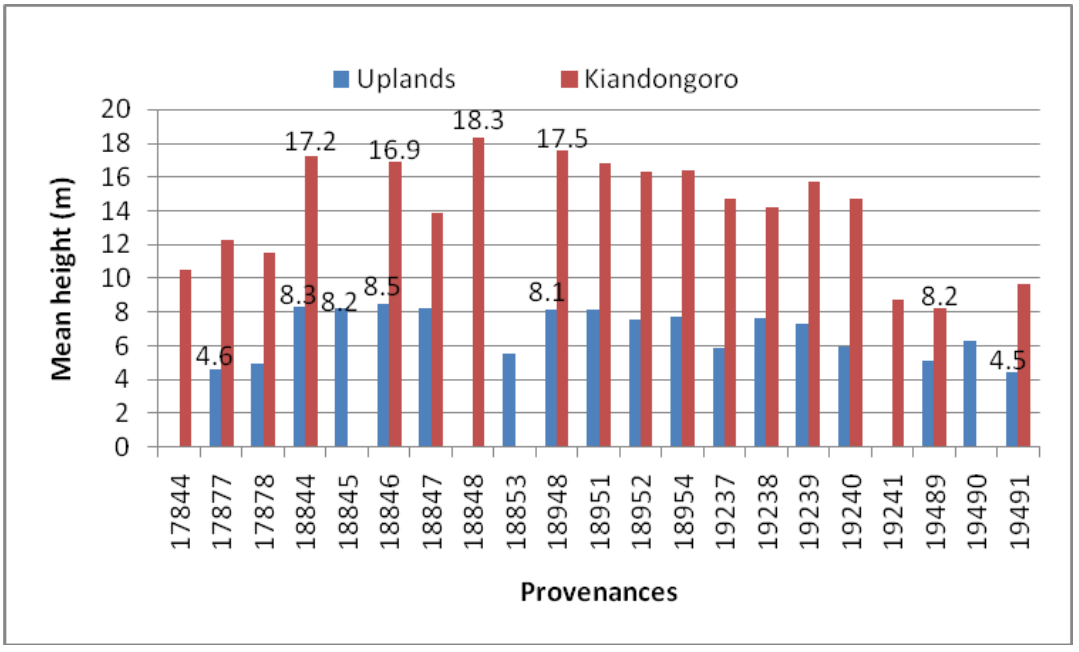

Fig. 3. Mean height growth of provenances at 4 years in Uplands and 6.5 years in Kiandongoro, Kenya.

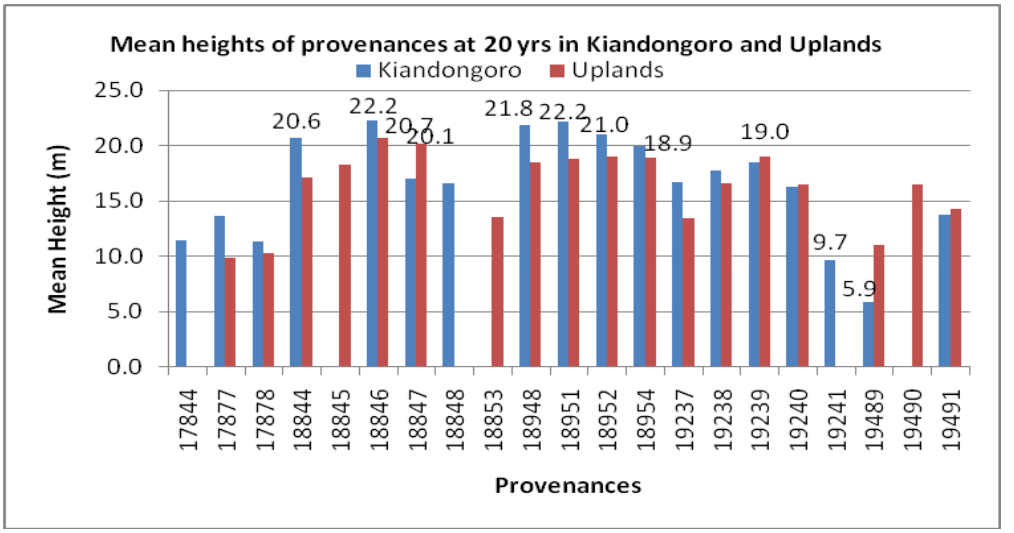

Fig. 4. Mean height growth of provenances at 20 years in Uplands and Kiandongoro, Kenya.

3.3 Survival of provenances

The survival rate of the provenaces was higher in Kiandongoro at 6.5 years than in Uplands which was assessed at four years (Fig. 5). In Kiandongoro, survival ranged from 60% to 93% among the provenances. A total of 14 out of the 18 provenances in the trial had survival rates over 70%. Uplands survival was lower, ranging between 26% and 86.7%, with only three provenances registering values higher than 70%.

3.4 Stem axis persistence, straightness and other qualititive parameters

In a score of 1-6, the following provenances were scored above a mean of 4: 18844, 18846, 18948, 18847, 18952 and 18954 in the two trial sites (Fig. 6). These provenances had no double leaders and generally no forking. In terms of stem straightness, there is very high variation among the provenances. The above

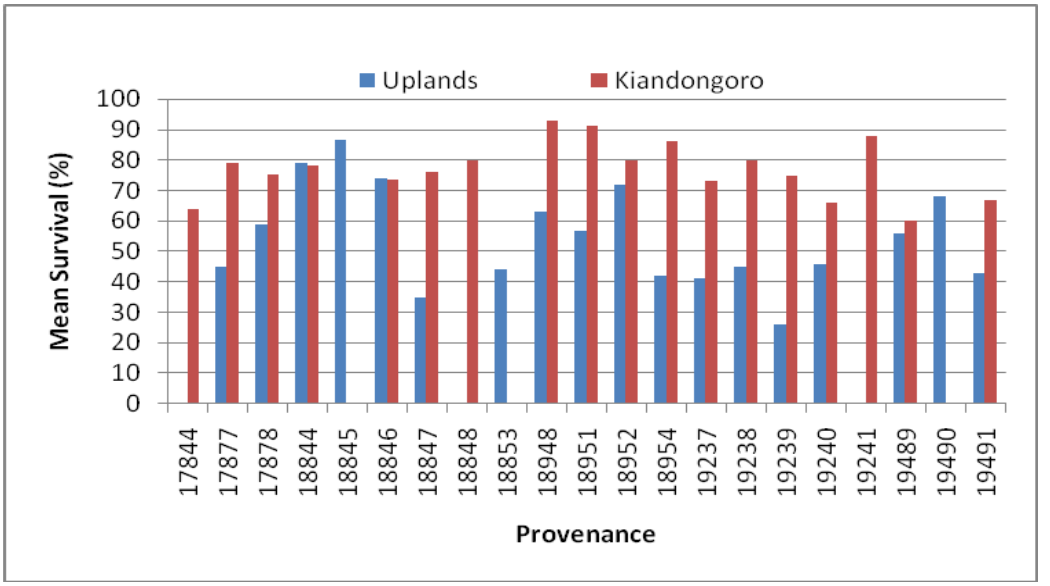

Fig. 5. Mean percent survival of provenances at 4 years in Uplands and 6.5 years in Kiandongoro, Kenya.

provenances recorded high values above 4 in 1-6 scale (Fig. 7).

In terms of branch density, provenances 18846 and 18954 had low values , a characteristic suitable for a tree targetted for interplanting with other crops. Among the provenances with high diameter and height growth, their branch length were above average with mean values between 1.8 and 2 (Fig. 8).

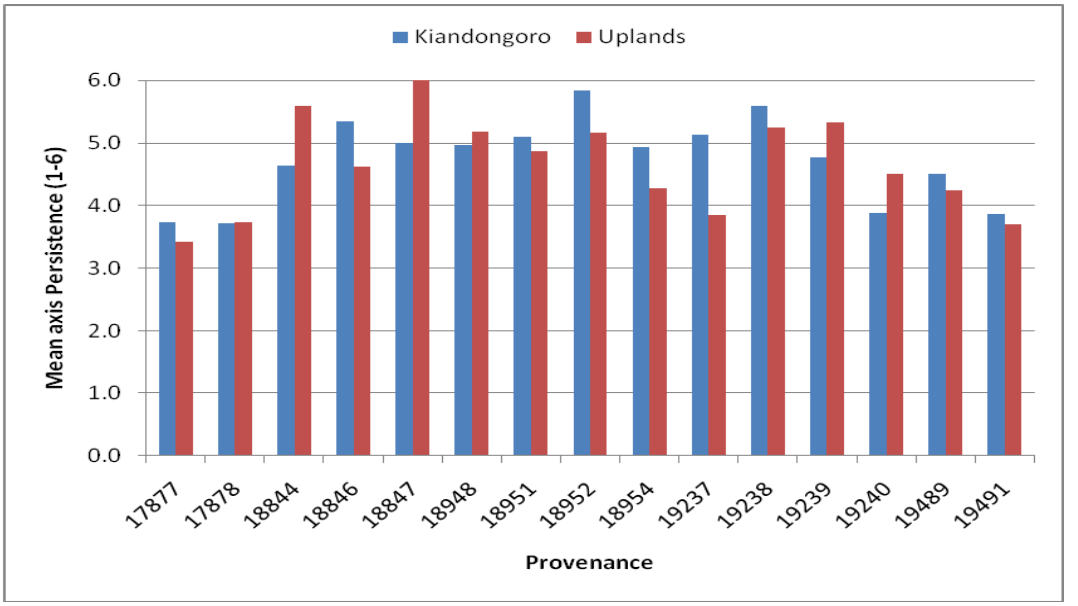

Fig. 6. Mean stem axis persistence among provenances at 20 years in Uplands and Kiandongoro, Kenya.

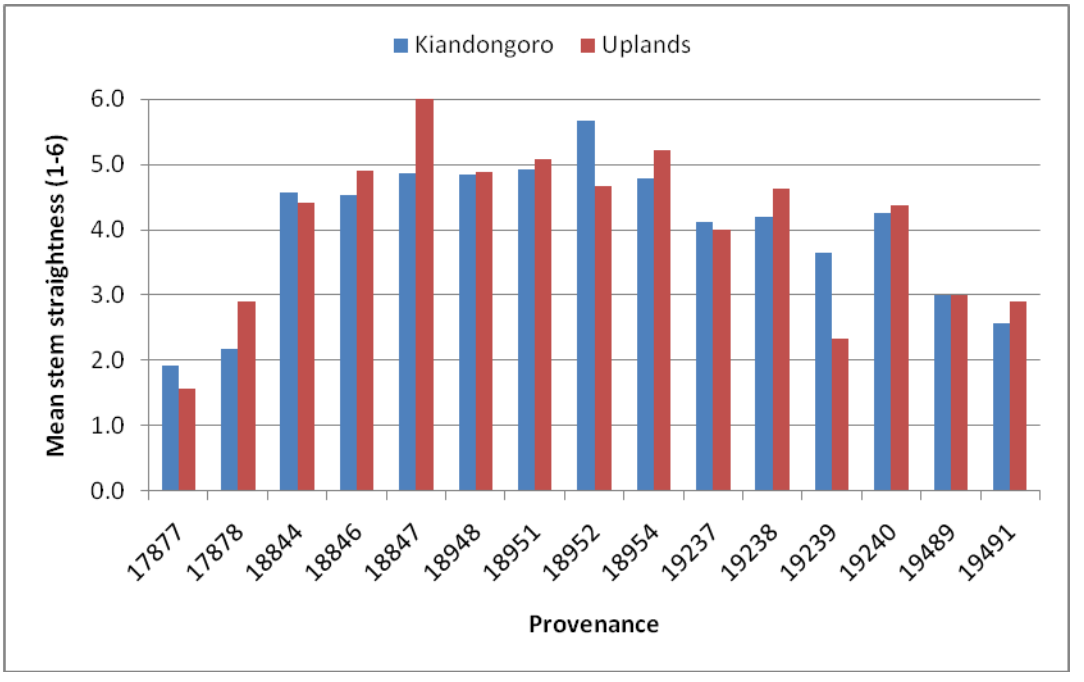

Fig. 7. Mean stem straightness of provenances at 20 years in Uplands and Kiandongoro, Kenya.

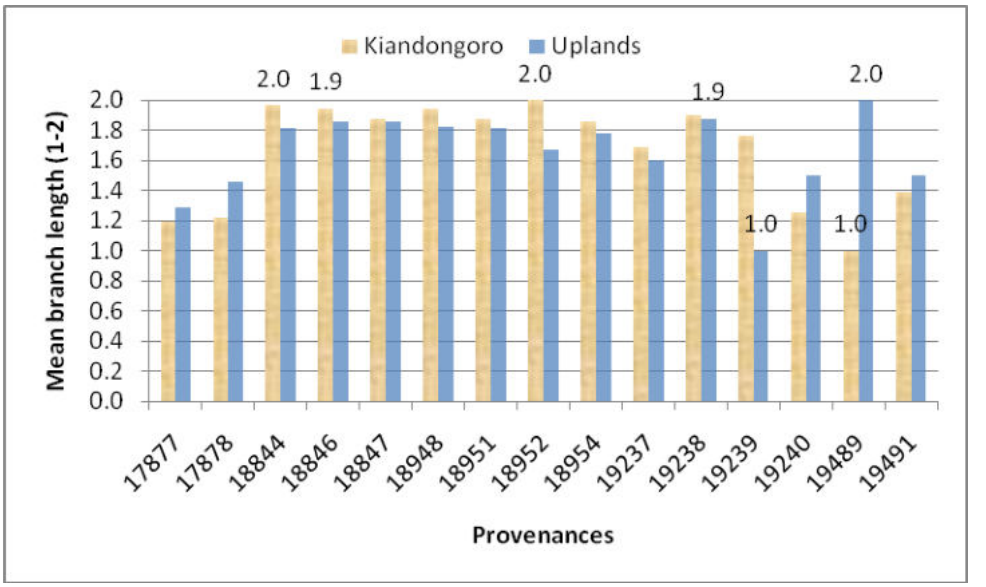

Fig. 8. Mean branch length of provenances at 20 years in Uplands and Kiandongoro, Kenya.

4 Discussion

The results indicated marked variation among populations of *C. junghuhniana* in many growth and morphological characteristics. From the results of the trial across the two sites, it has been observed that there is a close relationship between performance and altitude of source of any given provenance. There was a tendency for provenances from high altitudes to grow better than those from low altitudes. Provenances and land races from the lowlands were poorly adapted to the two trial sites that were above 2,300 m above sea level. These results are similar to those obtained from a provenance trial established in Tanzania with the similar seedlots. In Tanzania, high altitude provenances from Java, Bali and Lombok outperformed low altitude provenances from Wetar and Timor (Mwihomeke et al., 2002). The trial site in Tanzania was located at 1,500 m altitude. These results, also observed by Pinyopusarerk et al. (2005) in a wider set of trials, thus suggested a possible site-by-provenance interaction for *C. junghuhniana*. The land race from Tanzania may have performed poorly either because of inbreeding over successive generations associated with a possibly-narrow original range of introductions, because of its lowland origins, or both.

The trial revealed that there was considerable variation not only in growth performance but also in stem form and the branching habit. However, majority of the provenances with good growth had straight stems and light branches.

5 Conclusion

These trials demonstrated that there is great potential to use *Casuarina junghuhniana* as a commercial species in highlands of Kenya. However, considering the high variation among all provenances, selection of individual trees among the best performing provenances and land races is necessary to optimize plantation productivity for particular planting sites.

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Proceedings of the Sixth International Casuarina Workshop

Gains after one generation breeding cycle of *Casuarina junghuhniana* in Thailand

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Abstract

This paper reports gains in the growth performance of *Casuarina junghuhniana* after one generation of breeding in Thailand. Genetic materials included open pollinated seed from seedling seed orchard (SSO), seed production area (SPA) and two wild provenances (Wetar and Java) which had been planted in the first-generation breeding population/progeny trial. Of the SSO seed, two different bulks were made; one consisted of the top 30 trees ranked by breeding value (SSO BV) and the other consisted of the top 30 trees ranked by volume index (SSO PV). The SPA was developed through conversion of a provenance trial. Thus in total five seed sources of different genetic composition were compared. Field trials were established at three locations with different soils and climates. The trials were assessed for survival, height and diameter at breast height (DBH) at the age of 26 to 30 months after planting. An analysis of variance showed significant differences in growth traits among seed sources across the three sites. There were also genotype x environment interactions for height and DBH but not survival. Survival of plants of Java origin was consistently poorer than plants from the other sources while the differences among the other sources were generally not significant. Despite the significant interaction effects, SSO and SPA seed consistently outperformed unimproved wild provenances in height and DBH in the order SSO>SPA> Wetar> Java. Based on mean data across three sites, SSO BV and SSO PV were 54 and 52% respectively taller than Java, and 56 and 50% respectively larger in DBH. The results indicate significant gains in growth after one generation of breeding.

Keywords: *Casuarina junghuhniana,* genetically improved seed, genetic gain, growth, survival

1 Introduction

Casuarina junghuhniana was first introduced into Thailand for provenance trials in 1997-1998 (Pinyopusarerk et al., 2005). The species has shown promising growth for plantation development and

subsequently genetic materials from the species' natural distribution range in Indonesia and land races in Africa were introduced for assessment of genetic variation and establishment of seed orchard (Luechanimitchit et al., 2011). After completion of one breeding cycle, seed orchard seeds are now available for commercial deployment. It is often assumed that genetically improved seed from seed orchards will be more productive that those from unimproved materials. In order to quantify the success of the improvement programme, the superiority of seed orchard seeds must be evaluated relative to the original population sources. Such comparisons must be conducted under similar environmental conditions.

The objective of this study was to determine the increase in wood yield of seed from first generation seedling orchard in comparison with original wild provenances of *C. junghuhniana.*

2 Materials and methods

2.1 Plant materials

Open-pollinated seed from five populations were used to raise seedlings for field trials. These were:

(1) SSO PV. Seeds were bulked from the top 30 trees in a first generation SSO based on volume index data

(2) SSO BV. Seeds were bulked from the top 30 trees in the same SSO above but ranking based on breeding value calculated from volume index data. Fifteen trees in this seedlot were common in the SSO PV.

(3) SPA. Seeds were bulked from 15 outstanding trees in a provenance trial which has been converted to a seed production area.

(4) Wetar, Maluku Province, Indonesia. A wild provenance (CSIRO seedlot 17844) which was originally included in previous provenance trials and progeny trials in Thailand. It is considered the best provenance in Thailand.

(5) Java, central Indonesia. A wild provenance (CSIRO seedlot 18950) which was originally included in previous provenance trials and progeny trials in Thailand. It is considered a poor performing provenance in Thailand.

Seedlings were raised at a common nursery. They were five months old at the time of field planting.

2.2 Planting sites

The gain trials were established on three sites varying in soil and climate conditions in Thailand (Table 1).

2.3 Experimental design and layout

All trials were planted using randomised complete block design with 4 replicates, 49 (7x7) seedlings plot^{-1}. Spacing was 2 m x 2.5 m at Kanchanaburi and Lad Krating but 3 m x 3 m at Ratchaburi. One guard row of mixed provenances was planted surrounding each experiment. The experimental areas were disc ploughed twice prior to planting.

Table 1. Locations of *C. junghuhniana* gain trials in Thailand.

Site	Altitude (m)	MAR (mm)	No. dry months	MAT (°C)	Soil
Kanchanaburi (KB)	63	1031	4	28.7 (23.3-34.0)	Sandy loam, pH 6.3-6.8
Lad Krating (LK)	36	1875	3	27.0 (22.3-33.0)	Sandy loam, pH 4.1
Ratchaburi (RB)	49	1244	4	28 (23.0-33.0)	Sandy loam, pH 4.5-5.5

NB. MAR = mean annual rainfall; MAT = mean annual temperature (mean minimum and mean maximum in brackets). Monthly rainfall less than 40 mm is regarded as dry month.

2.4 Assessment and analysis

The trials were assessed for survival (S), height (H) and diameter at breast height (D) at the age of 26 months after planting at Kanchanaburi, 27 months at Lad Krating and 30 months at Ratchaburi.

Analysis of variance of trial data was implemented in Genstat version 16 (VSN International, Hemel Hempstead, UK). Least significant difference ($P<0.05$) was calculated to determine statistical differences among pairs of means.

3 Results and discussion

There were significant differences among seed sources ($P< 0.05 - 0.001$) in survival, height and diameter at breast height at each trial site.

In general, seed from genetically improved sources such as seedling seed orchard and seed production area performed consistently better than wild provenances from Wetar and Java in both survival and growth. SSO sources were also better than SPA likely due to more representations and intensive selection of genetic materials from which they were derived.

Seed from Java provenance was clearly the poorest in all growth parameters. This Java provenance was was found to be among the slowest growing provenances in initial provenance trials (Pinyopusarerk et al., 2005). The magnitude of the differences in height and diameter between plants derived from SSO seed and Java seed was greater than 50%.

As for plants derived from the SPA and Wetar provenance, although growth of SPA plants was never poorer than that from Wetar their differences were relatively small. This SPA was converted from a provenance trial in which superior individuals were retained during the heavy thinning process. As the best overall provenance in the trial, it is evident that a higher proportion of superior individual trees retained in the SPA belong to the Wetar provenance. Therefore, it would be unsurprising if the majority of the 15 trees bulked up in the SPA have a Wetar origin. This would explain the small difference in performance of plants from SPA and Wetar seed sources in these trials.

Differences between SSO PV and SSO BV were small and not significant. Although diameter growth of SSO BV was never smaller than that of SSO PV, there was no clear pattern in the differences in survival and height. The results suggest that it is not warranted to invest in resources to determine the breeding value. It is reasonable to assume that

Table 2. Mean survival, height and diameter at breast height in three *C. junghuhniana* gain trials. Seed sources differed significantly at $P<0.001$ except for survival at Lad Krating ($P<0.05$) and Ratchaburi ($P<0.01$).

Trial site	SSO PV	SSO BV	SPA	Wetar	Java	LSD (5%)
Survival (%)						
Kanchanaburi	88	89	86	92	38	21
Lad Krating	63	59	62	49	28	23
Ratchaburi	80	82	69	74	46	18
Height (m)						
Kanchanaburi	7.3	7.3	6.7	6.4	5.0	0.23
Lad Krating	5.9	6.2	5.8	5.5	4.0	0.28
Ratchaburi	7.9	7.8	7.2	7.2	4.7	0.28
Diameter at breast height (cm)						
Kanchanaburi	6.3	6.4	5.7	5.2	4.3	0.27
Lad Krating	4.7	4.9	4.5	4.1	3.1	0.27
Ratchaburi	5.1	5.4	4.9	4.8	3.2	0.25

selection of top ranking superior individual trees based on phenotypic values, e.g. volume index in this case, is sufficient to provide high quality genetic materials for *C. junghuhniana.*

4 Conclusion

Significant gain after one generation of breeding is clearly demonstrated for *C. junghuhniana.* The results are encouraging for large scale plantation development.

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Early growth performance of *Casuarina* clones as an alternative tree crop in eucalyptus growing areas of Karnataka State, India

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Abstract

Eucalyptus is the most preferred choice of species for planting in low rainfall (<700 mm) and degraded soil conditions in south India. In particular large area of government owned land is managed under eucalyptus cultivation by forest development corporations of different States. The wood produced from these plantations is mainly used as pulpwood for papermaking. Cultivation of eucalyptus is banned in Karnataka State since 2017 due to ecological concerns about monoculture involving a few clones in large areas. Following this, pilot plantations of casuarina clones were taken up in selected locations to assess their suitability for raising large scale plantations in areas previously planted with eucalyptus. This paper reports the early performance of three casuarina clones in nine locations of the State planted during 2017 and 2018. In low rainfall areas, the average height of one-year-old plant was 2 m and that of two-year-old was 4 m. In one location where the rainfall (>700 mm) and the number of rainy days were higher than rest of the locations, the height was 3 and 5 m during first and second years respectively. The height was substantially higher in two plantations located in nursery area and given protected watering (7 m at the age of two years). In general the interspecific hybrid (*Casuarina equisetifolia* x *C. junghuhniana*) clone IFGTB-CH-5 performed better than the other two clones, IFGTB-CJ-9 and CJ-01. Based on the early results observed in the pilot plantations, the strategies for deploying casuarina clones as an alternative to eucalyptus in dry and degraded areas are discussed.

Keywords: *Casuarina* hybrid clone, degraded land, low rainfall, growth, pilot plantations

1 Introduction

India is a world's largest eucalyptus planting country with an estimated 6.6 million hectares under its planting. It has a high socio-economic and environmental importance and a large number of farmers' livelihood is depended on eucalyptus cultivation particularly in the dry areas receiving an annual rainfall of <700 mm. Nearly two thirds of the pulpwood raw material need of the Indian paper industry is met from eucalyptus plantations. Large areas of government owned land is managed under eucalyptus cultivation by forest

development corporations of different States. Cultivation of eucalyptus is banned in Government-owned lands of Karnataka State since 2017 due to ecological concerns about monoculture involving a few clones in large areas (Anon, 2017; 2019). This sudden halt of the most dependable tree crop has affected the afforestation programmes in dry areas since not many trees are known to grow and yield commercially viable wood production in those areas. Since casuarina has a stable market as pulpwood and poles, it is the first choice of consideration as an alternate candidate for planting in former eucalyptus areas. This paper reports preliminary results from the field trials and pilot plantations raised in semi-arid regions of Karnataka State to evaluate its suitability for such low rainfall conditions.

2 Materials and methods

The performance of casuarina clones discussed in this paper has been sourced from the following three kinds of plantations located in different parts of the Karnataka State in India.

2.1 Genetic gain trial

This trial was established with five clones and one seedling accessions at Kunchenahalli, Shimoga District, Karnataka (14°02'22.9" N; 75°33'43.7" E, 705 m asl). The site of the trial was previously under eucalypt plantation. This area receives an annual rainfall of 750 mm and has a red loamy soil. Of the five clones, four (CH1, CH2, CH4 and CH5) were interspecific hybrids between *C. equisetifolia* and *C. junghuhniana* and the other (CJ9) belong to *C. junghuhniana*. The seedling accession was raised from seeds collected in a second-generation seedling seed orchard of *C. junghuhniana*. The trial was established in a randomized block design with 10 replications each having 49 trees accession $^{-1}$ planted at a spacing of 3 x 1.5 m. Land was cleared of previous growth and ploughed twice. Pits of the size of 30 cm x30 cm x30 cm were dug and clonal plants / seedlings planted during the monsoon rains in the month of July 2017. The plot was manually weeded twice after planting and raised under rainfed conditions. Height and diameter at breast height was measured for all trees at the age of 12 months. The data was subjected to ANOVA to determine the level of significance of differences between the accessions.

2.2 Clonal trial

A clonal trial was established with three clones (CH1, CH4 and CH5) to evaluate their performance under irrigated conditions. The trial was planted in the nursery area of Karnataka Forest Development Corporation (KFDC) at Kalenahalli (14° 15'15.5" N, 75°19'18.2" E, 620 m asl), Shimoga District, Karnataka during 2017. The planting site has black loamy soil and receives an annual rainfall of 760 mm. The trial was planted in a randomized block design with four replications each having 25 trees clone^{-1} planted at a spacing of 1.5 x 1.5 m. Planting and maintenance were carried out similar to the genetic gain trial described above except that irrigation was provided once in a week during the first year after planting. Height and diameter at breast height were measured for all trees at the age of 18 months. Data was subjected to ANOVA to understand the level of significance of differences between the accessions.

2.3 Pilot plantations

Seven pilot plantations of casuarina clones raised by the KFDC during 2017 and 2018 were assessed for height growth during April 2019. All these sites were under the cultivation of eucalyptus for several rotations. Plants of the five clones used for establishing the genetic gain trial were used for raising these plantations extending from ha 5 to 34 ha in each location with a rainfall of 600 mm to 720 mm. Field planting was carried out during the south west monsoon (June to September) and grown under rainfed conditions in all the locations.

3 Results and discussion

ANOVA showed significant ($p<0.05$) variation among the accessions in the genetic gain trial. The mean height and diameter at 12 months age was 3.29 m and 1.92 cm respectively (Table 1). This is an encouraging growth obtained under rainfed conditions. The seedling accession showed the best growth of 3.58 m height and 2.39 cm diameter. All interspecific hybrid clones showed a growth on par with that of the seedling while the pure species clone CJ9 recorded the lowest growth. It has been widely observed that casuarina seedlings grow faster than clones during first one or two years as observed in the genetic gain trial (Santhakumar et al., 2001; Warrier et al., 2010). Since *C. junghuhniana* is more drought tolerant than *C. equisetifolia*, the

performance of the pure species clone CJ9 under rainfed conditions needs to be observed in the coming years.

The effect of irrigation on the growth of casuarina hybrid clones was evident in the clonal trial at Kalenahalli nursery. The average height was 8.1 m and diameter 4.46 cm at 18 months showing the high potential of these clones (Table 2). There was no significant difference in the growth among the three clones tested in this location. Clonal tests conducted in other locations generally showed clones CH1 and CH5 growing faster than CH4 (Nicodemus et al., 2020). Clonal differences in growth may be more pronounced in the later years.

Pilot plantations raised in low rainfall areas showed an average height of 2 m at the age of nine months and 3 m at 21 months (Table 3). In two locations where the rainfall is 700 mm and above, the height was 5 m at 21 months age. The observed initial growth is promising

Table 1. Growth of clonal and seedling accessions at 12 months of age in a genetic gain trial of casuarina at Kunchenahalli, Shimoga, Karnataka State, India.

Accession	Height (m)	DBH (cm)
CH1	3.06	1.68
CH2	3.48	2.07
CH4	3.44	1.90
CH5	3.32	1.89
CJ9	2.93	1.61
Seedling	3.58	2.39
Mean	3.29	1.92
LSD ($p<0.05$)	0.214	0.181

Table 2. Growth of casuarina clones at 18 months age in Kalenahalli, Karnataka State, India.

Accession	Height (m)	DBH (cm)
CH1	7.9	4.59
CH4	8.1	4.33
CH5	8.3	4.48
Mean	8.1	4.46
SED	0.328	0.166
F-prob	0.586^{NS}	0.348^{NS}

Table 3. Details of pilot plantations of casuarina clones raised in Karnataka State, India.

Location and extent of plantation	Latitude / Longitude	Rainfall (mm)	Year of planting	Age (months)	Height (m)
Chakanakatte;20 ha	13°42′16.2″ N 76°23′70.1″ E	600	2017	21	3
Kamenahalli; 34 ha	13°26′54.4″ N 75°50′29.5″ E	700	2017	21	5
Salur; 25 ha	14°13′22.4″ N 75°18′50.0″ E	600	2017	21	2.5
Bikonahalli; 6.9 ha	14°00′12.3″ N 75°58′70.1″ E	720	2017	21	5
Dodda Metikurke; 20 ha	13°27′34.3″ N 76°24′13.5″ E	600	2018	9	2
Sindhigere; 29 ha	13°21′09″ N 75°55′11″ E	600	2018	9	2
Jambur; 5 ha	14°18′02.3″ N 75°17′25.7″ E	720	2018	9	2

to consider casuarina as an alternative to eucalyptus. However, the low rainfall and the short duration of rainy season (60 to 70 days), necessitate testing more number of accessions drawn from genetically diverse germplasm for effective matching between site and planting material. Considering their good performance in the genetic gain trial seedling-raised plants from genetically improved seeds of *C. junghuhniana* should be included in all the future tests. Their drought-tolerance and coppicing ability are valuable traits while selecting an alternate tree for eucalyptus planting areas in semi-arid zones (Nicodemus, 2003; 2015). Further research is also needed for soil and water conservation methods to increase the survival and growth in the casuarina plantations.

4 Conclusion

The early growth of casuarina seedling and clonal accessions in semi-arid areas of Karnataka State is promising to be considered as an alternative tree to eucalyptus. Multilocation testing of more clonal and seedling accessions from a broad genetic base is warranted to select site-specific planting material for former eucalyptus growing sites in semi-arid areas.

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Evaluation of growth and wood traits in clones from superior seedlings and coppiced plus trees of *Casuarina junghuhniana* in southern India

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Abstract

Twenty-eight superior trees selected from a three-year-old *Casuarina junghuhniana* plantation of seed orchard origin were evaluated in a clone trial along with 159 cloned seedlings of the same seedlot maintained in hedges. There was no significant difference in mean growth (height and diameter) between the two groups of clones at 24 and 48 months of age whereas clones varied significantly within each group. Clones originating from three-year-old coppiced trees had 12% higher overall survival (90%) and 0.4% higher absolute pulp yield (43.7%), but comparable wood density (636 kg m^{-3}) and stem straightness at 48 months. Significant improvement in growth and stem straightness was obtained by selecting the top ten clones of seedling origin compared to top ten clones from coppiced trees. Clonal repeatability ranged from 0.57 for survival to 0.67 for wood traits and 0.72 for growth at 48 months. There were more female clones, with a female to male ratio of 2.8 at 48 months.

Keywords: seed orchard, coppice, pulp yield, wood density, clones

1 Introduction

Industrial plantations of casuarina are a major source of wood for the pulp and paper industry in India. The demand for quality planting stock of *Casuarina junghuhniana* has increased in recent years due to attributes like tolerance to drought, blister bark disease and good coppicing ability relative to *C. equisetifolia* (Kumar et al., 2016). Improved seed of *C. junghuhniana* and *C. equisetifolia* was supplied to farmers from first generation seedling seed orchards established from natural provenances as per a breeding program implemented in southern India (Pinyopusarerk et al., 1996; Kumar et al., 2016). Seed is widely used by farmers for raising high density plantations in rotations of three to four years, while clonal plantations are promoted by the industry for enhancing wood yield. A hybrid clone (*C. junghuhniana* x *C. equisetifolia*) is grown in the eastern coastal regions of peninsular India, but it is not suited to dry inland regions. Clones of *C. junghuhniana* and interspecific hybrids are being developed by different agencies to suit dry inland regions of southern India (Kannan et al., 2016).

Phenotypically superior trees identified in plantations are generally cloned and evaluated at specific sites. The source or origin of the seedlot is

often not maintained when trees are selected from farmlands which often comprise a narrow range of genetic material, resulting in a narrow genetic base, as seen in commercial *Eucalyptus* clones deployed in India (Kulkarni, 2005). A casuarina improvement program was initiated by ITC Ltd by introducing native germplasm of *C. junghuhniana* from CSIRO, Australia and accessing improved seed from first generation seedling seed orchards established in inland region (Varghese et al., 2004; Nicodemus et al., 2016). As there are not many clones available for commercial deployment in dry arid regions it is necessary to fast track the clone development scheme for supply to farmers. The normal procedure for clone development involves selection of phenotypically superior trees in seedling origin plantations, cloning, and evaluation of clones, which takes about eight years. Though *C. junghuhniana* has better coppicing ability than *C. equisetifolia,* coppice production is quite uneven in arid regions and it is necessary to ensure that felling is taken up immediately after rains for coppicing. Some outstanding trees may not coppice or produce insufficient shoots even with supplementary irrigation. A study was therefore taken up to test the hypothesis that cloning improved progeny from seed orchards at nursery stage can be an alternative to the conventional scheme of cloning superior trees from plantations. This paper summarises the performance evaluation of cloned seedlings at an arid site in comparison to outstanding trees of same seedlot cloned from a plantation at the same site. The study discusses the scope of reducing the time taken for clone development, and enhancing gain after a generation of breeding, by cloning the best progeny from seed orchards of *C. junghuhniana.*

2 Material and methods

2.1 Genetic material

Seed procured from a *C. junghuhniana* seedling seed orchard located in the inland region of Tamil Nadu was raised at an ITC (LSTC) field station at Mettupalayam (latitude 11°19′ N, longitude 77°56′ E, altitude 426 m asl and 760 mm annual rainfall) in Tamil Nadu, India. The seedlings were graded in the nursery, and the top 200 seedlings selected at six months were planted in soil and pruned to hedges of 20 cm height. The remaining seedlings were established in a 3-acre plot in the field station at a spacing of 3 m between rows and 1.5 m between plants within a row. The plantation was evaluated for growth at three years and 50 phenotypically superior trees, in terms of growth and stem form, were selected for coppicing and cloning. Stem cuttings from coppiced trees and seedling hedges were rooted in a mist chamber following standard procedure, and raised in root trainers.

2.2 Evaluation of clones

A clone trial was established at the field station to evaluate clones from 28 coppiced plus trees (CC) and 159 seedling clones (SC) in randomised complete block design with seven replications in single tree plots. The clone trial was maintained weed free following standard silvicultural practices. Fertilizer application (NPK 17:17:17, 25 g plant^{-1}) was made annually to coincide with rains during July-August. The trees were evaluated for survival, growth (height and dbh), and stem straightness (St str, scale of 1-4) at 24 and 48 months. Two increment core samples (5 mm) were extracted at breast height from each tree at 48 months. One core sample was used to determine wood density using the water displacement method and the second sample was used to estimate pulp yield (PY) using a NIR (near infrared reflectance) calibration curve developed for *C. junghuhniana* (Ramadevi et al., 2011). The sex of each tree was assessed at 48 months based on visual observation of male inflorescence and female cones.

2.3 Statistical analysis

The values for height, dbh, stem straightness, wood density and pulp yield of each tree were subjected to analysis of variance using the following linear mixed model:

$$Y = \mu + REPL + GROUP + CLONE + RESIDUAL$$

where Y is the vector of observations, μ is the trial mean, REPL and GROUP are replicate and group effects fitted as fixed factors, CLONE within group is the vector of clone effects fitted as random factor, and RESIDUAL is the vector of residual effects remaining after fitting the model. Analysis was performed using the REML directive in the statistical software package Genstat Release 9 (VSN International Ltd, United Kingdom). Data analysis (ANOVA) was done to evaluate the performance of the two groups of clones and variation

between clones within each group.

The analyses for all traits were then repeated with groups omitted from the model, to obtain best linear unbiased predictions for all individual clones. Clonal repeatability (CR) of each trait was calculated using the formula $CR = \sigma_{clone}^2/(\sigma_{clone}^2 + \sigma_e^2)$, where σ_{clone}^2 and σ_e^2 are the clone and residual variance components respectively.

3 Results

3.1 Growth and stem form

At 24 and 48 months, there was no significant difference between the two groups – coppice origin (CC) and seedling (SC) clones in height and dbh (Table 1). There was however a significant difference between clones within each group. The CC clones had 7 and 12% higher overall survival (24 and 48 months respectively) compared to the SC clones but clones differed significantly within each group. The SC clones had better stem form at 24 months, but at 48 months there was no significant difference between the two groups of clones. When the top ten clones of the two groups were selected, the top ranking SC were significantly superior to the top ten CC clones in height (3%) and dbh (8%) at 24 and 48 months. Survival (%) of the top ten SC was better but not significantly different at both assessment ages than the CC. The top ten SC clones had better stem straightness (5%) than the top 10 CC clones. There were more female clones with a female: male clone ratio of 2.8.

3.2 Wood density and pulp yield

Wood density at 48 months was at par (636 and 639 kg m-3) in the two groups of clones but there was a significant difference between clones in each group. Pulp yield was generally low (around 43%) and only marginally higher (0.4% absolute difference) in the CC clones compared to SC clones. The trend was more or less same in the top 10 clones of each category. The top ten CC clones had almost same wood density (about 1% difference) and PY as the top SC clones.

3.3 Clonal repeatability (CR) and correlation of traits

CR was high for height and dbh (0.67 - 0.73) and stem straightness (0.75 and 0.79) at both assessment ages

Table 1. Mean values of growth and wood traits in *C. junghuhniana* clones.

Source	Ht 24 (m)	Dbh 24 (m)	Ht 48 (m)	Dbh 48 (cm)	Survival 24 (%)	Survival 48 (%)	St str 24 (1-4)	St str 48 (1-4)	Density (kg m^{-3})	PY (%)
Seedling clones (SC)-All	8.56	7.09	12.25	9.59	88.5	80.1	3.52	3.65	639.1	43.3
Coppice clones (CC)-All	8.55	7.06	12.33	9.73	94.6	89.8	3.34	3.59	636.4	43.7
SC- top10	9.40	8.51	13.31	11.39	96.7	96.7	3.47	3.62	630.0	43.4
CC- top10	9.08	7.78	12.94	10.54	95.0	95.0	3.31	3.43	638.9	43.9
Site mean	8.56	7.08	12.26	9.61	89.4	81.7	3.49	3.64	638.6	43.4
p_{source}	0.927^{ns}	0.800^{ns}	0.424^{ns}	0.308^{ns}	0.007	<0.001	<0.001	0.163^{ns}	0.464^{ns}	0.013
SE_{source}	0.062	0.115	0.102	0.145	2.28	2.76	0.042	0.044	3.64	0.153
p_{clones}	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
SE_{clones}	0.447	0.832	0.732	1.047	16.45	19.91	0.299	0.318	26.2	1.10

(Table 2) whereas it was comparatively low for survival (0.57). CR was around 0.67 for both wood density and PY. The phenotypic correlation between growth and wood traits was similar in male and female clones (Table 3). There was high correlation between height and dbh at 48 months (0.67 and 0.7) whereas correlation between growth and wood traits (density and pup yield) was quite low in clones of both sexes.

4 Discussion

Casuarina was formerly considered a crop suited to light soils of coastal regions. With domestication there is greater acceptance of *C. junghuhniana* in dry regions normally suited to eucalypts. *Casuarina junghuhniana* is seen to have better stem form in inland sites than in coastal regions (Kannan et al., 2016). Productivity of *C. junghuhniana* has increased with deployment of improved orchard seed which can further be enhanced by developing suitable clones (Kumar et al., 2016).

4.1 Clones for arid sites

Candidate trees are identified for cloning by intensive selection from plantations, considering growth, stem form and wood traits for different end uses. There is greater chance of getting superior clones when outstanding trees are selected from improved progeny of known pedigree or origin at a suitable age when the traits are expressed (Nicodemus et al., 2011). A rigorous clone evaluation process is however essential for clone development since the correlation between performance of the ortet (seed origin) and clonal ramets is low (Luechanimitchit et al., 2016) as reported in *Eucalyptus* (Reis et al., 2011).

Performance evaluation of seed crop, preferably a progeny trial or genetic gain trial is quite essential to start any cloning program as it not only gives information on the suitability of the crop to the site but also helps to identify the provenance, maternal and paternal effects of different traits. However, this testing is done with small populations (Luechanimitchit et al., 2016) which can be the basis for establishing large plantations from which genotypes can be screened for cloning. Genetic improvement programs of *Casuarina* recognise the need for separate breeding populations in arid and coastal locations (Kumar et al., 2016), as seed crops produced from inland and coastal orchards can be quite different due to fertility difference and adaptability of parent trees to the site (Varghese et al., 2011). A seed orchard crop from an inland site was the base material for the current study which ensured high survival and adaptability of the tested clones in dry inland conditions. Moreover, the top 11% (20 clones) were screened from 187 clones tested for adaptability and wood characteristics.

4.2 Genetic improvement for clone development

A breeding program with large number of families of suitable provenances will be required to ensure a wide genetic base in clones. A seed crop of limited diversity from a seed orchard of bulked provenance seedlots at an inland site (Varghese et al., 2004) was the genetic

Table 2. Clonal repeatability of traits in *C. junghuhniana* clones.

Ht 24	dbh 24	Ht 48	dbh 48	Survival 48	St str 24	St str 48	Wood Density	PY
0.713	0.677	0.735	0.721	0.578	0.756	0.792	0.673	0.667

Table 3. Phenotypic correlation of height, dbh, density and PY in male (above diagonal) & female (below diagonal) clones at 48 months.

Ht 48	*	0.672	-0.009	0.193
Dbh 48	0.704	*	-0.057	0.146
Den 48	-0.044	-0.062	*	0.012
PY 48	0.197	0.244	-0.045	*

base of the clones evaluated in the current study. Family seedlots of suitable provenances from Timor and Wetar regions of Indonesia should be introduced and tested to improve stem form and productivity. Small effective population size in orchards would reduce seed crop diversity and increase relatedness in progeny causing rapid genetic deterioration, as reported in *C. junghuhniana* landraces (Pinyopusarerk et al., 2005). Stem straightness, which is a concern in *C. junghuhniana* plantations, has improved in the tested clones compared to F_1 orchard progeny as reported earlier (Varghese et al., 2011). Variation in wood density and pulp yield (mean value, 638 kg m^{-3} and 43.4%) is quite low in the tested clones which limits the scope for improvement for better utilisation of the wood. Clones of lower wood density (556 kg m^{-3}) and higher pulp yield (51%) were reported from irrigated plantations in Tamil Nadu (Nicodemus et al., 2011). Higher wood density values (636 – 765 kg m^{-3}) have also been reported in *Casuarina* hybrid clones at an inland site in Tamil Nadu, and lower values (569 – 671 kg m^{-3}) at a coastal site (Kannan et al., 2016), which clearly indicates that genetic and site effects impact wood properties and utilisation of *Casuarina* wood.

4.3 Clone development from seedlings

Significant variation is reported in performance of *C. junghuhniana* clones based on their provenance origin (Luechanimitchit et al., 2016) which underlines the need for a good seed source as starting material for clone development. All plus trees cloned from the same seed source do not end up as good clones due to very low expression of traits compared to the original tree (Reis et al., 2011). In a clone development program, very few plus trees finally reach commercial deployment stage after rigorous evaluation of provenance, family, clone and GxE effects. As only 10% trees reach the top list after an elaborate screening process of over eight years involving plus tree selection in seed origin plantation, and evaluation of clonal performance, the entire process needs to be re-examined. -Introduction of more genotypes into the clone evaluation phase, based on intensive screening at the seedling stage, would be beneficial. Moreover, in conventional program, a plus tree is selected based on the performance of a single tree (ortet), whereas in the proposed new scheme, we have the opportunity to screen several ramets of an individual with known provenance and family background, thereby allowing the genotype-by-environment effect to be taken into account. In the current study it can be seen that there is a 20% difference in survival rate between the top 10 and the mean of 159 seedling clones (SC), compared to just 6% difference between the top 10 and the mean of 28 coppiced (CC) plus tree clones. The difference in stem straightness was less in SC clones (1%) compared to CC clones (4%), and top SC clones had 5% better stem straightness than the corresponding CC clones, which could be due to the juvenile nature of seedling clones. As there is no significant correlation of sex or growth with any other trait, selection at seedling stage will not adversely affect the traits of the clone. The top 10 SC clones had comparable wood density and pulp yield as top CC clones which clearly highlights the opportunity to reduce time, and enhance the efficiency of clone selection for each region, from improved seed for the target site. Seedlots can be developed to suit different climatic regions by sub-lining the main breeding population, and maintain diversity in the deployed clones by ensuring seed collection from unrelated families each year.

5 Conclusion

A new scheme for developing *Casuarina* clones is proposed by direct cloning of elite seedlings produced from seed orchards in a breeding program without conventional candidate plus tree selection for clone testing. Gain produced through breeding and clone deployment can be fast-tracked by this strategy, compared to the conventional method, maintaining greater diversity in clonal plantations as an insurance against pests and diseases. A comprehensive breeding program should provide the base for clonal forestry to develop high performing clones of desired traits.

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Selection of *Casuarina junghuhniana* clones in Thailand

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Abstract

Casuarina junghuhniana is an emerging species for plantation development in several tropical countries. In Thailand, breeding populations involving genetic material from a wide range of the species' distribution have been established to determine the extent of genetic variation within the species. Subsequently outstanding individuals have been selected and subjected to a two-stage process of clone testing, screening and proving. Following the first screening process, ten clones were selected for further proving tests at four locations across Thailand in the northern and central regions. A local commercially-deployed hybrid (*C. junghuhniana* x *C. equisetifolia*) was included as a benchmark clone for comparison. Trials were assessed at 3 years (33-38 months) after planting for height and diameter at breast height, and a derived volume index. Analysis of variance revealed considerable differences in the growth among clones and with no significant clone by site interaction. Four clones (19, 21, 22 and 25) were significantly more productive than the benchmark clone, and another three (4, 15 and 29) were better though not significantly. The results indicate that at least four of the new clones of *C. junghuhniana* are suitable for commercial plantation establishment in Thailand.

Keywords: *Casuarina junghuhniana,* clone trials, growth performance, G x E interaction

1 Introduction

Casuarina junghuhniana was first introduced into Thailand for provenance trials in 1997-1998 (Pinyopusarerk et al., 2005). The species has shown promising growth for plantation development and subsequently seed sources from the species' natural distribution range in Indonesia and land races in Africa were introduced for assessment of genetic variation and establishment of seed orchards (Luechanimitchit et al., 2011). An important step in the successful implementation of such a program is the identification of genetically superior clones that will bring about economic returns and related benefits to growers. Thirty superior individuals in a progeny trial/seedling seed orchard were propagated by root cuttings for first stage clone screening (Luechanimitchit et al., 2017): eleven clones were found to perform well. The objective

of this study was to further determine the growth performance of these clones in different regions of Thailand in order to identify and confirm those clones best suited for commercial deployment.

2 Materials and methods

2.1 Plant materials

Ten novel clones of *C. junghuhniana* were propagated by rooted cuttings for this study. They were a subset of 30 clones in a previous clone screening test and subsequently selected based on superior growth and stem form characteristics (Luechanimitchit et al., 2017). Among them, 7 clones were of Indonesian origin: Bali (1), Timor (1) and Wetar (6), and another 2 clones were of land-race sources from Kenya. In addition, a widely planted commercial hybrid clone of *C. junghuhniana* x *C. equisetifolia* was included as a performance benchmark. Thus, in total, 11 clones were included in the trial.

2.2 Planting sites

The clone trials were established on four sites varying in soil and climate conditions in Thailand (Table 1). These sites are considered representative of those where the clones might be commercially deployed.

2.3 Experimental design and layout

All trials were planted using randomised complete block designs with 4 replicates, 5 ramets in a single row per plot and 3 m x 2 m spacing at three sites and 3 m x 3 m at one site. One or two border rows of mixed clones were planted surrounding each experiment. The experimental areas were disc ploughed twice prior to planting.

2.4 Assessment and analysis

The trials were assessed for survival (S), height (H) and diameter at breast height (D) at the age of 33 to 38 months after planting. A stem conical volume index (V) was calculated for all trees as $V = 1/3\pi(D/2)^2H$.

Analysis of variance of trial data was implemented in Genstat version 16 (VSN International, Hemel Hempstead, UK). Least significant difference ($P<0.05$) was calculated to determine statistical differences among pairs of clones.

3 Results

There were significant differences ($P< 0.05 - 0.001$) in survival, height, diameter at breast height and stem volume index among the clones at each trial site. Clone performance patterns were similar across the four

Table 1. Locations of *C. junghuhniana* clone trials in Thailand.

Site	Altitude (m)	MAR (mm)	No. dry months	MAT (°C)	Soil
Fang (far north)	507	1660	5	18.8 (16.4-22.6)	Clay, pH 5.6-5.9
Phrae (north)	126	1244	4	22.3 (27.0-33.4)	Clay loam to Sandy clay loam, pH 5.1-5.6
Lad Krating (east)	36	1875	3	27.0 (22.3-33.0)	Sandy loam, pH 4.1
Kanchanaburi (west)	63	1031	4	28.7 (23.3-34.0)	Sandy loam, pH 6.3-6.8

NB. MAR = mean annual rainfall; MAT = mean annual temperature (mean minimum and mean maximum in brackets). Monthly rainfall less than 40 mm is regarded as dry month.

Table 2. Pooled mean values of survival, height, diameter at breast height (DBH) and stem volume index of *C. junghuhniana* clones over four sites in Thailand. Clones are ranked by volume index data.

Clone origin	Clone ID	Survival (%)	Height (m)	DBH (cm)	Volume index (m^3 x 10^{-4})
Wetar	22	87.5	9.7	8.2	18.14
Wetar	25	92.0	10.2	8.0	17.79
Wetar	21	87.9	9.5	8.2	17.67
Kenya	19	84.0	9.9	8.0	17.32
Wetar	15	81.5	10.1	7.4	14.23
Wetar	29	89.1	9.1	7.3	13.09
Timor	4	92.6	9.0	7.1	13.07
Hybrid	7	90.5	9.5	7.0	12.89
Kenya	17	86.5	9.4	6.6	11.54
Bali	14	66.4	8.6	6.1	9.81
Wetar	2	82.7	8.3	6.3	9.17
Overall mean		85.5	9.4	7.3	14.1
LSD (5%)		10.4	0.6	0.6	3.41

trials and clone by site interactions were not significant. Therefore we present the results based on pooled values of means of the four sites (Table 2).

Except one clone from Bali, survival rate was high (>80%) after three growing seasons. This Bali clone was also growing poorly; its stem volume index was only slightly better than the least productive clone from Wetar (ID 2).

The results displayed by clones from Wetar were somewhat unexpected. While three Wetar clones (21, 22, 25) were top three in the overall ranking, one clone (2) was last in height and volume index, while another two clones (15 and 29) were around the overall mean. All these point to considerable variation in growth performance of materials from this small Indonesian island.

Two Kenyan clones also exhibited opposite growth performance with one clone (19) ranked fourth, and the other (17) ranked ninth.

The benchmark, local commercial hybrid clone had high survival rate (90.5%) but its growth was on par or below trial average at most sites.

4 Discussion and conclusion

This study demonstrated marked variation in survival and growth among clones of *C. junghuhniana* planted in different regions of Thailand. Such variation was clearly observed despite the clones had been selected in the first stage clone screening based on their good performance. More than half of the clones included in this study had growth which was well below par. It should be noted that the initial clone screening test was conducted on one site only (Luechanimitchit et al., 2017). The results thus indicate the sensitivity of clones, and that clonal test over a range of sites different in soil and climate conditions must be carried out before the release of clones for large-scale planting.

In Thailand, a single hybrid clone of *C. junghuhniana* and *C. equisetifolia* has been planted commercially for many decades for production of poles (Chittachumnonk 1983). As shown in a previous study (Luechanimitchit et al., 2017) and in this study, this hybrid grows much slower than pure species clones of *C. junghuhniana*. Based on this study, at least four clones, three from

Wetar (clone 21, 22, 25) and one from Kenya (clone 19), can now be deployed for commercial planting in Thailand.

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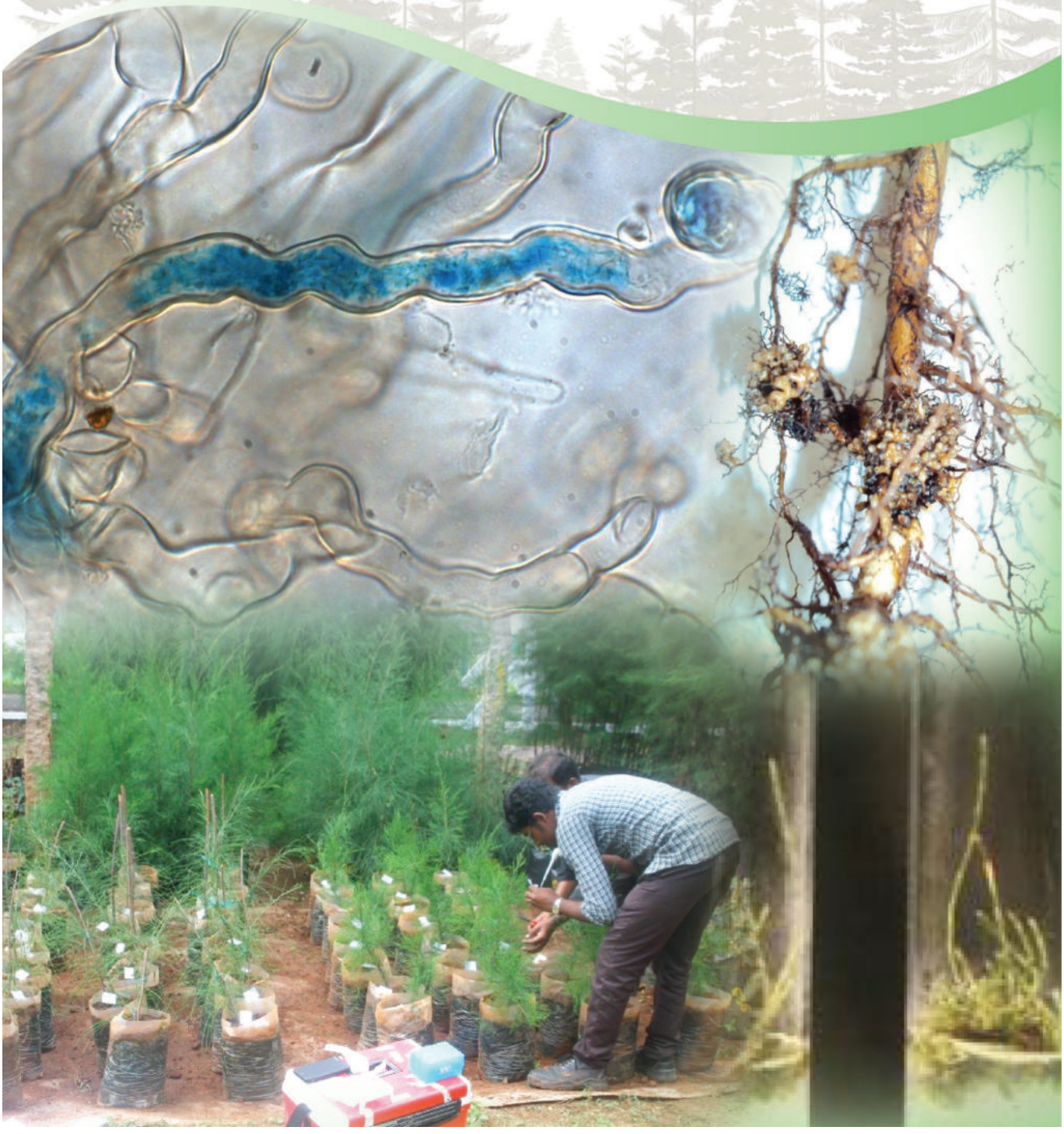
Nitrogen fixation, Physiology, Biotechnology



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Ion content and transport under NaCl salt stress in *Casuarina equisetifolia*

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Abstract

Associated with its high salt tolerance *Casuarina equisetifolia* is widely planted in coastal areas of tropical and subtropical regions as windbreaks or to stabilize dunes against wind erosion. In order to investigate the mechanisms responsible for its salt tolerance, *C. equisetifolia* Clone A8 plantlets were exposed to 200 mM NaCl treatment and their mineral compositions including Na^+, Cl^-, K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+} in roots and shoots were assessed over time. Results indicated that Na^+ and Cl^- content in roots and shoots significantly increased with treatment time while their increasing levels in roots were much higher than in shoots. K^+, Ca^{2+} and Mg^{2+} content in roots decreased while Fe^{2+}, Cu^{2+} and Zn^{2+} content in shoot decreased under salt treatment. Additionally, the transfer coefficients analysis showed that the transfer coefficients of Na^+, Cl^- and Cu^{2+} significantly decreased under salt stress while K^+, Ca^{2+}, Mg^{2+} and Zn^{2+} increased. And then *High-affinity Potassium Transporter1* (*HKT1*), *Na+/H+ antiporter* (*NHX*) genes expression analysis in roots was also examined. Most of *CeqNHX* gene obviously upregulated in roots under 24h and 168h salt stress, and *CeqNHX7* was specially induced at 168h salt stress. Hence, it concluded that salinity tolerance of *C. equisetifolia* mainly relied on sequestering excess Na^+ and Cl^- into root and preventing their translocation into shoots through NHX pathway gene inducing expression in roots and with high K^+ levles maintained in shoots.

Keywords: *Casuarina equisetifolia*, salt stress, gene expression, *CeqNHX*

1 Introduction

Salinity resulting mainly from NaCl is one of the most severe environmental stresses affecting plant growth and productivity. High salinity resulted in the ionic disequilibrium and the osmotic imbalance in the plant, which caused the metabolic alterations and high demand of energy and induced oxidative stress leading to plant death in non-halophytes (Türkan and Demiral, 2009). However, some plants referred to as halophytes and salt-tolerant non-halophytes can grow under high salinity stress owing to having the capacity to sequester and accumulate salt into the cell vacuoles, synthesize compatible solutes and possess a complex

antioxidant defense system, etc (Zhu, 2003). Hence, discovering the mechanisms of tolerance to salt stress and conferring these tolerances to crops and sensitive plants are essential for sustainable food and wood production. A number of studies have focused on elucidation of physiological, biochemical and molecular mechanisms, such as antioxidant resistance, ion exclusion and osmotic adjustment on stress-tolerant species (Munns and Tester, 2008). Many genes including for osmolytes synthesis and accumulation, regulation of signaling pathways, oxidative protection and regulating ion homeostasis were recognized as contributing to salt tolerance through complex genetic regulatory networks (Munns and Tester, 2008; Yang et al., 2009). Among them, maintaining cellular ion homeostasis genes such as salt overly sensitive (SOS) pathway proteins and Na^+/H^+ antiporters (NHX) were identified as the key genes to responses to salt stress by restricting the accumulation of toxic sodium. However, as model plants can only endure low concentrations of salt, their study may not elucidate key genes or pathways related to salt tolerance.

Casuarina equisetifolia is extensively cultivated in near-coastal areas and on limestone soils of tropical and subtropical regions for sand stabilization, soil rehabilitation, and as shelterbelts. Furthermore, *C. equisetifolia* has been reccognised as providing critical ecosystem services of coastal forests, particularly after the devastating 2004 Southeast Asian tsunami (De Zoysa, 2008). *Casuarina equisetifolia* is also an excellent raw material in the paper and pulp industries and is favored for use as poles and in scaffoldings (Zhong et al., 2010). As a salt-tolerant, non-halophytes species, there was particular interest in studying how this species responds and adapts to salinity stress. Recently, a few studies had selected higher salt tolerance clones of *C. equisetifolia* associated with proline, electrical conductivity (EC) and peroxidase (POD) activities and their seed germination under salt stress (Zhang et al., 2008; Wu et al., 2010). Meanwhile, it was reported that *C. equisetifolia* seedlings could survive in 500mM NaCl solution and proline was thought as a major compatible solute to adjust the osmotic pressure (Tani and Sasakawa, 2003). However, only a few studies have investigated ion transfer and transport in the period when plants of this species were responding and adapting to salinity stress.

For these reasons, ion contents were determined after exposure to a moderately high salinity (200mM NaCl) treatment for up to one week. Meanwhile, the *High-affinity Potassium Transporter1* (*HKT1*) and *Na^+/H^+ antiporter* (*NHX*) genes expression analysis in roots was also examined. The aim of the present study was to explore the effects of salinity on *C. equisetifolia* and identify the more critical mechanisms, such as osmotolerance and specific ion transfer and transport, that likely assist the species to adapt to high salt stress.

2 Materials and methods

2.1 Plant material and NaCl treatment

C. equisetifolia clone A8 plantlets (derived from cuttings), cultured in a growth chamber for 8 weeks, were prepared for the experiment. Then the plants transferred to clean water after the plant's roots were carefully washed clearly by using sterile ion free water. Next, the plants were transferred to solutions containing 200mM NaCl for 0, 1, 6, 24, and 168 hours of salt treatment. The roots and shoots were harvested and some for mineral elements content analysis and part of samples were stored at -80°C for RNA extraction.

2.2 Ion concentrations

All samples were finely ground separately, and corresponding 1.00 g plant material was digested with concentrated HNO_3 in a microwave system (CEM, Mars 5), respectively. The mineral elements contents in extracts were analyzed using inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian Vista-PRO RL). The concentrations were expressed as mg g^{-1} DW for Na, K, Cl, Ca, Mg, Zn, Cu and Fe. All experiments were repeated at least three times, and data were analyzed by one-way ANOVA test using IBM SPSS Statistics 19 for Windows. Means were compared using the least significant difference (LSD) test at $P < 0.05$. In all the figures, the spread of values is shown using error bars representing standard errors (SE) of the means.

2.3 RNA extraction and cDNA synthesis

Total RNA from each sample was isolated separately using the RN38 EASYspin plus Plant RNA kit (Aidlab Biotech, Beijing, China) following the manufacturer's instructions. RNA samples were treated with the RNase-Free DNaseI (Qiagen) to eliminate residual

genomic DNA. The purified RNA concentration was quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). For each sample, 2μg of total RNA were used as template in RT reactions with the SuperScript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Next, 2μg of total RNA was used as a template in cDNA synthesis with the SuperScript III reverse transcriptase (Invitrogen) and cDNA products were diluted 25-fold for qRT-PCR.

2.4 Quantitative real-time polymerase chain reaction (qPCR)

The sequences of HKT1 and NHX genes were obtained from the assembly of illumina high-throughput sequencing in *C. equisetifolia* (SRP064226 in NCBI SRA). The primers for the genes were designed using the Primer 3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). The gene sequences and primers are listed in Table 1. The qRT-PCR was performed on an Applied Biosystems 7500 Real-Time PCR using SYBR Premix Ex Taq™ kit (TaKaRa, Japan) following the manufacturer's instruction. Thermal cycling conditions were 30 s at 95°C following by 40 cycles of 5s at 95°C , 34s at 60°C. The dissociation curve was obtained by heating the amplicon from 60 to 95°C. The *CaeUBC* and *CaeEF1α* combination was used as internal control(Fan, et al., 2016). Each sample was analyzed three times. The relative expression level was calculated as ΔΔCT method. Meanwhile, NTC (non-template controls) and RT negative control were used for each sample.

3 Results

3.1 Ion content changes in shoots and roots under salt stress

In the roots, the sodium and chloride content increased immediately at 1 h of treatment and significantly increased while calcium concentration swiftly decreased and then continuously reduced with the extended treatment time (Fig. 1). Meanwhile, potassium and magnesium in roots also showed obvious decreases after 24 h and 168 h of NaCl treatment compared with the control group, which exhibited a little difference from calcium. Interestingly, zinc content was significant decreased only after 168h treatment. However, the copper and iron contents showed slight or no changes.

In the shoots, the sodium and chloride contents gradually increased with the treatment duration increasing and reached the highest point at 168h treatments, which was still much lower than the contents in roots (Fig. 1). Sodium and chloride concentration were significantly higher in roots compared with shoot tissues. This suggested that *C. equisetifolia* was able to sequester Na^+ and Cl^- in root tissues, which may contribute to preventing its transfer to the shoot or may be a consequence of a

Table 1. Primers of HKT1 and NHX genes used for quantitative real time-PCR analysis in *Casuarina equisetifolia.*

Gene	Gene ID and length	*Arabidopsis* ortholog locus	Primer sequences(Forward/reverse)
CeqHKT1	comp158245	AT4G10310	GCTTCTTATGGTGTGGGCCT/GTCTTAGCTCTCCGGGCATC
CeqNHX1	comp1309771	AT3G05030	TTCACGTCAATGGCTGCTCT/TCCAAGCCCGGTAATGACAC
CeqNHX2	comp169350	AT3G05030	CTTGGTGACCCTTCCGTTCA/GAAAGGAACAAAACCCCGGC
CeqNHX3	comp153028	AT3G05030	CGCCATTTTCAAGCATCGGT/AGTGCTGTTACGGCATTCCA
CeqNHX4	comp169117	AT3G05030	GGACTCGTTCATCCAGCGAT/CGACATCCGATCATGCCTCA
CeqNHX5	comp1368811	AT1G79610	CATGTCAAGAGCGGTCCAGT/ATCCTAGCCATGCTGCCATC
CeqNHX6	comp1415551	AT1G79610	GAGAGCGGCTTCTCCATCAA/AAGAGGAAGGTCTCGACCCA
CeqNHX7	comp2549756	AT2G01980	CGACGAGACCTTCGACGATT/CCAGCCGTCAAGTCCAAGAT
CeqNHX8	comp2331177	AT5G27150	CCCGAGAGCGACTTTAGCAT/AACGTGATGACGGCACTGAT

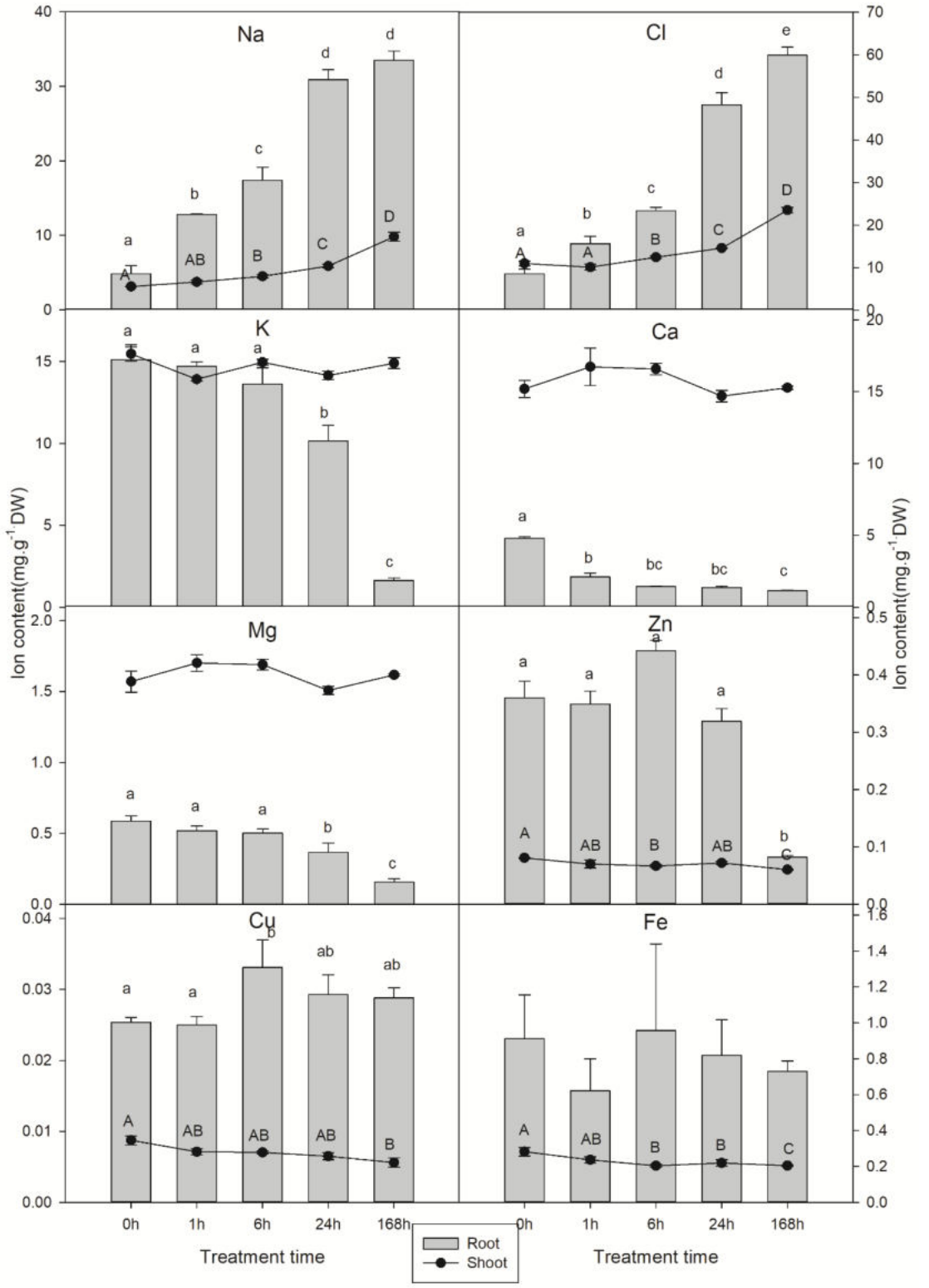

Fig. 1. Mineral elements content in *C. equisetifolia* at different treatment durations. Values are mean ± SE. Different letters indicate significant difference between treatments using the least significant difference (LSD) test at $P < 0.05$.

reduced transfer to the shoot. It was also found that zinc, copper and iron showed significant decreases with the increasing salt treatment duration. However, potassium, calcium and magnesium did not show significant changes with the increasing duration of salt treatment. Furthermore, it was found that zinc, copper and iron contents were higher in roots while calcium and magnesium mainly enriched in shoots.

3.2 Ion transfer efficiency changes under salt stress

Ion transfer efficiency represents the ion transport from root to shoot, which was calculated by ion content in shoot/root ratio. Sodium and chloride shoot/root significantly decreased under salt treatment (Fig. 2). However, sodium shoot/root did not continuously decrease while chloride shoot/root gradually decreased with the increasing of treatment duration. It indicated that chloride maybe contributing to the main ion damage. It was found that potassium, magnesium and zinc shoot/root showed no change under less than 24h salt treatment and significantly increased when *C. equisetifolia* exposed to 168h NaCl solution. Hence, it was concluded that high shoot cytosolic levels of potassium, magnesium and zinc under salinity stress would maintain enzymatic and photosynthesis reactions, which is a characteristic feature of salt tolerance in *C. equisetifolia*. Meanwhile, calcium shoot/root showed gradual increase while copper decreased with increasing treatment duration. However, iron shoot/root showed no significant changes under salt treatment. Additionally, the Na/K, Na/Ca, Na/Mg ratios show obvious increases in the root and shoot with salt treatment time increasing (Fig. 3). Besides, it was found that Na/K, Na/Ca, Na/Mg ratios show a markedly higher ratio in the root than in shoot with salt treatment time increasing (Fig. 3).

3.3 Differential expression of genes encoding Na^+/K^+ transport proteins in response to salt stress

To determine the mechanisms underlying differential Na^+ and K^+ accumulation in *C. equisetifolia*, expression profiles of the genes encoding Na^+ and K^+ transport proteins were analyzed in roots. *CeqHKT1* gene and *NHX* gene family member including *CeqNHX1, CeqNHX2, CeqNHX3, CeqNHX4, CeqNHX5, CeqNHX6, CeqNHX7/CeqSOS1* and *CeqNHX8* were annotated as Na^+ and K^+ transport related genes based on our previous transcriptomic sequencing for *C. equisetifolia* (SRP064226 in NCBI SRA). The results showed these genes were strongly regulated under salt stress (Fig.4). *CeqNHX1, CeqNHX5, CeqNHX6, CeqNHX7* and *CeqNHX8* were obviously upregulated with increasing salt treatment time courses, but the mRNA levels of *CeqNHX3* was reduced. It was interesting that *CeqNHX1, CeqNHX7* and *CeqNHX8* were especially responsive to salt stress and *CeqNHX7* only expressed after 168h salt treatment. However, *CeqHKT1* and *CeqNHX4* were slightly downregulated after 24h NaCl treatment and NHX2 downregulated after only 1h NaCl treatment.

4 Discussion

C. equisetifolia, as a salt-tolerant beach strandline species, was used in the present study to elucidate its mode of adaptation to salinity stress through physiological and transcriptional analyses. Under salt stress, sodium, chloride contents and Na^+/K^+ ratios in shoots were less than in roots. Meanwhile, salt stress increased the transcription of most of *NHXs* genes.

Among salt-tolerant traits, the ability to restrict the transport and accumulation of Na^+ in the shoots appeared to be the most significant to adapt to salinity (Munns and Tester, 2008). *C. equisetifolia* plants accumulated Na+ mainly in root with increasing NaCl concentrations and time courses. Meanwhile, *C. equisetifolia* plant exhibited lower shoot Na^+ content. Although Na^+/K^+ in the shoots and roots significantly increased with increasing NaCl concentrations and treatments time courses, *C. equisetifolia* still had higher levels of K^+ content. Meanwhile, the observed reduction in growth was caused by increased Na^+/K^+, which was not lethal for *C. equisetifolia*. It was similar with previous studies in barley and *Gypsophila oblanceolata* (Widodo et al., 2009; Sekmen et al., 2012). It was also found that many halophytic plants accumulate inorganic ions to concentrations equal to or greater than that of the surrounding root solution to facilitate water uptake from the medium (Bradley and Morris, 1991; Aghaleh et al., 2009). This is, because roots could accumulate Na^+ in the endodermis and exodermis by the deposition of apoplastic barriers and Na^+ was prevented the direct entry of external fluid into the stele and entered the shoot (Krishna Kumar et al.,

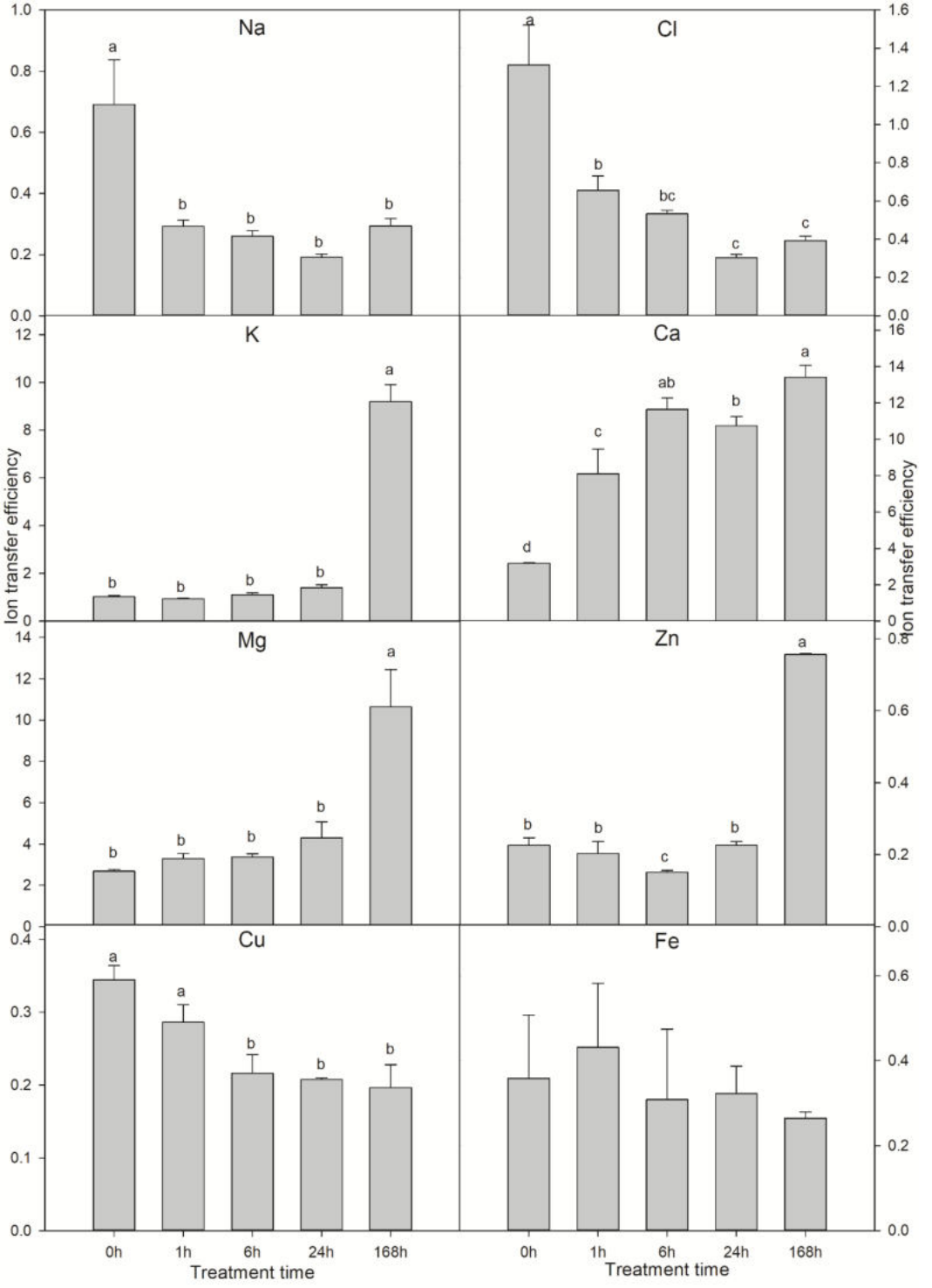

Fig. 2. Mineral elements transfer efficiency in *C. equisetifolia* at different treatment durations. Values are mean ± SE. Different letters indicate significant difference between treatments using the least significant difference (LSD) test at $P < 0.05$.

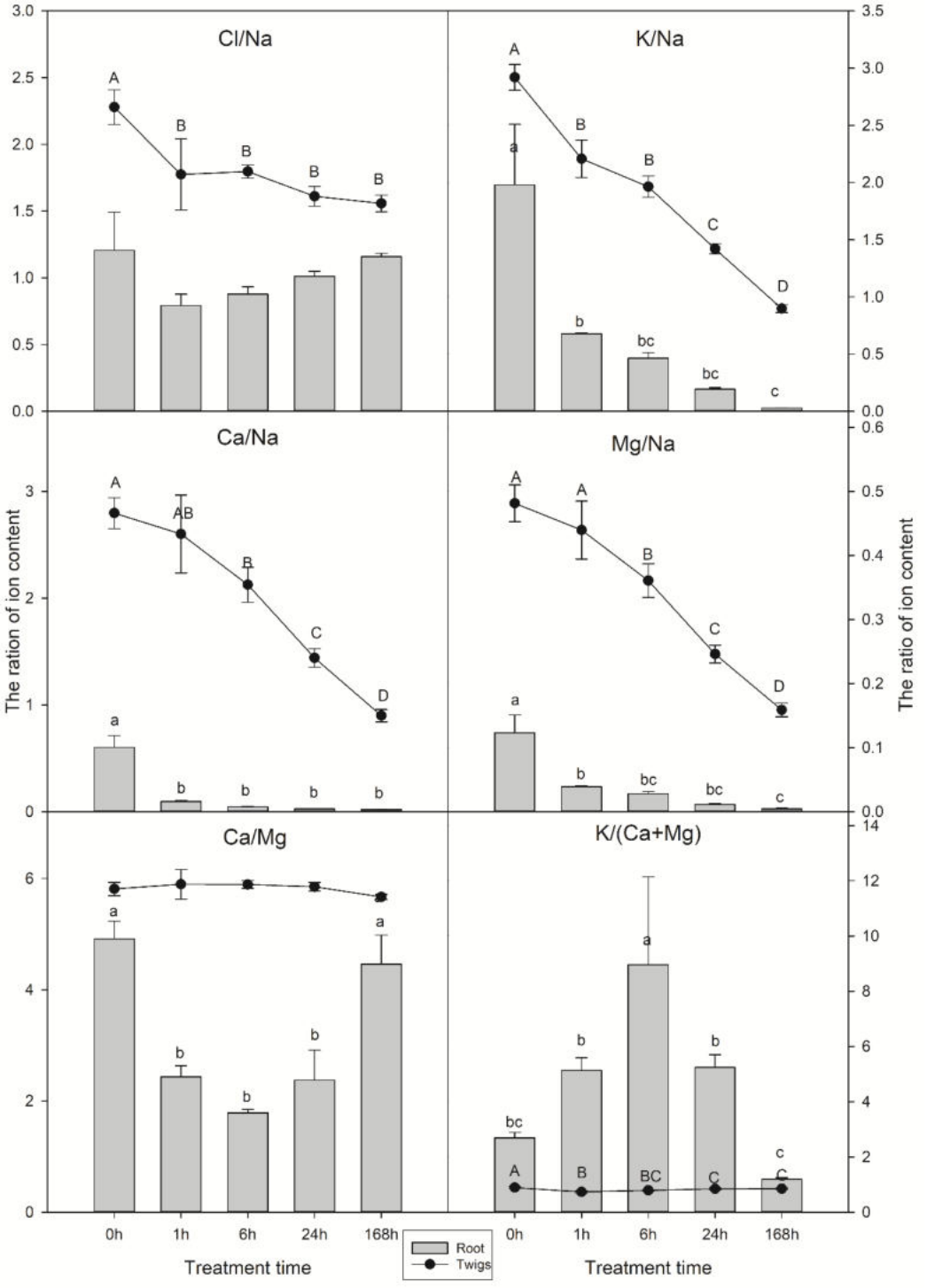

Fig. 3. The ratio of various mineral elements content in *C. equisetifolia* at different treatment durations. Values are mean ± SE. Different letters indicate significant difference between treatments using the least significant difference (LSD) test at $P < 0.05$.

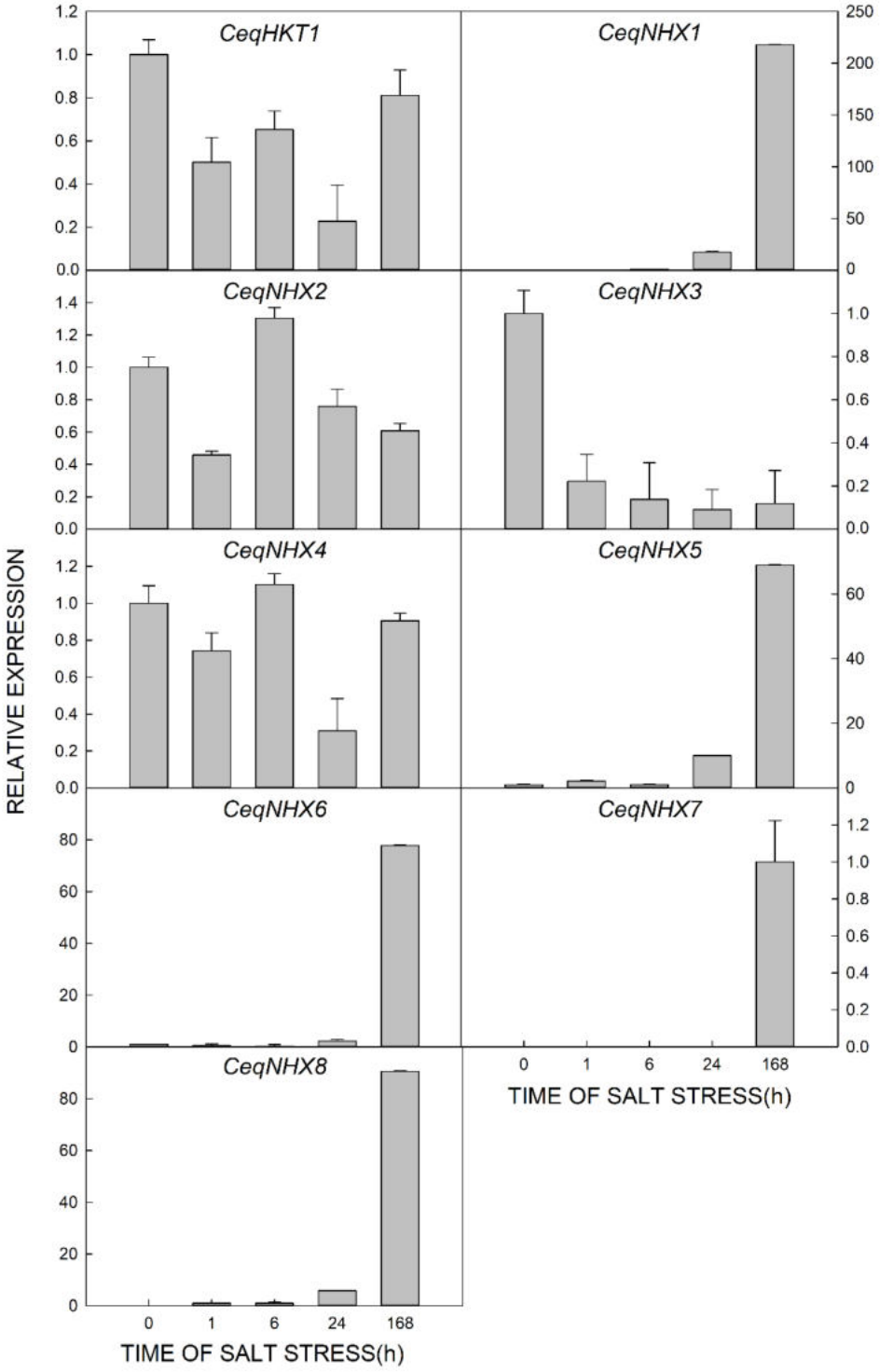

Fig. 4. Expression levels of *HKT1* and *NHX* genes in roots of *C. equisetifolia* at different treatment durations under 200mM NaCl solution. Error bars represent the standard errors among three independent replicates. Data are mean ± SD of three replicates.

2011). However, excessive Na^+ often interferes with K^+ functions, which resulted in impaired metabolic activities.

To restrict transport of Na^+ to the shoots, various Na^+ transport proteins were identified (Munns and Tester, 2008). Among them, Salt overly sensitive (SOS) pathway proteins and vacuolar Na^+/H^+ antiporters (NHX) were considered as two efficient ways to reduce ionic toxicity in plant cells by extrusion of Na^+ to the apoplast or external environment or sequestration Na^+ in vacuoles respectively (Munns and Tester, 2008; Yang et al., 2009; Ji et al., 2013). To understand the mechanisms underlying limited Na^+ transport to the shoots in *C. equisetifolia, HKT1, NHX* gene pathway genes expression was analyzed in this study. The expression of *NHX1, NHX5, NHX6, NHX7* and *NHX8* genes in roots were all up-regulated under salt stress and the expression of most of these genes was higher following 168h salt treatment. This may be an important reason for the reduced shoots Na^+ content and salt damage. In previous studies, the SOS signaling pathway activation was recognized as a key mechanism for Na^+ exclusion and ion homeostasis control (Zhu, 2000; Ramezani et al., 2012; Feki et al., 2013; Zhao et al., 2016). Meanwhile, the high expression of these sodium related genes after 168h NaCl treatment may be ascribed to some signals originating from the root under salt stress, suggesting that root could induce salt tolerant genes to respond to salt stress in *C. equisetifolia*.

5 Conclusion

Salt stress in *C. equisetifolia* were developed in this study. Salinity induced several mineral elements changes in *C. equisetifolia,* including increase in Na^+ accumulation and increase in Na^+/K^+ ratio. K^+ contents in shoots and roots showed progressive decrease with increasing duration of NaCl treatment, but levels stayed high in contrast to the control. Meanwhile, the expression of *NHX1, NHX5, NHX6, NHX7* and *NHX8* genes which are involved in Na^+ exclusion and ion homeostasis in roots were up-regulated under salt stress. This genetic regulation of Na^+ exclusion from the shoot in *C. equisetifolia* is considered to be an important factor contributing to the species high level of salt tolerance.

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An assessment of the adaptive potential in four *Casuarina* species in southern China

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Abstract

Casuarinas have been introduced into China and become widely used for shelterbelt in coastal areas. We report on the adaptive variation of different *Casuarina* species in southern China and evaluated their genetic potential for forest plantations and shelterbelts. Field trials of *Casuarina glauca, C. equisetifolia, C. junghuhniana* and *C. cunninghamiana* were established at three sites in Raoping, Wuchuan and Xuwen. In this study, we compared tree height, diameter at breast height, wood volume and survival rate at 3 years of age. Survival and growth rates of the four *Casuarina* species were best in Xuwen and poorest in Raoping. Both precipitation and high temperature affected adapative traits, with significant adaptive divergence observed among the four species at each site. *C. equisetifolia* exhbited the best growth rate, while *C. glauca* had better growth in the more northern site. An interaction between environment and species was observed for growth and survival, and underscores the importance of appropriate species/germplasm selection. Significant genetic variation among and within the four *Casuarina* species could also be found and will be useful for future introductions and breeding of casuarinas.

Keywords: *Casuarina* species, adaptive variation, multi-site field experiments, Southern China

1 Introduction

Plant adaptation is an important evolutionary process, impacting distribution, speciation, hybridization and transplantation (Egan and Ott, 2007; Arnold, et al., 2008; Otto, 2018). Adapation can be affected by genetics, environment and their interaction.

Casuarinaceae is a family of woody species with four genera and more than 90 species (Der Moezel et al., 1989). The species are naturally found in South-east Asia, Australia and the South-west Pacific Islands, but have been widely planted in tropical, subtropical and semiarid regions of the world for economic and ecological purposes (Zhong et al., 2010; Diagne et al., 2013; Hu et al., 2016). The first introduction of *Casuarina* into China was likely in the 1860s for landscaping purposes, but now *Casuarina* is an important pioneer tree species for coastal shelterbelts in southern China (Zhong et al., 2005; Han, 2017). As an exotic tree, the tree adaption of different species of *Casuarina* is crucially important and has been given attention by forestry department and tree breeders

(Luo, 2005; Ye et al., 2005; Wei et al., 2019). There have been more than 20 species of *Casuarina* and *Allocasuaria* introduced into China (Zhong et al., 2010; Jiang, 2011), but studies of their adaptation to different sites have been limited, and possibly leading to poor species/provenance/site matching and suboptimal deployment of *Casuarina* germplasm.

Casuarinas have been planted along about 10,000 km of coastline from Zhejiang to Hainan in southern China. A comprehensive assessment of germplasm adaptation covering the main planting areas is needed to inform future plantings and introductions and tree breeding programs (Nie, 2009; Yu et al., 2019). In this study, we conducted a multi-site field trials to compare the sapling(tree) adaptation of four species (*C. glauca, C. equisetifolia, C. junghuhniana* and *C. cunninghamiana*) on the coast of China. These four species are most widely used in shelterbelt plantations (Han et al., 2017; Ye et al., 2019), and through these experiments we aimed to (1) evaluate the adaptive variation of the four species and their potential in different parts of southern China coastline, and (2) identify the most promising germplasm resources for coastal shelterbelt and forest plantations.

2 Materials and methods

2.1 Study system

The multi-site field experiments were conducted in Xuwen, Wuchuan and Raoping. These sites are separated by a distance of more than 300 km from each other, with Xuwen in the southwest, Raoping in the northeast, and Wuchuan in between (Fig. 1). Meteorological data of the three sites including the temperature, precipitation, and sunshine duration were collected during the experiment from the ClimateAP2.03 (Table 1; Wang et al., 2017).

The field trials were established with seedlings from 57 families of four species. The seeds used to represent the populations of *C. glauca, C. equisetifolia, C. junghuhniana* and *C. cunninghamiana* were collected from a germplasm resource nursery with 64 provenances in Lingao, Hainan covering the main distribution of the four species. All families were represented in the Wuchuan trial, viz. 29 of *C. equisetifolia,* 11 of *C. glauca,* 11 of *C. cunninghamiana* and 6 of *C. junghuhniana,* while some families were missing in the Wuwen and Raoping trials (Table 2). Seeds were collected in spring 2015, and then cultivated in seedling beds with forest soil.

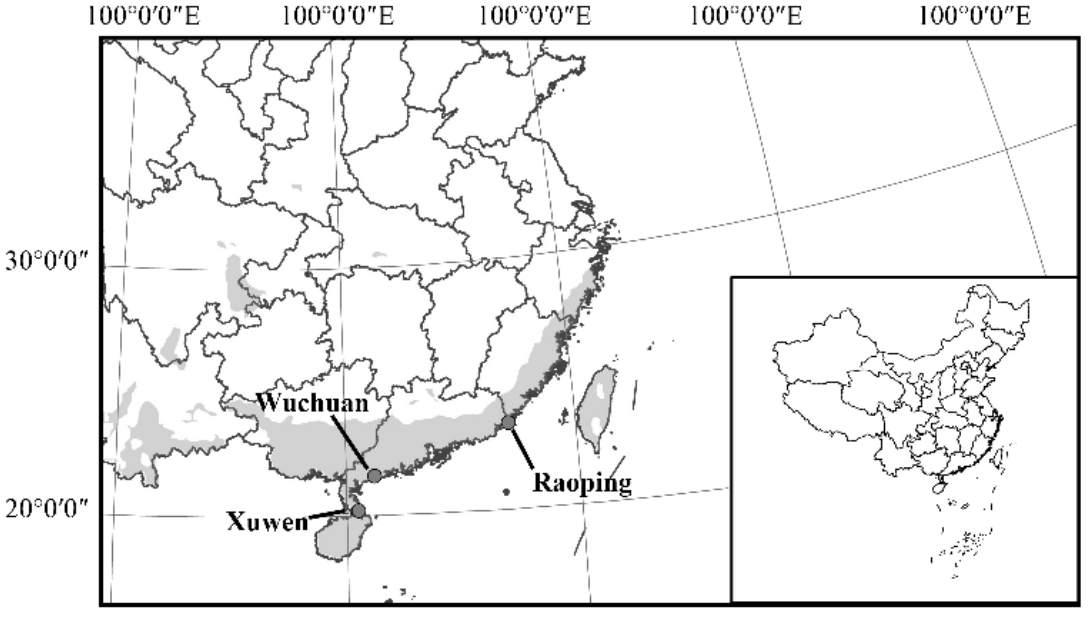

Fig. 1. The three experimental sites in this study.

Table 1. Meteorological data for the field trial sites.

	LAT	LON	MAT	MWMT	MCMT	MAP	AHM	DD>5	DD>18
Raoping	117.13	23.57	21.4	28.6	13.3	1528	20.5	5924	1714
Wuchuan	110.73	21.39	22.8	28.7	14.5	1671	19.6	6425	2070
Xuwen	110.54	20.48	23.3	27.6	17.4	1612	20.6	6588	2044

MAT, mean annual temperature; MWMT, mean warmest month temperature; MCMT, mean coldest month temperature; MAP, mean annual precipitation; AHM, annual heat:moisture index; DD>5, degree-days above 5°C; DD>18, degree-days above 18°C

Table 2. *Casuarina* species and families used in the field trials.

Site	Species	Number of families	Number of saplings
Wuchuan	*C. glauca*	11	220
	C. equisetifolia	29	580
	C. junghuhniana	6	120
	C. cunninghamiana	11	220
Xuwen	*C. glauca*	9	180
	C. equisetifolia	29	580
	C. junghuhniana	6	120
	C. cunninghamiana	8	160
Raoping	*C. glauca*	10	200
	C. equisetifolia	27	540
	C. junghuhniana	6	120
	C. cunninghamiana	8	160

2.2 Experimental design

The experiments were established at the same time in 2015 in Raoping, Xuwen, and Wuchuan. The experiments were located on barren, sandy coastal sites. Six-month old saplings were transplanted from the nursery for the field experiments. The experiments were laid out in randomized complete block design, with four replications, 5 seedlings plot^{-1}, and 2 m ×2 m spacing.

In spring 2018, three years after planting, height (SH, m), diameter at breast height (DBH, cm) and survival rate (SR, %) were assessed. An individual tree stem volume (VOL, m^3 tree^{-1}) was calculated by the equation:

$$VOL = \frac{1}{12} \pi \times DBH^2 \times SH$$

2.3 Statistical analysis

Analysis of variance (ANOVA) was used to assess the adaptive divergence among the four species. Variables were log-transformed to ensure data had normal distribution prior to analysis. Mean value and standard deviation were used to describe the genetic difference as well as Duncan's analyses in each site. Interaction of environment and species/family was examined by variance component and describes the variation trend of four species. Analyses were conducted in the R (version 2.13.0) statistical and programming environment using the nlme package (Pinheiro et al., 2007).

Pearson correlations were used for measured growth and meteorological variables at each site to examine habitat effects and adaptability variation. Meteorological data covering the last 50 years was

collected for mean annual temperature (MAT, °C), mean warmest month temperature (MWMT, °C), mean coldest month temperature (MCMT, °C), annual heat:moisture index (MAT+10)/(MAP/1000)) (AHM), mean annual precipitation (MAP, mm), degree-days above 5°C (DD>5), degree-days above 18°C (DD>18). This multivariate correlation analysis was performed using SPSS 18.0 (Peter, 2010).

3 Results

3.1 Adaptive variation among different sites

The ANOVA revealed significant differences among the test sites for all traits (Table 3), suggesting the major environmental effects. The four species grew in Xuwen had the highest value in all four traits (Fig 2), indicating Xuwen was the most suitable environment for growth of *Casuarina*. Species in Raoping had the lowest values for SH, DBH and VOL, while species in Wuchuan had the lowest SR except for *C. junghuhniana*. Furthermore, we found that the SR of *C. junghuhniana* in Raoping and Xuwen was the lowest but much higher than the other species, *C. cunninghamiana* had the second highest values in SH, DBH and VOL in Xuwen, but in Raoping and Wuchuan, the rank in lower, indicating a strong G×E interaction.

3.2 Adaptive variation among the four species

Significant genetic variations were observed among the four species in each site (Table 4). The variance component among species ranges from 0.04 (SR) to 0.24 (SH) which was much higher than for families, indicating that the genetic variations were greater among species than among families.

In Raoping, *C. equisetifolia* had the highest values in all traits, showing the fastest growth rate and survival compared with the other three species. *C. glauca* possessed the second highest values for SR, DBH and VOL. The SR in *C. junghuhniana* was the lowest, while *C. cunninghamiana* plants had the lowest volume.

In Wuchuan, the survivial rate of the four species ranged from 52% to 67% and *C. junghuhniana* had the highest value in SR. *Casuarin equisetifolia* saplings grew faster than other species with the highest values in SH, DBH and VOL. *C. junghuhniana* and *C. cunninghamiana* had similar values for all four traits, which were much lower than *C. junghuhniana* and *C. equisetifolia*.

In Xuwen, plants of three species had greater than 80% survival while plants of *C. junghuhniana* had 71% survival. *Casuarina equisetifolia* had the highest values for SH, DBH and VOL and *C. glauca* had the highest survival. *C. cunninghamiana* had much greater diameter growth and wood volume than *C. junghuhniana* and *C. glauca*.

3.3 Environment association

In *C. equisetifolia*, mean annual precipitation (MAP) and DD>18 were positively correlated with the four traits, while mean annual temperature (MAT) and DD>5 were negatively correlated (Table 5). The correlation between growth rate (SH, DBH and VOL) and

Table 3. ANOVA of four traits in the multi-site trials.

Traits	Mean Squares				Variance Component			
	SR	SH	DBH	VOL	SR	SH	DBH	VOL
Site (2)	6.28**	2055.74**	770.49**	77.15**	0.19	0.40	0.33	0.23
Species (3)	0.87**	825.68**	182.72**	25.97**	0.04	0.24	0.12	0.11
Site:Species (6)	0.38**	91.26**	46.96**	11.49**	0.03	0.05	0.06	0.10
Family (57)	0.13**	14.54**	10.32**	1.31**	0.11	0.08	0.13	0.11
Site:family (95)	0.06**	5.09**	3.58**	0.94**	0.09	0.05	0.06	0.12
Residual (779)	0.05	2.29	2.14	0.34	0.55	0.17	0.30	0.33

NB. SR=survival; SH=tree height; DBH=diameter at breast height; VOL=tree volume; Data in the brackets is degree of freedom. *=significant difference at P <0.05; **=significant difference at P <0.01.

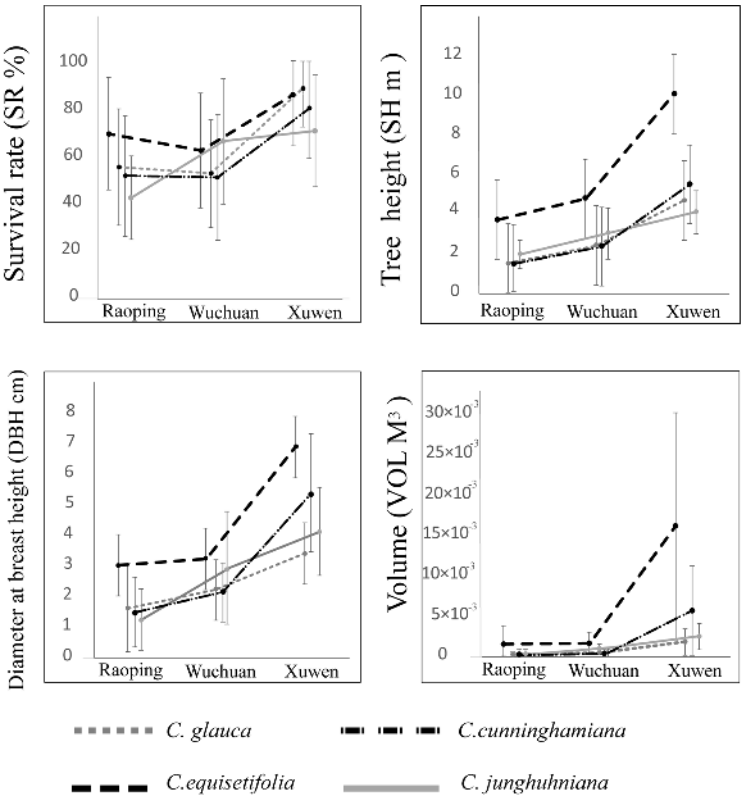

Fig. 2. Traits variation among three experimental sites.

Table 4. Growth and survival of the four *Casuarina* species at the three trial sites.

Site	Species	Survival rate (%)	Tree height (m)	Diameter at breast height (cm)	Tree volume ($\times10^{-3}$ m^3 $tree^{-1}$)
Raoping	*C. glauca*	56.00±24.91^b	1.53±0.81^b	1.62±1.72^b	2.85±6.59^b
	C. equisetifolia	70.10±23.96^a	3.75±1.74^a	3.04±1.78^a	16.24±22.54^a
	C. junghuhniana	42.90±17.84^c	1.98±0.72^b	1.27±1.20^b	2.44±5.22^b
	C. cunninghamiana	52.10±25.68^b	1.51±0.72^b	1.51±1.16^b	1.97±3.00^b
Wuchuan	*C. glauca*	53.10±22.88^b	2.44±0.92^c	2.23±0.92^b	4.53±5.14^c
	C. equisetifolia	63.00±24.36^a	4.80±1.38^a	3.25±1.17^a	17.06±13.83^a
	C. junghuhniana	66.90±26.69^a	3.05±1.29^b	2.95±1.36^a	10.54±10.27^b
	C. cunninghamiana	51.60±26.80^b	2.38±0.98^c	2.16±0.96^b	4.29±4.60^c
Xuwen	*C. glauca*	89.40±16.16^a	4.72±1.49^{bc}	3.43±1.07^c	18.54±16.34^b
	C. equisetifolia	86.80±21.72^{ab}	10.1±3.36^a	6.90±2.47^a	162.14±140.94^a
	C. junghuhniana	71.40 ±23.90^c	4.12±1.08^c	4.13±1.82^c	24.93±25.04^b
	C. cunninghamiana	81.20±21.64^b	5.53±1.99^b	5.42±1.93^b	57.33±55.44^b

Data are presented as mean ± SD. Mean with different superscript letters (a, b, c) are significantly different ($P < 0.05$) as determined by Duncan's Multiple Range Test.

Table 5. Environmental association between the meteorological data and the measured traits.

Species	Traits	MAT	MWMT	MCMT	MAP	AHM	DD>5	DD>18
C. glauca	Survival	0.253**	0.307**	-0.068	0.546**	-0.617**	0.263**	0.417**
	Tree height	0.059	0.577**	-0.332**	0.507**	-0.752**	0.073	0.292**
	DBH	0.013	0.410**	-0.250**	0.323**	-0.504**	0.023	0.173*
	Tree volume	0.141	0.344**	-0.146	0.432**	-0.549**	0.151*	0.298**
C. equisetifolia	Survival	0.197**	0.137**	0.004	0.372**	-0.366**	0.204**	0.299**
	Tree height	0.067	0.548**	-0.319**	0.549**	-0.766**	0.082	0.316**
	DBH	0.136**	0.436**	-0.215**	0.539**	-0.686**	0.149**	0.350**
	Tree volume	0.152**	0.381**	-0.172**	0.514**	-0.631**	0.164**	0.347**
C. junghuhniana	Survival	-0.282**	0.483**	-0.439**	0.035	-0.342**	-0.275**	-0.139
	Tree height	-0.163	0.588**	-0.449**	0.276**	-0.592**	-0.151	0.050
	DBH	-0.220*	0.596**	-0.482**	0.213*	-0.548**	-0.209*	-0.014
	Tree volume	-0.053	0.426**	-0.296**	0.287**	-0.493**	-0.043	0.117
C. cunninghamiana	Survival	0.146	0.311**	-0.134	0.432**	-0.510**	0.156	0.305**
	Tree height	0.069	0.592**	-0.352**	0.564**	-0.781**	0.086	0.332**
	DBH	0.102	0.557**	-0.314**	0.577**	-0.771**	0.118	0.356**
	Tree volume	0.144	0.389**	-0.185*	0.492**	-0.604**	0.156	0.335**

DBH Diameter at breast height; MAT, mean annual temperature; MWMT, mean warmest month temperature; MCMT, mean coldest month temperature; MAP, mean annual precipitation; AHM, annual heat:moisture index; DD>5, degree-days above 5°C; DD>18, degree-days above 18°C

MAP and DD>18 was high, showing that the precipitation and accumulated temperature strongly correlated with faster growth rate for plants of *C. equisetifolia.* For *C. cunninghamiana* the correlation between DBH and the environment was strong, and for *C. glauca,* MAP was strongly correlated with the four traits, while DD>18 mainly affected plant height. For *C. junghuhniana,* both latitude and longitude were positively correlated to SR, SH and DBH showing a different geographic variation trend from the other species. The DD>18 was strongly correlated with the four traits, while the MAP correltion was slight.

4 Discussion

4.1 Adaption of four species on coastal area in southern China

In our study, the survival and growth rate of four *Casuarina* species were greatest in Xuwen and least in Raoping, reflecting a gradual geographical variation trend on the adaptability of *Casuarina.* Casuarinas behave like many other tropical and subtropical trees, whereby growth is enhanced by higher termperatures and precipitation (Zhong et al., 2005; Gaskin et al., 2009). As shelterbelt species, casuarinas are usually cultivated on barren coastal sands, which may be subject to drought. Thus, precipitation and soil fertility were usually considered the key factors on cultivation of the shelterbelt (Li and Ye, 2008; Zhang and Qian, 2011).

Based on the association analysis, the sapling growth and survival rate of *Casuarina* were positively correlated with temperature and precipitation. However, the correlation coefficient with preciptation was the highest, indicating that an increase of precipitation was more effective than warmer temperatures in improving *Casuarina* survival and growth in the coastal environment. Xuwen—located on the the southernmost peninsula of the Chinese mainland—received the highest annual precipitation and was the hottest field trial site. However, Raoping was located further north—near the Tropic of Cancer—and its precipitation showed a strong seasonal effect. The better adaption of *Casuarina* to Xuwen likely shows the importance of increased precipitation for the afforestation of *Casuarina* in sandy coastal sites.

Additionally, we found that the DD>18 had a higher correlation to *Casuarina* adaption than DD>5 (except the *C. junghuhniana*). *Casuarina* plants showed a seasonal growth rhythm in Chinese coastal areas, such that growth rate was reduced in the autumn and winter seasons, and much faster in spring and summer seasons. DD>18 could more effectively reflect the duration and intensity of high temperature climate than DD>5. Thus, the seasonal rhythm of growth of *Casuarina* may be affected by the higher biogenetic temperature limit.

4.2 Evaluation of species for suitability in coastal shleterbelts

Different species of *Casuarina* species occupy different ecological niches in natural forests, suggesting adaptive divergence (Wei and Tan, 1990). Accrodingly, a comparison of the adaptive variation among species in the main afforestation area can inform further *Casuarina* introduction and germplasm utilization.

Traits for survival and grow rates are important indicators for afforestation projects (Fenner, 1985). In this study, the survival rate of the four species varied considerably among the three sites, whereby *C. equisetifolia* had highest survival (70%) in Raoping cf. 43–56% survival for the other species, *C. junghuhniana* had highest survival (67%) in Wuchuan cf. 52–63 % survival for the other species, and *C. glauca,* had highest survival (89%) in Xuwen cf. 71–87 % survival for the other species. The three trial sites in this study were more than 300 kilometers apart and the habitats markedly different. *C. equisetifolia, C. junghuhniana* and *C. glauca* had an advantage in saplings survival in different habitats, suggesting the adaptive divergence of these species and a significant species x site interaction.

Growth rate of *Casuarina* strongly influences the efficacy of shelterbelts, and is also an important indicator in germplasm selection (Ma et al., 2014; Han et al., 2017). In this study, the plant height, DBH and stem volume of *C. equisetifolia* were significantly higher than those of other species, showing superior growth rate across all sites. *C. equisetifolia* was introduced to China at the earliest period, and had been widely cultivated in southern China (Zhong et al., 2010). The high growth rate of this species is an important factor in its extensive cultivation. Additionally, *C. glauca* had also been widely distributed in Zhejiang and Fujian provinces, which are north of the Raoping site (He et al., 2011; Zhang et al., 2015). In this study, *C. glauca* has

the second highest ranking in height and stem volume at the Raoping test site, showing a good potential growth rate in this high latitude area. Although the comparison test in Zhejiang and northern Fujian was lacking, the consequence in our study was that *C. glauca* is worth testing in near-coastal areas further north.

5 Conclusion

Casuarina is one of the most successfully introduced tree species in southern China, and adaptive test covering the main planting area is necessary to aid in its wider application. The findings from the present study reveal that the environmental effect on the adaption variance of four species, and climate factors of DD>18 and MAP affects the sapling adaptation. The results of our experiments can only partially identify adaptive variation of the four species. Significant genetic variation within and among the four *Casuarina* species could also be found and will help inform future introductions and breeding projects.

Acknowledgements

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Role of helper bacteria (*Paenibacillus polymyxa*) on *Frankia* and AM fungal interaction for sustainable nutrient management of *Casuarina equisetifolia* in farm forestry

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Abstract

Field experiment was conducted using a Completely Randomized Block Design with 4 treatments and 3 replicates, each replicate containing 6 m x 6 m block (36 trees block^{-1}) and treated with *Casuarina equisetifolia* needle litter compost and helper bacteria *Paenibacillus polymyxa*. Totally 432 trees were used for data collection to find out the role of helper bacteria (*P. polymyxa*) on nodular development, growth, biomass and carbon sequestration potential of *C. equisetifolia* in a farm forestry at semi-arid zone of southern Tamil Nadu, India. Three-year-old trees of *C. equisetifolia* inoculated with a combination of casuarina needle litter compost and helper bacteria recorded growth in mean height of 13.29 m, diameter at breast height (dbh) of 8.36 cm, and total biomass of 42.905 kg tree^{-1}, these are equivalent to a 10.8% increase in total biomass and 56% increase in nodular biomass over the control. The carbon fixation in the above plantation was 21.453 kg tree^{-1}. However, control treatment trees (without *P. polymyxa*) exhibited comparatively poorer growth performance (height 11.9 m, dbh 6.8 cm, total biomass 38.53 kg tree^{-1}) resulting in lesser carbon accumulation of 19.263 kg tree^{-1}. Nutrient distribution and uptake was higher in *P. polymyxa* treated trees compared to the control. This shift in the growth, nodular development, accumulation of biomass, carbon and nutrient uptake is attributed to the application of plant-growth promoting helper bacteria *P. polymyxa* which helps in sustainable nutrient management of *C. equisetifolia* through nitrogen fixation, phosphate solubilization and mobilization.

Keywords: biomass, casuarina, carbon sequestration, AM fungi, *Frankia*, farm forestry, *Paenibacillus polymyxa*, sustainable nutrient management

1 Introduction

Trees play a vital role to fight against climate change and global warming because they absorb and store key greenhouse gases before they reach the atmosphere. While all living plants absorb CO_2 as part of photosynthesis, short rotation fast-growing trees absorb more CO_2 due to their size and extensive root structure. Hence, afforestation through agroforestry and farm forestry systems has been suggested as a cost-effective approach to mitigate the predicted effects of global climatic change. Therefore, any increase in forest biomass will reduce the build-up of atmospheric carbon dioxide. Agroforestry and farm forestry are widely considered as a potential way of improving environmental and socioeconomic sustainability (Costa, 1996) and can reduce the pressure on existing natural forests by providing fuel, fodder, timber and wood products directly to the farmers. On the other hand it may provide many indirect environmental benefits such as soil, water and biodiversity conservation (Singh and Lodhiyal, 2009). Long-term sustainability in plantation forestry is possible only through the use of low-cost farm grown inputs, which works in harmony with nature. Bio-inoculants act as perpetually renewable inputs, which help in better nutrient management in tree crop and maintenance of soil health and water management leading to improved silvicultural practices.

Casuarina (*Casuarina equisetifolia* Forst.) is a fast-growing multipurpose actinorhizal tree species and commonly used for soil reclamation and wasteland development (Kondas, 1983; NRC, 1984; Ponnampalam et al., 1999; Karthikeyan et al., 2009). It is well suited to sandy soil, resistant to saline spray and has the ability to grow in high density planting. Casuarina is highly suitable for farm forestry and agroforestry plantation, due to its ability to form symbiotic relationship with nitrogen fixing microorganism of *Frankia* and arbuscular mycorrhizal association and is also used for soil reclamation and widely adopted by farmers (Rajendran and Devaraj, 2004). Casuarina also produces high biomass, more needle litter and accumulate more carbon in the soil (Rajendran, 2001; Uma et al., 2011; Uma et al., 2014). Casuarina growth is significantly improved, especially in poor and nitrogen-deficient soils, through its symbiotic relationship with the nitrogen-fixing actinomycetes, *Frankia* through tripartite association. (Rajendran et al., 1999; Barritt and Facelli, 2001; Dutta and Agrawal, 2001; Rajendran and Jothibasu, 2006). Plant growth promoting rhizobacterium (*Paenibacillus polymyxa*) has attracted considerable attention because of the demonstrated abilities of its various strains to encourage crop growth via one or more mechanisms, as well as to produce lignocellulose-modifying enzymes (Lal and Tabacchioni, 2009). A strain of *P. polymyxa*, P2b-2R promotes plant growth by fixing nitrogen (Anand et al., 2013; Puri et al., 2016). Hence, the present experiment was conducted to study the role of helper bacteria (*P. polymyxa*) for sustainable biomass production, carbon sequestration potential and nutrient uptake of *C. equisetifolia* in farm forestry plantation.

2 Materials and methods

2.1 Study area

The study was conducted in the farmland at Panjamadevi Villianur village in Villupuram District in Tamil Nadu State, India, where casuarina is predominantly cultivated. The semi-arid area is located at 11° 59′ - 12° 48′ N latitude and 78° 60′ - 80° E longitude, at an elevation of 45 m asl. The temperature of the site ranges from 20 °C to 36 °C and the mean annual precipitation ranges from 1001 mm to 1200 mm.

2.2 Experimental design and planting material

Six-month-old plants of *C. equisetifolia* clone (CH2) were obtained from Tamil Nadu Newsprint and Paper Limited (TNPL), Kagithapuram, Karur district, Tamil Nadu, India and transplanted in 30 cm x 30 cm x 30 cm pits at a spacing of 1 m x 1 m. The experiment was set up in a completely randomized block design for four treatments and three replicates, total 432 plants (36 plants replicate plot^{-1}). After planting, the following four treatments (T1 - Control; T2 - Casuarina needle compost; T3 – *P. polymyxa*; T4 - Casuarina needle compost + *P. polymyxa*) were imposed in the rhizosphere of casuarina. The field was kept free from weeds through periodical hand weeding and hoeing every two months during the study period. Watering was done twice a month during the summer season. The casuarina stand in this study at aged 6 months and two years after planting is shown in Fig. 1

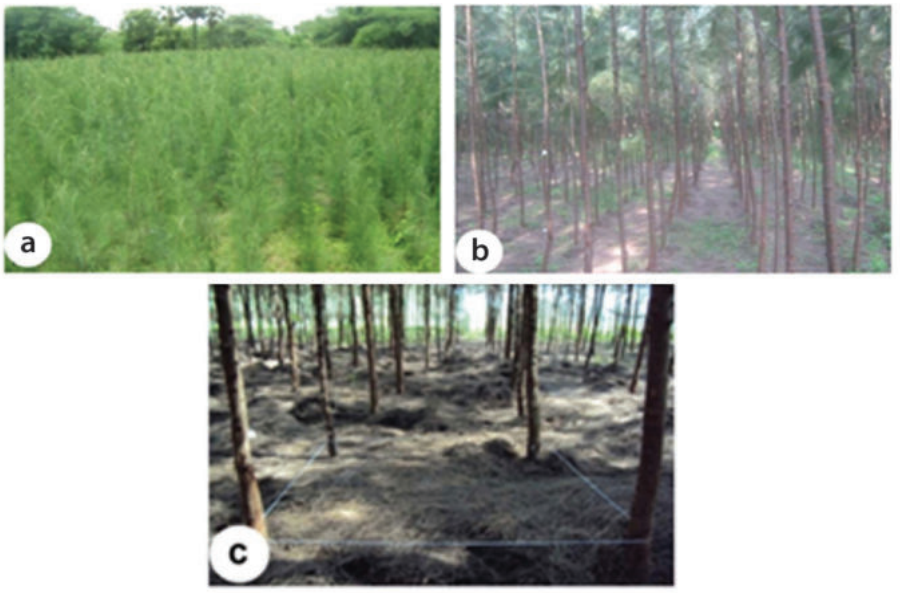

Fig. 1. *C. equisetifolia* in the study plot at aged 6 months (a) and 24 months (b) at Panjamadevi Villianur Village in Villupuram District, Tamil Nadu. Considerable amount of needle litter (c) was produced two years after planting.

2.3 Soil physico-chemical analysis

Soil sample was collected at the depth of 60-90 cm. The sample was taken randomly by using a 45-mm-diameter hand auger in the experimental plot. Visible roots and organic residues were removed during sampling and each soil sample was separately air-dried, ground and stored in cloth bags before analysis. Soil pH was determined in 1: 2.5 ratio of soil and water suspension using a pH meter (Jackson, 1973). Electrical conductivity was determined at 1: 2.5 ratio of soil and water suspension by a conductivity meter (Jackson, 1973).

2.4 Estimation of growth and biomass

Girth at breast height (1.37 m), total height, and basal area were assessed. Random sampling method was adopted to estimate the biomass. Biomass components were separated into bole wood, branch, twig, bark, needle, fruiting cones and roots. The roots were excavated from 1 m^3 soil volume for three randomly chosen trees at the end of the third year and fresh weights were also recorded. Fresh samples were collected in replicates for each of the components and oven dried at 80°C to constant weight. The dry matter of all the components was calculated from fresh weight and percent dry weight.

2.5 Estimation of nutrients and carbon in plant samples

The oven-dried plant samples were ground and sieved through a 0.5 mm plastic sieve before digestion. Total biomass carbon was measured using the dry combustion with a CHNS analyzer (VARIO-ELIII elementar) (Nelson, 1982; Kraenzel et al., 2003). Nitrogen (N) was analyzed by colorimetrically using a Kjeldahl auto analyser 1030 (Jackson, 1973). Phosphorus (P) was estimated colorimetrically using a spectrophotometer (Jackson, 1973). Potassium (K) in the extract was determined by using a flame photometer (Jackson, 1973). Calcium (Ca) and Magnesium (Mg) were determined by the Versenate method as described by Jackson (1973).

2.6 Statistical analysis

Statistical analysis (mean, standard error, analysis of variance) was carried out to determine the statistical differences in tree growth, tree component and total biomass, and carbon sequestration using SPSS 11.5.

3 Results and discussion

Cultivation of nitrogen-fixing tree species like *C. equisetifolia* in farm-forestry and agroforestry systems provides high biomass in a short rotation and improves soil fertility through a tripartite association of casuarina with *Frankia* and mycorrhizae (Rajendran et al., 2000; Rajendran and Devaraj, 2004). Statistical analysis of growth and biomass data of the present field experiment shows that the improved growth and biomass production of casuarina plants were attributed to high amount of microbial exopolysaccharides (EPS) production inoculated with both *Frankia* and *P. polymyxa* (Fig. 2 a&b). on EPS play an important role in the *Frankia* invasion process, infection threads formation, nodule development and plant defense response. These EPS also confer protection to *Frankia* when exposed to extreme environmental stress condition. EPS also has ability to form gels that promote the adhesion of soil particles, forming stable aggregates that contribute to better plant growth and development. In the present study the role of *Frankia* EPS in soil aggregation is clearly demonstrated. Inoculation with *Frankia* and *P. polymyxa* stimulated the growth and biomass of *C. equisetifolia* trees.

In the present experiment using 3-year-old trees of *C. equisetifolia* planted at ca. 10,000 trees ha^{-1} in a semi-arid zone, the results showed that *P. polymyxa* significantly improved the mean height, (13.28 m), dbh (8.36 cm), and total biomass (42.905 kg $tree^{-1}$) (Table 1). These growth data were equivalent to 10.4% increase in height, 18.5% increase in diameter at breast height and 10.8% increase in total biomass increase over the control. The carbon fixation through above plantation was 21.452 kg $tree^{-1}$. However, control trees (without treatment) exhibited comparatively poorer growth performance (mean height 11.9 m, mean dbh 6.82 cm, total biomass, 38.526 kg $tree^{-1}$) resulting in lesser carbon accumulation of 19.263 kg $tree^{-1}$ (Tables 1, 2).

The improvement of growth and biomass could be attributed to the application of suitable plant-growth promoting helper bacteria *P. polymyxa* and casuarina needle litter compost. Similarly, Muthukumar and Udaiyan (2009) reported improved growth and quality seedlings of *C. equisetifolia* in nursery condition inoculated with *P. polymyxa*. Casuarina needle litter compost may help in microbial assimilation of organic matter, regular irrigation, and intensive care of the experimental field. The above results are consistent

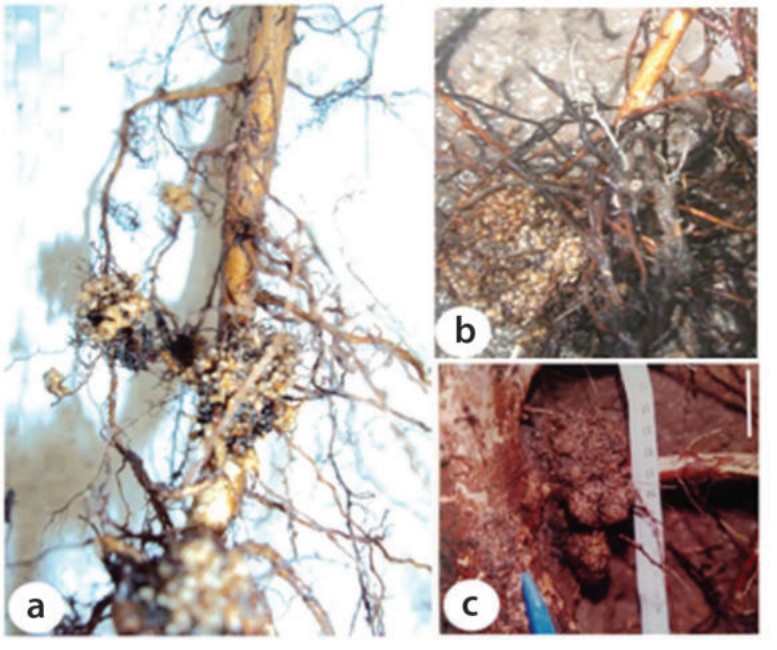

Fig. 2. Root nodules (a & b) covered by biofilm of exopolysaccharides (EPSs) produced by *P. polymyxa*; A well-developed *Frankia* nodule after early inoculation is shown in (c).

Table 1. Growth of 3-year-old *Casuarina equisetifolia* inoculated with helper bacteria (*Paenibacillus polymyxa*) in a farm forestry plantation.

Treatment	DBH (cm)	Height (m)	Basal diameter (cm)
T1	6.81±0.165a	11.86±0.262a	25.38±0.730a
T2	7.39±0.137b	12.38±0.255b	26.53±1.517b
T3	7.81±0.185b	12.66±0.233b	26.88±0.370b
T4	8.35±0.127c	13.28±0.244c	27.13±1.467c

T1- Control; T2- Casuarina needle compost; T3 - Helper Bacteria; T4 - Casuarina needle compost + helper bacteria. Means followed by a common letter in the same column are not significantly different at the 5 % level by DMRT

with earlier report of Rajendran and Devaraj (2004) in that the growth of trees was markedly improved when adopting systematic cultivation method using suitable combination of biofertilizers (Azospirillum, Phosphobacterium, AM fungi and *Frankia*). Excellent growth was observed in the height of *C. equisetifolia* which ranged from 9.87 m to 11.90 m and girth at breast height from 16.8 cm to 23.2 cm in two years after planting.

Biomass and carbon accumulation

Trees promote sequestration of carbon into soil and plant biomass. Therefore, tree based land use practices could be viable alternatives to store atmospheric carbon dioxide due to their cost effectiveness, high potential of carbon uptake and associated environmental as well as social benefit (Costa, 1996). Analysis of biomass data revealed that inoculation of helper bacteria with casuarina needle litter compost produced higher nodular and total biomass when compared to individual inoculation either needle compost or helper bacteria (Fig. 2c). The increased biomass accumulation due to decomposed needle litter (Fig. 1c) influenced the physical-chemical properties of soil and made nutrient availabille to both beneficial microorganisms and casuarina trees (Uma et al., 2014). In the present study, we found higher carbon accumulation in trees treated with helper bacteria in the field condition. These results are in accord with our earlier studies which showed that sustainable development of casuarina with high biomass production could be achieved by the application of ecofriendly and economically viable bio inoculants (Biofertilizers) along with decomposed organic substrate (Rajendran et al., 2000; Rajendran

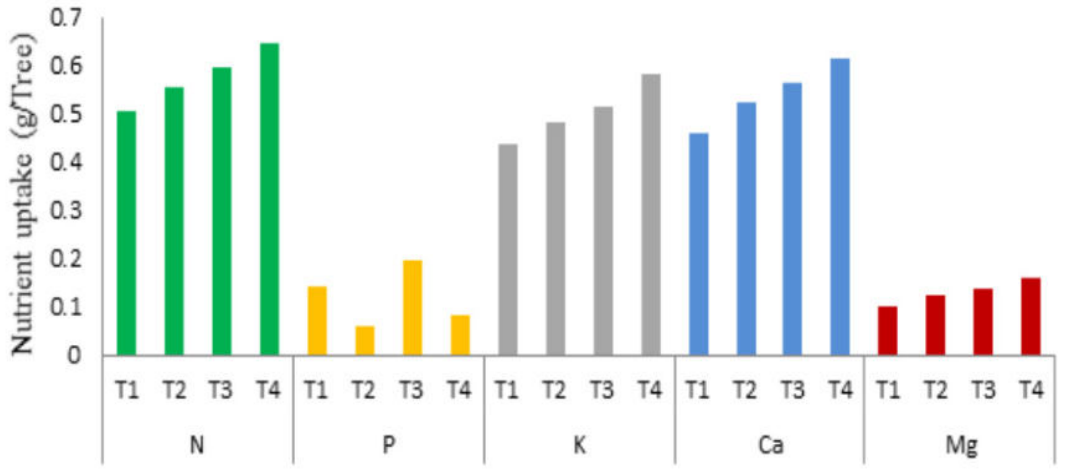

Fig. 3. Nutrient uptake of *C. equisetifolia* as influenced by helper bacteria (*P. polymyxa*). T1 - Control; T2 - Casuarina needle compost; T3 - *P. polymyxa*; T4 - Casuarina compost + *P. polymyxa*.

Table 2. Biomass (kg/tree) of different components of 3-year-old *Casuarina equisetifolia* plantation inoculated helper bacteria (*Paenibacillus polymyxa*) in farm forestry plantation.

Treatments	Stem	Bark	Branch & Twig	Needle	Fruit	Total AGB	Root	Root nodule	Total BGB	Total Biomass
T1	23.724^{a} ±0.863	2.445^{a} ±0.092	2.954^{a} ±0.094	4.876^{a} ±0.204	1.339^{a} ±0.012	35.356^{a} ±1.142	2.997^{a} ±0.228	0.173^{a} ±0.012	3.170^{a} ±0.236	38.526^{a} ±1.368
T2	24.031^{b} ±0.771	2.737^{b} ±0.09	3.024^{a} ±0.111	5.146^{b} ±0.192	1.639^{b} ±0.014	36.577^{b} ±1.115	3.165^{b} ±0.200	0.268^{b} ±0.018	3.433^{b} ±0.192	40.013^{b} ±1.254
T3	24.041^{b} ±0.587	2.887^{c} ±0.08	3.034^{b} ±0.132	5.345^{c} ±0.181	1.639^{b} ±0.019	36.946^{d} ±1.143	3.567^{bc} ±0.200	0.364^{c} ±0.014	3.931^{bc} ±0.132	40.87^{b} 7±1.291
T4	24.341^{c} ±0.575	2.987^{d} ±0.09	3.064^{b} ±0.111	5.545^{d} ±0.194	1.739^{bc} ±0.015	37.676^{c} ±1.165	3.765^{d} ±0.200	0.394^{c} ±0.016	4.157^{d} ±0.156	42.90^{c} 5±1.321

T1- Control; T2- Casuarina needle compost; T3 – *P. polymyxa*; T4-Casuarina needle compost+ *P. polymyxa*. Means followed by a common letter in the same column are not significantly different at the 5 % level by DMRT

and Devaraj, 2004; Munisamy et al., 2010; Saravanan et al., 2012; Uma et al., 2012). Growth and biomass of *C. equisetifolia* is enhanced by high levels of N and P and K (Fig. 3). The results of the present experiment also support the application of helper microbes (*P. polymyxa*) which helps to improve the growth, biomass and carbon sequestration potential of casuarina.

Tree Carbon

In east coast districts of Tamil Nadu the carbon sequestration potential of *C. equisetifolia* was displayed by the total carbon accumulation in bole of 16.7 MT ha^{-1} year^{-1} of C in Alfisols soil and 4.7 MT ha^{-1} year^{-1} of C in Vertisols soil (Ravi et al., 2010). Estimated total biomass carbon of *C. equisetifolia* plantation recorded after one year was 1.350 kg tree^{-1}, after two years was 4.687 kg tree^{-1} and after three years was 8.452 kg tree^{-1} (Uma et al., 2011). In contrast, in the present experiment under similar agro-climatic region, the average total biomass carbon was 21.452 kg tree^{-1} in three-year-old *C. equisetifolia* treated with combined inoculation of casuarina needle litter compost and *P. polymyxa*. This shift in the accumulation of biomass carbon are likely related to inoculation of helper bacteria (*P. polymyxa*) helps *Frankia* in nitrogen fixation (Muthukumar and Udaiyan, 2009), and intensive silviculture management such as appropriate spacing between trees, regular watering, weeding and manuring. Energy flow and nutrient cycling are major ecosystem processes that lead to fixation of CO_2 and production of organic compounds that store energy. In East Timor, *C. equisetifolia* (≥30 years) contains up to 200 mg C ha^{-1} in above-ground biomass carbon (Lasco and Cardinoza, 2007). Casuarina plantation (four years old) in northern Atlantic coast of Puerto Rico was reported to store tree biomass carbon of 48.2 mg ha^{-1} in above ground biomass and 10.5 mg ha^{-1} in roots (Parrotta, 1999). Similarly, Uma et al. (2011) reported that 10.37 kg tree^{-1} of carbon was accumulated in three-year-old *C. equisetifolia* in sandy loam soil of tropical coastal region in India.

4 Conclusion

The results of the present study provide valuable information to support the establishment of casuarina plantations in farm forestry and baseline data regarding suitable species, growth, biomass, nutrient

accumulation, litter production and nutrient cycling for site quality management. Casuarina in farm forestry not only helps increase wood and fodder but also protects existing forest where unemployed and poor people rush to earn their livelihood. It is therefore important that the policy makers, NGOs and Government Organizations promote sustainable tropical farm forestry in developing environmental-friendly and cost-effective technologies for poverty reduction in India by providing quality planting material, training programs for casuarina cultivation methods like trickle irrigation, suitable bio-inoculants and weed management.

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Effect of *Frankia* inoculation on photosynthetic activity and growth of *Casuarina junghuhniana* under elevated CO_2

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Abstract

Despite the importance of climate change there is only limited information on the role of microbes in increasing resilience of plants to climate change and carbon sequestration. To understand the effect of elevated CO_2 in *Casuarina junghuhniana* with *Frankia* (a nitrogen-fixing microbe) this study has been conducted. The rooted stem cuttings of *C. junghuhniana* were selected for this study as they propagated in inert media (vermiculite) where microbes are absent. These cuttings were treated with 2000 ppm of Indole Butyric Acid for rooting and inoculated with *Frankia*. The inoculated cuttings along with unincoulated cutting as control were placed in the open top chambers with elevated CO_2 facilities. The *Frankia* inoculated and non-inoculated cuttings were replicated at 10 times contains five cuttings per replicate in the open top chambers Ten replications each with five inoculated and uninoculated cuttings were used for three levels of CO_2 viz. ambient control, 600 ppm and 900 ppm and grown in open top chamber for one year. After one year, the photosynthetic activity and growth parameters of all the cuttings were assessed. The analysis of data showed that under elevated CO_2 conditions cuttings inoculated with *Frankia* showed higher number of nodules and better growth and biomass production particularly at 900 ppm of CO than the uninoculated cuttings. Nitrogen analysis showed higher tissue nitrogen content under elevated CO_2 levels than ambient control. The photosynthetic rate was also high under elevated CO_2 levels at 9.2 µ mol m^{-2} s^{-1} in 900 ppm followed by 14.6 µ mol m^{-2} s^{-1} under 600 ppm whereas it was only 7.8 µ mol m^{-2} s^{-1} under ambient CO_2 control. Results from this study revealed that *C. junghuhniana* inoculated with *Frankia* are resilient to high atmospheric CO_2 conditions as *Frankia* helps *C. junghuhniana* to thrive and improve the growth and biomass.

Keywords: *Casuarina junghuhniana, Frankia,* CO_2, climate change, biomass

1 Introduction

Green house gases including carbon dioxide (CO_2) accumulated in atmosphere due to air pollution and depletion of forests results in global warming. Afforestation is one possible solution to mitigate the

global warming through growing suitable tree species. Studies are being undertaken worldwide on different tree species to understand their carbon sequestration potential. Many studies on microbes showed that they can reduce the CO_2 level significantly contributing towards consumption of greenhouse gases such as CO_2, methane (CH_4), nitrous oxide (N_2O), and nitric oxide (NO) (Wiley et al., 2009). The microbes specifically the microbial symbionts associated with plants have contributed in Carbon sequestration by increasing nutrient uptake in plants (Garcia et al., 2011). It was also reported that N fixing plants respond positively to elevated CO_2 than other plants due to their high nutrient demand (Temperton et al., 2003) and the N fixing plants improved their nutrient supply through N fixing bacteria under elevated CO_2 (Vogel et al., 1997). Trees under elevated CO_2 also showed increased growth and photosynthesis due to high nutrient supply through microbial symbionts (Ceulemans et al., 1999). It has been reported that the microbial symbionts facilitate to sequestrate the Carbon in plants. This was also confirmed by Tissue et al. (1997) as they found increased photosynthetic rates and carbon storage in *Gliricidia sepium* inoculated with *Rhizobium* sp. under elevated CO_2. *Frankia* is a symbiotic actinomycete which is associated with *C. junghuhniana* and form N fixing root nodules. As part of the symbiotic relationship with *Frankia,* fixes N up to 300 kg ha^{-1} $year^{-1}$ (Wheeler and Miller, 1990) and in return for the 'fixed' N, this tree supplying Carbon to the symbiotic bacteria (Santi et al., 2013). *C. junghuhniana* has high calorific value with 7150 kcal kg^{-1}, and it gives the productivity from 30 to 36 m^3 ha^{-1} $year^{-1}$ (Jayaraj, 2010). But no reports found on effect of CO_2 on *Frankia* and *C. junghuhniana.* The present study has been undertaken to determine the effect of *Frankia* inoculation in *Casuarina junghuhniana* under elevated CO_2 to find out the response of growth, photosynthesis, N fixation, and biomass improvement.

2 Materials and methods

2.1 Culture of *Frankia*

Root nodules of *C. junghuhniana* were collected from the matured trees at farm fields of Coimbatore, India. The nodules were transported in an ice box and stored at -4 °C and surface sterilized with 30% H_2O_2. Later the nodules were kept at room temperature for 30-40 min. Under aseptic conditions the nodules were rinsed in sterile water and 0.2 gram of nodule was ground manually in a sterile mortar and pestle. The nodule solution was centrifuged at 1000 rpm for 20 min and the supernatant was filtered through Whatman No.1 filter paper. After that the suspension was placed on P media (Shipton and Burgraff, 1983) plates and incubated at 25°C for 3-4 weeks. (NB: one litre of P medium contained: 10g $CaCl_2$,$2H_2O$, 20g $MgSO_4$, 0.46g propionic acid, 0.15g H_3BO_3, 0.15g $ZnSO_4$.$7H_2O$, 0.45g $MnSO_4$.H_2O, 0.004g $CuSO_4$.$5H_2O$, 0.028g Na_2MoO_4.$2H_2O$, 0.009g $CaCl_2$.$6H_2O$, 0.04g Biotin, 100g K_2HPO_4, 67g NaH_2PO.$2H_2O$, 0.1g FeNa EDTA, and 8 g agar; pH of the medium is 6.8). After 25 days of incubation, the *Frankia* growth occurred as fluffy white cloudy colonies in P media plates and these colonies were transferred into P media broth for scaling up the inoculum.

2.2 Propagation of rooted stem cuttings

The stem cuttings of *C. junghuhniana* were collected from a casuarina germplasm bank at Model Nursery of Institute of Forest Genetics and Tree Breeding, Coimbatore. Uniform sized (5 cm length: 1 mm thick) stem cuttings with 10 g (±0.8) of total biomass were treated with 0.1% carbendazim fungicide for 3 min and after applied with 2000 ppm of IBA (40 mg of IBA + 20 g of talcum powder) at the basal end of the cuttings for 0.5 min by dip method. After the treatment with IBA, the cuttings were placed in 100 cc root trainers containing the inert media vermiculite. The rooted stem cuttings were thereafter placed in polytunnels made of polythene sheets (180 cm × 90 cm) and maintained with temperature of 32–35°C and 60–65% relative humidity for 30 days to develop roots.

2.3 Inoculation of *Frankia*

After development of lateral roots the cuttings were inoculated with *Frankia* culture at the rate of 10 ml $cutting^{-1}$. They were grown in 100 cc root trainers with/without inoculation of *Frankia* inside Open Top Chambers (OTC) and maintained for 12 months. These OTC are cubical structures of 3 m x 3 m x 3 m dimension fabricated with galvanized iron pipe frame and covered with polyvinyl chloride sheet. The upper portion of chamber is kept open to maintain the atmospheric conditions and to allow natural sunlight. A software facility called supervisory control and data acquisition

(SCADA) was used to control the CO_2 supply. The control (uninoculated) and *Frankia* inoculated cuttings were replicated 10 times consists of 5 rooted stem cuttings replicate^{-1}. All were watered daily and no fertilizers were added. Three OTC were used for this study viz. (i) OTC with 600 ppm CO_2 supply day^{-1}; (ii) OTC with 900ppm CO_2 supply day^{-1}; and (iii) an ambient CO_2 controlled chamber. 598 (±2.2) ppm of CO_2 was provided throughout the day in 600 ppm chamber and 899 (±1.7) ppm of CO_2 was provided in 900 ppm chamber. These CO_2 levels were supplied using CO_2 cylinder in the chambers for the entire study period and monitored through SCADA. The ambient CO_2 chamber showed 380 (±1.1) ppm of CO_2. The average temperature in the chambers was 36.8±1 ºC and the average relative humidity was 65±1.2%. The mean annual rainfall recorded (Coimbatore, India) during the period of study was 796.8 mm.

2.4 Harvest and analyses

All the plants were harvested after 12 months in the OTC chambers and measured for their growth in terms of shoot length, root length, number of nodules, collar diameter and biomass. The tissue N content was analysed by the method of Jackson (1971).

2.5 Photosynthetic rate

At the end of 12 months study period the light saturated photosynthetic rate (A_{sat}, µ mol CO_2 m^{-2} s^{-1}) was measured on 15-day-old needles from the top of the stem using a photosynthetic meter (Li 6400 XT, Licorlinc, USA). The leaf chamber of photosynthetic meter was set at 380 ppm CO_2, 24°C temperature and saturating photosynthetic rate of 1500 µ mol m^{-2} s^{-1}. All the inoculated and uninoculated plants placed in OTC kept under ambient, 600 ppm and 900 ppm CO_2 conditions were measured for photosynthetic rates.

2.6 Statistical analyses

Each measured variable in the OTC experiments were statistically analyzed using Duncan's multiple range test (SPSS ver. 17).

3 Results

3.1 *C. junghuhniana* rooted stem cuttings

At the end of the 12 months study period, the effect of elevated CO_2 on *Frankia* inoculated rooted stem cuttings of *C. junghuhniana* showed the highest growth and biomass were improved under 900 ppm of elevated CO_2 conditions. The shoot biomass (62.3 g plant^{-1}), root biomass (44.59 g plant^{-1}) and number of nodules (26.3 g plant^{-1}) of these plants were significantly (P = 0.05) increased in *C. junghuhniana* rooted stem cuttings inoculated with *Frankia* under of elevated CO_2 conditions than uninoculated control seedlings (Table.1). Root nodules were observed in all the rooted stem cuttings of *C. junghuhniana* inoculated with *Frankia* culture although they were grown in the inert media (vermiculite). None of the uninoculated plants showed presence of root nodules. Nodule number was higher under 600 ppm of CO_2 conditions than ambient CO_2 conditions. However, the uninoculated control plants under 900 ppm of elevated CO_2 conditions showed poor growth, biomass than ambient and 600 ppm of elevated CO_2 conditions. The collar diameter was significantly (P<0.05) increased with inoculation of *Frankia* under 600 ppm and 900 ppm of CO_2 conditions than control seedlings. Under ambient CO_2 conditions the *Frankia* inoculated plants showed significantly (P<0.05) increased growth and biomass and number of nodules than control plants (Table 1). In overall the results showed that the rooted stem cuttings inoculated with *Frankia* could improve the growth and biomass under elevated CO_2 conditions whereas the uninoculated control plants showed poor performance under both elevated CO_2 conditions (particularly under 900 ppm) and ambient conditions.

3.2 Photosynthetic activity

C. junghuhniana rooted stem cuttings showed higher photosynthetic rates in 600 ppm and 900 ppm of elevated CO_2 conditions and *Frankia* inoculation. The photosynthetic rate found increased (19.2µ mol m^{-2} s^{-1}) in 900 ppm level of elevated CO_2 conditions than 600 ppm (14.6 µ mol m^{-2} s^{-1}) and ambient control (7.8 µ mol m^{-2} s^{-1}) conditions. The control plants showed poor photosynthetic rates than *Frankia* inoculated plants particularly under 900 ppm of elevated CO_2 conditions (Fig.1).

3.3 Tissue nitrogen content

The tissue N content (mg g^{-1}) was significantly higher (P<0.05) in *Frankia* inoculated cuttings at 900 ppm (4.3mg g^{-1}) of elevated CO_2 conditions than those at

Table. 1. Response of *C. junghuhniana* rooted stem cuttings inoculated with *Frankia* under three levels of CO_2 conditions (mean of 10 replicates) after growing for 12 months in open top chamber. Means followed by same letters are not significantly different at 5% level of DMRT.

CO_2 treatment	Inoculation treatment	Shoot length (cm)	Root length (cm)	Collar diameter (cm)	No. of nodules plant^{-1}	Shoot biomass (g)	Root biomass (g)	Total biomass (g)
Ambient CO_2	*Frankia*	52.6b	28.3b	3.4b	12.2	22.5b	16.3b	38.8b
	Control	36.3a	18.6a	2.8a	0	18.6a	15.4a	34.0a
CO_2 600ppm	*Frankia*	59.3b	30.8b	4.4b	18.4	42.1b	32.2b	74.3b
	Control	40.1a	24.6a	3.2a	0	14.3a	11.5a	25.8a
CO_2 900ppm	*Frankia*	85.2b	55.4b	5.6b	26.8	62.3b	44.59b	76.89b
	Control	33.3a	19.3a	1.9a	0	10.03a	9.58a	19.61a

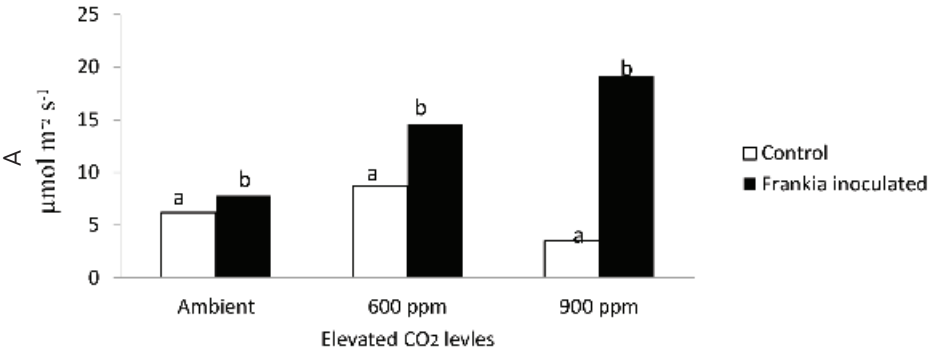

Fig. 1. Photosynthetic rates of *C. junghuhniana* rooted stem cuttings under elevated CO_2 conditions (mean of 10 replicates). Bars indicating same letters are not significantly different according to DMRT (P<0.05).

ambient CO_2 level. Further it was observed that under 900 ppm of CO_2 conditions, *Frankia* inoculated cuttings showed significantly (P = 0.05) higher N content (4.3 mg g^{-1}) than control seedlings under elevated CO_2 conditions (Fig. 2).

4 Discussion

The microbial symbionts like *Frankia* can contribute to carbon sequestration by increasing nutrient uptake by plants (Garcia et al., 2011) as found in this study. In the present study elevated CO_2 greatly influenced the growth biomass, nutrient content and photosynthesis in *C. junghuhniana* inoculated with *Frankia*. The rooted stem cuttings of *C. junghuhniana* grown in soilless media (vermiculite) without any fertilization the plants have responded well in growth and biomass under elevated CO_2 due to inoculation of *Frankia*. It was also confirmed that the inoculation of *Frankia* has only promoted the growth of *C. junghuhniana* rooted stem cuttings through N fixing root nodules as the other microbes were absent in the inert media (vermiculite). Inoculation of *Frankia* mitigate the temperature and drought stress of the *C. junghuhniana* and may be induced the oxidative stress under the elevated CO_2 (Abd. Elgawad et al., 2015) which may be the reason of growth improvement in *C. junghuhniana*. The rooted stem cuttings of *C. junghuhniana* of present study responded positively to elevated CO_2 in growth, biomass, photosynthetic rates and nutrient accumulation with microbial symbionts which are in accordance with earlier studies on *C. equisetifolia* and

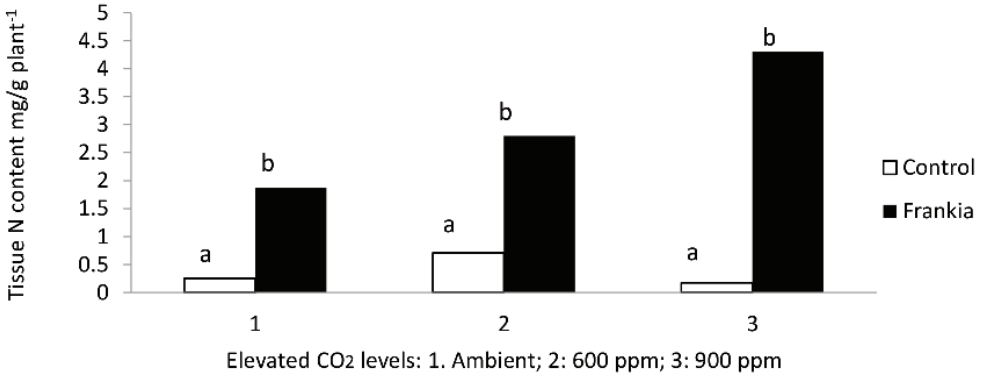

Fig. 2. Tissue N content in rooted stem cuttings of *C. junghuhniana* inoculated with *Frankia* under elevated CO_2 conditions. Bars indicating same letters are not significantly different according to DMRT (P<0.05).

Acacia auriculiformis (Karthikeyan, 2017; 2019). The increased number of root nodules observed in this study may be due to increased nitrogenase activity that led to higher fixation of N. In extreme CO_2 elevated conditions (900 ppm) the rooted stem cuttings of *C. junghuhniana* without *Frankia* found poor growth due to low N fixation and temperature stress. Nigom et al. (2016) also reported the successful tolerance of casuarinas to environmental stress with *Frankia*. Song et al. (2014) found enhanced growth and biomass in *Loliumperenne* inoculated with *Trichoderma* under ambient CO_2 conditions. Elevated CO_2 conditions (900 ppm) increased collar diameter compared to ambient CO_2 which agreed with Yazaki et al. (2004). In overall this study showed that *C. junghuhniana* along with *Frankia* responded positively at 900 ppm of elevated CO_2conditions.

5 Conclusion

Nitrogen fixing microbial symbiont, *Frankia* plays important roles in improving adaptability and growth of casuarinas. In this present study under high atmospheric CO_2 conditions the *Frankia* facilitate the casuarinas for growth and biomass improvement.

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A novel compound for inducing grazing tolerance in *Casuarina junghuhniana* clones

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Abstract

Casuarina saplings are browsed by herbivores causing economic loss in unguarded plantations. The current study is aimed at developing a novel compound, which can provide protection to plants from grazing. Our hypothesis is that certain enzymes present in animal saliva induce secondary metabolite production in plants, which deter further grazing. Novel compounds synthesised from identified enzymes in animal saliva were treated directly on plants along with animal saliva as control. The investigation methodology was to capture protein response in treated casuarina saplings in controlled nursery conditions using mass spectroscopy. Saliva of different domestic animals analysed using mass spectrometry revealed a total of 181 proteins of which, the expression of bovine serum albumin (BSA) and Lactoperoxidase was high compared to other compounds. The results indicated that proteins produced during stress and stimulus were up regulated in plants, treated with Lactoperoxidase and BSA. The protein expression pattern was similar to that of plants treated with animal saliva (control). Lactoperoxidase in animal saliva is found to induce defence pathway in plant system, which is expressed during grazing. We conclude that casuarina needles respond to elicitors in animal saliva resulting in changes in phytohormone levels and defence-associated secondary metabolites. Lactoperoxidase treatment in casuarina can be used to prevent grazing, as treated needles showed 83% reduction in feed intake in controlled animal studies.

Keywords: Lactoperoxidase, animal grazing, secondary metabolites.

1 Introduction

Casuarina is an important plantation species cultivated for various purposes, highly suited for agrarian ecosystem in South and South East Asia. Major advantages of this species are short rotation period, ability to biological nitrogen fixation which enhances soil fertility and ready marketability. Currently crop improvement is through selection of outstanding phenotypes from provenance trials. (Nicodemus et al., 2001; Pinyopusarerk et al.,

2004). Genetic improvement of the species has led to development of high yielding clones, which need to be characterized for effective utilization of the resource in harsh sites. In India, farmers are shifting to casuarina cultivation in inland areas, where it is not traditionally planted, due to shortage of farm labour, insufficient water availability for agriculture, non-remunerative prices for farm produce and growing tendency of absentee farming. (Nicodemus, 2009). Movement of coastal tree species to inland region results in stress due to new environment, pest and disease and susceptibility to animal browsing. Tree vitality is severely impacted due to mammalian herbivory. In temperate broadleaved forests, sapling growth and development is severely affected due to deer browsing (Kuijper et al., 2010). Species composition and ecosystem processes is changed due to animal browsing (Horsley et al., 2003). Deer browsing affects sapling performance and mortality due to damage of leaves and buds (Augustine and McNaughton, 1998; Cote et al., 2004; Ammer et al., 2010). Most of the trees are having thorns or spines, but temperate deciduous tree species lack such mechanical defence against browsing animals.

Herbivory attack made many plants to evolve mechanism to perceive and respond to external stimulus (Coley, 1983; Bryant et al., 1989; Rautio et al., 2012). Insect-herb interactions revealed that phytohormones such as jasmonates and cytokinins are triggered (Erb et al., 2012; Giron et al., 2013). Expression of jasmonates activates defence metabolite pathway in the plants (Fonseca et al., 2009; De Geyter et al., 2012). Similarly, growth response regulation, plant interaction with environment and defence metabolites induction is regulated by cytokinins (Sakakibara, 2006; Argueso et al., 2009; Großkinskyet al., 2011). Due to different morphogenesis, seasonal effects and complex growth pattern, trees' response to insects may be more complex phenomenon (Laitinen et al., 2005; Boege et al., 2007). Response in plant system is similar to insect attack as it produces jasmonates as a chemical response (Frost et al., 2008; Boeckler et al., 2013). Plant response to mammalian attack could be similar to insect attack, which requires detailed study of plant response to mammalian infestation. Plants are capable to receive elicitors peculiar to herbivore feeding habits (Alborn et al., 1997; Halitschke et al., 2003; Schmelzet al., 2006; Schafer et al., 2011; Falk et al., 2014). Oral secretions of insects produce elicitors which triggers phytohormone production in the plant (reviewed in Erb et al., 2012). Hormone such as salicylic acid responsible for defence against pathogen inhibit jasmonate-related defences (Gilardoni et al., 2011) and trade off happens for plant defence against herbivores. A study was conducted by removing leaves and application of saliva significantly increases the number of newly formed branches in moose saliva on sallow (*Salix caprea* L.) (Bergman, 2002). Similarly, goat saliva has shown significant shoot growth on *Combretum apiculatum* Sonder (Combretaceae) in Botswana (Rooke, 2003). Current study aimed at identifying, functional compounds present in animal saliva and make casuarina plants tolerant to animal grazing.

2 Material and methods

2.1 Proteomics of animal saliva

2.1.1 Animal saliva collection

Feed was withheld from animals for 12 hours prior to all anesthetic procedures. To achieve partial sedation, Xylaxin (0.1% kg^{-1} body weight) was administered intramuscularly in the thigh by using Syringe. After 20 minutes using 15-mL polypropylene centrifuge tube, saliva was collected from oral cavity of different animals. Immediately tubes were labelled and stored in dry ice, shifted to laboratory for downstream analysis.

2.1.2 Sample preparation for proteomics

Protein quantification of saliva samples were carried out using Bicinchoninic Acid (BCA) assay method, after quantification 47 µl (6M Urea, 50 mM ammonium carbonate) of solution was added 50 µg of animal saliva. Samples were mixed with gentle tapping, centrifuged for 5-10 min at 8,000 rpm. Suspend pellet with 1µl solution (250 mM Tris(2-carboxyethyl) phosphine (TCEP) prepared in milliQ water) for denaturation. Again samples were mixed and spin down, incubated at 37 °C for 30 min. Added 2 µl of 625 mM Iodoacetamide (IAA), incubated in room temperature in dark condition. In solution digestion was done by adding 450 µl of activated trypsin (in 50 mM ammonium bicarbonate). Samples were incubated at 37 °C for 12-16 hours. Trypsin-digested proteins were purified by Waters Oasis HLB cartridge (1CC/30 mg, Part No. WAT094225) chromatography and collected

in protein low-bind Eppendorf microcentrifuge tubes for mass spectrometry as per standard protocol with 0.1% Trifluoroacetic acid and 100% Acetonitrile. Proteins from Oasis cartridge were eluted in 5µl of Elution buffer (70% Acetonitrile in 0.5% formic acid) twice in a fresh 1.5 ml tube, vacuum dried and stored at -80 °C.

2.1.3 Mass spectrometry

Samples were dried and re-suspended in 0.1% formic acid with 5% acetonitrile in MS water. The peptides were loaded onto an EASY-nano LC system (Proxeon). Peptide mixtures were separated on a C18 reverse phase column (PicoFrit capillary column, 75µm×10cm, New Objective) using a linear gradient of Solvent A (0.1% formic acid in 5% acetonitrile) and Solvent B (0.1% formic acid in 95% acetonitrile) at a flow rate 250 nL min^{-1} directly into a LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, CA, USA) with nano-ESI source with spray voltage was set at 1.6 KV. Stable spray condition was maintained prior to sample loading. The instrument was operated in data-dependent mode with dynamic exclusion enabled. The MS/MS spectra on the top 20 most abundant peptide ions in full MS scan were obtained. The normalized CID was set at 35% for MS/MS.

2.1.4 Data analysis

The data were processed using Proteome Discoverer, version 1.4. The files were subsequently submitted to an in-house Python based tool Pro^{t}Lyzer server for database searching through the Proteome Discoverer ss1.4 programme. The data were searched against the bovine protein database of UniProt. The search was performed choosing trypsin as specific enzyme. A maximum of, one missed cleavage was allowed. Peptide (parent ion) tolerance of 2.5Da and fragment ion tolerance of 1Da were allowed, Oxidation (M) as variable modifications and fixed modification of carbamidomethylation (C) were used. Based on the set parameters in the Proteome Discoverer workflow, the final results have list of mapped proteins with its abundance / intensities measured by the respective number of peptides, the peak value estimation measured by area and the peptide matching score in Pro^{t}Lyzer tool.

2.2 Plant response to saliva and chemicals

2.2.1 Saliva and chemical treatments to plant

Plant study was conducted at Mettupalayam (11°14′43.6″ N, 76°52′17.1″ E) near Coimbatore, Tamil Nadu, India. The region has an average annual temperature of 26.9 °C with 751 mm mean annual rainfall. One clone of *C. junghuhniana* (PL143) was used for the study with three replications in a randomized complete block design). Clones were vegetatively propagated from mother bed. Later plants were transplanted to 30cm covers filled with red soil mixed with FYM. After 15 days of transplantation, plants were used for studies. We used plants, which were free from scars and growth deformation, confirming that plants were not previously grazed by any animals. Each sapling was assigned to following treatments: (i) control (no treatment), (ii) clipping of apical buds along with side branch and water application (iii) clipping of apical buds along with side branch and different animal saliva, and (iv) clipping of apical buds along with side branch and functional chemicals (Table 1, Fig.1-3).

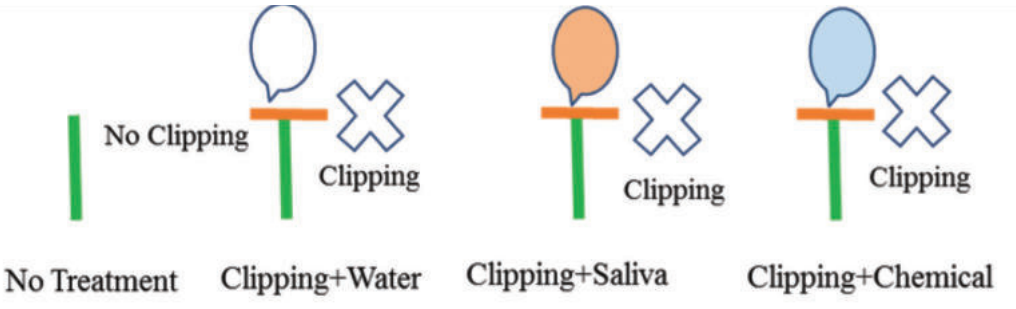


Fig. 1. Treatment design for studies.

Table 1. Treatment details used for this study.

Treatment	Details
T1	No treatment
T2	Clipping and water
T3	Cow saliva
T4	Buffalo saliva
T5	Sheep saliva
T6	Goat saliva
T7	BSA
T8	Lactoperoxidase

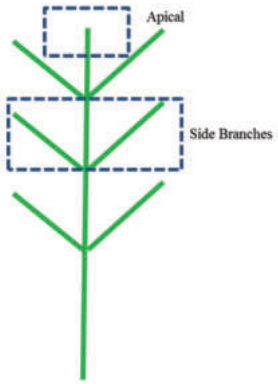


Fig. 2. Clipping positions on the plants for studies.

Fig. 3. Saliva treatment to plants: (a) clipping at apical region, (b) clipping at side branches.

Animal saliva and chemical compounds were applied at 20 µl plant^{-1}. Samples were collected immediately after treatment, 7 days after treatment and 15 days after treatment to capture protein expression changes. Samples tubes were labelled and stored in dry ice, shifted to lab for downstream analysis.

2.2.2 Protein isolation and analysis

Proteins were isolated from samples collected after animal saliva and chemical treated plants using P-PER® plant protein extraction kit (Cat No.89803, Thermo Scientific) as per standard protocol. Sample preparation; mass spectroscopy and data analysis were performed as mentioned in Section 2.1.1.

2.3 Animal studies

2.3.1 Effect of treated casuarina on feed intake of sheep and buffalo

This study was conducted at the Department of Animal Science, University of Agricultural Sciences, Dharwad, Karnataka, India. (15°29′05.6″ N; 74°58′52.7″ E). For animal studies, UASD breed of the sheep (Ram) and Murrah breed of buffalo (Heifers) were used. The study was conducted in two parts. The first part feed acclimatization to animals, sheep and buffalos were fed with casuarina leaves for four weeks to determine the acceptance and actual intake by the animals. During the study, intake of casuarina leaves, body weight changes and health parameters were monitored. The second part of the study was to evaluate the effect of lactoperoxidase enzyme on the intake of casuarina leaves for weeks. Eight sheeps placed in different pens were fed with treated casuarina, eight sheeps placed in different pens were fed with casuarina untreated and eight sheeps placed in different pens were fed with normal feed (Fig. 4). Similarly, four buffalos were fed with treated casuarina, four buffalos were fed with untreated casuarina and four buffalos were fed with normal feed for 4 weeks. In animal studies, 1 kg treated

casuarina and untreated casuarina along with 500 g green fodder was fed to sheep every day for four weeks. Sheep fed with normal fodder (1.5 kg day^{-1}) was considered as control. Everyday fodder wastage was monitored after 24 hours of supply. In case of buffalos 10 kg of treated casuarina and untreated casuarina along with 10 kg of green fodder was supplied for four weeks. Buffalos fed with normal fodder (20 kg day^{-1}) was considered as control. Everyday fodder wastage was monitored and recorded after 24 hours of supply.

3 Results and discussion

3.1 Proteomics of animal saliva

Mass spectrometry results revealed that cow saliva has got the highest level of protein expression compared to other animals (Fig. 5) with the total number of approximately, 181 proteins, of which Serum albumin, Lactoferrin, Lactoperoxidase, Cathelicidin are commonly found saliva functional compounds in all animal saliva. Commonly expressed functional compounds were synthesised and used for inducing similar changes in plant as of actual animal saliva.

Treatment	No. of Pens		No. of animals/ Pen	
	Sheep	Buffalo	Sheep	Buffalo
Casuarina treated + green fodder	4	4	2	4
Casuarina control + green fodder	4	4	2	4
Normal feed	4	4	2	4

Fig. 4. Experimental design for pens arrangement; (a) for sheep and (b) for buffalos.

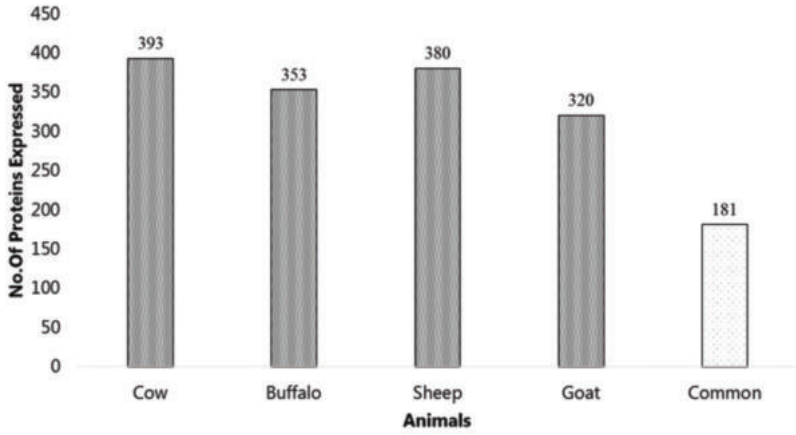


Fig. 5. Proteins identified in different animal saliva.

3.2 Plant response to saliva and chemicals

3.2.1 Protein expression in plants response to animal saliva and Lactoperoxidase

Mass spectrometry results showed that 55% more proteins are expressed after 15 days of treatment compared to immediately after treatment in animal saliva. Similarly, 71% more proteins are expressed after 15 days compared to immediately after treatment in Lactoperoxidase (Fig. 6). Results revealed that functional compound is stable up to 15 days after treatment. Experiment also suggested that same proteins are expressed in animal saliva and Lactoperoxidase (Table 2).

3.2.2 Protein expression in major pathways of plants in response to animal saliva and Lactoperoxidase

Mass spectrometry studies revealed that proteins in response to stress, stimulus, many cellular process pathways are triggered in animal saliva treated plants (Fig. 7). Similar proteins are also triggered in plants treated with Lactoperoxidase enzyme, which suggests that Lactoperoxidase is the functional compound commonly found in animal saliva, which triggers similar pathways of animal saliva and inducing secondary metabolite production in response to animal grazing. (Fig. 8). Comparison of proteins expressed in plants treated with animal saliva, Lactopeoxidase, BSA and no treatment suggests that animal saliva treated plants

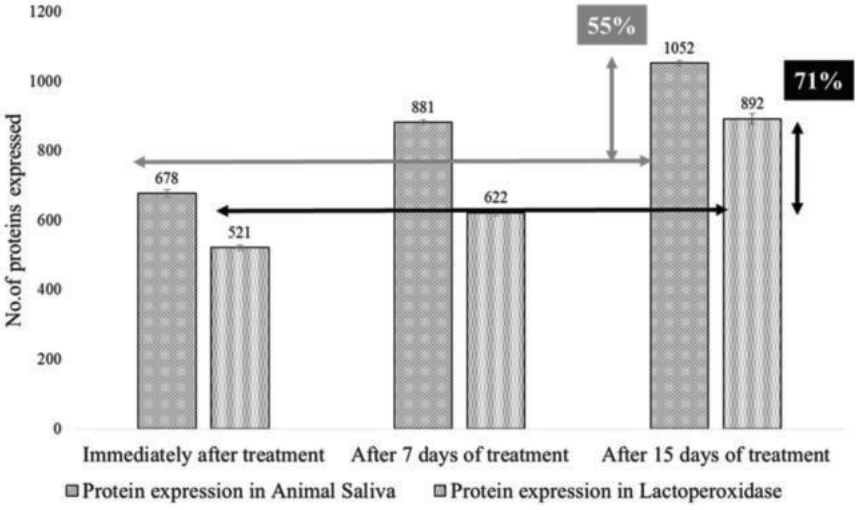
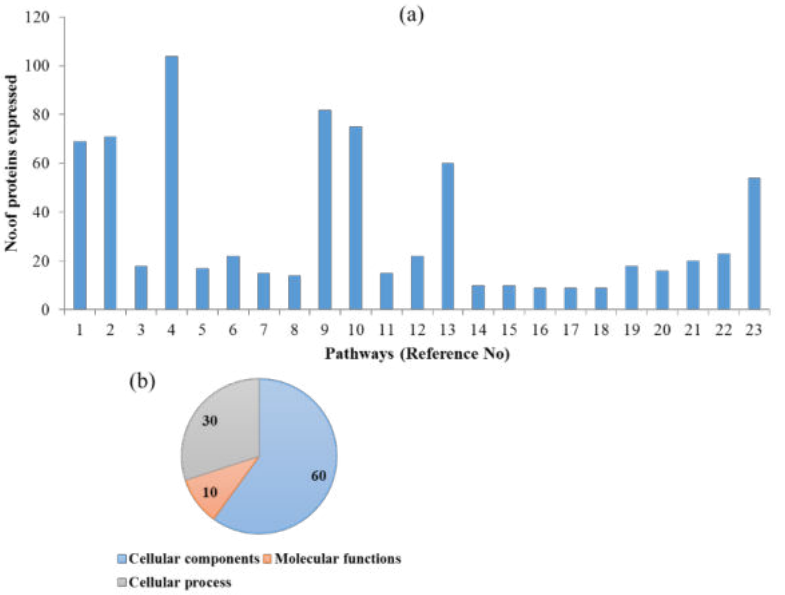


Fig. 6. Proteins expression in plants to animal saliva and Lactoperoxidase.

Table 2. Phytohormones and defence metabolites expressed in animal saliva and Lactoperoxidase.

Phytohormones
Jasmonic acid
Jasmonic acid-isoleucine
Salicylic acid
Trans zeatin
Trans zeatin riboside
Hydroxy jasmonic acid

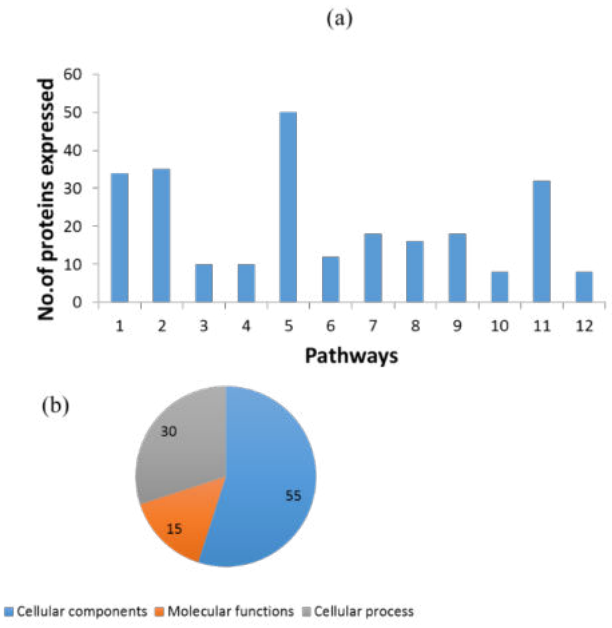
Defence metabolites	Compounds
Condensed tannins	Catechin, Epicatechin, proanthocyanidin dimers and trimmers
Flavonols	Quercetin, Quercetin glucoside, Kaempferol glucosides and Kaempferol rhamnoside, Quercetin rhamnoside
Phenolic acids	Caffeic acid derivative Caffeoyl quinates
Hydrolysable tannins	Maplexin, Corilagin, Geraniin isomers, Ellagitannins



(7a)

Reference No	Pathways
1	Resonse to stress
2	Response to stimulus
3	Protein folding
4	Cellular process
	Generaton of precursor metabolites and energy
5	Catabolic process
6	Cofactor metabolic process
7	Photosynthesis
8	Metabolic process
9	Cellular metabolic process
10	Cellular component assembly
11	Small molecule metabolic process
12	Single organism process
13	Macromolecular complex subunit organization
14	Macromolecular complex assembly
15	Protein complex assembly
16	Protein complex biogenesis
17	Protein complex subunit organization
18	Cellular component biogenesis
19	Carbohydrate metabolic process
20	Carbohydrate metabolic organization
21	Single organism metabolic process
22	Nitrogen compound metabolic process
23	

Fig. 7. Plant response to animal saliva treatment (a) Number of proteins expressed in different pathway (Refer Table:7a); (b) Classification of pathways.



(8a)

Reference No	Pathway
1	Resonse to stress
2	Response to stimulus
3	Cofactor metabolic process
4	Generaton of precursor metabolites and energy
5	Cellular process
6	Carbohydrate metabolic process
7	Small molecule metabolic process
8	Catabolic process
9	Single organism metabolic process
10	Protein folding
11	Single organism process
12	Photosynthesis

Fig.8. Plant response to animal saliva treatment (a) Number of proteins expressed in different pathway (Refer Tabe:8a); (b) Classification of pathways.

and Lactoperoxidase treated plants are expressing similar number of proteins involved in pathways related to stress, stimulus and cellular process compared to control and BSA. This experiment indicates that Lactopeoxidase is inducing defense mechanism similar to animal saliva. So Lactopeoxidase can be used to induce defense mechanism in plants against animal grazing (Fig. 9).

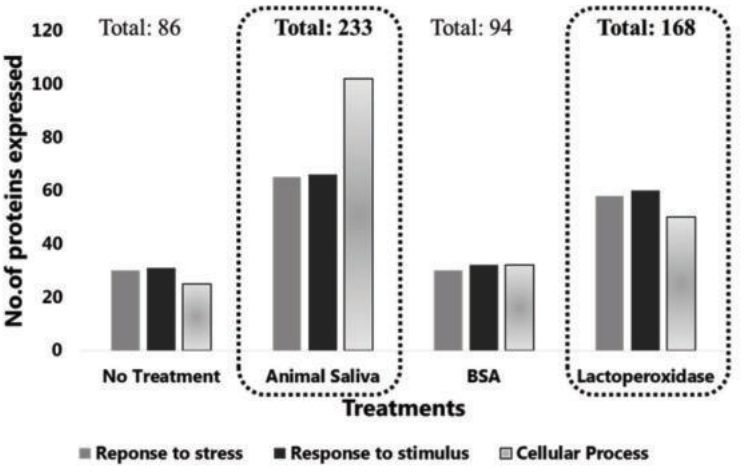


Fig. 9. Protein expression changes in plant to animal saliva and Lactoperoxidase treatments.

3.3 Animal studies

3.3.1 Effect of treated casuarina on feed intake of sheep

Fodder wastage was monitored every 24 hours for two weeks. Wastage of casuarina treated is around 83% more compared to untreated casuarina, which lead to less wastage of green fodder as animal maintains intake of fodder for its survival. This clearly indicates casuarina treated feed is not preferred by animals. Fodder wastage is less in untreated casuarina and green fodder, revealing acceptance of fodder by animals (Fig. 10).

3.3.2 Effect of treated casuarina on feed intake of buffalos

Everyday 10 kg casuarina and 10 kg green fodder were supplied per animal per day. Wastage is recorded after 24 hours, which showed that more than 90% wastage in treated casuarina compared to untreated casuarina. Wastage is optimum in case of untreated casuarina and green fodder alone. This study further showed that, Lactoperoxidase is inhibiting feed intake in animals due to activation of defense pathway by producing indigestible compounds (Fig. 11).

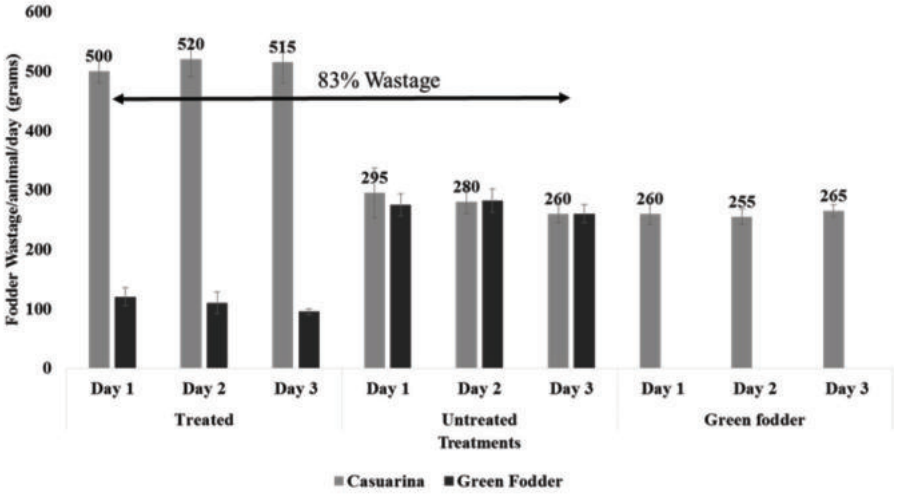


Fig. 10. Fodder wastage per animal per day (sheep).

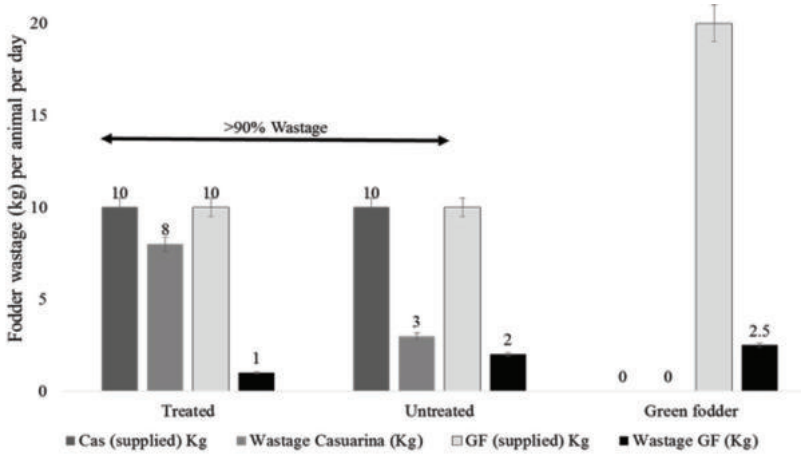


Fig. 11. Fodder wastage (kg) per animal per day.

Generally, casuarina growth in coastal environment is relatively better compared to inland due to its coastal adaptation. Despite its advantages, casuarina consumption as a pulp wood is insignificant in India, due to the shortage of raw material at affordable cost. In dry site, casuarina is not popular due to non-availability of drought tolerant varieties and animal grazing, while the recent tree improvement programs are geared up for development of drought tolerant varieties, however animal grazing is still an important issue to be addressed.

Animal grazing at early stage of growth results in mortality of plants because of removal of stem, leaves and buds. Some adaptable tree species have thorns as a defensive mechanism against grazing. Currently, the animal grazing can be managed by fencing or border – trenching, but both methods are expensive. Earlier studies suggested that animal grazing induces defense pathway in plants in general, while the current study clearly shows that Lactoperoxidase is inducing defence pathways, similar to animal saliva and making plants to develop secondary metabolites to combat possible animal grazing. Looking at the cost effectiveness, the identified commercial formulation of Lactoperoxidase could be sprayed on the border plant to prevent animals from entry.

4 Conclusion

Here we present the first attempt to translate measured chemical responses into product for preventing/ escaping animal browsing at early stage of plant growth. Based on understanding of different animal saliva at protein level, functional compound was derived. This compound would induce similar secondary metabolite pathways, similar to animal saliva. This entire study would help farming community to make casuarina plantations tolerant to animal grazing at early growth stage to prevent crop loss due to plant mortality.

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Pests and diseases, Climate change adaptation

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Ecology of Guam's *Casuarina equisetifolia* and research into its decline

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Abstract

On Guam, the estimated lifespan of *Casuarina equisetifolia* subsp. *equisetifolia* is 35 to 90 years and trees may reach a height of 24 m and a diameter at breast height of 92 cm. The majority of its trees are monecious. In 2002, it was noticed that many trees were in a slow decline and when felled, droplets often formed on their fresh stumps. In 2009, this condition was coined ironwood tree decline (IWTD). The droplets which consisted of various mixtures of plant exudates and bacterial ooze were found to contain *Ralstonia solanacearum* and various wetwood bacteria. Reported in 2015 and confirmed in 2020, Guam's *R. solanacearum* strains are of the phylotype 1 (Asia). Cross-sections of IWTD trees from Guam are very similar in appearance with those of bacterial wilt from China. In 2012, a project was initiated to diversify the gene pool and identify resistance of *C. equisetifolia* in Guam trees. Using the 1991-1993 international provenance trials of *C. equisetifolia,* 11 geographically paired seedlots were planted at a farm where IWTD was present. Over the succeeding years, none of these trees have developed IWTD nor tested positive for *R. solanacearum.* Over the years, the following pests were investigated and ruled out as contributors to IWTD: *Protaetia orientalis, Protaetia pryeri, Selitrichodes casuarinae, Helicotylenchus* sp. and *Phellinus* sp. Between 2010-2019, data analyses identified three prominent variables as predictors of IWTD: bacterial wilt pathogen *R. solanacearum,* butt and root rot fungus *Ganoderma australe* and termites. In 2019, *Nasutitermes takasagoensis* complex was found to be the dominant termite species infesting Guam's *C. equisetifolia.*

Keywords: *Casuarina equisetifolia, Ralstonia solanacearum, Ganoderma australe,* wetwood, ironwood tree decline on Guam, bacterial wilt China, bacterial wilt India

1 Introduction

Much of the information presented in this article is contained in a general review of Guam's *C. equisetifolia* (Schlub, 2019).

1.1 History

Casuarina equisetifolia, subsp. *equisetifolia,* is tightly integrated into the local culture and the Guam's environment, where it is locally known as ironwood (in English) and "gago" (in the native Chamorro language). It has been continually propagated on Guam since the 1600s. It is a hardy, pioneer, salt-resistant tree that occurs on the island's main soil types: limestone, volcanic, and coral sand. It is propagated for windbreaks, erosion control, and urban landscapes. Because *C. equisetifolia* is the dominant tree species on many of the sandy beaches of the Mariana Islands, it has become an important perching tree for the white-collared kingfisher (*Halcyon chloris*), the Mariana fruit-dove (*Ptilinopus roseicapilla*), and the white fairy tern (*Gygis alba*), which commonly lays eggs in ironwood trees. Since the 1980s and prior to appear of tree decline in the 2000s, the Guam Department of Agriculture provided approximately 250,000 seedlings to farmers, the public, and government agencies for various tree planting projects.

1.2 Ecology

On Guam, estimated lifespan of *C. equisetifolia* is 35 to 90 years, and it may reach a height of 24 m and DBH of 92 cm. Its population on Guam is estimated to be 80% monecious, 3% male, and 10% female. Ironwood thickets are a component of Guam's forestland, where *C. equisetifolia* is considered a secondary forest species. In the Mariana Islands, it grows in the clay volcanic soils of savanna grasslands and in the calcareous and loamy sands of coastal strands. On Guam, *C. equisetifolia* is only one of eight tree species larger than 28 cm in diameter at breast height (DBH). In addition, *C. equisetifolia* is a prominent member of the halophytic (sea-salt adapted) vegetation type. This vegetation type is found along beaches in northern and southern Guam, where it may be composed solely of *C. equisetifolia* or a mixture of other species, including *Cocos nucifera, Guettarda speciosa, Hernandia sonora, Pandanus tectorius, Scaevola taccada, Thespesia populnea,* and *Tournefortia argentea.*

1.3 Ironwood tree decline (IWTD)

Symptomatic *C. equisetifolia* began appearing in tree stands across Guam in 2002. In one farm location, five 10-year-old trees planted as part of a windbreak exhibited symptoms of rapid yellowing (chlorosis) and mortality. At the same time, trees at this location and elsewhere on Guam were exhibiting symptoms of thinning foliage and a lethal progressive dieback. Age of affected trees ranged from 10 years to several decades. A study was commissioned in 2004, after Natural Resources personnel with Commander Navy Region Marianas (COMNAVMAR) observed high mortality among trees at the Naval Station. The study failed to identify a cause for the mortality but did rule out two invasive beetles, *Protaetia pryeri* (Janson) and *Protaetia orientalis* (Gory and Percheron). By 2005, one third of all trees at the Naval Station were dead. In 2008-2009, the condition of foliage thinning and dieback on *C. equisetifolia* was referred to as ironwood tree decline (IWTD) (Schlub et al., 2011). At that time a visual tree scoring system was developed based on five ordinal categories of branch fullness and dieback: 0= symptomless, 1=slight damage, 2=distinctly damaged, 3=heavily damaged, and 4=nearly dead) (Schlub et al., 2011). As of today, trees continue to die, although the rate of loss appears less than that in 2005.

2 Materials, methods and results

2.1 International provenance trial

To identify potential resistance to IWTD, 11 geographically paired seedlots obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Australian Tree Seed Centre were planted on Guam in 2012. The majority of these seedlots were used in the 1991-1993 international provenance trials of *C. equisetifolia.* The fastest growing pairs were those from Solomon (CSIRO 18402), Vanuatu (CSIRO 18312) and Papua New Guinea (CSIRO 20586 and CSIRO 18153), while the slowest pairs were from Australia (CSIRO 19821 and CSIRO 18378), Thailand (CSIRO 18297 and CSIRO 18299), and Guam. Though these trees were planted in the immediate area where IWTD occurs, today these trees remain healthy and free of infection by *R. solanacearum* (bacterial wilt pathogen) or *G. australe* (wood-rot fungus).

2.2 Gall-inducing wasp

After discussions among attendees of Guam's IWTD conference in 2009 (Schlub et al., 2011) and subsequent surveys, the gall-inducing wasp (*Selitrichodes casuarinae*) was discovered. Subsequently, this gall-inducing was found to be widespread across Guam (Fisher et al., 2014). On healthy trees, its impact is likely negligible, but may be significant on trees in decline.

2.3 Nematodes

In 2011, over a 5-day period, nematodes were extracted from *C. equisetifolia* roots and associated soils from five sites with healthy trees, and nine sites with declining trees. Soil samples from sites with decline contained a higher number of nematodes and nematode species than samples from sites with healthy trees. *Tylenchus* and *Helicotylenchus* were the most common genera isolated, with both genera found on 50% of samples from healthy sites, and 44% and 78%, respectively, of samples sites with declining trees. The highest average nematode counts from 10 g samples from both sites (healthy and declining trees) were those of *Helicotylenchus*, with 10 (healthy tree sites) and 39 (declining tree sites). It was concluded that *Helicotylenchus* sp. was likely the only nematode isolated with a potential to negatively impact *C. equisetifolia.*

2.4 Termites

In 2015, termites from 48 infested *C. equisetifolia* trees across Guam were collected. *Nasutitermes takasagoensis* or a closely related species from the *Nasutitermes takasagoensis* complex was found to have infested 45 trees. *Coptotermes gestroi* and *Microcerotermes crassus* were found to infested two trees (Park et al., 2019).

2.5 Conk forming basidiomycetes

Tree surveys during 2008-2009 found that 65% of *C. equisetifolia* were nearly dead (DS=4) and conks (fruiting bodies/basidiocarps of wood-decay fungi) were visible on most of these trees. In 2010, five conk-forming basidiomycete genera of the class Agaricomycetes, belonging to the orders Polyporales (*Ganoderma, Favolus, Pycnoporus*), Hymenochaetales (*Phellinus*) and Thelephorales (*Sarcodon*), were identified based on macro- and micro-morphology and DNA sequencing (e.g. ITS rDNA). A short survey for conk-forming basidiomycete was conducted in 2012 in healthy and IWTD sites on Guam and the nearby island of Saipan where IWTD does not occur.

Conks found growing on live trees belonged primarily to two species: *Ganoderma* sp. (*australe* group), which fruits on the tree roots, butt, and less commonly bole, and *Phellinus* spp., which primarily fruited on the butt. Both species were commonly found on Guam and infrequently found on Saipan. *Phellinus* does not appear to be a primary contributor to IWTD by itself. In contrast, *Ganoderma* appears more likely as a factor that contributes to IWTD on Guam, because it is a consistent indicator of IWTD (or a tree in decline) and its occurrence is irrespective of tree size. The *G. australe* complex identification was confirmed from samples collected at three sites on Guam and one site on Saipan in 2013. ITS sequences of a sample (*G. australe* complex) from Guam showed a 99% identity with sequences of *Ganoderma* sp. from China (GenBank GU213473), and a sample from Saipan showed a 99% identity with other sequences of *Ganoderma* sp. from China (GenBank FJ392286). Appropriate taxonomy within the *G. australe* complex remains unclear.

2.6 Detection and isolation of *R. solanacearum* on Guam

Within minutes of felling a tree in decline, droplets often formed on the cut surface. The droplets, hereafter referred to as ooze, consist of various mixtures of plant exudates and bacterial ooze. In 2011, it was discovered that ooze and tissue samples from decline trees tested positive using *R. solanacearum* specific Immunostrips (Agdia, Inc.) (Ayin et al., 2015; Ayin et al., 2019).

Three forms of ooze were observed: white to off-white viscous ooze (VO), watery amber ooze (WO), and a mixture of the two (MO). Drops of VO commonly appear in sapwood and sapwood-transition zones, may occur in unstained tissue, and frequently tested positive for *R. solanacearum*. Drops of WO commonly appear in the sapwood-transition and heartwood zones, always appear in stained tissue, and frequently tests negative for *R. solanacearum*. Drops of VO and MO were not randomly distributed, but appeared to coincide with growth rings (Fig. 1).

Though *R. solanacearum* could be detected from wood chips and drill shaving from roots, stems and branches of trees, attempts to isolate these bacteria from these same drill shavings failed. The only means

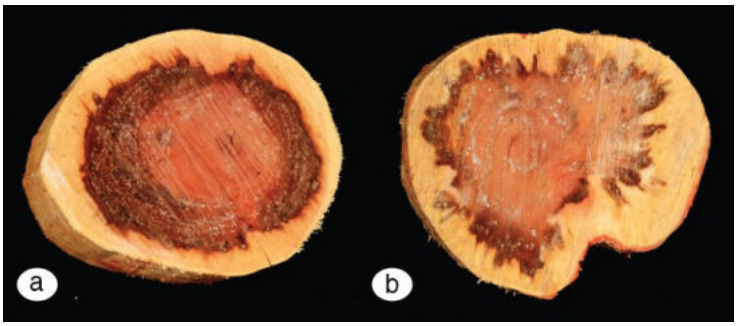

Fig. 1. Cross-sections of *Casuarina equisetifolia* trees on Guam with decline symptoms: (a) decline level DS=1 (slight damage), 63% wetwood symptomatic tissue present in sapwood and sapwood-heartwood transition zones, extensive bacterial ooze formed within minutes of felling, the majority of which is viscous, positive for *R. solanacearum*, positive for *Klebsiella oxytoca*, and negative for *Ganoderma australe*; (b) decline level DS=3 (heavily damaged), 58% wetwood symptomatic tissue present in sapwood and sapwood-heartwood transition zones, extensive bacterial ooze, of which the majority is viscous, positive for *R. solanacearum*, and negative for *Ganoderm australe*.

by which *R. solanacearum* could be isolated was by streaking ooze that formed on slices of stems, roots, or large branches from infected trees onto selective medium. To enhance the production of ooze, slices were placed on saturated paper-towel in a moisture chamber for 24 hrs. Once formed, the ooze was streaked on Engelbrecht's semi-selective medium (SMSA) (Ayin et al., 2015; Ayin et al., 2019). Colonies were re-streaked on to SMSA, which was followed by streaking onto modified Kelman's tetrozolium chloride medium (TZC) before subculturing on TZC.

2.7 Statistical links to decline

Various statistical methods have been applied to variables in search of predictors for IWTD. These variables were related to tree growth (e.g. diameter), abiotic factors (e.g. management practices and site density), or biotic organisms (e.g. termites and bacteria). In 2008-2009, 1,427 trees were surveyed for decline severity (DS) using an ordinal scale consisting of five categories (0=no damage, 1=slight damage, 2=moderately damaged, 3=heavily damaged, and 4=nearly dead). In addition 13 predictive variables were measured: latitude, longitude, altitude, number of tree stems, tree stand density, tree site location, typhoon damage, fire damage, CBH, intensity of management practices (none, moderate, or high), whether the tree was naturally planted or not, and whether a tree had conks or termites (Schlub et al., 2011). Through the application of multinomial modeling, three variables were determined to be significant (Schlub, 2010) (Table1).

In 2012, variables derived from 16 GIS map characteristics were added to the survey data. These GIS-derived variables included: cemetery buffer, fire risk, fires per year, proximity to golf courses, land cover, management areas, school buffer, soil available water at 150 cm, available water at 25 cm soil depth, soil depth to restrictive layer, soil series, vegetation, and the 2002 USDA Forest Service Inventory Analysis (FIA) map of trees with conks. The soil series was dropped from the analysis because of correlations with regressors. Nine GIS-derived variables were determined to be significant (Table 1).

After establishing in 2013 that *R. solanacearum* (bacterial pathogen) and wetwood bacteria were present in declining trees (Ayin et al., 2015), trees were surveyed to determine the relationship of bacteria and *G. australe* (wood-rot fungus) with IWTD. Using data collected in 2015 from a set of 77 whole trees, a proportional odds logistic regression model was fit with the following covariates: DBH, height, and presence/absence of *R. solanacearum* and *G. australe*. In addition, nine covariates (percent wetwood area, ooze initiation, ooze quantity, ooze type, and presence/absence of *Klebsiella* colony types, *K. oxytoca, R. solanacearum*, and *G. australe*) were applied univariately to data from a 30-tree subset of the original 77. From the two studies, four explanatory variables or covariates were determined to be significant (Ayin et al., 2019)

Table 1 Explanatory variables that were found to be significant positive (+) or negative (-) predictors of ironwood tree decline (*Casuarina equisetifolia*) (IWTD) from three statistical studies, variables are listed in order of significance.

Study*	Variable	Significance
2010	When conks were present	+
	When termites were present	+
	With increases in landscape management practices	+
2012	When conks were present	+
	When trees were intentionally planted	+
	When located where soil water is available at 25 cm	-
	With increases in altitude of location	+
	When located on a golf course	-
	When located in a forested areas	-
	With increases in tree circumference	+
	When termites were present	+
	When located in an urban landscape area	+
2019	When *Ralstonia solanacearum* was detected	+
	When *Ganoderma australe* was present	+
	With increases in percent wetwood in tree cross-sections	+
	When ooze bacterial ooze forms within 24 hrs of tree felling	+

*Analyses (Schlub 2010; 2019 and Ayin et al., 2019).

(Table 1).

2.8 Comparison between IWTD in Guam and bacterial wilt in China and India

Though IWTD in Guam and bacterial wilt in China and India have the pathogen *R. solanacearum* in common, IWTD differs with respect to symptomatology and abiotic and biotic contributors (Table 2).

3 Discussion

Based on our current information, it appears that IWTD is unique to Guam and it has no single cause. Though *R. solanacearum* occurs in Guam, China and India, it only accounts for 65% of the trees with IWTD symptoms in Guam, whereas in China and India is accounts for 100% of trees with bacterial wilt symptoms. Another stark difference between Guam and China and India is that the symptoms are nondistinctive and gradual with IWTD in Guam, whereas in China and India thedisease symptoms are distinct and sudden.

The identification of several significant explanatory variables strengthens the concept that IWTD is not solely caused by *R. solanacearum,* but it is instead the result of a disease complex comprising multiple biotic and abiotic factors in which biotic factors play a dominant role. The presence of *R. solanacearum* and *Klebsiella* colony types in symptomless trees suggests that at least some trees could possibly remain symptomless when infected with *R. solanacearum* and wetwood bacteria, provided its sapwood is not compromised through the formation of wetwood or ooze (Ayin et al., 2019).

4 Conclusion

On Guam, where IWTD is prevalent 20% of the outwardly asymptomatic trees test positive for *R. solanacearum* and 50% have some degree of wetwood. Only a few trees exhibit no internal discoloration or bacterial ooze, and test negative for *R. solanacearum* (Fig. 9).

Table 2. Comparison between ironwood tree (*Casuarina equisetifolia*) decline (IWTD) in Guam and bacterial wilt caused by *Ralstonia solanacearum* in China and India.

	Guam	China	India
Age at onset	10 years and older	Several months to 10 years (Fig. 4)	Sapling to one year
Foliage symptoms	Gradual thinning and die-back of branches (Fig. 3a)	Rapid wilt and death of branchlets (Fig. 4, 5)	Rapid yellowing of lowest branchlets and progressing upward
Onset to tree death	Months to years	Weeks to months but heavy rain can extend the period	Weeks
Appearance of freshly cut stomp or stem cross-sectional disc/slice	Those in severe decline usually produce ooze (Fig. 1), others may (Fig. 2) but often do not (Fig. 3b), wetwood may occur in the sapwood, sapwood heartwood transition zone (Fig. 1) or heartwood (Fig. 2), asymptomatic trees may (Fig. 2) or may not (Fig. 3b) have wetwood	Usually produce ooze (Fig. 6, 7) and may have evidence of wetwood in the sapwood or the sapwood-heartwood transition zones (Fig 6.)	Often show ooze and discoloration
Occurrence	Roughly 5% of the tree population, dense tree cohorts often <0.5 ha or as a few scattered trees across several hectares	Roughly 5,000 hectares mainly in Guangdong province, occurring dense tree cohorts close to agricultural lands (Fig. 4)	Dense plantations
Maximum Incidence	Approximately 85%	>90% within 1-2 months following a typhoon	40%
Phylogenetic Analysis	phylotype 1 (Ayin et al., 2015)	phylotypes 1, biovar 3 or 4 and race 1 (Jiang et al., 2017)	Phyloptype I, biovar 3 & 4, race 1 (Ramesh et al. 2014; Singh et al., 2018)
Contributing factors	In sites with decline approx. 65% of the symptomatic trees are infected with *Ralstonia solanacearum*, other factors linked to IWTD included site conditions, management practices, bacterial wetwood, the termite *Nasutitermes takasagoensis*, and the butt and root rot fungus *Ganoderma australe*	100% of the symptomatic trees are infected with *R. solanacearum*, appears sporadically at first and gradually reaches >90%, sudden death often follows typhoon (Fig. 4,5), occurs in plantations, disease level increases with repeated plantings in infected sites, disease is greatest near crops infected with bacterial wilt	Usually occurs under wet conditions, most of the plantation are adjacent to tomato fields, biocontrol organisms such as *Trichoderma* and *Micromonospora* controlled the disease and reversed the symptoms if applied during early stages of infection, revived trees showed no discoloration of stem or oozing when tested at the age of 18 to 24 months (Fig. 8)

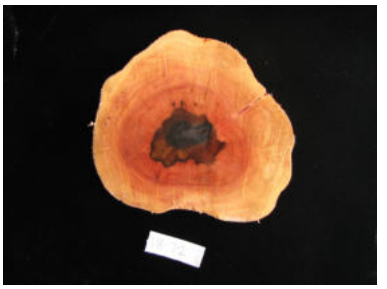

Fig. 2. Cross-section of a *Casuarina equisetifolia* tree on Guam with no outward symptoms of decline: DS=0 (symptomless), 14% wetwood symptomatic tissue present in heartwood, slight viscous and watery bacterial ooze, negative for *Ralstonia solanacearum* and *Ganoderma australe*

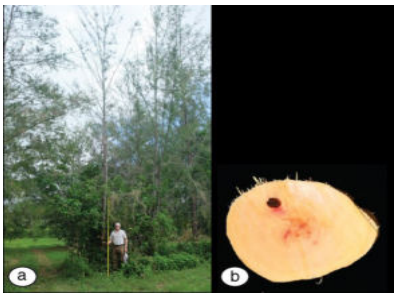

Fig. 3. *Casuarina equisetifolia* tree on Guam with decline symptoms: (a) level DS=2 (distinctly damaged) (b) tree 'a' cross-section, 0% wetwood symptomatic tissue, no bacterial ooze, positive for *Ralstonia solanacearum*, and negative for *Ganodera australe*.

Fig. 4 Mortality of various aged clonal *Casuarina equisetifolia* trees in China, following a strong typhoon in 2015.

Fig. 5. Mortality of bacterial wilt-infected *Casuarina equisetifolia* trees in a clonal trial in Wuchuan, China, 2 months after a strong typhoon in 2015.

Fig. 6. Cross-section of bacterial wilt-infected *Casuarina equisetifolia* tree in Xuwen, China in 2019: severe bacterial wilt, 38% wetwood symptomatic tissue present in sapwood-heartwood transition zone, extensive viscous bacterial ooze is formed within minutes of felling, *Ralstonia solanacearum* positive.

Fig. 7. Cross-section of bacterial wilt infected *Casuarina equisetifolia* tree in Xuwen, China in 2019: severe wilt, 0 % wetwood symptomatic tissue, slight viscous ooze bacterial exudate is formed within minutes of felling, *Ralstonia solanacearum* positive.

Fig. 8. Cross-section of an 18-month-old tree in India that recovered from bacterial wilt with no signs of wood discoloration or bacterial ooze, after showing symptoms at 6 months and following treatment with *Micromonospora maritima*.

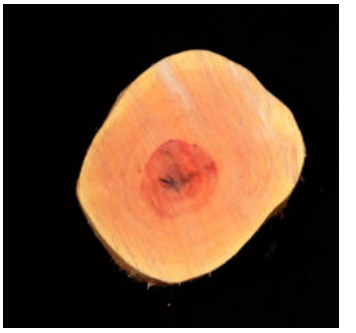

Fig. 9. Cross-section of a *Casuarina equisetifolia* tree on Guam with no outward symptoms of decline: IWTD level DS=0, symptomatic tissue 3%, no wetwood or bacterial ooze, negative for *Ralstonia solanacearum*, negative for *Klebsiella oxytoca*, and negative for *Ganoderma australe*.

These healthy trees tend to occur in natural stands, at low altitude or areas not prone to drought. Due to the slow progression of IWTD and its general sporadic nature, it is likely that IWTD could be reduced through the following measures: (1) increasing the genetic diversity and species diversity of *C. equisetifolia* in Guam, (2) removal of *R. solanacearum* / *G. australe* infected trees, (3) prevention of root-grafts, and (4) the application of cultural practices that promote healthy growth.

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Micromonospora: A promising biocontrol agent for bacterial wilt disease in casuarina

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Abstract

A rapid method for biocontrol of wilt disease in casuarina clonal plantations noticed in the State of Tamil Nadu, India. The disease showed the symptom of chlorotic sudden wilt of foliage particularly in young plantations of an interspecific hybrid (*Casuarina equisetifolia* x *C. junghuhniana*) clone CH5. The causal organism was identified as a bacteria, *Ralstonia solanacearum*. Earlier reports noted that this disease is difficult to control since the pathogen in soil borne. In the present study, the effectiveness of *Micromonospora* to control the disease under field conditions was tested. *Micromonospora* is an actinomycetous bacteria and recognized as a source of secondary metabolites for controlling pathogens. *Micromonospora* was isolated from the root nodules of *C. equisetifolia* and cultured in ISP -2 medium. The isolated strain was identified as *M. maritima* by 16s rRNA sequence and mass multiplied. Pathogenecity test of *R. solanacearum* in *C. equisetifolia* seedlings and antibiosis test with *M. maritima* were conducted under laboratory conditions. In the pathogenecity test the infected *C. equisetifolia* showed chlorotic and wilting of cladophylls due to infection of *R. solanacearum*. The antibiosis test results showed suppression of *R. solanacearum* by *M. maritima*. Based on these findings the *M. maritama* broth was applied in the root zone of infected casuarina clones (CH5) at 20 ml plant^{-1}. After 30 days of application the symptoms disappeared in the infected CH5 clones and the plants developed new sprouts of foliage. The entire infected plantation was recovered up to 95% and also showed improvement in growth. It is concluded from this study that *M. maritima* was able to control the soil borne pathogen *R. solanacearum* by secretion of secondary metabolites and its antagonistic activity. It was also deduced from this study that *Micromonospora* is an effective biocontrol agent for controlling the bacterial wilt disease in casuarinas.

Keywords: *Micromonospora*, bacterial wilt, *Casuarina, Ralstonia solanacearum*, biocontrol

1 Introduction

Micromonospora is an actinomycete which has been recognized as a significant source of secondary metabolites and for bio controlling the fungal pathogens. It is associated with another actinomycete *Frankia* which fixes nitrogen in actinorhizal plants such as species of *Alnus, Ceonthus* and *Casuarina* through symbiotic relationship. *Micromonospora* is reported to be isolated from roots of wheat (Combs and Franco, 2003), nitrogen-fixing root nodules of *Casuarina equisetifolia* (Valdés et al., 2005) and *Coriaria myrtifolia* (Trujillo et al., 2006). It was recognized as a biocontrol agent against pathogens in the recent years (Hirsch and Valdes, 2009) and known for synthesizing antibiotics especially aminoglycocide, enediyne and oligosaccharide (Berdy, 2005). In the present study *Micromonospora* was employed to control the bacterial wilt disease caused by *Ralstonia solanacerum* (Sun et al., 2014).in young trees of an interspecific hybrid clone of *Casuarina equisetifolia* x *C. junghuhniana* (CH5). Wilt disease in clonal plantations was reported by casuarina farmers from Tindivanam, Cuddalore and Villupuram regions of Tamil Nadu, India. Affected plants showed the symptom of chlorotic and brownish cladophylls resulting in sudden death particularly in young plantations. Since in a clonal plantation all plants have the same level of susceptibility to the disease, the disease causes high levels of mortality leading to significant economic loss to farmers. This paper reports the effectiveness of *Micromonospona* as a biocontrol agent in arresting the bacterial wilt disease in young casuarina clonal plantations.

2 Materials and methods

2.1 Isolation and culture of *Micromonospora*

Root nodules of *Casuarina equisetifolia* were collected and transported in an ice box and stored at -4 °C in the laboratory. The nodules were surface sterilized with 30% H_2O_2 and kept at room temperature for 30-40 min. Under aseptic conditions the nodules were rinsed in sterile water and 0.1 g of nodule was ground manually in a sterile mortar and pestle. The nodule solution was centrifuged at 1000 rpm for 20 min and the supernatant was filtered through Whatman No.1 filter paper and the suspension was placed on ISP -2 media. After 15 days of incubation, *Micromonospora* growth was observed as orange colonies in ISP -2 media plates and these colonies were transferred to ISP -2 media broth for multiplication. This culture was characterized at molecular level through DNA extraction, PCR and sequencing of 16s rRNA. Based on the molecular data the strain of *Micromonsopora* was identified as *M. maritama* and deposited in the National Center for Biotechnology Information, USA, vide accession No. MK 271738.1 (Karthikeyan et al., 2018).

2.2 Isolation of *Ralstonia solanacearum*

Collected 100g soil sample and root portions infected by the bacteria from a *Casuarina* hybrid (CH5) clonal plantation in Tamil Nadu, India. The infected root and soil samples were placed in Casamino acid peptone glucose medium plates and incubated at 35 °C for 10 days. White colonies of *R. solanacearun* appeared after the incubation period. The cultures were again mass multiplied in Casamino acid peptone glucose broth using culture shakers. The multiplied *R. solanacearum* was stored at -4 °C.

2.3 Antibiosis test

Antibiosis test was carried out between *R. solanacearum* and *M. maritima* in the nutrient agar medium. The nutrient agar medium plates (9 cm diameter) were bifurcated and a colony of *R. solanacearum* was placed on one side and other side colony of *M. maritima* was placed. The growth incidence (%) of *R. solanacearum* with and without *M. maritima* was calculated as follows (Karthikeyan et al., 2003).

$$\text{Growth incidence (\%)} = \frac{\text{Growth area}}{\text{Total area}} \quad X\ 100$$

2.4 Pathogenecity test

The seedlings of *C. equisetifolia* were raised and maintained in the glass house. They were later inoculated with *M. maritima* and *R. solanacearum* individually and in combinations at the age of one month. These treatments were replicated 10 times with 5 seedlings replication^{-1}.

2.5 Field application

20 ml of *M. maritima* was applied at the root zone of six-month old plants of clone CH5 in the plantation affected by bacterial wilt at Tindivanam, Villupuram

and Cuddalore, Tamil Nadu, India. After the application the disease severity was noticed in the plantations at regular intervals.

2.6 Statistical analysis

All the data of experiments carried out in this study were statistically analyzed by ±SE of mean.

3 Results

3.1 Antibiosis test

The antibiosis test results showed that *R. solanacearum* growth was suppressed by *M. maritima* after seven days of incubation. The *R. solanacearum* spread over 6.5 cm^2 growth area in nutrient agar medium whereas *M. maritima* suppressed its growth to 1.8 cm^2 due to inhibition of. The growth incidence (%) of *R. solanacearum* was 42.1 in control and 6.4 in the presence of *M. maritima* (Fig.1).

3.2 Pathogenecity test

The pathogenecity test showed white pustules initially on cladophylls of *C. equisetifolia* and that changed to brownish after 15 days. (Fig. 2, 3).

3.3 Field application

The field application of *M. maritima* against the bacterial wilt causing pathogen *R. solanacearum* showed up to 98% recovery in CH5 clone under field conditions (Table 1). Interestingly new cladophylls emerged at the base of the stem of infected CH5 clones (Fig. 4, 5) after 30 days of application. Their growth was also found to be improved (Table 1).

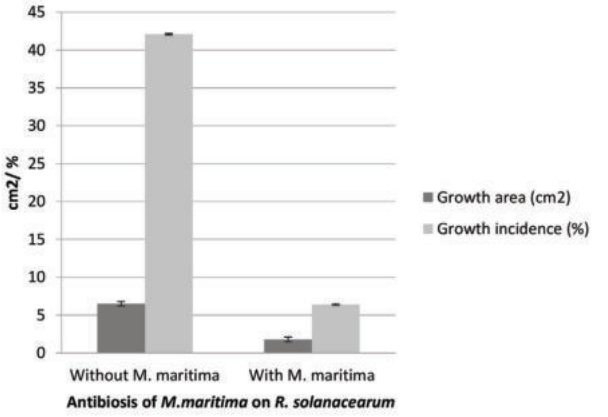

Fig. 1. Growth f *R. solanacearum* in the presence and absence of *M. maritima.*

Fig. 2. Pathogenecity of *R. solanacearum* in *Casuarina equisetifolia.* Inoculated seedlings showing white pustules in cladopylls (left) and the healthy unincoulated seedling (right).

Fig. 3. Cladophylls with white pustules changing brownish in brown under pathgenecity studies.

Table 1. Growth improvement and bacterial wilt recovery in CH5 clone of casuarina 30 days after application of *M. maritima* (mean of 10 replicates).

Place	Recovery (%)	Growth improvement (cm)	
		Before application of *M. maritima*	After application of *M. maritima*
Tindivanam	95.6 (±2.2)	95.2 (±2.5)	120.6 (±2.1)
Villupuram	96.3 (± 2.1)	100.5 (±2.2)	135.3(±2.3)
Cuddalore	98.2 (±2.2)	91.3 (±2.3)	128.4(±2.2)

Fig. 4. A casuarina clonal plant (CH5) infected by *R. solanacearum* showing wilt disease symptom of yellowing of needles before application of *M. maritima.*

Fig. 5. A recovered plant of casuarina clone CH5 infected by *R. solanacearum* after application of M. maritima.

4 Discussion

To date, a number of actinomycetes isolated from soil and the rhizosphere of certain plants have shown the activity of plant growth promotion and disease control (Hallmann et al., 1997; Compant et al., 2005; Joseph et al., 2007). In the present study, *M. maritima* effectively controlled the wilt disease causing pathogen *R. solanacearum* in casuarina clone. This antagonistic activity of *M. maritima* is possible due to secretion of secondary metabolites especially aminoglycosides (Berdy, 2005). Smith (1957) successfully isolated *Micromonospora* sp. from tissue sections of an apparently healthy tomato and found that this actinomycete showed a strong inhibitory effect to *Fusarium oxysporum f.* sp. l*ycopersici*. *Micromonospora* is known to be a versatile degrader of complex organic matters such as cellulose, lignin, xylan, chitin and other complex polysaccharides (Macagnan et al., 2008). This helps to invade the root system and arrest the pathogen. *Micromonospora* sp. form thread-like filaments in the soil which gives them an advantage

in colonizing the rhizosphere effectively. As a rhizobacteria, *Micromonospora* sp. influence plant growth, antagonize plant pathogens and make nutrients available for the plants (Maheshwari and Shimizu, 2011). Cell wall of *Micromonospora* sp. contain meso-diaminopimelic acid (DAP) and/or its 3-hydroxy derivatives and glycin. Due to their ability to produce hydrolytic enzymes like xylanases and chitinases, they are able to degrade a wide range of organic matter in their natural habitat (Hirsch and Valdes, 2010). This is the reason for *M. maritima* controlling the soil pathogens including the bacteria causing wilt disease in casuarina clonal plantations. Based on these supportive earlier studies and the results from this study it was proved that *M. maritima* is an effective biocontrol agent to bacterial wilt disease in casuarina.

5 Conclusion

Micromonospora, a widely distributed actinomycetes is an efficient biocontrol agent for bacterial wilt disease of casuarina. Identification of efficient *Micromonspora* strains for biocontrol and biofertilization activity will be very useful for increasing productivity of casuarina plantations.

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Changes of phenols contents in *Casuarina equisetifolia* provenances with different resistibility infected by *Ralstonia solanacearum*

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Abstract

In order to understand the resistance mechanism of *Casuarina equisetifolia* against *Ralstonia solanacearum*, changes in tannin and phenol contents in casuarina plants infected by *R. solanacearum* were examined. The results showed that the change trends of tannin and phenol contents varied with different source materials. The tannin and phenol contents in the high-resistant provenance peaked two days after inoculation and declined rapidly while the contents in the moderate-resistant provenance reached the peak four days after inoculation then also declined rapidly. In contrast the susceptible provenance showed highest tannin and phenol contents five days after inoculation and the contents tended to remain stable for a few more days. After infected with *R. solanacearum*, the condensed tannin content increased steadily from Day 1 to Day 7 in all three resistant categories. The content in the high-resistant provenance was 70% greater than that in the susceptible provenance. Results obtained for flavonoid content showed that in the highly resistant provenance, the content increased steadily from Day 1 to peak at Day 5 then decreasing thereafter. While in the moderately resistant and susceptible provenances, the flavonoid content increased slowly but steadily from Day 1 to Day 7. This study revealed different defense mechanisms between resistant and susceptible plant materials. The greater the content of secondary compounds, the stronger bacteriostasis and antioxidant activities which reflect the resistibility of *C. equisetifolia* against bacterial wilt. Variation in disease resistance among different provenances suggests potential for selection for bacterial wilt resistance.

Keywords: *Casuarina equisetifolia, Ralstonia solanacearum,* resistant, susceptible, tannin, flavonoid

1 Introduction

Casuarina is a pioneer species for construction of coastal protection forest in southeast of China. It can withstand typhoon winds and thrive in infertile soils. In some sandy beach sites, casuarina is the only tree species suitable for afforestation (Zhong et al., 2010). Casuarina also plays an important role in ecological

and economic aspects (Zhong et al., 2010; Zhang et al., 2016).

Bacteria wilt caused by *Ralstonia solanacearum* is a devastating soil-borne disease which has high infectivity and lethality. It could affect more than 200 species in 54 families, including many plants with great economic and social value, such as *Nicotiana tabacum, Lycopersicon esculentum, Solanum tuberosum, Musa nana* and *Eucalyptus* spp. (Qiao et al., 2013). It is one of the most difficult bacterial diseases for prevention and control (Salanoubat et al., 2002). Bacterial wilt is a critical casuarina disease, affecting coastal shelterbelt forest and ecological security in southeast of China. *R. solanacearum* has high transmission speed and large variation. A great deal of work has been done in the aspect of chemical and biological controls, but the spread of the bacterial wilt continues. In order to effectively control this bacterial wilt it is necessary to understand the disease resistance mechanism in casuarinas. Among the factors influencing disease resistance in plants, secondary metabolites play an important role in physiological and biochemical aspects, which can effectively inhibit the spread and proliferation of pathogens in the host, or even directly kill pathogens (Sun et al., 2018).

Phenolics are important secondary metabolites of plants that participate in many physiological and biochemical processes. Many studies have shown that phenolics play an important role in the process of plant resistance to pathogens (Teng et al., 2007). After invasion of pathogen, plants produce a large number of soluble phenols, which inhibit the migration and expansion of the pathogen (Guo et al., 2003). A large amount of phenolic substances in the cell wall are also closely associated with the disease resistance of plants (Chen et al., 1996). Flavonoids can also inhibit the activities of polyphenol oxidase and cytochrome oxidase, thus affecting the disease resistance of plants (Hu et al., 2002; Wu et al., 2007; Huang et al., 2017). Hence, phenolics and flavonoids are closely related to resistance mechanism in plants. Therefore studying the phenolic and flavonoid contents can help understand the disease resistance characteristics of plants.

In this study, provenances with varying bacterial resistibility were used as plant materials to investigate the changes in phenolic substances and flavonoids in the branches of *C. equisetifolia* after inoculation with *Ralstonia* bacterial wilt. The physiological mechanism of resistance in *C. equisetifolia* against bacterial wilt and the differences between resistant and susceptible provenances were studied. Results of this study could be used to develop future breeding programmes of *C. equisetifolia* with a particular focus on bacterial wilt resistance.

2 Materials and methods

2.1 Plant materials

Plant materials of *C. equisetifolia* were collected in May 2017 from a provenance trial established in 2014 in Xuwen, Guangdong Province. The provenances in the trial were divided into three groups based on bacterial wilt resistance: high resistance (R), moderate resistance (M), and susceptible, non-resistance (S). Each group was represented by one provenance. Fifty plants were sampled from each group. Ten young branches were taken from each plant, and propagated by cuttings. After 15 days, the cuttings with 5 cm roots were transplanted into growing containers and given water once a day. The potting mix was composed of equal part of humus, sand and loess.

2.2 Inoculation and sampling

Ralstonia solanacearum strain GL-3 obtained from the Research Institute of Tropical Forestry in Guangzhou was used to inoculate casuarina plants in this study (Wang et al., 2007). The GL-3 strain was cultured on TTC medium (2,2,3-triphenyltetrazolium chloride) for 48 h according to the method of Liu et al. (2010). A single colony was isolated from the culture and inoculated in protein agar medium for 24 h, and then inoculated in glucose-peptone liquid medium for another 24 h in culture shakers at 30 °C. The culture was allowed for 30 min to collect the bacteria, and then diluted to 3×10^{9} cfu ml^{-11} with distilled water for inoculation.

Uniformly grown and vigorous seedlings (rooted cuttings) 40 cm in height were selected for inoculation with GL-3. The seedlings were removed from the growing containers and the roots were carefully washed with tap water. One-third of the roots was cut off. The seedlings of each provenance group were then divided into two parts. The first part was immersed in bacterial suspension (I, inoculation) for 30 min at 30°C. The second part was immersed in distilled water as control (W). The inoculated plants of each group were transplanted into plastic trays (40 cm x 25 cm and 8 cm

deep) filled with equal part of loess, peat soil and sand. The plants were arranged in a randomized block design for 6 treatments (R-I, R-W, M-I, M-W, S-I, and S-W) and 3 replicates. Each treatment replicate consisted of four trays (80 plants). The plants were watered regularly. Relative humidity was maintained at 80%.

The plants of each treatment were carefully monitored and the infected plant parts (stem, cladophylls) were collected and stored in a deep freezer at -20°C.

2.3 Index determination

Tannin and flavonoid contents of each treatment were extracted and analysed following the method described in Wang et al. (2012; 2015). The total phenol content of the plants was determined by Folin-Ciocalteu Reagent Colorimetry (Makkar et al., 2010). Condensed tannin was extracted by butanol / hydrochloric acid (95:5) (Makkar, 2003). These analyses were repeated three times for each replicate, and the average calculated as mean data for replicate.

2.4 Data analysis

SPSS ver.19.0 and Excel 2007 were used to analyze the plot mean data. Duncan multiple range test (5%) was used to compare statistical differences between means.

3 Results

3.1 Total phenol content

The total phenol content in the branches of *C. equisetifolia* seedlings inoculated with *R. solanacearum* showed varying disease resistance among provenance groups (Table 1). The content in the highly resistant provenance increased rapidly and reached the maximum value of 126.89 mg g^{-1} on Day 2 after inoculation, then decreased continuously thereafter, while the content in the moderately resistant provenance reached the peak (126.16 mg g^{-1}) on Day 4 after inoculation. In contrast, the phenol content in the susceptible provenance did not reach the maximum (109.57 mg g^{-1}) until Day 5. The phenol content of the inoculated plants was consistently higher than that in the un-inoculated plants of all three provenance groups.

There were no differences in the phenol content among the un-inoculated seedlings of different provenances. Mean value fluctuated within the range 73.28-86.2, 76.01-93.92, and 77.34-96.41 mg g^{-1} for highly resistant, moderately resistant, and susceptible provenances respectively.

3.2 Tannin content

Results obtained for tannin content in *C. equisetifolia* seedlings after inoculation with bacterial wilt pathogen are presented in Table 2. In general the pattern of responses was similar to that shown in the phenol content. The tannin content of highly resistant and moderately resistant provenances peaked on Day 2 and Day 4 respectively after inoculation, while that of the susceptible provenance reaches the peak on Day 5. Tannin content in the seedlings of high and moderate disease resistance was also higher than that in the susceptible provenance.

There were no differences in the tannin content among un-inoculated plants of the three resistant categories.

3.3 Condensed tannin content

In all casuarina seedlings inoculated with *Ralstonia* bacterial wilt the condensed tannin content increased consistently from Day 1 and reach the peak on Day 7 (Table 3). Highly disease resistant provenance recorded the highest value of 13.03 mg g^{-1} followed by 10.36 and 10.2 mg g^{-1} respectively in the moderately resistant and susceptible provenances.

There were no statistical differences among the un-inoculated seedlings although the value was lowest in the susceptible provenance.

3.4. Flavonoid content

Results for the flavonoid content in the branches of *C. equisetifolia* seedlings inoculated with bacterial wilt pathogen are presented in Table 4. The increment was relatively small in the three provenance groups during the first two to three days. There was a steep increase from Day 3 to Day 4 for the highly resistant and moderately resistant provenances and the flavonoid content tended to plateau thereafter. In contrast, the susceptible provenance showed an exponential increase in the flavonoid content from Day 3 to Day 7. Despite the continuous increase, flavonoid content in the susceptible provenance was much lower than that in the highly resistant and moderately resistant provenances.

Table 1. Total phenols content (mg g^{-1}) in *Casuarina equisetifolia* seedlings infected by *Ralstonia solanacearum*.

Day after inoculation	R-I	R-W	M-I	M-W	S-I	S-W
0	81.77±5.68a	77.75±13.58a	89.73±6.71a	86.18±12.52a	84.58±8.81a	85.86±13.66a
1	96.25±19.27a	85.56±4.36a	94.32±11.27a	88.04±4.98a	87.89±13.13a	88.15±9.99a
2	**126.89±12.75a**	86.2±16.04bc	109.45±12.88ab	76.01±13.52c	91.23±18.81bc	96.41±12.87bc
3	100.65±14.48ab	73.28±10.91c	120.06±18.84a	81.99±4.45bc	99.53±11.21ab	89.1±11.48bc
4	93.64±11.33b	82.33±18.41b	**126.16±8.87a**	90.62±14.03b	100.71±13.62b	84.45±9.11b
5	94.78±20.3a	84.81±14.18a	97.66±11.98a	93.92±11.43a	**109.57±8.53a**	91.66±11.75a
6	86.99±7.58a	76.96±10.25a	84.84±11.69a	89.96±18.98a	100.01±12.83a	84.54±9.44a
7	85.38±5.32ab	78.87±4.89ab	90.22±15.31ab	91.17±19.03ab	102.5±12.87a	77.34±11.3b

R – highly resistant provenance; M – moderately resistant provenance; S – susceptible provenance;
I – inoculated; W – control, uninoculated.
On a given day after inoculation, data followed by different letters indicate significant difference at $P<0.05$ level.

Table 2. Tannin content (mg g^{-1}) in *Casuarina equisetifolia* seedlings infected by *Ralstonia solanacearum*.

Day after inoculation	R-I	R-W	M-I	M-W	S-I	S-W
0	80.78±8.13a	76.07±7.37a	88.76±10.33a	85.45±8.54a	83.44±18.38a	84.69±5.43a
1	95.26±8.24a	84.72±13.79a	93.37±7.98a	87.21±9.58a	87.17±6.42a	87.14±13.53a
2	**125.92±7.02a**	85.63±4.92cd	108.17±7.89b	75.37±9.93d	89.84±16.77bcd	95.52±8.28bc
3	99.72±3.89ab	81.55±14.54b	119.35±12.78a	80.78±7.67b	98.45±13.32ab	88.37±11.45b
4	92.79±3.05b	83.65±15.79b	**125.41±8.76a**	89.85±6.06b	99.89±17.05b	83.79±11.85b
5	93.26±8.33a	75.2±6.74b	96.52±4.64a	93.13±10.07a	**108.29±13.95a**	90.57±9.52ab
6	85.11±10.37a	76.05±11.53a	83.64±12.73a	88.7±15.89a	99.3±13.11a	83.48±13.72a
7	84.7±8.9ab	77.41±10.05b	89.21±7.46ab	89.86±9.53ab	101.07±8.88a	76.68±7.82b

R – highly resistant provenance; M – moderately resistant provenance; S – susceptible provenance;
I – inoculated; W – control, uninoculated.
On a given day after inoculation, data followed by different letters indicate significant difference at $P<0.05$ level.

Table 3. Condensed tannin content (mg g^{-1}) in *Casuarina equisetifolia* seedlings infected by *R. solanacearum*.

Day after inoculation	R-I	R-W	M-I	M-W	S-I	S-W
0	8.61±1.4a	8.15±0.82a	7.42±2.21a	7.69±1.21a	5.68±1.93b	5.88±1.23b
1	9.45±2.22a	7.41±1.15ab	7.8±1.72ab	8.62±1.53ab	5.7±2.06b	6.72±2.01ab
2	9.74±1.2a	7.96±1.45ab	7.96±1.26ab	7.77±2.09ab	6.18±0.66b	5.69±1.34b
3	11.71±1.85a	8.82±2.12b	8.34±1.62b	8.26±1.11b	6.47±0.99b	6.21±1.04b
4	12.27±1.96a	7.71±1.44bc	9.33±1.77b	7.82±1.88bc	6.64±1.43bc	5.37±1.08c
5	12.55±1.28a	8.8±0.66bc	10.24±0.7b	8.95±2.15bc	7.25±1.01cd	5.7±1.07d
6	12.69±0.66a	7.91±1.56bc	10.34±1.75ab	8.97±1.92bc	8.4±0.68bc	6.68±1.13c
7	**13.03±1.27a**	7.65±2b	**10.36±1.71ab**	8.68±2.15b	**10.2±0.65ab**	7.18±1.66b

R – highly resistant provenance; M – moderately resistant provenance; S – susceptible provenance;
I – inoculated; W – control, uninoculated.
On a given day after inoculation, data followed by different letters indicate significant difference at $P<0.05$ level.

Table 4. Flavonoid content (mg g^{-1}) in *Casuarina equisetifolia* seedlings infected by *Ralstonia solanacearum*.

Day after inoculation	R-I	R-W	M-I	M-W	S-I	S-W
0	40.01±3.92a	43.07±3.08a	41.56±4.84a	41.21±1.94a	39.22±3.6a	38.85±4.56a
1	40.08±3.76a	42.65±2.06a	41.98±3.42a	42.2±3.7a	40.81±2.92a	40.92±6.22a
2	40.82±4.34a	40.12±4.44a	44.36±6.2a	40.89±4a	41.96±4.08a	39.91±1.98a
3	52.81±4.14a	41.62±4.02b	50.07±3.96a	40.82±1.78b	42.16±3.82b	42.57±3.16c
4	51.62±1.96a	45.24±3.7b	51.93±3.48a	42.53±4.76bc	43.24±4.18bc	38.16±2.66c
5	**56.66±8.46a**	41.76±3.74c	52.38±4.08ab	43.49±3.5bc	43.92±3.94bc	39.29±4.6c
6	54.8±4.54a	43.92±3.58b	52.36±4.76a	40.25±3.8b	44.38±3.32b	39.71±4.94b
7	53.23±4.62a	41.82±2.28b	**54.08±1.7a**	42.42±4.78b	**46.94±4.82ab**	40.98±4.9b

R – highly resistant provenance; M – moderately resistant provenance; S – susceptible provenance;
I – inoculated; W – control, uninoculated.
On a given day after inoculation, data followed by different letters indicate significant difference at $P<0.05$ level.

4 Discussion

It was demonstrated from this study that the bacterial wilt caused by *R. solanacearum* in *C. equisetifolia* changed the physiological and biochemical substances systematically. The current planting of *C. equisetifolia* employs a few or even single clone which limits the progress of breeding for disease resistance. At the same time, due to the various pathogenic bacterial diseases, the selection and application of resistant varieties are not much improved. This appears to have caused dieback *C. equisetifolia* plantations in large areas. Therefore, new resistant germplasm materials are urgently required for coastal shelterbelt planting in southern China. This study showed the resistance mechanism of *C. equisetifolia* at provenance level. The disease resistant material promised a scope for selection of genetic resources.

Identification of resistance in *C. equisetifolia* against bacterial wilt is a long-term management strategy. According to the mechanism of disease resistance it can be divided into component resistance and induced resistance (Wang et al., 2014). The plants, when induced by pathogens, could produce some substances to protect themselves as defense mechanism (Wu et al., 2001). In this study, both tannin and flavonoid content of resistant and susceptible *C. equisetifolia* increased after being infected by bacterial wilt disease. The tannin content increased initially and then decreased whereas flavonoid content of the moderate resistance and susceptible provenances increased steadily over the 7 days. The increase of tannin and flavonoid content in high resistant provenances was more pronounced than that in the susceptible provenances. These results indicated that *C. equisetifolia* induced resistance after infected by bacterial wilt. Therefore, the content of tannin and flavonoid can be used as physiological index to evaluate the resistance of *C. equisetifolia* against bacterial wilt. Tannins and flavonoids are representative components of polyphenols. They are two important secondary metabolites produced by *C. equisetifolia* after being attacked by pathogenic bacteria. They are derived from shikimic acid pathway and phenylalanine pathway. They have many functions such as bacteriostasis and antioxidation, and are the main defense measures in plants (Xu et al., 2017). Wang et al. (2015) reported that the tannin content of resistant *Casuarina* clones before and after inoculation with bacterial wilt was higher than that of susceptible clones. Lin et al. (2014) studied the selection of resistant *C. equisetifolia* clones against *Anoplophora chinensis* and found higher content level of tannin, flavonoids and other secondary metabolites in the disease resistant plants. This is in accordance with the results of the present study. There was no significant difference in tannin and flavonoid content between different provenances on the day of inoculation which may be due to the genetic characteristics of different provenances of *C. equisetifolia*. The different values of tannin content in the resistant provenances may be due to the different expression of some genes.

There is a long incubation period of *C. equisetifolia* after infected by bacterial wilt disease in the field. In this study 3 $x10^{-9}$cfu ml^{-1} inoculation was used to detect the physiological and biochemical changes of *C. equisetifolia*. There were constitutive and inducible tannins and flavonoids in *C. equisetifolia* found in this study. The induced tannins and flavonoids were mainly produced after the infection of pathogenic bacteria. Further study on the changes in other defense substances will help reveal the resistance mechanism of *C. equisetifolia* against *R. solanacearum*.

5 Conclusion

In this study, there were significant differences in disease index with physiological contents among provenances infected by the bacterial wilt pathogen. The changes of tannin and phenol contents in provenances with different resistibility were noticed. The contents of tannin and phenols in highly- and moderately-resistant provenances increased initially and then decreased later. The susceptible provenances showed a gradual increase of phenol and other substance contents. The content of flavonoids in the highly resistant and moderately resistant provenances showed unpredictable increase whereas the susceptible provenance increased slowly. These results provide a scientific platform for breeding of *C. equisetifolia* varieties against *R. solanacearum*.

Acknowledgements

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Possible benefits for future climate change adaptation from genetic diversity in the Casuarinaceae

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Abstract

The trends of increasing global temperature rise and atmospheric CO_2 since 1960 has led the United Nations Intergovernmental Panel on Climate Change (IPCC) to conclude that well-mixed greenhouse gases are the main cause of global warming. However, no direct empirical evidence exists for this conclusion, which is based on laboratory measurements regarding infrared absorption and emission by greenhouse gases and correlated trends, but with no supporting empirical field measurements. The climate sensitivity in terms of temperature increase for a doubling in CO_2 in the atmosphere is still not known precisely, given that it depends on a positive feedback from increased water vapour as temperature rises. Plant research has shown that most C_3-plants have higher yields with increased water use efficiency as CO_2 levels rise, although some crops may be more nitrogen-deficient. An interesting question is whether the productivity of *Casuarina* species is also increasing with higher levels of CO_2 and whether nitrogen levels are maintained, as could be predicted for N_2-fixing systems. It is prudent for effective climate management that other possible causes of climate change also be considered. We have recently claimed that increasing application of irrigation water is also making a significant contribution to direct warming in dry regions. Another potential cause of localised surface heating we are investigating is the effect of frictional vorticity and turbulence in the boundary layer resulting from increasing roughness of the Earth's surface. Given these uncertainties, this review considers how the natural properties of casuarinas in CO_2 sequestration to form carbonaceous products, biological N_2 fixation (BNF) and architecture of landscapes might be advantageous in management of future climates, but with immediate economic benefits in contrast to carbon neutral practices that offer benefits only well after 2050.

Keywords: IPCC; global warming; vortical entropy; nitrogen fixation; hydrogen generation; farm forestry

1 Introduction

As ancient natives of Australia and southern Asia dating from the era of Gondwana land, the many *Casuarina* species are naturally diverse, adapted to a wide range of Australian ecosystems. These adaptations range from wetlands to highly arid ecosystems (Ganguli and Kennedy, 2012). For example, the desert oak (*Allocasuarina decaisenana*) is the most prominent tree in the natural vegetation near Uluru (Ayers Rock) in central Australia. Its deep-rooted characteristic as well as its independence of fixed nitrogen gives it a unique ability to thrive in such an adverse environment, perhaps promoted by runoff from the surface microclimate of Uluru. The genotypic and phenotypic characteristics of casuarinas - fast growth and high CO_2 fixation rates, energy-sparing N_2 fixation, pioneering in adding organic matter to sandy soils, high N-content of foliage, high calorific value of wood, strong and flexible tree structure with facilitation of air flow through foliage and a role in landscape design - provide a unique set of benefits in managing climate change.

2 Global warming

2.1 Effect of irrigation

Kennedy and Hodzic, (2020) estimated that a 3-4% increased application of water to dry land of about 4000 km^3 since 1960 to grow extra food (Plusquellic, 2002; de Marsily et al., 2016) may have increased the concentration of water vapour in the global atmosphere by 1% (Fig. 1). An area in moisture deficit about half that of Australia is now irrigated, often using ground water. This area increases directly with the need for food for the world population, unlike the logarithmically diminishing effect of increasing atmospheric CO_2. Since water vapour causes more than 80% (27-28 °C) of the 33 °C that the Earth is warmed from having an atmosphere, a 1% increase would be expected to cause ca. 0.2-0.3 °C of global warming since 1960. This is consistent with the 1 °C variation in the El Nino-La Nina cycles (Fig. 1) where the atmosphere varies by about 4% in water content (Trenberth and Smith, 2005). We estimate that the primary sensitivity for warming predicted by water vapour from the area irrigated generated substantially between 1960 and 2000 is 0.98 W m^{-2}, providing an increased global temperature without positive feedback of over 0.20 °C. This depends on the extra irrigation water maintaining sufficient extra water vapour in air to contribute to all three sources of warming – direct absorption of sunlight at 6.5 µm, evapotranspiration transferring heat from the surface and greenhouse absorption. CO_2 is attributed about 20% of the natural greenhouse effect, some 6.6 °C of natural warming derived from 31.0 W m^{-2} of climate forcing, assuming temperature increases occur by mechanisms independent of the primary solar source of forcing. The extra forcing caused by the increase in atmospheric CO_2 from 319 ppmv to 370 ppmv between 1960 and 2000 is 0.79 Wm^{-2} (5.3*ln*[370/319]; Andrews, 2010), also equivalent to an increased global temperature without feedbacks of a little more than 0.20 °C.

This possible warming effect of irrigation on the atmosphere was not included (Kennedy and Hodzic, 2019) in the assumptions of the IPCC-approved global climate models (GCMs). Water vapour from evapotranspiration is often dismissed as raining out after mixture with oceanic air. But van Ent and Tuinenberg (2017) have shown that the residence time for water vapour in semi-arid regions approaching two weeks is twice that over the ocean. We have recently contrasted evapotranspiration in the Ord River basin in irrigated land versus surrounding land in the dry season using Modis satellite data based on the Penman-Monteith equation with other spectral data. In our modelling we found (Kennedy et al., 2020) this is consistent with about a 0.2 C increase, taking into account the extra land irrigated globally since 1960. Unlike the effect of CO_2, which cannot be differentiated geographically, the effect of water on outgoing longwave radiation is manifest, as shown in Table 1 for moist and dry areas on the same latitude.

2.2 Vortical entropy and resultant heating of the boundary layer

Using theory developed in Kennedy et al. (2019) regarding the link between action and entropy, we have proposed that the heat capacity of the atmosphere is increased by an order of magnitude (Kennedy and Hodzic, 2020) as a result of the vortical action of anticyclones and cyclones (Fig. 2). In this theory, entropy is interpreted as an index when multiplied by temperature indicates the total heat required to bring a system to the given temperature from absolute zero. This consists of vibrational, rotational and translational

action that yields the entropy (Kennedy et al., 2019) as a logarithmic function (Fig. 2). From frictional impacts of anticyclones and cyclones turbulence in the boundary layer of the atmosphere and at the surface, heat previously used to generate vortical action without raising temperature can now be released. A greater heat capacity of air would be expected to diminish the climate sensitivity from doubling CO_2; it would also provide unexplained sources of heating when air masses collide (Hirsch, 2020), as occurred on Black Saturday in 2010 when over 100 people lost their lives.

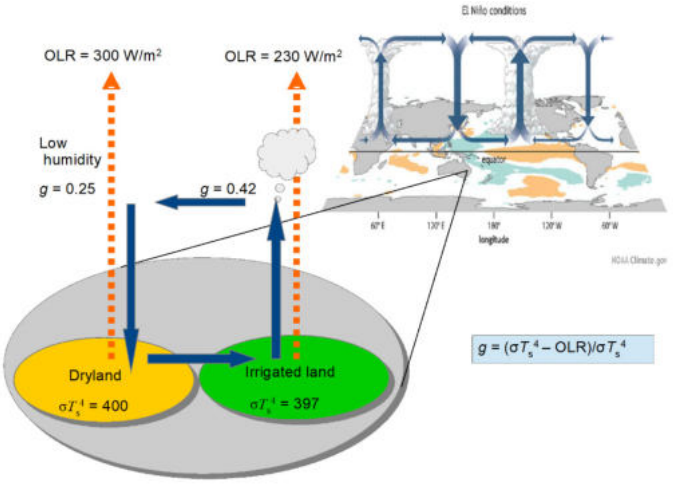

Fig. 1. Model for greenhouse warming by irrigating dryland. This is likened to the El Nino, in that evapotranspiration leads to a reduction in the outgoing longwave radiation leading to greater retention of heat in the atmosphere. To this must be added the increased heat trapped by direct absorption of sunlight at 6.3 μm wavelength due to water. Irrigated regions have a surface temperature cooled by about 1°C.

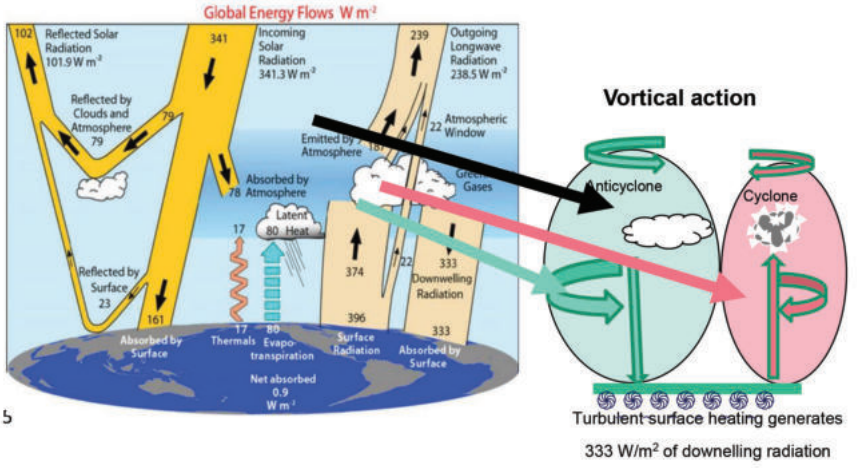

Fig. 2. Generation of downwelling radiation by vortical action in anticyclones and cyclones. Additional heat stored in the circulating atmosphere is released near the surface as a result of surface friction. Modified from Kiehl and Trenberth 1997 (left hand side) with turbulent surface heating as a result of friction from vortical action. Heat absorbed in air from sunlight and by latent heat of condensation is considered to drive the vortical motion, when is then released nearer the surface as a result of friction associated with vorticity, shown as a gradient in wind velocity in the boundary layer nearer the surface.

The Ningaloo Nino is an event in the eastern Indian Ocean of Western Australia (Marshall et al., 2015) in which an unusual southwards, warm Leeuwin current is generated. Both ocean and land may have coincident warming events, providing a test case for the effect of vortical entropy and release of latent heat in the atmosphere if ocean current and prevailing winds are opposed in direction. Such a collision of sea and air currents would be predicted to give excessive warming. So, given these uncertainties regarding causes and respective contributions to anthropogenic global warming, we need to consider how the genetic diversity of casuarinas can nonetheless contribute positively to sustainable climates in the future. Four such ways dependent on these advantages, that could also be immediately cash-generating for the Australian economy, are described below.

3 Potential role of casuarinas

3.1 Re-forest productively yielding carbon offsets on a major scale in cleared agricultural zones

Farmers can grow N_2-fixing trees such as water-efficient casuarinas or acacias on, say, one-quarter of their land, harvesting after 4-5 years; this would offer rehabilitation of acidified soils and chemical products from sunlight (wood, carbon chemicals and ammonia) for local industry, as well as inputs to Lovegrove's electrical power generators (see below).

In India, large scale breeding of better clones of casuarinas selected for homogeneous and rapid growth has been achieved (Nicodemus et al., 2010; Warrier et al., 2012; Karthikeyan et al., 2013), even yielding paper pulp (Parthiban et al., 2012) from trees grown in rice paddies. This work will need to be repeated in Australia, with new selections to examine the range of diversity. In all this research, quantitative information will be sought, including the relative value of products such as timber, paper pulp, charcoal, biomass pellets, organic volatiles, N-fertilisers, ammonia, hydrogen and soil enrichment or depletion. The feasibility to establish an ethical supply of renewable energy able to complement the use of coal or even replace it eventually must be assessed. For example, based on this Indian data, 7.5 million ha of casuarinas on favoured farmlands could produce 150 million tonnes of dry wood or cogeneration pellets for electricity per annum, of the same order as the current consumption of coal in power stations. This biotechnology would help keep any CO_2 problem in check by reducing atmospheric CO_2 in a measured fashion. Fossil fuels such as natural gas and coal might even be used provided the re-forestation provides an excess of C-offsets. Farmers would benefit from increased, more reliable cash-flow, the local community from secondary economic benefits and the environment in sustaining its biota in well designed ecological corridors. Attention should also be paid to smoothing landscapes to limit turbulent surface heating in the boundary layer.

3.2 Generate electrical power using the reversible Haber process chemistry

Such reversible reactions can store solar energy as thermodynamic work, raising the entropy of reactants, such as in the Haber reaction used to fix atmospheric N_2. This reaction is currently the largest industrial chemical process on the planet. This reaction forms ammonia from N_2, but in this case requires a source of priming H_2. Keith Lovegrove's group (2004) working at the Australian National University has shown that the reversible reaction [$3H_2 + N_2 \Leftrightarrow 2NH_3$ + heat] can be used alternately as a chemical battery storage by day and a heat generator at night, using underground gas storage in pipes. This process is driven connected to solar mirror-collectors on the surface, with continuous steam generation from turbines to provide electrical power. This could be fed with natural gas for priming with H_2, or forest products from casuarinas, also allowing off-take of the safer fuel ammonia, or hydrogen. Regional plants producing say 100 MW of power would remove any need for energy storage by pumping limited water uphill or installing expensive batteries for unreliable solar-cell or wind-farms. Such a system provides constant power output proportional to capital input and can continue to operate, even in prolonged periods of low or no sunlight. Once charged, there is no need to provide more H_2 unless withdrawals of H_2 or ammonia as fuel is required.

Ammonia can also be used as feedstock for H_2 generation. The *Frankia*-containing root nodules of N_2 –fixing casuarinas are clearly a major source of ammonia, normally used for plant growth (Kennedy and Ganguli, 2016). H_2 is often proposed as a clean fuel that would overcome many environmental problems related to pollution. But its main sources such as steam and gas are now considered to be too expensive.

Electrolysis of water to $2H_2$ and O_2 uses large quantities of electricity and is employed only where cheap hydroelectric sources are available. However, ammonia itself can also be used as a viable fuel to directly power motor vehicles. Less known is the fact that the Haber reaction normally used to prepare N-fertilisers can be reversed to produce hydrogen gas, consuming a considerable amount of heat.

$3H_2 + N_2 <=> 2NH_3 + 66.8$ kJ (per mole N_2 at 20 MPa, 293 K)

This reaction, usually catalysed using Fe-based catalysts and other metals and high pressures, is unusual in that it is poised and able to be conducted in either direction, depending on the conditions of reaction. As an exothermic reaction, it releases heat normally used to help generate H_2 by hydrolysis of methane by steam. The technology to allow solar energy to be stored in a system operating in sunlight and then to be reversed at night has already been well developed at a pilot scale (Lovegrove et al., 2004) and awaits investment. This approach could be very appropriate for decentralised power generation, using portable components able to be installed from pre-fabricated units. In principle, casuarinas could offer a relatively inexpensive source of ammonia from their foliage, harvested on a continuous basis perhaps by coppicing or in farms operating on a 3–4 year rotation cycle. It is not unreasonable to assume that 100 kg of ammonia could easily be harvested per ha on an annual cycle without loss of the forest resource or even more using rotations; 1000 ha would yield a minimum of 100 tonnes of ammonia of value $50,000 at $US50 ha^{-1}. While there is a prospect for making H_2 which is a highly desired clean fuel, ammonia itself is an excellent fuel, releasing 317 kJ mol^{-1} of ammonia burnt (ΔH = -317 kJ mol^{-1}).

$NH_3 + 0.75O_2 => 0.5N_2 + 1.5H_2O + 317$ kJ

By comparison, the reaction of H_2 with oxygen releases 285 kJ mol^{-1} of H_2 burnt (ΔH = -285 kJ mol^{-1}). The specific energy cost in 2004 was $US13.3 GJ^{-1} (Lovegrove et al., 2004). Thus, one tonne of ammonia is estimated to yield 19.2 GJ of energy, worth $US255, or $US25.5 yield ha^{-1} as electricity.

Hence, ammonia from farm-forests has the potential to be used in small scale electricity stations, forming H_2 by day while generating solar power and releasing heat by night allowing continuing power generation from steam (Lovegrove et al., 2004). Such flexibility can be contrasted with the very large economic inertia when chemical products are manufactured in super-large installations by the Haber process or power is generated in facilities able to supply whole states with millions of consumers.

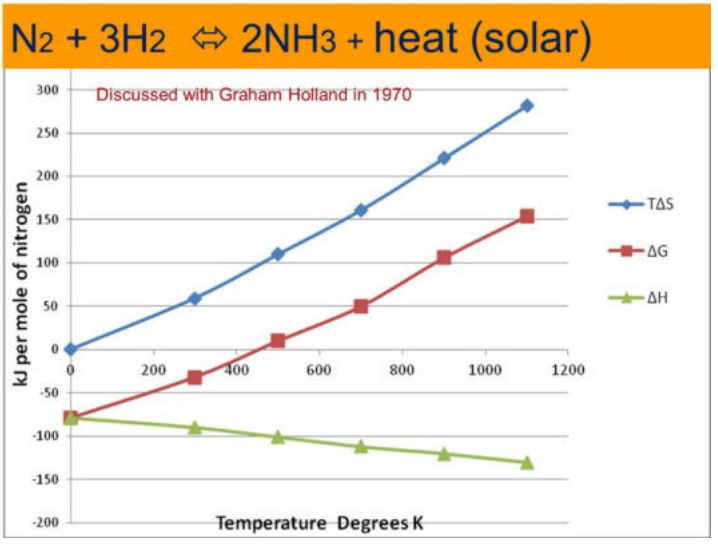

Fig. 3. Thermodynamics of the Haber process reaction, potentially using sunlight to reverse the reaction. Kp is equal to 1.0 at 450 K (ΔG=0). As temperature increases, more heat is released.

This technology, when suitably scaled, would solve any possible problem from burning fossil fuels, also allowing for planned periods of transition, retaining base power for the grid as regional Lovegrove units are activated.

3.3 Re-humidify the landscape

We should take all measures possible to raising the water content of soil and increasing its residence time before being taken off-shore in runoff or the atmosphere. The late Peter Andrews was a proponent of this approach, effectively increasing the residence time of water falling locally as rain by reducing runoff. However, this long-term project would gradually increase the proportion of deep-rooted trees in the landscape. Casuarinas are water efficient yet fix large amounts of carbon using sunlight that can improve the water-holding capacity of soil by increasing soil organic residues. It has been shown that the decline of rainfall in WA is partly because of the extensive land clearing of mallee in the 20th century. Given that most of the rainfall in the Amazon is generated from the forest, we should take advantage of this local recycling as far as possible in our more arid climate. This goal has many potential promoters and if encouraged by policy makers would reduce the intensity of droughts. By regarding farmlands as agents in the environment, farmers would be enlisted in joint activities benefiting both agriculture and the environment.

3.4 Promote water use efficiency

Granted greenhouse credits as a matter of policy, a greater proportion of water could remain in storages, whether in soil available for casuarinas or in catchment dams. It could be possible to grow food with half as much water using trickle irrigation and this improvement in important given the declining stocks of ground-water that has now been moved towards the ocean. Waste plastic has been proposed by the Visy recycling company as an economical source of piping and flexible sacs to reduce evaporation from widely used open channels. Systems of irrigation based on plant demand are favoured to limit water requirements and evapotranspiration (see B. Omodei systems www.measuredirrigation.com.au).

4 Concluding remarks

4.1 Generate integrated regional industry based on farm-forestry

Forest products have many uses as beneficial inputs for other outputs. Thus, tree foliage could be reprocessed biologically for ammonia or urea. A regional industry could use Fischer-Tropsch technology to manufacture carbon products such as degradable plastics and structural materials (Fig. 4). There would be prospects for encouraging employment in regions, involving the diversity of productive systems and providing less in the supply of the products of labour.

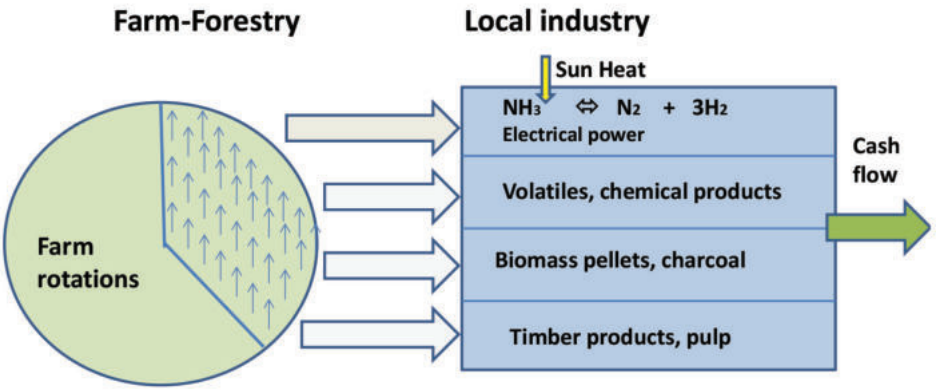

Fig. 4. The combination of farm forestry, electricity generation and associated chemical industry is shown. To minimise transport costs, this would require the planned development of regional centres providing more diversity in industry and employment.

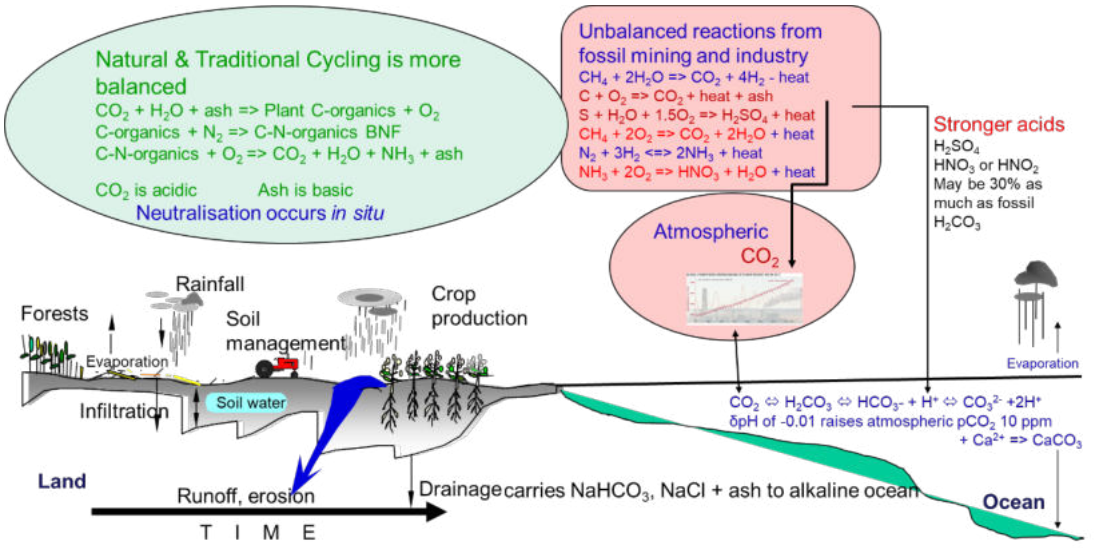

Fig. 5. Ecosystem cycling of CO_2 involves neutralisation of its weak acidity by the alkalinity of BNF. The alkalinity of the ocean is generated by leaching of bicarbonate in river flows to the ocean, with a steady state quasi-equilibrium between bicarbonate and CO_2 in the atmosphere, influencing its atmospheric concentration. Sequestration of CO_2 by agroforestry of casuarinas will act to reduce the CO_2 in the ocean, raising the pH value of seawater. This may protect coral reefs.

4.2 Ecosystem acid-base balancing achieved

The interaction of the carbon and the nitrogen cycles occurs from photosynthesis and BNF (Kennedy, 1992), summarised in Fig. 5. Global carbon and nitrogen cycling is strongly integrated with the consumption of mildly acidic CO_2 in the atmosphere being offset by BNF providing an alkaline product in ammonia so that key compartments in ecosystems are effectively in pH balance. The Earth's soils through runoff provide chemical interaction between the land surface and the ocean. So while the atmosphere is inherently acidic through its CO_2 content and its O_2, which generates sulphuric and nitric acids (Kennedy, 1992), the alkaline constituents of the ocean maintaining its alkaline pH are carbonate and bicarbonate leached into rivers from alkaline soils (Fig. 5). Agroforestry with casuarinas can provide an excellent model of such neutralising reactions in nature.

Thus, irrespective of the true causes of climate variation, the casuarina species can have an important role in environment and landscape management. No other family important in silviculture can combine so many advantages. However, research and strategic planning will be needed to realise these benefits.

Acknowledgements

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Industrial applications

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Chemical and morphological characterization of *Casuarina equisetifolia*

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Abstract

Bangladesh is very much successful in coastal afforestation programmes, which protects from frequent cyclones. *Casuarina equisetifolia* has shown a climate resilient and promising species. No study has been done on industrial application of *C. equisetifolia* in Bangladesh. In this study, *C. equisetifolia* was characterized in terms of chemical, morphological and anatomical properties. It is characterized with higher α-cellulose, similar hemicelluloses and lignin as compared to other hardwood species grown in Bangladesh. The lignin contained mainly of syringyl unit followed by guaiacyl unit. The fiber of *C. equisetifolia* was shorter in length with very thick cell wall and narrow lumen, consequently the wood density and runkel ratio were very high (2.89). *C. equisetifolia* was also fractionated by formic acid (FA) at atmospheric pressure to pulp, dissolved lignin and hemicelluloses. Pulp yield was 50% with kappa number of 40 at the conditions of 3 h treatment with 90% FA followed by 2 h peroxyformic acid treatment. But in the kraft process, *C. equisetifolia* produced pulp yield of 44% with kappa number 17. Both pulps showed good bleachability. The papermaking properties were in acceptable range. Finally, it can be said that *C. equisetifolia* is a promising species for pulping.

Keywords: *Casuarina equisetifolia*, cell wall thickness, Lignin, Pulping, Papermaking properties

1 Introduction

The importance of pulp industries in Bangladesh is increasing with the country's economic growth. All paper industry in Bangladesh is dependent on imported pulp. In 2018, the paper industry in Bangladesh faced severe problem in importing pulp as the price of pulp hike to the highest level. Therefore, entrepreneur has interested to produce pulp in Bangladesh. Unfortunately, Bangladesh is a forest deficient populated country. In the light of fibrous raw material crisis, increasing attention has been given on fast growing species and non-wood (Ferdous et al., 2019, Haque et al., 2019). Therefore, many studies have been conducted on different hardwood species grown in Bangladesh (Jahan et al., 2010; 2011; 2019).

Casuarina equisetifolia can grow in poor soil throughout coastal and mountainous areas in

subtropical and tropical regions (Asakawa, 1983). This species has been introduced outside its natural range for sand dune stabilisation and shelterbelts (Prasad and Dieters, 1998). In Bangladesh, it is being used for coastal protection (Islam, 2003) and is recommended for use in agroforestry program (Jashimuddin et al., 2006). Forest Department Plantation Program in Bangladesh suggested to plant *C. equisetifolia* as short rotation species (Islam, 2003). This species can be an important lignocellulosic resource in Bangladesh. A very few reports are available on it utilization as pulping raw material.

Guha and Sharma (1970) studied on proximate chemical analysis, fiber dimensions and pulping of *C. equisetifolia* and showed that it was not suitable for mechanical pulps as the energy consumption for grinding was high and the pulps were dark. Maan et al. (2019) studied kraft pulping of *C. equisetifolia* with varying sulfidity, cooking time, and cooking temperature on screened pulp yield and Kappa number. Handsheets made from these pulps showed a vessel picking problem during printing that was reduced by treating the pulp with 0.1% cellulase and beating the pulp at high consistency. To better understand the potential utilization of this species, the knowledge of chemistry of *C. equisetifolia* is essential. No reports are available on the lignin chemistry of this species. Therefore, anatomical, chemical and morphological characteristics of *C. equisetifolia* grown in Bangladesh have been studied in this paper. The lignins isolated from *C. equisetifolia* extractive free milled wood by acidic dioxane method (Jahan and Mun, 2007) and characterized in terms of elemental analysis, phenolic hydroxyl, molecular weight, ^{1}H-NMR, FTIR and UV. It is well documented that organic acid fractionation of biomass is suitable to develop integrated biorefinery (Jahan et al., 2006; 2007b). In the paper, formic acid (FA) and kraft pulping of *C. equisetifolia* was also done.

2 Materials and methods

2.1 Materials

C. equisetifolia was collected from the Jamuna river side in Sirajgong District. Three trees were selected for this experiment. Sixty cm from top and bottom and branches of these trees were discarded, and the remaining portion was debarked and chipped to 0.5 cm x 0.5 cm x 2 cm size. The chips were ground in a Wiley mill and 40-60 mesh size was used for chemical analysis.

2.2 Physical, morphological and chemical properties

The basic wood density of *C. equisetifolia* was determined according to PAPTAC Standard A. 8P. For the measurements of fiber length, sample was macerated in a solution containing 1:1 HNO_3 and $KClO_3$. A drop of macerated sample was taken on a slide. The fiber diameter and length was measured in image analyzer Euromex-Oxion using Image Focus Alpha software. The fiber diameter and length were also measured at each annual growth ring.

The extractive (T204 om88), 1% alkali solubility (T 212 om98), water solubility (T207 cm99), Klason lignin (T211 om83) and ash content (T211 os76) were determined in accordance with Tappi Test Methods. Holocellulose was determined by treating extractive free wood meal with $NaClO_2$ solution. The pH of the solution was maintained at 4 by adding CH_3COOH-CH_3COONa buffer and α-cellulose was determined by treating holocellulose with 17.5% NaOH.

2.3 Isolation of lignin from *C. equisetifolia*

C. equisetifolia milled wood (40-60 mesh) was extracted with acetone solvent. The extract free milled wood was refluxed with acidic dioxane (9:1) solution. The concentration of HCl in dioxane solution was made 0.2 N by using concentrated HCl. The detail method was described in elsewhere (Jahan and Mun, 2007).

2.4 Pulping

2.4.1 Formic acid (FA) treatment

The *C. equisetifolia* were refluxed with formic acid in a hotplate under the following different conditions of : Formic acid concentration was 90% (v/v), Reaction time: 1, 2, 3 and 4 h at boiling temperature and Liquor ratio, 5.

After desired reaction time, pulp was filtered in a Buchner funnel and washed with fresh formic acid followed by peroxyformic acid treatment. FA treated pulp was further delignified with peroxyformic acid (PFA) at 80 °C. The reaction was carried out in a thermostatic water bath. The PFA was prepared by adding 90% formic acid with 4% H_2O_2 (on o.d. FA pulp). The treatment was continued for 120 min. After completion of the PFA treatment, the pulp was filtered

off and washed with 80% fresh formic acid, and finally with water. Pulp yield was determined gravimetrically on raw material. The kappa number of the resulting pulp was determined in accordance with Tappi Test Methods (T 236 om-99). All experiments were carried out thrice and average reading was taken.

2.4.2 Kraft pulping

Pulping was carried out in a thermostatically controlled electrical oil bath contained four vessels made of stainless steel having 1.5 litres of volume of each vessel. The capacity of the digester was 1.5 litres. The normal charge was 200 g of oven dried (o.d.) *C. equisetifolia.* Pulping conditions of kraft are as follows: Active alkali was 16, 18, 20 and 22% on oven-dry raw material as Na_2O, Cooking time was 2 h at maximum temperature 170 °C, Liquor to material ratio was 4 and Sulphidity 30% for kraft process.

After digestion, pulp was washed till free from residual chemicals, and screened by flat vibratory screener (Yasuda, Japan). The screened pulp yield, total pulp yield and screened reject were determined gravimetrically as percentage of o.d. raw material. The kappa number (T 236 om-99) of the resulting pulp was determined in accordance with Tappi Test Methods. Three replicates of all experiments were done and average reading was taken.

2.5 Bleaching

Total chlorine free bleaching process was applied to bleach the FA/PFA pulp. FA/PFA pulp was bleached in two stages alkaline peroxide bleaching at 10% pulp concentration. The pH was adjusted to 11 by adding NaOH. The hydrogen peroxide was varied to 4% on o.d. pulp. The bleaching temperature was 80 °C for 1 h. A similar procedure was followed in the 2nd stage of peroxide bleaching. Kraft pulp was bleached by $D_0(EP)D_1$ bleaching sequences. (where D represents chlorine dioxide and Ep represents peroxide reinforced alkaline extraction). The chlorine dioxide charge in D_0 stage was 2%. The temperature was 70 °C in D_0 stage for 60 min. Pulp consistency was 10%. The pH was adjusted to 2.5 by adding dilute H_2SO_4. In the alkaline extraction stage, temperature was 70 °C for 60 min in a water solution of 2% NaOH and 0.5% H_2O_2 (on od pulp) were used. In the D_1 stage, pH was adjusted to get end pH 4.5. The ClO_2 charge in the D_1 stage was 1.0. The brightness and viscosity of the bleached pulp were determined in accordance with Tappi Test Methods.

2.6 Evaluation of formic acid pulp/ kraft pulp

Bleached FA and kraft pulps were beaten in a PFI mill at different revolutions to different freeness (SR) and hand sheets of about 60 g m^{-2} were made in a Rapid Kothen Sheet Making Machine. The sheets were tested for tensile (T 494 om-96), burst (T 403 om-97) and tear strength (T 414 om-98) according to TAPPI Standard Test Methods.

2.7 Isolation of lignin from FA liquor

The lignin dissolved from *C. equisetifolia* during the delignification with formic acid was precipitated from the concentrated spent liquor with five volumes of water. The detail procedure was described by Jahan et al. (2007b).

2.8 Acetylation of lignin

An amount of 100 mg purified lignin was added in 1.5 ml of dry pyridine- acetic anhydride (1:1) for 72 h. The method is described in elsewhere (Jahan et al., 2007b).

2.9 Elemental analysis

C and H analyses of *C. equisetifolia* lignin were carried out in Institute National Analytical Research and Service, BCSIR, Dhaka, and the oxygen was determined by difference. The methoxyl content in dioxane lignin was determined in accordance to *Zeisel–Viebock–Schwappach method.*

2.10 Spectroscopy

Ultraviolet: 7-8 mg of *C. equisetifolia* lignin was dissolved in 100 ml 2-methoxyethanol followed by two times dilution and absorbance was recorded in Shimadzu UV-1800 240V spectrophotometer.

Fourier Transformed Infrared Spectroscopy (FT-IR): FT-IR spectroscopy was performed using a PerkinElmer FT-IR spectrometer (Model: Frontier, Perkin Elmer, USA) with GAAS detector.

^1H NMR: Spectra of lignin solution (100 mg of acetylated lignin contained in 0.5 ml $CDCl_3$) were recorded in a Bruker 400 spectrometer. Solvent was used as internal standard (ppm 7.25). For quantification of protons, the signal in specified

regions of the spectrum were integrated with respect to a spectrum-wide baseline drawn at the level of the background noise, and the results were referred to the signal for methoxyl protons, whose average number per C_9 unit was established as described above.

3 Results and discussion

3.1 Chemical, morphological and physical properties

Table 1 shows the average chemical composition and morphological properties of *C. equisetifolia* and compared with a native fast-growing species *Trema orientalis* grown in different parts of Bangladesh. It is seen that extractives soluble in acetone was only 0.52, which was slightly lower than *T. orientalis* (Jahan et al., 2010) and much higher than *Acacia auriculiformis* grown in Bangladesh (Haque et al., 2019). The holocellulose and α-cellulose contents were 74.2% and 45.7%, respectively, those were very close to *T. orientalis*. As shown in Table 1, pentosan content was much lower than *T. orientalis* (17.1% vs 21.2-23.5%). The pentose sugars content in *C. equisetifolia* was very close to the pentosan content in *Eucalyptus* (Ribeiro et al., 2019).

As shown in Table 1, the klason lignin content in *C. equisetifolia* was slightly higher than *T. orientalis* (25.6% vs 23.6-24.1%) but similar to other hardwoods in Bangladesh (Jahan et al., 2010; 2011).

The average basic density of *C. equisetifolia* was 0.947 g cm^{-3}, which was higher than the other hardwood species (Jahan et al., 2010; Al-Maruf and Jahan, 2015; Haque et al., 2019). The density near to pith was 0.84 g cm^{-3}, which was increased to 1.1 g cm^{-3} near to bark (data are not shown).

The morphological characteristics and its derived values of *C. equisetifolia* are shown in Table 2 and compared with *Acacia auriculiformis*. The average fiber length of *C. equisetifolia*, was 0.69 mm, which is considered as short fiber of tropical hardwoods (0.7-1.5 mm) (Hale, 1959). As compared to *T. orientalis* also *C. equisetifolia* had shorter fiber 0.83-1.34 mm (Jahan et al., 2010). The fiber wall thickness (5.09 µm) was wider and lumen was narrower (3.52 µm) than the common hardwood species. Maan et al. (2019) also found wider wall thickness and narrower lumen of *C. equisetifolia*. Fibers with larger lumen is easier for beatabilitty and collapsability consequently increase better bonding. Thick wall is also evident in

Table 1. Chemical, morphological and physical properties of *C. equisetifolia*.

	C. equisetifolia	*Trema orientalis* (Jahan et al., 2010)
Extractive (%)	0.52	0.81-0.89
Holocellulose (%)	74.2	74.4-78.5
α-cellulose (%)	45.7	42.5-45.1
Pentosan (%)	17.1	21.2-23.5
Klason lignin (%)	25.6	23.6-24.1
Acid soluble lignin	3.1	2.2-2.9
Ash Content (%)	0.22	1.1-1.2
Density (g cc^{-1})	0.947	0.357-0.380

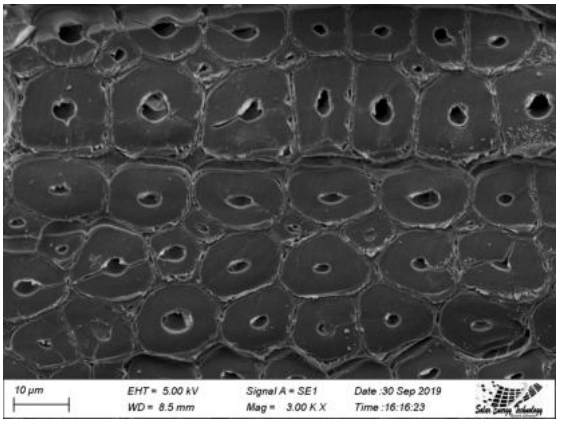

Fig. 1. Cross section of *C. equisetifolia* in SEM.

SEM image of *C. equisetifolia* (Fig. 1). This wider wall thickness and narrower lumen explains higher wood density (Table 2). Fiber width and wall thickness affected fiber flexibility. Thick-walled fibers adversely affected the bursting strength, tensile strength and folding endurance of paper. According to Bektas et al. (1999) *C. equisetifolia* fiber is highly rigid having flexibility coefficient less than 30.

The slenderness ratio of *C. equisetifolia* fiber was 49.15, which was close to that of *A. auriculiformis*. The fibers with higher slenderness ratio show lower collapsibility and conformability, consequently, produce paper of high tear, porosity, bulk and opacity. The

Table 2. Morphological properties and its derived value.

Morphological property	*C. equisetifolia*	*Acacia auriculiformis* (Jahan et al., 2019)
Fiber length (mm)	0.69	0.91
Fiber diameter (µm)	14.04	18.27
Lumen diameter (µm)	3.52	15.71
Wall thickness (µm)	5.09	2.46
Slenderness ratio	49.15	49.86
Flexibility coefficient	25.07	85.9
Runkel ratio	2.89	0.31

slenderness ratio also affects flexibility and resistance to rupture of the fibers (Maiti, 1997). The flexibility coefficient of *C. equisetifolia* fiber was only 25.07, which was much lower than *A. auriculiformis* fibres. Fibers of low flexibility coefficient do not collapse easily thus retain their tabular structure on pressing consequently lesser surface for bonding. Such type of fibers produces paper sheets of higher tear, bulk and opacity. Due to wider cell wall thickness, the runkel ratio of *C. equisetifolia* fibers was 2.89, while the same for *A. auriculiformis* was 0.31 only, consequently affect paper density (Pillow et al., 1941).

Table 3. Formic acid (90%) followed by peroxiformic acid pulping of *C. equisetifolia*.

FA treatment time (h)	Pulp yield (%)	Kappa Number
1	71.48	57.25
2	52.84	44.77
3	50.71	40.3
4	50.55	40.4

3.2 Formic acid (FA)/peroxyformic acid pulping

Casuarina equisetifolia was cooked with formic acid by varying temperature followed peroxyformic acid treatment. As shown in Table 3, pulp yield and kappa number decreased with FA treatment time. FA cooking for 1 h followed by 2 h peroxyformic acid treatment produced pulp yield of 71.5% with kappa number 57.3, which decreased yield to 50.7% with kappa number 40.3 with increasing FA cooking time to 3 h followed by 2 h PFA treatment. Further increase of FA cooking time did not reduce kappa number. But the kappa number was still quite high to make a bleachable pulp. A higher pulp yield in organic acid pulping was also observed in case of *Trema orientalis* pulping with pulp yield of 56% and kappa number 34 (Jahan et al., 2007a).

3.3 Kraft pulping

Table 4 shows the effect of active alkali charge on *C. equisetifolia* pulping. At 16% alkali charge, *C. equisetifolia* was delignified to Kappa number 19.5, which was quite acceptable for pulp bleaching. However, screened reject was very high (6.2%) resulted in high total pulp yield (48.35%) and low screened pulp yield (42.15%). The screened pulp yield increased to 43.8% and kappa number decreased by 1.9 points with increasing active alkali charge to 18%.

It is seen from Fig. 2 that total pulp yield decreased and screened pulp yield increased with increasing active alkali charge up to 20%, but further increase of active alkali charge decreased screen pulp yield. At 22% AA charge, both pulp yield and kappa number decreased rapidly. Therefore, active alkali charge of 20% was considered as an optimum active alkali charge for *C. equisetifolia* for 2 h cooking at 170 °C. The pulp yield in *C. equisetifolia* was very similar to *Acacia* hybrid, where pulp yield was 42.7% and Kappa number of 23.5 at the conditions 20% for 2 h at 170 °C (Haque et al., 2019).

Table 4. Kraft pulping of *C. equisetifolia*.

Alkali charges (% as Na_2O)	Pulp yield (%)			Kappa number
	Screened	Reject	Total	
16	42.15	6.20	48.35	19.50
18	43.82	1.40	45.33	17.61
20	44.20	0.82	45.02	17.00
23	42.10	0.80	42.90	14.79

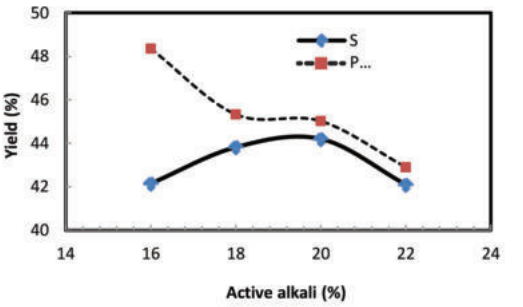

Fig. 2. Screened pulp yield (SPY) and total pulp yield (TPY) of *C. equisetifolia*.

3.4 Bleaching

FA/PFA and kraft pulps were bleached by alkaline peroxide and $D_0(EP)D_1$ bleaching, respectively, and results are shown in Fig. 3. Bleachability of *C. equisetifolia* kraft pulp was very good. In three stages ($D_0(EP)D_1$), kraft pulp brightness reached to 86.9%, while *C. equisetifolia* FA/PFA pulp reached 74.6% in alkaline peroxide bleaching. The *C. equisetifolia* pulp bleachability was similar to *Trema orientalis* kraft pulp (Jahan et al., 2008, 2010) and better than acacias grown in Bangladesh (Haque et al., 2019a). As the brownstock kappa number FA/PFA pulp was higher (Table 3), therefore, required bleaching chemicals was higher to remove the remaining lignin entering the bleach plant.

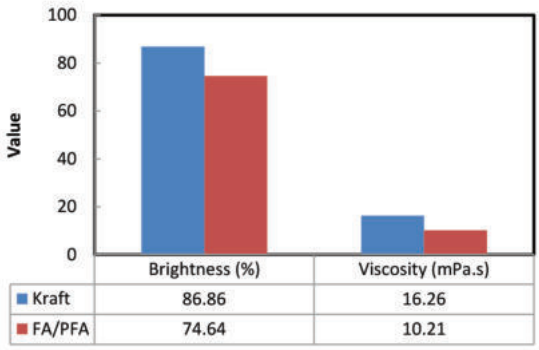

Fig. 3. Bleached pulp properties of *C. equisetifolia*.

3.5 Papermaking properties

FA and kraft pulps were beaten in a PFI mill to different revolution and handsheets were prepared for tensile, tear and burst indexes. The papermaking properties of kraft pulp were superior to FA pulp (Table 5). As shown in Table 5, tensile and burst indexes increased with increasing drainage resistance as expected. At SR value of 30, the tensile index of kraft pulp was 54% higher than FA pulp. Similarly, 154% higher burst index was observed for kraft pulp. The tensile and tear of *C. equisetifolia* pulp were inferior to other hardwood pulp grown in Bangladesh (Jahan et al., 2010, 2011; Al-Maruf and Jahan, 2015; Haque et al., 2019).

Tear index of both pulps increased initially followed by slow decreased. The maximum tear index of kraft pulp 7.7 $mN.m^2\ g^{-1}$ was obtained at SR value of 21, while the maximum tear index of FA pulp was 5.8 $mN.m^2g^{-1}$ at SR value of 32. The lower tear index *C. equisetifolia* pulp can be explained lower fiber length (Table 2).

3.6 UV spectra of *C. equisetifolia* lignin

UV spectra of *C. equisetifolia* and FA lignin are very similar to wood lignin (data are not shown). The characteristic absorption maximum of lignin occurs at 280 nm. This slight shift of FA lignin at 282 nm was due to the removal of methoxyl group during FA pulping. The absorptivity of *C. equisetifolia* lignin at 280 nm was 13.53 L g^{-1} cm^{-1} and for the FA lignin at 282 nm 13.93 L g^{-1} cm^{-1}.

3.7 FTIR spectra of *C. equisetifolia* and FA lignin

As shown in Fig. 4, both lignins were broad band at 3300, which was due to hydroxyl groups of aromatic and aliphatic structures. It is clearly seen from the spectra that FA lignin was stronger band than

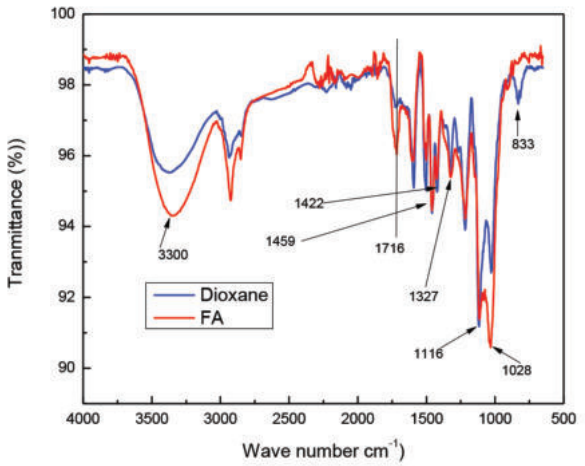

Fig. 4. FTIR spectra of *C. equisetifolia* and FA lignin.

C. equisetifolia lignin. Strong band of carbonyl group at 1716 cm^{-1} was found in FA lignin. Similar result was obtained in organosolv lignin (Rossberg et al., 2015). The strong intensity of the band supported the process of esterification during formic acid (Ede and Brunow, 1989). The band at 1459 and 1422 cm^{-1} are attributed for methoxyl groups, which were slightly stronger in dioxane lignin than acid lignin. A clear difference between FTIR spectra of dioxane and FA liquor lignins are observed from the intensities of peaks around 1327 and 1271 cm^{-1} (ring breathing of S and G units, respectively). The band at 1325 cm^{-1} is associated with the C=O stretching of syringyl structure in lignin molecules. Peaks at 1216 cm^{-1} are assigned to the guaiacyl structure in lignin molecules, which is prominent in FA lignin. The out-of-plane C-H bending of syringyl content was observed at 833 cm^{-1}. Organic acid cleaves β-O-4 and α-O-4 linkages leaving a lot of non-etherified phenolic OH groups in lignin, visible.

3.8 Empirical formula and NMR

The empirical formula of *C. equisetifolia* lignin was $C_9H_{8.90}O_{3.48}(OCH_3)_{1.54}$ (data are not shown), which was close to aspen lignin (Jahan and Mun, 2007). The methoxyl content in *C. equisetifolia* lignin was 1.54/C_9 unit while the same for *Trema orientalis* lignin was 1.27-1.32 (Jahan and Mun, 2007).

Table 5. Physical properties of bleached *C. equisetifolia* pulp in FA/PFA and kraft processes.

Pulp	SR	Tensile index ($N.mg^{-1}$)	Burst index ($kPa.m^2g^{-1}$)	Tear Index ($mN.m^2g^{-1}$)
Kraft	12	37.36	0.68	4.36
	18	32.86	1.58	5.6
	21	3.53	2.05	7.72
	26	37.83	2.60	7.23
	40	44.70	2.81	6.0
FA/PEA	21	19.6	0.54	4.7
	33	26.8	1.1	5.8
	40	30.9	1.5	3.74
	56	40.3	2.0	3.1

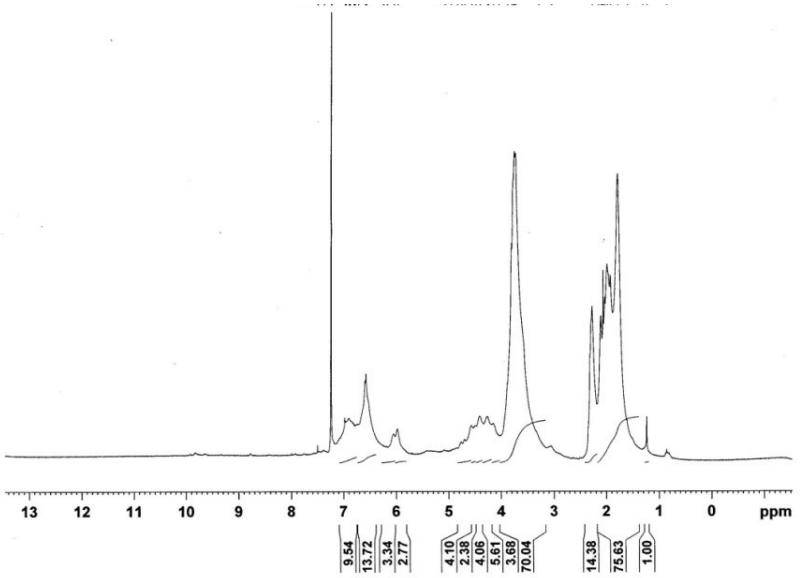

Fig. 5. ^{1}H-NMR spectrum of *C. equisetifolia* lignin.

The numbers of free aliphatic and phenolic hydroxyl groups per C_9 unit were determined from the corresponding acetate signals. The proton of phenolic hydroxyl group of *C. equisetifolia* lignin was 0.31/C_9, which was very similar to *T. orientalis* lignin a native species of Bangladesh. The proton of aliphatic hydroxyl group was 1.66/C_9.

There were two peaks of *C. equisetifolia* lignins in the aromatic proton region, one was for syringyl units at δ 6.6 and another was guaiacyl units at δ 6.9. Syringyl units shows stronger peak than the guaiacyl units (Fig. 5). This result is consistent with FTIR result. NMR integration suggests that *C. equisetifolia* lignins had 0.91 + 0.63 = 1.54 aromatic protons per C_9 units. Considering 100 C_9 units and their methoxyl contents (154/C900 from empirical formula), the proportion of the syringyl and guaiacyl units was calculated to be 46% and 54% respectively. The detail calculation was shown in elsewhere (Islam and Sarkanen, 1993). The β-*O*-4 aryl ether linkage constitutes the main intermonomeric connection in lignin (Lundquist, 1979). As in other hardwood, *C. equisetifolia* lignin shows both *erythro* and *threo* configurations due to the presence of proton at the C-α position of the side chain. The *erythro* protons (Hα) give stronger peak at 6.01 ppm than the corresponding peak for *thero* form at δ 6.09.

4 Conclusion

C. equisetifolia has very high-density wood with wider cell wall and narrow lumen diameter. Its α-cellulose content was 45.7% and lignin 25.7%. The lignin isolated from *C. equisetifolia* showed higher syringyl to guaciayl ratio. Formic acid (FA) treatment followed by peroxyformic acid treatment produced very high pulp yield but high kappa number. The dissolved lignin in FA treatment was higher phenolic hydroxyl group and lower methoxyl group. Kraft pulping produced pulp yield of 45.02% at kappa number 17.0. The bleachability of *C. equisetifolia* was very good. Produced pulp needs higher beating degree to produce good strength.

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Mechanical and physical properties of *Casuarina junghuhniana* wood

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Abstract

Physical and mechanical properties of *Casuarina junghuhniana* wood from a 6-year-old clone trial in Kanchanaburi Province, Thailand were studied during 2015–2017. Wood samples were taken from four clones. Three clones were of *C. junghuhniana* species: one originating from Kenya, and two originating from Bali and Wetar, Indonesia. In addition, a local commercial hybrid clone of *C. junghuhniana* and *C. equisetifolia* was included for comparison. The physical properties were examined on dry and wet wood samples, while the mechanical properties were determined on 12% moisture content wood samples. Clones differed markedly in many wood traits. By using the MOR values at 12% moisture content to categorize hardness, the wood of Thailand, Bali and Wetar clones was regarded as hard while that of Kenya clone is considered medium hard. Overall results indicated that the wood of Kenya clone was less strong and less hard than wood from the other three sources.

Keywords: *Casuarina junghuhniana,* wood properties, mechanical properties, physical properties, Thailand

1 Introduction

Mechanical properties are the properties of woods or materials that react to the application of loads or external forces. There are four categories of mechanical properties. *Strength* determines the ability to resist or absorb loads. *Stiffness* is concerned with the ability to resist deformation when force is applied. *Toughness* indicates the ability of wood in absorbing energy and breaking down completely or at a certain point. Lastly, *hardness* shows the ability to resist permanent change in shape from being scratched, pierced or drilled.

Physical properties are the characteristics and behavior of woods towards external factors besides from applied forces. (Winandy, 1994). Examples of such factors include shrinkage and swelling, moisture content, density, specific gravity, thermal and pyrolytics properties, electrical conductivity, and durability.

Casuarina junghuhniana Miq., known locally as Iron Wood Horsetail, in the family Casuarinaceae is a native of Indonesia and Timor Leste. It is a medium-sized evergreen tree up to 20 m in height. It is usually found in places ranging from beaches to mountains of up to 3,100 m above mean sea level. It grows on soils of pH 3 to 8. Its stem is straight, with a symmetric conical crown. Its branchelets are small, orderly, and acutely angled towards the trunk. The green, needle-like branchlet are 10–20 cm long. The blackish brown

bark is shallowly divided into tiny plates, hanging by the trunk. The leaves are reduced to scale-like teeth arranged in whorls of 9–10. The scale leaves are green with a yellow tip. It is dioecious species with male and female inflorescenses on separate trees. The seeds are small and round in shape (Bounyaprapatsorn and Chokchaicharoenporn, 1999). Its timber has many uses such as: poles, masts, construction crutches, timbers and charcoals. The wood has a high calorific value similar to mangrove species. Large-sized logs can be used as planks. It can also be processed into smaller pieces to make furniture, parquet and timbers. Moreover, it is widely grown for ornamental purposes, wind protection, and along roadsides.

The purpose of this study is to provide basic information for other research in the "Economical Value-added of *Casuarina junghuhniana* Miq. in the Future" series.

2 Materials and methods

2.1 Wood samples

Wood samples were taken from a six-year-old *C. junghuhniana* clone trial located in Kanchanaburi province, Thailand (latitude 14°24′ N, longitude 98°56′ E, altitude 370 m asl). The trial was managed by the Central Silvicultural Research Centre of the Royal Forest Department. A subset of four clones was used in this study. Three clones were of *C. junghuhniana,* of which one from a landrace in Kenya, and two were from natural stands in Bali and Wetar in Indonesia. The fourth clone, included for comparison purposes, was a local commercial hybrid clone of *C. junghuhniana* and *C. equisetifolia.*

A 30-tonne Testomatric MICO 500 at the Forest Research and Development Office, Royal Forest Department in Bangkok was used for mechanical property testing. Other equipment included a vernier caliper to measure the size of wood samples; a conditioning cabinet which could be set to 100°C; and a digital balance with precision to one milligram.

2.2 Processing of wood samples

For each clone, six trees were felled and the bottom portions of the stem, 2 m in length were cut for the study. The wood samples were taken and stored at the Northeastern Forest Utilization Development Center in Khon Kaen Province, north-eastern Thailand until they reached the same moisture content (air-dry). Then, they were processed according to the British Standard of BS 373:1957 Methods of Testing Small Clear Specimens (British Standard, 1957). Wood samples were cut into 2 cm square of different lengths for mechanical property test. These tests were conducted in both air-dry condition and wet condition. The samples for air-dry condition test were desiccated until their moisture content was the same as that for air-dry condition. The samples for wet condition test were soaked in water until their moisture content exceeded the fiber saturation point.

Fig. 1. *Casuarina junghuhniana* trees in the clone trial which were felled for wood property study.

2.3 Mechanical property tests

The following tests were conducted:

(1) Static bending test central point loading. Wood samples (30 cm long x 2 cm square) were cushioned with supporters which were 28 cm span. The velocity of loading was fixed at 6.604 mm min^{-1}. The radial side of the samples was used. The results were expressed as Modulus of Rupture (MOR)–the stress at proportional limit, and Modulus of Elasticity (MOE)–the stiffness of wood.

(2) Impact bending test. Wood samples (30 cm long x 2 cm square) were cushioned with supporters which were 24 cm span. A pendulum was released and its force was applied to the radial side of samples at the centerpoint. The value observed at the cross section was the force that broke the wood. The impact bending strength was expressed in Nm.

(3) Compression parallel to grain test. wood samples (6 cm long x 2 cm square) were put in the testing machine at the fixed velocity of push at 0.035 mm min^{-1}. The values were observed on both the radial and tangential sides.

(4) Shear parallel to grain test. Cube samples 2 cm x 2 cm x 2 cm were put in the testing machine at the fixed velocity of push at 0.635 mm min^{-1}. The values were observed on both the radial and tangential sides.

(5) Hardness test. Wood samples of 2 cm square regardless of length were put in the testing machine at the fixed velocity of push at 0.635 mm min^{-1}. The pinhead of the machine was round and had a diameter of 11.20 mm. It was pressed in the samples until half of it was submerged into the samples on both the radial and tangential side. Calculation of the mechanical properties was based on 12% moisture content (modified from Sono, 1973). In this study, tension test was not conducted because the small sample size was considered not safe for processing.

2.4 Physical property tests

The following tests were conducted:

(1) Density and specific gravity, which indicate the amount of actual wood substance present in a unit volume of the wood. These were determined using the previously tested cube wood specimens (2 cm x 2 cm x 2 cm).

(2) Shrinkage, also determined using cube specimens. Care was taken to ensure no defects on the radial and tangential sides. The weight, radial, tangential and length sides were measured to calculate the volume continuously while the samples were being dried in the incubator at 103±2 °C until they reached constant weight and size. The results were the extent of the shrinkage of each side.

(3) Fiber saturation point. This was measured from plotting a graph relating to moisture content and radial side shrinkage (modified from Sono et al., 1974).

3 Results and discussion

3.1 Mechanical properties

3.1.1 Mechanical properties of local commercial hybrid clone (see Table 1)

(1) The MOR of the samples in wet and air-dry condition was 75.8 and 115 MPa respectively. At 12% moisture content, the MOR of the samples was 119 MPa. According to the Royal Forest Department (RFD)'s Standards, MOR value higher than 100 MPa is considered hardwood. Thus, the wood of this hybrid is in the hardwood category.

(2) The stress at proportional limit in wet and air-dry conditions was 45.9 and 64.3 MPa respectively. At 12% moisture content, the value was 66.0 MPa. Compared with MOR, the r-ratio (minimum stress and maximum stress ratio) is 55%. Accordingly, *Casuarina* hybrid clone has a medium elastic range.

(3) The MOE in wet and air-dry conditions was 9,580 and 13,300 MPa, respectively. At 12% moisture content, the MOE was 13,630 MPa. The MOE is a value indicating how hard it is to deform when forces are applied to.

(4) The impact bending values of the samples in wet and air-dry conditions was 34.32 and 29.62 Nm, respectively. At 12% moisture content, the value was 34.72 Nm. This value indicates how much wood is able to be impact bended into tough. Each one has a different value as each has different make up.

(5) The compression to parallel grain of the samples in wet and air-dry conditions was 29.3 and 49.3 MPa respectively. At 12% moisture content, the value was 51.1 MPa. The samples are mostly deformed by crushing and shearing, which is a natural deformation that happens to wood when tested in this way (Jullalerk, 2001).

Fig. 2. Wood property tests conducted by the project.

(6) The radial side hardness of the samples in wet and air-dry conditions was 3,540 and 4,585 Newton (N) respectively. At 12% moisture content, the value was 4,979 N. The tangential side hardness was 3,422 and 4,928 N respectively. At 12% moisture content, the value was 5,066 N.

Table 1. Mechanical properties of *Casuarina* hybrid clone from Thailand.

Mechanical property	SI unit	Adjusted at 12% MC	Conditions in SI unit	
			Wet	Air dry
Modulus of rupture (MOR)	MPa	119	75.8	115
Stress at proportional limit	MPa	66.0	45.0	64.3
Modulus of elasticity (MOE)	MPa	13,630	9,580	13,300
Shearing (radial)	MPa	13.5	7.78	13.0
Shearing (tangential)	MPa	19.8	11.3	19.1
Compression parallel to grain	MPa	51.1	29.3	49.3
Impact bending	Nm	34.72	29.62	34.32
Radial hardness	N	4,979	3,540	4,858
Tangential hardness	N	5,066	3,422	4,928

3.1.2 Mechanical properties of Kenya clone (Table 2)

(1) The MOR of the samples in wet and air-dry condition was 42.0 and 71.8 MPa respectively. At 12% moisture content, the MOR was 74.2 MPa. As such, it is categorized as a medium hardwood according to the RFD Standards, based on MOR of 60–100 MPa.

(2) The values of stress at proportional limit of the samples in wet and air-dry conditions was 22.1 and 37.2 MPa respectively. At 12% moisture content, it was 38.4 MPa. Compared with MOR, the r-ratio is 55%. This means that *C. junghuhniana* clone from Kenya has a medium elastic range.

(3) The MOE of the samples in wet and air-dry conditions was 5,020 and 7,420 MPa respectively. At 12% moisture content, the MOE was 7,620 MPa.

(4) The impact bending of the samples in wet and air-dry condition was 12.36 and 16.08 Nm respectively. At 12% moisture content, the value was 16.38 Nm. This value indicates the ability to resist immediate maximal loading. It is suggested that *C. junghuhniana* clone from Kenya has a low impact bending value, i.e. its wood is relatively brittle.

(5) The compression to parallel grain of the samples in wet and air-dry conditions was 18.5 and 34.0 MPa,

Table 2. Mechanical properties of *C. junghuhniana* clone from Kenya.

Mechanical property	SI unit	Adjusted at 12% MC	Conditions in SI unit	
			Wet	Air dry
Modulus of rupture (MOR)	MPa	74.2	42.1	71.8
Stress at proportional limit	MPa	38.4	22.1	37.2
Modulus of elasticity (MOE)	MPa	7,620	5,020	7,420
Shearing (radial)	MPa	12.8	6.08	12.2
Shearing (tangential)	MPa	13.7	7.84	13.3
Compression parallel to grain	MPa	35.3	18.5	34.0
Impact bending	Nm	16.38	12.36	16.08
Radial hardness	N	2,604	1,937	2,553
Tangential hardness	N	2,874	1,931	2,802

respectively. At 12% moisture content, the value was 35.3 MPa. The samples were mostly deformed from crushing and shearing.

(6) The radial side hardness of the samples in wet and air-dry conditions was 1,937 and 2,553 N respectively. At 12% moisture content, it was 2,604 N. The tangential side hardness for wet and dry samples was 1,931 and 2,802 N respectively. At 12% moisture content, the value was 2,874 N.

3.1.3 Wood properties of Bali clone (Table 3)

(1) The MOR of the samples in wet and air-dry condition was 74.3 and 111 MPa respectively. At 12% moisture content, the MOR was 114 MPa. As such, it is categorized as hardwood, based on RFD Standards of MOR greater than 100 MPa.

(2) The stress at proportional limit of the samples in wet and air-dry condition was 45.9 and 64.3 MPa, respectively. At 12% moisture content, the value was 66.0 MPa. Compared with MOR, the r-ratio was 55%. Accordingly, *C. junghuhniana* wood from the Bali source has a medium elastic range.

(3) The MOE of the samples in wet and air-dry conditions was 9,290 and 10,350 MPa, respectively. At 12% moisture content, the MOE was 10,440 MPa.

(4) The impact bending of the samples in wet and air-dry condition was 43.25 and 39.32 Nm respectively. At 12% moisture content, the value was 38.94 Nm.

(5) The compression to parallel grain of the samples in wet and air-dry conditions was 34.1 and 56.4 MPa, respectively. At 12% moisture content, the value was 58.4 MPa. The samples were mostly deformed by crushing and shearing.

(6) The radial side hardness of the samples in wet and air-dry conditions was 3,824 and 5,249 N respectively. At 12% moisture content, the value was 5,363 N. The tangential side hardness was 4,057 and 5,557 N respectively. At 12% moisture, the value was 5,689 N.

3.1.4 Mechanical properties of Wetar clone (Table 4)

(1) The MOR of the samples in wet and air-dry condition was 61.6 and 97.5 MPa respectively. At 12% moisture content, the MOR was 102 MPa. As such, it is categorized according to the RFD Standards as hardwood, which has a MOR of above 100 MPa.

(2) The stress at proportional limit of the samples in wet and air-dry conditions was 35.6 and 53.3 MPa respectively. At 12% moisture content, the value was 55.3 MPa. Compared with MOR, the wood of the Wetar clone also has a medium elastic range.

(3) The MOE of the samples in wet and air-dry conditions was 8,150 and 10,110 MPa respectively. At 12% moisture content, the MOE was 10,330 MPa.

(4) The impact bending value of the samples in wet and air-dry condition was 22.75 and 24.22 Nm,

Table 3. Mechanical properties of *C. junghuhniana* clone from Bali.

Mechanical property	SI unit	Adjusted at 12% MC	Conditions in SI unit	
			Wet	Air dry
Modulus of rupture (MOR)	MPa	114	74.3	111
Stress at proportional limit	MPa	60.8	46.1	59.6
Modulus of elasticity (MOE)	MPa	10,440	9,290	10,350
Shearing (radial)	MPa	13.9	9.06	13.5
Shearing (tangential)	MPa	16.0	10.7	15.6
Compression parallel to grain	MPa	58.4	34.1	56.4
Impact bending	Nm	38.94	43.25	39.32
Radial hardness	N	5,363	3,824	5,239
Tangential hardness	N	5,689	4,057	5,557

Table 4. Mechanical properties of *C. junghuhniana* clone from Wetar.

Mechanical property	SI unit	Adjusted at 12% MC	Conditions in SI unit	
			Wet	Air dry
Modulus of rupture (MOR)	MPa	102	61.6	97.5
Stress at proportional limit	MPa	55.3	35.6	53.3
Modulus of elasticity (MOE)	MPa	10,330	8,150	10,110
Shearing (radial)	MPa	17.4	10.8	16.8
Shearing (tangential)	MPa	20.8	10.4	19.7
Compression parallel to grain	MPa	50.0	31.8	48.2
Impact bending	Nm	24.42	22.75	24.22
Radial hardness	N	5,232	3,537	5,035
Tangential hardness	N	5,411	3,953	5,262

respectively. At 12% moisture content, the value was 24.42 Nm.

(5) The compression to parallel grain of the samples in wet and air-dry conditions was 31.8 and 48.2 MPa respectively. At 12% moisture content, the value was 50.0 MPa. The samples are mostly deformed by crushing and shearing.

(6) The radial side hardness of the samples in wet and air-dry conditions was 3,537 and 5,035 N respectively. At 12% moisture content, the value was 5,202 N. The tangential side hardness was 3,953 and 5,262 N, respectively. At 12% moisture content, the value was 5,411 N.

The results obtained for mechanical properties showed that the wood of six-year-old *C. junghuhniana* clones from Thailand, Bali and Wetar is considered hardwood according to the RFD Standards, that is the MOR is greater than 100 MPa. The wood of Kenya clone has a medium strength according to the RFD Standards, i.e. MOR 60–100 MPa. Deformation of the wood of all four clones was caused mostly by simple tensions, while some was due to cross grain tensions or splintering tensions. Overall, the mechanical properties of Kenya clone are inferior compared to the other three clones.

3.2 Physical properties (Table 5)

(1) The dry density of six-year-old wood from Thailand, Kenya, Bali and Wetar clones was 542, 552, 672 and 670 kg cm^{-3}, respectively. These values are within the range of medium density wood.

(2) The specific gravity of six-year-old wood from Thailand, Kenya, Bali and Wetar clones was 0.49, 0.53, 0.64 and 0.62, respectively.

(3) The radial side shrinkage of wood samples from Thailand, Kenya, Bali and Wetar clones was 2.64, 2.65, 3.84 and 2.57%, respectively. The tangential side shrinkage was 5.17, 6.05, 5.01 and 4.19%, respectively. The length shrinkage was 0.32, 0.45, 0.13 and 0.12%, respectively. The volumetric shrinkage was 14.73, 9.94, 6.65 and 7.04%, respectively. The results suggest Thailand, Kenya, Bali, and Wetar clones have medium to high shrinkage in green to dry conditions.

(4) The fiber saturation point of the six-year-old wood samples from Thailand, Kenya, Bali and Wetar was 25.1, 26.4, 25.6, and 22.8 %, respectively.

4 Conclusion

This study demonstrated marked differences in the mechanical and physical properties among *C. junghuhniana* clones. By using the MOR values at 12% moisture content to categorize hardness, the wood of Thailand, Bali and Wetar clones was regarded as hardwood (i.e. MOR greater than 100 MPa) while that of Kenya clone was considered medium hardwood (MOR 60–100 MPa). Results obtained for other mechanical properties (compression, shear strength, MOE, toughness and hardness) suggest that the six-year-old wood samples from Thailand, Bali and Wetar clones have a moderately strong structure. However,

Table 5. Physical properties of *Casuarina* clones.

Physical property	Unit	Thailand	Kenya	Bali	Wetar
Shrinkage					
Radial	%	2.64	2.45	3.84	2.57
Tangential	%	5.17	6.05	5.01	4.19
Length	%	0.32	0.25	0.13	0.12
Volume	%	14.73	9.94	6.65	7.04
Specific gravity		0.49	0.53	0.64	0.62
Dry density	kg m^{-3}	542	552	672	670
Fiber saturation point	%	25.1	26.4	22.8	22.8

the wood of hybrid *C. junghuhniana* and *C. equisetifolia* grown in Thailand has a low natural durability of 4.6 years (Jullalerk et al., 2005). Accordingly, for use in low decay situations, such as in above-ground construction or use as tools, they may still be useful. If treated with preservatives, their woods will be more durable and useful.

However, the wood of *C. junghuhniana* used in this study was only six years old. Their properties are not as strong compared to the properties obtained from old trees (Jullalerk et al., 2005). There is a need to determine the age for acceptable wood quality of this species when grown in plantation.

The physical properties of wood samples in this study indicate that the species has moderate wood density, for example specific gravity 0.49–0.64 and dry density 522–672 kg m^{-3}. Their fibre saturation point is 22–26% which is lower than that reported for other species which is about 28% (Hirizoglu, 2004). However, the shrinkage characteristic is similar to other species.

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Short communications

Proceedings of the Sixth International Casuarina Workshop

Current status and prospect for future development of casuarina in Vietnam

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Casuarina equisetifolia Forst. was first introduced to Vietnam in 1896. It is fast-growing and has been planted mainly in coastal area in central Vietnam. This species plays important ecological and economic roles for local people as it is often used as a pioneer in coastal afforestation to protect against winds and moving sands, and for improving soil fertility and supplying timber and fuel wood. The total area of *C. equisetifolia* in Vietnam in 2018 was estimated to be about 21,500 ha, a considerable reduction from 120,000 ha in the 1990s. Productivity of most plantations is poor because they have been planted by unimproved seed from local seed sources or scattered trees although good provenances and some selected clones were introduced from China. Most of the current plantations are coppiced plantations which were planted more than 20 years ago and subjected to logging and harvesting several times for fuel wood. The area of casuarina plantations in coastal area has decreased due to high land demand for agriculture, tourism and mining. Although casuarina is facing problems from socio-economic development in Vietnam at present, it may still play important roles thanks to its strong wind and typhoon tolerance, and suitability of wood for pulp, paper and fiberboard. It may be an alternative species to acacia in central coast of Vietnam which is now facing serious diseases such as *Ceratocystis*. Therefore, it is necessary to develop good seed sources as well as test new clones in Vietnam in anticipation of possible demands in future.

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Twenty years of research on functional analysis of the actinorhizal symbiosis *Casuarina glauca-Frankia*

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Because nitrogen is a critical element for tree growth and development, understanding of the molecular basis of actinorhizal root nodule symbiosis is a major issue. The development of genomics both in *Frankia* and in some actinorhizal plants such as the tropical tree *Casuarina glauca,* together with the possibility to obtain transgenic actinorhizal plants and develop some functional analyses, have offered valuable tools to achieve significant progress in the understanding of the actinorhizal symbiosis.

Transcriptomic data were used to identify genes potentially important in actinorhizal nodulation process and the function of candidate genes was then examined in *C. glauca* by RNAi-based gene silencing and promoter studies. So far more than 10 plant genes expressed during the interaction with *Frankia* have been characterized and about 15 transcriptional fusions between promoters from symbiotic genes and the reporter genes *GUS* or *GFP* have been studied in transgenic *Casuarinaceae* plants.

We have established that:

- the Common Symbiotic Signaling Pathway (CSSP) required for endosymbiosis with arbuscular mycorrhizal fungi and rhizobia is also involved in actinorhizal nodulation;

- key symbiotic genes expressed in nodule cortical cells infected by *Frankia* are also expressed at the prenodule stage;

- two promoters were shown to be induced by diffusible *Frankia* factors, thus providing a biological test for the isolation and further characterization of *Frankia* molecules perceived by the root system;

- the hormone auxin plays an important role during plant cell infection in actinorhizal symbiosis; and

- although the actinorhizal nodule is comparable with a symbiotic lateral root, the molecular mechanisms involved in primordia initiation in lateral roots may differ from those in actinorhizal nodules.

The major challenges in the coming years will be 1) the biochemical characterization of *Frankia* symbiotic signal(s); 2) the isolation of the *Frankia* symbiotic signal(s) receptor(s); and 3) understanding how *Frankia* triggers lateral root program to initiate actinorhizal primordium. The application of genome editing technologies in *Casuarina* will undoubtedly contribute to accelerate our knowledge on the molecular mechanisms underlying the actinorhizal symbiosis.

Proceedings of the Sixth International Casuarina Workshop

The socio-economic and ecological importance of *Casuarina equisetifolia* to the coastal region of Kenya: A case of Haller Park rehabilitation project

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Casuarina equisetifolia is one of the species that has become widely spread in Kenya and appreciated by most small growers both for commercial and non-commercial purposes. The species is found growing in woodlots, plantations, boundary planting, streets and agroforestry systems both inland and the coastal zone but has gained more popularity in the coastal zone. It is an exotic species that has been well entrenched into the ecosystem merging significantly well with other indigenous species. In the coastal region, *C. equisetifolia* has in a big way dominated and filled many socio-economic and ecological gaps. It is a species that has been able to endure high temperatures, high salt concentrations, sandy soils as well as low rainfall. The tree has provided a perfect raw material for the residents in a number of uses. The wood is used in making fishing canoes, building posts, beam work for high end beach hotels and resorts worked together with palm leaves for roofing and Banda making. It is a main source of fuelwood given its excellent burning properties with low smoke emission. It also forms a main source of income for small growers with standing trees sold per inch of diameter at breast height. One of the remarkable impacts of the species in coastal Kenya is its use in rehabilitation of once highly degraded and ugly Bamburi cement quarry sites. The species emerged to be one of the few species that managed to survive on the site and due to its nitrogen fixing ability and its symbiotic relationship with other organisms was able to fully rehabilitate the site to a most sought-after tourist site in the coastal region. The presentation shall highlight the different socio-economic roles and contributions of *C. equisetifolia* in the coastal strip and how it was used to rehabilitate the Bamburi quarry sites into an integral ecosystem full of biodiversity. It shall also cover the emerging challenges and gaps in its breeding as well as GATSBY AFRICA's effort in commercially promoting casuarina.

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Casuarinas and commercial wood supplies

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The capacity of casuarinas to offer considerable community benefit and contribute to the improvement of household incomes and support national industries has been clearly demonstrated in India and China; particularly in sandy coastal systems where site conditions offer few alternatives for profitable land use.

The narratives around casuarinas and their important protective role in landscape and agroforestry systems cannot be divorced from the dialogue surrounding wood production and society's expanding consumption of wood. Regional industries need greater supplies of wood fibre and within the Asia Pacific there have been recent "game-changing" market developments which offer expanded opportunities for commercial supplies of casuarina wood.

Globally, a 300% increase in demand for industrial wood demand is anticipated to 2050 and planted trees will be needed to meet this demand; clearly reflected by the substantial predicted shortages of wood fibre in the region. Recent independent assessments of Asian supply and demand for hardwood woodchip by market analysts suggest a looming annual deficit of 7 – 10 million cubic metres for the pulp sector alone. Faced with looming shortages of wood fibre availability has become a risk for Asian pulp producers; can projects get enough chips?

Expanding demands for wood chips for biomass energy production are contributing to solid demand and attractive prices. Japan and South Korea's biomass project pipelines are substantial and growing. In 2017, energy chip demand increased by 42% in Japan and 15% in Korea and there is not enough regional biomass supply (chips, palm kernel shells (PKS) or pellets) to meet the expected demand. Domestic markets for biomass will inevitably have an impact upon casuarina cultivation in both India and Thailand.

Casuarinas already play an important role in commercial wood supplies in the region, offering some 2.5 million gmt of fibre to seven pulp mills in India. Demand for construction poles remains strong with prices about three times those for pulp.

In expanding the role of casuarinas in regional wood supplies, issues of sustainability need to be considered; growers wish to maintain and increase their production from finite land resources; industry needs reliable supplies of wood, and: consumers are increasingly asking for evidence of legality and certification.

There remains a strong need for ongoing R&D to ensure that casuarinas can play their role in this emerging environment of regional shortages for commercial wood for fuel, fibre and solid wood. It encourages us to *revisit* our commitment towards this amazing genus *Casuarina*.

Cost-effective mass production of casuarina clones to meet the need of smallholding farmers in India

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A major part of casuarina plantations in India is raised by smallholding farmers. They heavily depend on the limited land area (average one hectare) for their livelihood needs. The short rotation period of casuarina helps them to have quick turnover and regular cash flow. Since casuarina has to be replanted after every harvest, a large amount of planting material is needed to establish new plantations. Till recently farmers have been using seedlings raised from traditional nurseries using locally available seeds. These seedlings are supplied as bare-rooted to reduce production and transport cost. In order to compensate for low survival and large growth variation among trees, up to 10,000 trees are planted in a hectare. The advent of fast growing clones has changed many cultivation practices adopted so far by the farmers most importantly the stocking has been reduced by around 50%. Farmers are now convinced about the benefits of using genetically improved planting material but are unable to afford the cost of such material. In order to make the new clones available to farmers at an affordable cost, we have been undertaking cost-effective mass-multiplication and supply for over a decade. Cuttings needed for rooting are produced from an intensively managed vegetative multiplication garden. The production cost is kept low through use of trays with 72 cells, composted coconut coir pith as growing medium and rooting in mistless tents. The small cells also help in saving transport cost by accommodating up to 50,000 plants in a standard truck. Our nursery has supplied 12 million plants during 2018 and the target for 2019 is 15 million. All plants are inoculated with *Frankia*, VAM and Trichoderma to ensure high survival, fast juvenile growth and disease resistance in the plantations.

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Large scale multiplication of *Casuarina* hybrid clones using mini-cuttings technique for farm forestry plantation in South India

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Casuarina is one of the important fast growing short rotation tree species used as a best alternative to meet the raw material requirements of wood based paper industries in India and Asian countries. The recent thought of meeting the customer's requirements in better products at low costs in a sustainable manner is possible only through innovative methods. The apical cuttings technique is the most widely used method for large scale propagation of casuarina and other pulpwood species in India. The present study to find an innovative technique for mass multiplication was carried out at Clonal Propagation and Research Centre, Tamil Nadu Newsprint and Papers Limited, Kagithapuram (11° 03' N, 77° 59' E), Karur, Tamil Nadu, South India. The *Casuarina* hybrid clones such as CH1, CH2, CH5 and control clone CJ 9 were used for the present study. TNPL has started mass multiplication of *Casuarina* inter species hybrid clones using mini-cutting technique from indoor clonal mini hedges raised in sand beds. When compared to nodal cuttings, indoor clonal mini hedges raised in sand beds improves the rooting potential, quality of root systems, save time and cost. The productivity of cuttings is increased by 5 times in indoor clonal hedge orchard than conventional apical cuttings. The *Casuarina* hybrid clones rooting percentage, root length and number of lateral roots also improved to 93-95%, 18 cm and 55 numbers respectively without rooting hormone whereas the same is only 63-65%, 14 cm and 40 numbers respectively in stem cuttings. The plant developed through mini-cuttings technique has tap root system which helps the plants/trees to withstand during heavy winds. Enhancement of cutting capacity was evident from the production of quality plants in much shorter period with easier and cheaper maintenance. The study reveals that mini-cuttings technique is ideal for large scale propagation of *Casuarina* inter species hybrid clones in Tamil Nadu, South India.

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Results of second-generation progeny test of *Casuarina junghuhniana* in Thailand

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Second-generation progeny tests of *Casuarina junghuhniana* Miq. were established at two sites in Thailand. Sai Yok Experimental Station, Saiyok district, Kanchanaburi province, represents the moist area, while Kamphaeng Phet Experimental Station, Mueang district, Kamphaeng Phet province, represents the drier part of the range where the species might be deployed. The trials were planted in 2013, with 43 families selected from the first-generation progeny test of *C. junghuhniana* in Thailand and 37 new families from West Timor, Indonesia.

The results showed highly significant differences ($P<0.001$) in height and diameter at breast height (DBH) in both experimental plots. At Kanchanaburi, the average height was 10.18 m (range 5.91-11.38 m) and and DBH was 8.26 cm (range 3.21-10.61 cm). The top-five-ranking families in the experimental area at Kanchanaburi were 84, 12, 15, 16 and 18. At Kamphaeng Phet average height was 4.75 m (range 3.22-7.19 m) and DBH 3.47 cm (range 1.91-5.65 cm). The top-five ranking family ID numbers were 68, 74, 81, 78 and 76.

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