The Fourth International Conference of the IUFRO Unit 2.09.02:

Somatic Embryogenesis and Other Vegetative Propagation Technologies

Proceedings

Development and Application of Vegetative Propagation Technologies in Plantation Forestry to Cope with a Changing Climate and Environment

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Professional Council of Economic Sciences

La Plata, Province of Buenos Aires, Argentina

Hosting Organizations

Facultad de Ciencias Agrarias y Forestales

FCAyF

UNIVERSIDAD NACIONAL DE LA PLATA

CIEFAP
Conocimiento e Innovación en Bosques Patagónicos

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NATIONAL INSTITUTE OF FOREST SCIENCE

IUFRO Unit 2.09.02:
Somatic Embryogenesis and Other Vegetative Propagation Technologies

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Preface

La Plata 2016 was the fourth successful international conference of the IUFRO 2.09.02 Unit since its inception (2008)! We were particularly pleased to have this conference organized this time in South America where there is so much interest, from scientists to industrial practitioners, in tree multiplication and plantation forestry. The conference was held in La Plata, the capital city of the Province of Buenos Aires, and a model example for modern urban planning during the XIX century.

The conference was co-hosted and mainly supported by the Faculty of Agriculture and Forestry Sciences (FCAyF) of the National University of La Plata (UNLP, Argentina), the Center for Forest Research and Extension Andean Patagonian (CIEFAP, Argentina) and, for the third time (after Suwon 2010 and Vitoria-Gasteiz 2014), by the NIFoS (National Institute of Forest Science) of the Korea Forest Service, Republic of Korea. The Ministry of Science, Technology and Productive Innovation (MINCyT, Argentina) and UNLP also provided funding support to this conference and various other sponsors provided helpful organizational support. We are so fortunate that we had again such strong supporters for our international IUFRO 2.09.02 activities! Thank you very much Mr. Raúl Aníbal Perdomo, President of UNLP, Dr. José Daniel Lencinas, Director of the CIEFAP, Dr. Nam Sung Hyun, Director General of NIFoS, Minister Dr. Lino Barañao and Dr. Alejandro Mentaberry (MINCyT), Minister Leonardo Sarquís (Ministry of Agroindustry, Province of Buenos Aires) and Dr. Sandra Sharry, Dean of the FCAyF-UNLP.

The La Plata 2016 conference was attended by 114 participants and received contributions from 135 IUFRO 2.09.02 members from around the world (32 countries). A total of 50 public institutes, organizations or universities were represented (from 24 countries) as well as 17 private organizations (from 10 countries). It was organized into 6 thematic sessions: (i) Strategies for integration of vegetative propagation into breeding programs in the context of global warming and associated stresses; (ii) Towards multivarietal (or clonal forestry): environmental factors affecting vegetative propagation of trees; (iii) (Epi)genomics of embryo or other vegetative propagule development; (iv) Preservation and adaptation of wild and selected genetic resources to environmental and socio-economic changes; (v) Lessons from *in vivo* growth of vegetative propagules, especially in various pedoclimatic conditions; and (vi) Reducing socio-economic and environmental costs of plantation forestry. A total of 51 oral presentations (6 key, 24 invited and 21 voluntary speakers) were made and, equally important, 48 posters were presented and introduced during an oral plenary session. All the scientific sessions provided excellent opportunities to discuss the most recent developments and also to network with colleagues.

The Local Committee guided by Sandra Sharry (FCAyF-UNLP) organized flawless scientific sessions (Sept. 20-23) and one satellite workshop on "*Current status and prospects of vegetative propagation technologies in Argentinean Patagonia*" (Sept. 21, chaired by María Laura Vélez & Javier Grosfeld, CIEFAP) at the Professional Council of Economic Sciences and also one introductory public session on "*Innovation and challenges in the forestry sector*" (Sept. 19, chaired by Sandra) at FCAyF-UNLP. The Local Committee also arranged a fantastic social program and outside trips: Opening reception by hosting authorities & welcome cocktail (Sept. 19), city tour (Sept. 20), Gala Dinner at the beautiful central hall of the Presidency building of UNLP with Argentine Asado, Tango & Salsa (Sept. 21), a visit at the (relaxing!) astronomy observatory "Planetario" with cocktail (Sept. 22), and the last day (Sept. 23), a visit to the Nursery "Charles

Darwin" at the Pereyra Iraola Park (UNESCO "Biosphere reserve" since 2008, Province of Buenos Aires) supervised by Sebastián Galarco (Forest Director of the Province of Buenos Aires). We discovered both exotic and native trees that are of strong interest for wood/biomass production or as ornamentals in Argentina for plantation forestry or landscaping engineering. The tour ended with typical Argentinean food, folklore dance and beer tasting in a relaxed atmosphere and also by a visit to the Children Republic, a proportionally-sized children's theme park that represents the entire workings of a democratic city. Thank you so much Sandra, Claudia, Maite, Sebastián, Corina, Elina, Javier, Patricia, María Laura, Fernando, Manuela and Pedro and also to the wonderful student team (Elizabeth, Julián, Julieta, Juan and Tatiana)!

Young scientists are a high priority for the IUFRO 2.09.02 Unit and La Plata 2016 was the opportunity to organize the Second Biennial Student's Scientific Competition chaired by Dr. Mariano Toribio (IMIDRA, Spain). The selected competition themes were "Advances in vegetative propagation technologies" and "Application of somatic embryogenesis in tree breeding and biotechnology". The winner, João Filipe da Silva Martins (Portugal), has been invited to present his work during the conference with full support. All 5 runners-up were invited to contribute to the scientific program and make an oral presentation (Kanagaraj Suganthi, India; Anna Maria Wójcik, Poland) or a poster presentation (Giovanna Campos Mamede Weiss de Carvalho, Brazil; Evelyn Raquel Duarte, Argentina; Taiane Pires de Freitas de Oliveira, Brazil). Anna Maria Wójcik could be partially supported, whereas Kanagaraj Suganthi fulfilled all the selection criteria for full support through the IUFRO-SPDC program.

The IUFRO 2.09.02 Unit is proud to present during each conference some recognition of distinguished colleagues considering their high expertise and exemplary career and significant (sometime pioneering) contribution in vegetative propagation of trees. We think it is of prime importance to recognize outstanding contributions and scientific endeavors and ensure some intergenerational exchanges and "cultivate" a collegial spirit in our Unit. Our Honorees during previous conferences were: Drs. Antonio Ballester (Spain), Jan Bonga (Canada), Vladimir Chalupa (Czech Republic), Don Durzan (USA), André Francletł (France), Inger Hakman (Sweden), Pramod Gupta (USA), Krystyna Klimaszewska (Canada), Heung-Kyu Moon (Republic of Korea), Yill-Sung Park (Canada), David Thompson (Ireland), Ana Vieitez (Spain), and Sara von Arnold (Sweden). During La Plata 2016, the 2.09.02 Unit had the opportunity to express recognition to Drs. M. Raj. Ahuja (USA), Jenny Aitken (New Zealand), William J. Libby (USA), Gale H. McGranahan (USA), Scott A. Merkle (USA), Gerald S. Pullman (USA), Marguerite Quoirin (Brazil) and Mariano Toribio (Spain).

These proceedings contain the full articles (18), extended abstracts (17) and short abstracts (19) that were submitted for publication. Abstracts that were not submitted for editing are found in their original format in the "Book of Abstracts" that was available to participants during the conference. Both the Proceedings and the "Book of Abstracts" can be freely downloaded at the IUFRO website: http://www.iufro.org/science/divisions/division-2/20000/20900/20902/publications/.

Thanks to all contributors to the Proceedings of the 4th Conference of the IUFRO Unit 2.09.02!

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

July 25, 2017

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"Development and application of vegetative propagation technologies in plantation forestry to cope with a changing climate and environment"

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Obituary

Jens Iver Find

Our colleague Jens I. Find, member of the IUFRO 2.09.02 Working Group, suddenly passed out in early December 2016, a few weeks after his participation as an Invited Speaker to La Plata 2016 where he made an oral presentation about "results from the first full rotation of growth in clonal field trials of nordmanns fir (Abies nordmanniana)".

Jens I. Find during the 4th IUFRO 2.09.02 Conference, La Plata, Argentina

Jens got his PhD in 1997 from the Botanic garden, University of Copenhagen. After a post-doc (1997), he became an Associate Research Professor (1999-2000, 2004-2009) and Head (2000-2001) of the Tissue Culture laboratory, Botanic garden, University of Copenhagen. He spent previously much time as a researcher at the Department of Tissue Culture and Molecular Genetics (1997-1998, 2001-2004) and also as a member of the management team at the CellWall Biotechnology Centre (2003-2004), Forest Research/Scion, New-Zealand.

Since 2009, Jens was Associate Professor at the Natural History Museum of Denmark (SNM) and Head of the Tissue Culture Laboratory at IGN (Forest, Nature and Biomass, Department of Geosciences and Natural Resource Management), University of Copenhagen, Denmark. He was also Administration Head of ScienceLab and coordinator of the establishment of common laboratory facilities at SNM for zoology, geology, botany and the National Facilities - ScienceLab.

Jens' research was focused on plant physiology and development of methods for in vitro propagation of a large variety of different plant species. An area of special interest was the development of methods for clonal propagation of conifers by somatic embryogenesis (SE), especially Nordmanns fir (Abies nordmanniana) and Sitka spruce (Picea sitchensis) as model species but he was also involved in projects on other conifer and angiosperm species. The SE systems in conifers was used as the basis by Jens' lab for investigation of epigenetic effects and for development of advanced tissue culture methods such as protoplast cultures, in vitro fertilization (IVF), treachery elements, reprogramming/rejuvenation of mature tissue, and genetic transformation of conifers. He was currently investigating the possibility of selecting cell lines with special focus on organic farming of Christmas trees. Jens was testing the Nordmanns fir SE system in commercial scale by production of plants for clonal field testing and by production of large quantities of plants from selected cell lines. Jens reported during the conference that SE trees were accepted recently for organic production with perspectives for 500 new clones tested each year and setting-up of large scale production facility for 50.000 plants/year/selected clone.

Our IUFRO 2.09.02 Unit wishes to emphasize the outstanding contribution and scientific endeavours of Jens I. Find in the vegetative propagation of trees, especially somatic embryogenesis in conifers and scaling-up the process in commercial scale.

Our Unit lost a Great Scientist and, even more importantly, a Friend. Jens was highly and unanimously appreciated for his kindness and so generous nature. On behalf of all members of our IUFRO 2.09.02 Unit (currently 742 scientists from 65 countries worldwide), and with a very heavy heart, we would like to express all our sympathy to his close family.

July 25, 2017, Bordeaux, France.
Jean-François Trontin, IUFRO 2.09.02 Coordinator

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Full articles

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Status of forest resources in Argentina

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Purpose of this paper

To provide the readership of the Proceedings of the IUFRO 2.09.02 conference held in La Plata with some information about current state of the forest resources in Argentina.

Keywords: Argentina, forest resource, status

Brief summary of the forest regional situation

In order to situate Argentinean forest resources status, it is important to understand the actual regional forest situation. Latin America and the Caribbean, contain 22% of the global forest area, and 14% of the global land area. In this region, the largest contiguous block of tropical rainforest in the world is the Amazon basins. According to FAO statistics (FAO 2015), between 1990 and 2015, the region lost almost 80 million hectares, 12% of its forest area, mainly due to conversion of forest land to agricultural uses. On the other hand, the total area of planted forests has increased by 5 % per year over the last decade.

Latin America and the Caribbean have about 12.5 million ha of planted forests. The region is emerging as a leader in high productivity plantations. Argentina, Brazil, Chile and Uruguay possess 78% of planted forests in the region (Brazil, (2015): 6 million ha; Chile (2013): 2.9 million ha; Uruguay (2015): 1.1 millions ha; Argentina (2015): 1.2 million ha). The plantation development is led by the private sector, supported by governments, through favourable policies and financial incentives. Among them we can list: the partial reimbursement of plantation costs, tax exemptions and reduced interest loans for small land owners. These factors have made South America a very important investment destination for pulp and cellulose companies, and have positioned the area globally in that role. In order to explain this fact, the key features of forest plantations in the region are as follows:

1) investment in technologies to improve productivity, especially clonal propagation, with which is achieved, in some cases, a productivity of more than 50 m^3 per ha per year;

2) the use of fast-growing species, such as *Eucalyptus spp.*, *Pinus radiata*, *Pinus taeda*, *Pinus elliottii*, and *Pinus elliotti x Pinus caribaea* var *hondulensis* (hybrid pine) intensively managed;

3) the integration of the management of wood processing from these plantations, especially the production of pulp and paper as well as panels. Current projections suggest an increase in the area of planted forests in the region from 12.5 million ha in 2015 to 17.3 million ha in 2020.

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Status of forest resources in Argentina

Over the almost 278 million ha of the continental area of Argentina, more than 30 million ha are used for agriculture and almost 10 million ha for cattle raising. The native forest ecosystems area reaches 31.5 million ha while the rest of the country is occupied with desert or semi desert lands, used mainly for sheep and goat raising. A vast amount of land, ideal weather, rich soil, and species diversity, make Argentina a very competitive country in the forestry sector.

Primary forest production is divided in two different major sectors. Forest native resources and cultivated forest, like in the rest of region, Brazil, Uruguay and Chile.

Native forests

According to the First Inventory of Native Forests (PINBN 2005), the total area of Native Forest in the Argentine Republic amounts to 31.500.000 ha that are distributed into 5 principal ecosystems as shown in **Fig. 1**.
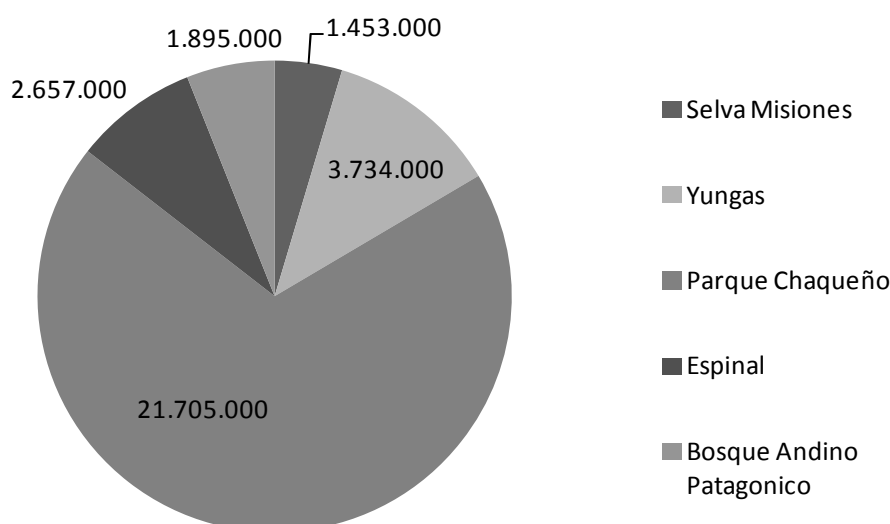
Figure 1. Distribution of the total Native Forest area of the Argentine Republic (31.500.000 ha) into the 5 principal ecosystems.

Wood from the native forest is mainly used as firewood, poles and to a lesser extent as logs that are used for the manufacture of furniture and other remanufacturing such as flooring and panels. Native forest logs extraction for 2015 was about 1.2 million m³. Considering native forest use, mainly in Bosque Chaqueño, Selva Misionera, Yungas and Bosque Andino-Patagónico, these ecosystems are characterized by no rational harvest and without management. Forest producers who use this resource mostly live in very precarious social conditions, without access to the latest technology, which places them in a condition of social and economic vulnerability to buyers. An aggravating circumstance is that during the last decades the

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agricultural frontier has advanced, primarily by soybean crops and indiscriminate and unplanned logging. Only a small group of enterprises, technologically well-equipped, produces high quality products with native wood, using smaller timber to make products with high added value. Nowadays, except by using "lenga" (*Nothofagus pumilia*) and "algarrobo" (*Prosopis sp.*), possibilities of large-scale manufacture of products based on native forest wood are limited.

Regarding negative aspects of Argentinean native forest resources, the deforestation process is a reality; almost 300.000 hectares are converted annually to agriculture, forage and infrastructure. Since the beginning of the 20th century, the country lost almost 70% of its native forest ecosystems. This process seems to be unmanageable even though there are national and provincial legislations that forbid deforestation. Therefore, the decreased natural area resulted in loss of diversity and quality. This causes unavailability of propagation quality material and quantity (domestication).

On the other hand, opportunities are based on using the extended native forests areas properly; lots of native forest germplasm and non-wood products are available for potential markets. Native forests provide numerous advantages such as:

➢ Environment sustainability: thanks to native forests' biodiversity, the system has more ability to respond to disturbances. Crop pests are managed through biological control, avoiding the use of big quantities of chemical products. Native forests also improve water infiltration and retention, with a better distribution of water and by control of erosion. Furthermore, they absorb carbon dioxide, related to global warming and help with contamination reduction. Moreover, they enhance the soil fertility by providing it with nutrients.

➢ Development of herding areas for cattle, thanks to trees acting as shelters, protecting cattle from wind and storms, and provision of a good growing environment for pastures.

➢ Tourism activities: forests landscapes offer excellent opportunities for touristic activities.

Cultivated forest

Argentina has 1.2 million ha of cultivated forests of which 80% are located in the Mesopotamia Region (Provinces of Misiones, Corrientes, and Entre Ríos) as well as in other areas such as Buenos Aires Delta, Buenos Aires, Neuquén, and Río Negro. Argentina's forestry sector has recently implemented a new silvapastoral productive system. This system produces food and raw materials and combines livestock with annual and perennial cropping, making both to interact on the same land. The main goals are to provide light or shade, boost soil enrichment by retention of nutrients, increase land productivity, reduce soil erosion, increase biodiversity, and protect the land from further degradation, maintaining its sustainability. There are ten million tons of wood per year produced in the country. The general distribution of planted area per genera has been established by the National Ministry of Agro industries (**Fig. 2**).

Main cultivated species are:

➢ Pines: (54%) - *Pinus elliottii*, *Pinus elliotti x Pinus caribaea* var *hondulensis*, and *Pinus taeda*. There are others such us *Araucaria angustifolia*, *Pinus ponderosa* and *Pseudotzuga menziesii*.

➢ Eucalyptus: (32%) - *Eucalyptus grandis* and *Eucalyptus dunnii* are the most cultivated species. *Eucalyptus camandulensis*, *Eucalyptus tereticornis*, *Eucalyptus viminalis* and *Eucalyptus globulus* can also be found.

➢ Salix - Populus: (11%) - *Populus deltoides* and *Populus x canadensis* are the most cultivated species among Populus. *Salix babilónica var. sacramenta*, *Salix nigra*, *Salix babilónica x Salix alba* and *Salix matsudana x Salix alba* are the *Salix* species most cultivated.

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➢ Others: (3%) - *Grevillea* sp., *Paulownia* sp., *Melia* sp., *Robinia* sp., *Prosopis* sp and *Toona* sp.

The National Ministry of Agro industries proposed that about 20 million ha be used as forestry potential land, without competing with agricultural production. This potential area estimation is based on soils, climate and a requirement to study the suitability of various species.

Cultivated forests - mainly pine eucalyptus, poplars and willows - are destined for the production of logs and ground wood industry, mainly for the production of cellulose pulp and boards.
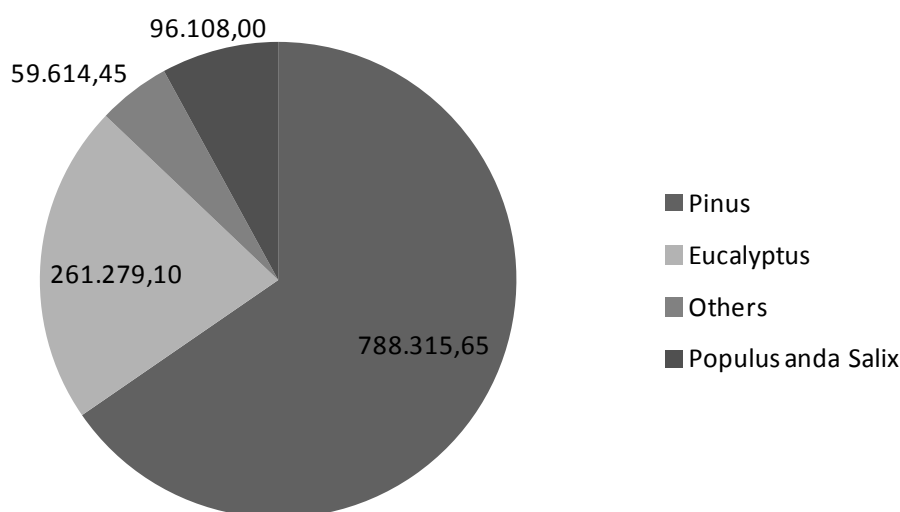
Figure 2. Distribution of the cultivated forest area of the Argentine Republic (1.205.317,10 ha) per genera (data from the National Ministry of Agro industries).

Nurseries

There are about 250 nurseries that produce about 50 million seedlings in the country, most of them located in the Mesopotamia region. Transplants for the planting of new forests and the restocking of harvested areas in existing forests are grown in forest nurseries. Today about 50 millions transplants are required for the annual planting. Transplants of *Eucalyptus, Pinus, Grevilleas* and other species are produced by three main methods: bare-rooted plants, container-grown plants and rooted cuttings. *Populus* and *Salix* transplants are produced in cutting nurseries. According to INASE (Seed National Institute), from a national total, i.e., 1.500 nurseries all around the country, almost 250 nurseries are recognized as forestry material producers.

Most of the material is made from selected seed; a small amount, about 10-15%, is produced by rooting cuttings from selected trees, mainly in *Eucalyptus*, improving significantly volume rates almost to 60 m3/ha/year. Cuttings dimensions are from 4 to 10 cm in length. The production of clones of *Eucalyptus* in northern Corrientes began in 1997 by the Company Forestadora Tapebicuá. In 2005, Posadas Biofactory, in Misiones Province began the construction of laboratories and greenhouses for a production target of 1,000,000 *Eucalyptus* clonal seedlings. Considering, Populus and Willows, commercial nurseries produce

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cuttings from selected trees (clonal propagation) through macro propagation, using cuttings of 40 – 50 cm in length and 1 inch in diameter (2.54 cm).

Privates companies, such as Arauco, Tapebicuá, Bosques del Plata, Pomera, Paul Forestal, Fiplasto, among others, carry out their own breeding programmes. Recently, Arauco obtained the permission to import pine embryos from Chile.

Production

The national annual plantation rate is about 40.000 ha. Of the consumption of saw logs from the total planted (exotic species) forests, almost 50% is destined for the manufacture of pulp, 30% for producing lumber and the rest is divided between the production of panels and veneer. The main destination of the logs from native forests is sawmilling**.** The proportion of forest products for industries, provided by planted forests, is significantly superior to that produced by the native forests. The main genus annual timber growth rate is:

> ➢ *Pinus*: 25 – 33 m^3/ha (twice the average in other countries)
>
> ➢ *Eucalyptus*: 38 - 50 m^3/ha
>
> ➢ *Populus*: 25 -35 m^3/ha
>
> ➢ *Salix*: 20 – 25 m^3/ha

Annual allowable cuts vary significantly between producing regions and the final destination of the wood.

While property ownership of the native area is highly fragmented, the wooded acreage is heavily concentrated in the hands of a group of large companies, many of them foreign-owned, vertically integrated with the subsequent steps (cellulose, sawing and boards). Among them are Arauco (belonging to the Arauco / Chilean group) with 127,000 ha, Bosques del Plata (CMPC / Chilean Group) with 60,000 ha, Forestal Argentina (Masisa/Chilean Group) with between 35 and 40,000 ha, Tapebicuá Group (Argentinean) with 21,000 ha, Group Las Marias 12,000 ha and Pomera (Group Insud / Argentinean) with 10,000 ha. Second, there are a set of 50 or 60 medium producers while there also are sawmills and re-manufacturers (e.g., Laharrague Chodorge, Don Guillermo, Leipzig, Pindo, Zenni, Fiplasto and Queiroz) and finally a group of about 8,000 small producers (up to 500 ha) that destine their production to smaller sawmills.

There is an inverse relationship between area and production of native and cultivated forest. Although the native forests area is 31.5 million ha, its contribution to total wood production is only 10 percent.

Industries

The forest-industrial chain of value in Argentina, linked to forest plantations, starts in log extraction, i.e., raw material undergoing a first industrial process by means of mechanical or chemical transformation, and the resulting products subsequently go through another industrial process. The products obtained are then destined to other industries or final consumption, in the domestic and international markets. Most of the sawmilling, wood re-manufacturing and board production, are close to the centres of the raw material supply; the Mesopotamian region is the largest developed forest-industrial pole. The industries that incorporate higher added value, such as furniture, are located near or in major urban centres such as Buenos Aires, Cordoba and Santa Fe. The number of main forestry industries established in Argentina are indicated in **Tab. 1**.

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Table 1. Number of production units for the main forest industries established in Argentina

Industry	Number of production units
Sawmill	> 2000
Paper and cellulose	69
Particle board	5
Fiber Board	6
Plywood	7
Impregnation	36

Historically, Argentina has imported many wood products with high added value (laminates, sawn wood from native forests and furniture) and exported primary goods (logs, sawn wood). As a result, there is a trade deficit. According to the Under Secrariat of Ambiente y Desarrollo Sustentable - Programa Nacional de Estadística Forestal, considering INDEC (National Institute of Census and Stathistics) data, the national forestry trade balance is historically negative.

The main destinations for the exported products are the MERCOSUR countries, Chile followed by the NAFTA countries and those of the European Union.

Sustainable forest management

The National forestry authorities promote the sustainable management of their forests. In order to have a sustainable forest management, it is fundamental that the different stakeholders, in an inclusive and participating way, drive development and enforcement of criteria and indicators of management best practices, forest certification and legal commerce. With the purpose of strengthening the length of the commercial chain of the forestry sector, and promoting a responsible management of the forests, the government, encourages the use of forest certification, such as FSC® (Forest Stewardship Council), and CerFoAr (Argentine System of Forest Certification).

Policy & forestry laws

In Argentina, forest competencies at the national level are divided into two main institutions responsible for the elaboration and implementation of national forest policies. The management of the Protected Areas is the responsibility of the National Parks Administration. The Ministry of Agro industries has responsibility for establishing policies for the cultivated forests. The Under secretariat of Forest Industrial Development is the office in charge of the management related to the cultivated forests within the national territory. The Ministry of Environment and Sustainable Development of the Nation has responsibility in the area of native forests. The Forest Office develops national policies and programs for the protection, conservation, recovery and sustainable use of native forests.

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Each province can develop its own rules or follow the national laws, since there is no national planning body, and coordination between the sectors is usually carried out through the Ministry of Finance or the National Cabinet Office.

Several laws apply in Argentina to enhance and preserve sustainability of the forestry production (**Tab. 2**). One of the most recent laws approved is Law number 26.331, which provides a description on how to prevent land deforestation and issues subsidies and grants to producers in order to maintain sustainability.

Table 2. Main laws applying to forest management in Argentina

Law number	Comment
26.331	This law establishes the minimum standards for environmental protection, restoration, conservation, development and sustainable management of native forests, as well as regime criteria for promotion and distribution of funds for environmental services provided by the native forests. The main goals are to promote conservation, regulate expansion of the agricultural frontier, and encourage restoration and management improvement activities.
25.080	This law was implemented in 1999 and is intended to increase land development by the Argentine forestry industry to 3 million ha of cultivated forest in 10 years. It has been extended for another 10 years in 2009. It establishes an inventory process for planted forests and it has provided for the establishment of agreements with international organizations that focus on development and technology transfer for this industry. This law also provides tax benefits and economic support for Argentine and foreign investors.
13.273	The foundational law for forestry activities in Argentina. It aims to preserve forests, prohibit deforestation, and the irrational use of forestry products. It also specifies that any investment project carried out in natural forests needs to be approved by the Argentine government.
26.815	Establishes minimum budgets for environmental protection regarding forestry and rural fires within the national territory.

National capacity in Research & Education

There are five National Universities that teach Forestry engineering: La Plata (UNLP), Misiones (UNM), Formosa (UNF), Santiago del Estero (UNSE) and Comahue (UNCOMA).

The actual public Forestry Research Projects are led by INTA (Instituto Nacional de Tecnología Agropecuaria), Universities, CIEFAP (Centro de Investigaciones y Extensión Forestal Andino Patagónico, located in Patagonia), CEPROVE (Centro Experimental de Propagación Vegetativa located in La Plata, UNLP), CADIC (Centro Austral de Investigaciones Científicas localted in Usuhaia), and LISEA (Laboratorio de Investigación de Sistemas Ecológicos y Ambientales, located in La Plata, UNLP). INTA National Forestry Program includes the following main objectives:

➢ Domestication of native forest species.

➢ Sustainable management of planted forests for timber production quality and environmental services.

➢ Forest Genetic improvement of forestry species.

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➢ Silvopastoral systems.

The Ministry of Agro industries and the Ministry of Environment and Sustainable Development executes through the Unidad para el Cambio Rural Office (UCAR) management of the "Forest Sustainable and Competitiveness Project" financially supported by international loans. The overall aim is to contribute to the sustainable management of forest plantation and the improvement of competitiveness of micro, small and medium forest enterprises, facilitating their access to supply chains and markets.

The germplasm banks currently operating are:

➢ Banco Nacional de Germoplasma de Prosopis, Córdoba. "Prosopis National Germoplasm Bank" (Córdoba).

➢ Banco de Especies Forestales Nativas de la Prov. De Buenos Aires (FCAyF, UNLP). "Native species Germoplasm Bank" (La Plata).

➢ Banco de Germoplasma Vegetal-Biofábrica Misiones. Biofábrica Vegetal Germoplasm Bank (Misiones).

➢ Banco de Germoplasma Forestal, Formosa. Forest Germoplasm Bank (Formosa).

Considering the private sector, the major forestry companies have their own research program (e.g., Pomera), most of the time with agreements with the public sector already mentioned. The Centre for Research and Forest Experiences (CIEF) is a private institution, supported by contributions from its member companies. This centre is run by a board of directors and provides service in obtaining progeny and local seed of superior genetic quality, based primarily on increased productivity. The centre also provides guidelines for appropriate management in order to raise the economic and technological impact of the materials generated.

Identified intervention areas of native species

1) To make available specific technologies for forest species native to Argentina: aiming to achieve base material (plant scaling) with native species to promote investment, improve the supply for industry, and have a strategic look at those species that have no commercial value, e.g., Caldén and native cedar, among others.

2) To achieve a set of validated norms to improve knowledge and produce compatible native species for differential market niches: aiming to be used for environmental services (carbon sequestration), ornamental and woodland roads, agro -forestry systems pastoral and phyto medicine.

Actual situation: included in the Forestry Research Projects, many efforts have been made to domesticate some native forest species and there are some partial results, but these have not yet resulted in recommendations ready to be implemented in the establishment of plantations or to the oriented use of wood, for example for furniture technology packages. Some protocols have been developed for propagation and for use by nursery-level Universities.

Identified intervention areas of cultivated forest species

➢ Clonal sylviculture (use of clones tested and validated in the field).

➢ Precision sylviculture (clone-site interaction).

➢ Wood energy and biomass waste from the forest.

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Three innovative alternatives, based on the current situation of the sector, focused mainly on the production of fast growing timber, low unit value, low biological diversity, low economic and environmental sustainability, as in the case of pure plantations of *Eucalyptus* sp. and *Pinus* sp.

The first alternative is the genetic variation of the species; focusing on the use of species of high wood quality and of landscape and environmental value. This requires research and development aimed at testing and improving different local and introduced sources. Among these we can name European oak (*Quercus robur*), American ash (*Fraxinus pensylvanica*), Walnut (*Juglans regia*), Cherry (*Prunus avium*) and *Acacia melanoxylon*, among the exotic ones. It is also very important to develop native species of value for each area where the investment is raised.

The second alternative is the variation of sites, which involves choosing sectors that do not compete with agriculture, such as the Salado Basin in Buenos Aires. This requires developing genetic improvements that allow resistance to adverse factors such as temporary flooding, salinity, etc.

Finally, it is proposed to use agro forestry systems in which the production of high-value wood in mixed forests, livestock production and agricultural production is effective.

Each of the proposed alternatives presents an innovative challenge, since they represent small steps with each requiring a lot of R&D investment, which ensures the strategic impact on the sector.

Final comments

This paper aimed at explaining and promoting the main aspects of forest plantations in Argentina. Undoubtedly, the country offers a huge potential for expansion of the industrial sector, due to the environmental conditions and the productivity of the main planted species, as well as due to the scientific and technological developments that have been achieved in later years, combined with the economic and fiscal benefits granted by the State. In brief, some main Argentinean forests indicators are:

- ➢ Native forest area: 31.443.873 h.
- ➢ Cultivated forest area: 1.216.000 ha.
- ➢ GDP forestry participation: 3-4 %.
- ➢ Annual plantation: 40.000 ha.
- ➢ Annual deforestation: ~300.000 ha.

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Current status of forest tree biotechnology in a changing climate

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Abstract

Woody plants have been cultured *in vitro* since the 1930s. After that time much progress has been made in the culture of tissues, organs, cells, and protoplasts in tree species. Tree biotechnology has been making strides in clonal propagation by organogenesis and somatic embryogenesis. These regeneration studies have paved the way for gene transfer in forest trees. Transgenics from a number of forest tree species carrying a variety of recombinant genes that code for herbicide tolerance, pest resistance, lignin modification, increased woody biomass, and flowering control have been produced by *Agrobacterium*-mediated and biolistic methods, and some of them are undergoing confined field trials. Although relatively stable transgenic clones have been produced by genetic transformation in trees using organogenesis or somatic embryogenesis, there were also unintended unstable genetic events. In order to overcome the problems of randomness of transgene integration and instability reported in *Agrobacterium*-mediated or biolistically transformed plants, site-specific transgene insertion strategies involving clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) platform offer prospects for precise genome editing in plants. Nevertheless, it is important to monitor phenotypic and genetic stability of clonal material, not just under greenhouse conditions, but also under natural field conditions. Genetically modified poplars have been commercialized in China, and eucalypts and loblolly pine are expected to be released for commercial deployment in USA. Clonal forestry and transgenic forestry have to cope with rapid global climate changes in the future. Climate change is impacting species distributions and is a significant threat to biodiversity. Therefore, it is important to deploy strategies that will assist the survival and evolution of forest tree species facing rapid climate change. Assisted migration (managed relocation) and biotechnological approaches offer prospects for adaptation of forest trees to climate change.

Keywords: clonal forestry, organogenesis, aspens, coast redwood, frost-tolerance, genotypic control, genetic stability, gene transfer, transgenic trees, climate change adaptation, conservation

Introduction

Theoretical basis for cell culture and concept of totipotency in plant cells were first formulated by Gottlieb Haberlandt in 1902. It was almost three decades later when Roger Gautheret in 1934 was the first to culture pieces of cambium cut from trees (*Salix capraea*, and *Acer pseudoplatinus*, and *Sambucus nigra*) in culture media. Initially, he did not expect much success, but two months later when he examined his culture, he noticed callus growth on them. Unfortunately, Gautheret's cultures ceased to grow after 6 months, because of some deficiency in the culture medium. Some years later, Gautheret (1940) was able to obtain bud formation from cambial tissues of *Ulmus campestris* and *Populus nigra*. A decade later, Ball (1950) was able to induce buds on callus cultures of *Sequoia sempervirens*. Long-term callus cultures in triploid quaking aspen, *Populus tremuloides*, were established by Mathes (1964a), and he was able to induce buds and roots on the same callus (Mathes 1964b). But it was not certain whether shoots and roots were directly attached to each other to give rise to plantlets. However, Winton (1968) was the first to achieve plantlet formation from callus cultures of triploid quaking aspen. That was the beginning of clonal propagule production in angiosperm forest trees by tissue culture technology. In a sense, it was the beginning of

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vegetative propagation by micropropagation in forest trees. Clonal propagation by organogenesis, that is, development of organs from plant tissues *in vitro*, has been successfully exploited in a number of angiosperm forest tree species (Bonga and Durzan 1987, Ahuja 1988a, 1991, 1993a, Ahuja and Libby 1993a, 1993b, Ahuja et al. 1996, Pardos et al. 1994, Ramawat et al. 2014). And in some forest tree species field trials have been conducted to ascertain the phenotypic and genetic fidelity of clonal trees.

Although clonal propagation by organogenesis in conifers has often been difficult because of recalcitrance and maturation problems (Bonga et al. 2010, Bonga 2012), there have been a number of reports on the plantlet regeneration by organogenesis in conifers. The first success to clonally propagate a conifer by organogenesis was in *Pinus palustris* by Sommer et al. (1975). This was subsequently followed in Douglas fir (Gupta and Durzan 1987), *Pinus* spp. (Diego et al. 2008, 2010; Cortizo et al. 2009; Bello-Bello et al. 2012; de Oliveira et al. 2012), *Larix decidua* (Ewald 2007), and *Sequoia sempervirens* (Boulay 1978; Arnaud et al. 1993; Ahuja 1996; Korban and Sul 2007). However, somatic embryogenesis has been more successfully exploited for the clonal propagation of a large number of conifer tree species using embryonal tissues and other explants. The first successful reports on somatic embryogenesis appeared in 1985, using immature embryos in *Picea abies* (Hakman et al. 1985; Chalupa 1985), and *Larix decidua* (Nagmani and Bonga 1985). After these reports, there has been an explosion of somatic embryogenesis in a large number of conifers, and angiosperm trees (Jain and Gupta 2005; Park et al. 2010; Park and Bonga 2012, 2014; Park et al. 2016; Bonga 2016; Guan et al. 2016; Trontin et al. 2016). Although somatic embryogenesis has been obtained for many conifer species, it has achieved an industrial level of propagation with only a few (Park et al. 2016). However, clonal propagation from mature trees *in vitro* still remains a problem in some forest tree species, mainly in conifers, but in others micropropagation has been achieved from mature trees. Therefore, rejuvenation of organs/tissues from recalcitrant mature trees is a necessary prerequisite for clonal propagation *in vitro*, and has been accomplished in some tree species. Subsequently much progress has been made in the culture of tissues, organs, cells, and protoplasts in tree species. Tree Biotechnology has been making strides in clonal propagation by organogenesis and somatic embryogenesis (Bonga and Durzan 1987; Ahuja 1993a; Park et al. 2016). However, somatic genetic control of differentiation and genetic stability of clones produced by organogenesis and somatic embryogenesis are important aspects of *in vitro* regeneration that need to be addressed in a clonal forestry program. Although there are limitations in cost-effective production of clonal propagules by organogenesis and somatic embryogenesis in forest trees, constructive and realistic biotech approaches are underway to overcome the limitations for large scale plantations of micropropagated clonal trees.

In this review, I shall discuss tree biotechnology and climate change in the framework of the following topics:

- Regeneration and clonal forestry
- Genetic engineering in trees, and
- Clonal forestry and transgenic forestry facing climate change

In other words, in this review, I shall briefly describe clonal forestry by vegetative propagation (macropropagation), and then discuss micropropagation. This will be followed by genetic transformation and confined field trials of transgenic trees, and finally I will discuss strategies for mitigation of global warming and adaptation of clonal forestry and transgenic forestry to cope with climate change.

Regeneration and clonal forestry

Clonal forestry involves production and use of vegetative propagules by vegetative propagation. Clonal propagation has been achieved by macropropagation or micropropagation. Vegetative propagation by macropropagation involves traditional vegetative propagation techniques, while micropropagation is carried out by tissue culture technology.

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Macropropagation

In the past several decades serious consideration has been given to genetic improvement of forest tree species. This involves hybridization between superior individuals and then establishment of seed orchards. In order to achieve genetic gain in forest tree species, it would be necessary to cross breed elite trees for at least a few generations, each requiring anywhere from 10 to 50 years. One way to capture genetic gain more readily in the same generation is to clonally propagate selected genotypes by vegetative means. In this respect, clonal forestry has certain distinct advantages over zygotic forestry. Clonal forestry is not new, Vegetative propagation has been part of the evolutionary history of herbaceous plants and forest trees, and ancient clones have existed for thousands of years. Clonal forestry involves efficient management and increase of genetic gain by selection and maintenance of additive and non-additive gene effects. On the other hand, through sexual reproduction only additive gene effects can be utilized; the non-additive gene effects arising from interaction of genes are not normally transmitted through generative reproduction (Ahuja and Libby 1993a). The non-additive gene effects can give rise to exceptional individuals within superior families (in outcrossed plants, such as forest trees), and these can be captured along with additive gene effects by vegetative propagation, or by crossing homozygous individuals. The alternative strategy of producing homozygous trees by progeny testing for a specific favorable combination of genes, and then operationally producing heterozygous F_1 families is very difficult in long-lived forest trees.

Methuselahs and potentially immortal clones

Some of the forest trees have long life spans of thousands of years, and have become Methuselah trees. The oldest living forest trees in the world are the bristlecone pines (*Pinus longaeva*) first described to be 4845 years old (Schulman 1958), but later revised to be 5062 years old by 2012 (www.rmtrr.org/oldlist.htm). The family Cupressaceae has also some of the oldest living conifers. These include *Fitzroya cupressoides* (3622 years old), *Sequoiadendron giganteum* (3266 years old) and *Sequoia sempervirens* (2200 years old) (see www.rmtrr.org/oldlist.htm). Other than *Sequoia sempervirens*, which can vegetatively propagate by stump sprouts and burls, other Methuselahs in this group do not normally propagate in nature by vegetative propagation. However, there are tree species that have produced ancient clones that are thousands of years older than these Methuselahs.

Natural clones of some forest trees have been around for thousands of years, and in a sense, clones of such trees may be considered potentially immortal! Vegetative propagation has been part of the evolutionary history of plants. There are a number of plant species in which ancient clonal colonies have survived for thousands of years. One of the oldest living clone is known as "Pando" (a Latin name meaning I spread). This ancient clone of quaking aspen (*Populus tremuloides*) belongs to the family Salicaceae, and its age has been estimated to be around 80,000 to 1,000,000 years. The Pando clonal colony is located in the Fishlake National Forest, north of Bryce Canyon National Park in central Utah. Vegetative propagation occurs in aspens from root suckers (or basal sprouts), and the clones can live up to thousands of years. Pando clonal colony has around 47,000 tree trunks, each with a normal complement of leaves and branches. Pando covers 43 hectares (106 acres) of the forest area (Grant, 1993; Mitton and Grant, 1996). Pando originated from a male tree and its clonal colony shares the common root system, and the same genetic constitution (DeWoody et al. 2008); and Pando most likely is a triploid clone (Mock et al. 2008; DeRose et al. 2015).

Another ancient forest tree clone belongs to a disjoint population of Palmer's oak (*Quercus palmeri*) in the Jurupa mountains of Riverside county in California, which is estimated to be more than 13,000 years old. Morphological homogeneity, allozyme polymorphism, and fire induced sprouting supports the hypothesis that this is a single ancient clone (May et al. 2009). Widespread fruit abortion suggests that Palmer's oak may be a triploid clone.

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Although spruces do not normally reproduce by vegetative means, there is an exceptional Norway spruce that also seems to be an ancient clone. This spruce clone was nick-named by Leif Kullman (2008) as "Old Tjikko" (*Picea abies*) after his late dog (Siberian Husky) and considered to be many thousand years old. This ancient Norway spruce clone is about 5 meters (16 ft) tall, and is located in the Fulufjället Mountains of Dalmarna Province of Sweden. This ancient clone has survived for thousands of years due to vegetative cloning. The present visible tree is relatively young, around 600 years old, but it is a part of a much older root system. The trunk of this clone may live up to 600 or more years, and when it dies, another trunk grows back in its place from the old root system by a process of layering. According to Kullman, heavy snow can bring branches close to earth, and new shoots can sprout from the contact point. Fossil wood pieces and cones have been recovered from this ancient clone, and their carbon dating suggests their age to be around 9,500 years, and have the same genetic constitution as the root system (Olberg and Kullman 2011).

In a changing world, clonal plants would be at a disadvantage, because of their narrow genetic base and genetic homogeneity, limited mobility, and capacity for adaptation. So how have these ancient clones survived and outlived the previous environmental changes, and persisted over the millennia. One possible hypothesis suggests that gene regulation through epigenetic mechanisms (changes) might provide the optimum phenotypic variation for clonal spread, adaptation and survival in a changing climate (Douhonikoff and Dodd 2015; Dodd and Douhonikoff 2016).

In vitro regeneration

Regeneration of forest trees has been routinely achieved by organogenesis and somatic embryogenesis. Genetic control of regeneration and morphological and genetic stability of regenerants by organogenesis and somatic embryogenesis have been investigated in a number of forest tree species.

In vitro regeneration potential of tissues depends on several parameters. These include:

- Explants source (axillary bud, nodal segment, cotyledon, zygotic embryo, shoot tip, bud meristem)
- Explants physiology, developmental stage
- Time of the year explants cultured
- Age of the donor tree
- Genotype of the donor tree
- *In vitro* environment: light regimes, temperature, media composition, and exogenous phytohormone concentrations.

The use of regeneration technology in forestry offers prospects for: (1) manipulation of the mature state of tissues, (2) germplasm preservation by long-term *in vitro* cultures, and cryopreservation, and (3) somaclonal variation for isolation of useful genotypes, and (4) gene transfer and production of transgenic trees.

Although, somaclonal variation in trees is usually undesirable (Tremblay et al. 1999; Breton et al. 2006; Marum et al. 2009), a few useful somaclonal variants have been reported in tree species (Ahuja 1998; Bairu et al. 2011; Krishna et al. 2016). Some of these useful variants include: height and morphological traits in poplar (Fry et al. 1997) and olive (Leva et al. 2012); disease resistance in poplar (Ostry and Ward 2003), apple, citrus, and mango (Krishna et al. 2016); different growth rates in poplar (Gamburg and Voinikov 2013); and stress tolerance in radiata pine (Montalbán et al. 2014).

These regeneration studies have paved the way for genetic transformation and production of transgenics carrying a variety of transgenes that code for herbicide tolerance, insect resistance, lignin modification, increased woody biomass, and reproductive manipulation, in a number of different forest tree species. Clonal forestry and transgenic forestry have come of age. However, more research is needed for their application in forestry.

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Micropropagation from mature trees

We have employed tissue culture technology for rapid clonal propagation of a number of forest tree species. Our aim was to optimize cost-effective protocols for micropropagation of selected genotypes, whose clones exhibited phenotypic and genetic stability not only in greenhouse, but also under field conditions. The second objective was to find out the ability of the propagules of an introduced species to adapt to a new environment. In both cases phenotypic and genetic fidelity of clones was also an important consideration. Here I shall focus on micropropagation of selected trees of aspens (*Populus tremula*, *P. tremuloides*, and their hybrids), and coast redwood (*Sequoia sempervirens*) introduced into Germany for its ability to survive under temperate and cold climates. We have employed direct organogenesis for micropropagation of mature trees.

Populus-Aspens

At the Institute of Forestry Genetics at Grosshansdorf, Germany, elite genotypes had been selected in European aspen (*Populus tremula*) and quaking aspen (*P. tremuloides*), and their hybrids following controlled crossings between European aspen and quaking aspen (*P. tremula* x *p. tremuloides*). The hybrid aspens have exhibited good form and growth under field trials (Melchior 1985). Aspens are difficult to propagate vegetatively by woody stems cuttings, because of lack of preformed root primordials. On the other hand, poplars can be easily propagated by stem cuttings (hardwood cuttings), which root readily in soil or peat-perlite substrate. However, aspens can be vegetatively propagated by root suckers, grafting, and juvenile green shoots. Because of cost factors, propagation by grafting, green shoots, and root suckers had limited commercial applicability in the aspen regeneration programs. For this reason, aspens have so far been mostly propagated by seed (zygotic forestry). However, tissue culture technology offered prospects of rapid large scale micropropagation of aspens. We undertook clonal propagation by organ culture of selected mature aspen and hybrid aspen genotypes by direct organogenesis by tissue culture technology. Dormant buds from 17 to 40 years old aspen and hybrid aspen were collected during winter months and stored at $0°C$. Bud meristems from more than 100 aspen and hybrid aspen clones were cultured on Aspen Culture Medium (ACM) (Ahuja 1983), which is a slightly modified version of Woody Plant Medium (WPM) (Lloyd and McCown (1981) for their regenerative potential. Initially, a 4-step micropropagation method (Ahuja 1983) was employed for clonal propagation of aspens (**Fig. 1**). This method consisted of: (1) Bud break and conditioning of bud meristems on ACM-1 (ACM + 20 mg/l adenine sulfate + 0.5 mg/l BA), (2) growth proliferation on ACM-2 (ACM + 0.2 mg/l BA + 0.02 mg/l NAA), (3) rooting of microshoots on ACM-3 (ACM + 0.5 mg/l IBA + 0.1 mg/l NAA), and (4) transfer of plantlets derived from ACM-3 to pots (after sloughing off agar from the roots) for hardening and acclimatization in controlled chambers and greenhouse (Ahuja 1983, 1986, 1987). Subsequently, the 4-step method was simplified so as to reduce the number of steps to make the micropropagation method cost-effective. First the 4-steps were reduced to three, by eliminating step 1, and accomplishing both meristem conditioning and shoot proliferation on ACM-2, without any appreciable impact on growth and differentiation. Later step-3 (ACM-3) was also eliminated from the scheme, and microshoots were rooted directly in once autoclaved soil-free potting mixture under high humidity controlled environment (Ahuja 1984). Thus, it was possible to evolve a 2-step micropropagation procedure (**Fig. 2**) from the original 4-step method, not only to reduce costs, but also to minimize the exposure of tissues to prolonged cultural conditions for minimizing somaclonal variation. There was a very or negligible amount of callus formation in our direct organogenesis method for micropropagation. The micropropagated aspens showed phenotypic and genetic stability when grown under greenhouse and field conditions (Ahuja, 1983, 1987, 1993b). We have randomly checked chromosome numbers of some of the micropropagated plants. Aspens have a diploid chromosome number of 2n=38, and our tested plants had the same genetic diploid constitution (Ahuja 1984). By employing the 2-step micropropagation method, we regenerated several thousand plantlets from more than 30 selected

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mature aspen and hybrid aspen clones. Twelve hybrid aspen clones have been approved under the category "Tested Material" in the multiclonal variety 'Grosshansdorf', Germany. We have established a number of field trials for testing micropropagated aspens and hybrid aspen clones, along with seedlings, and some of these are now more than 25 years old (**Fig. 1**) and are still undergoing phenotypic and genotypic evaluations (Wuhlisch and Ahuja, unpublished).

Figure 1. Top row, left to right: hybrid aspen bud meristems cultured in ACM-1; Shoot regeneration in ACM-2; plantlet regeneration in ACM-3. Bottom row, left to right: hybrid aspen clones in nursery, and more than 25-year-old trees (photograph by Dr. Georg von Wühlisch) in field trials in Germany.

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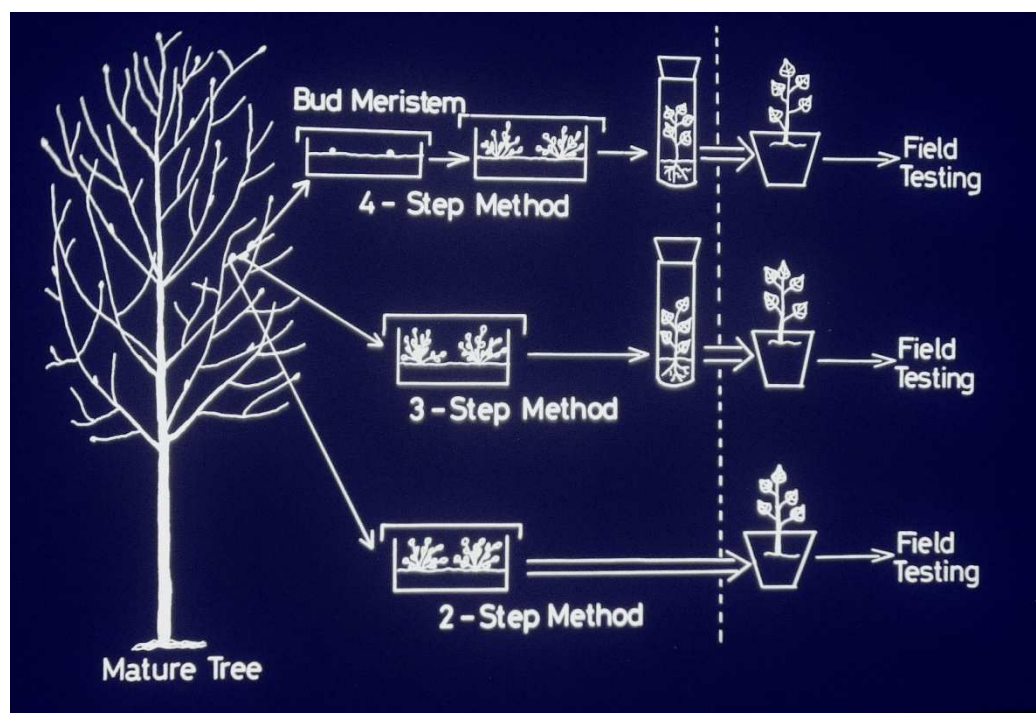
Figure 2. Diagrammatic representation of 4-step, 3-step, and 2-step methods for micropropagation of aspen clones from mature trees. By simplification of this procedure from 4-step to 2-step protocol, it is possible to reduce costs for clonal propagation.

Initial data from 48 clones revealed that regeneration of shoot differentiation was achieved in about half (22 of 48) of the clones tested (Ahuja 1983). Subsequently, additional data on 70 aspen clones also revealed that half (37 of 70) tested showed good microshoot differentiation (Ahuja 1984). Similarly, there was a 1:1 segregation in 39 clones (17 of 39) for shoot differentiation vs. no differentiation in the female and male hybrid aspen clones (Ahuja 1993b). Genetic control of microshoot differentiation by organogenesis and somatic embryogenesis in tissue cultures were also observed in both angiosperm (Wann and Einspahr 1986; Coleman and Ernst 1989; Coritzo et al. 2009; Erst et al. 2014), and conifer (Klimaszewska et al. 2007; Trontin at al. 2016) tree species.

Coast redwood (*Sequoia sempervirens*)

Coast redwood (*Sequoia sempervirens*) is an interesting and unique conifer. Sequoia is a long-lived conifer belonging to the family Taxodiaceae, (now included under Cupressaceae). It has the world's tallest trees, one measuring ~113 m in height (Koch et al. 2004). However, recently another Sequoia tree called Hyperion, which measured ~115 m in height, has become the tallest tree in the world (Sillett et al. 2015). Sequoia is the only conifer that is hexaploid (2n=6x=66), and origin of its enigmatic polyploidy still remains unresolved. It is not known when the polyploidy *Sequoia* evolved from its diploid ancestors, and which really are its putative progenitors (Stebbins 1948; Ahuja and Neale 2002; Ahuja 2009). Sequoia reproduces by both sexual (seed) and vegetative methods (from basal sprouts and burls) to maintain heterozygosity and adaptability for survival. It is a resilient conifer that presumably originated as a polyploid during Cretaceous and Tertiary periods (~ 135-65 mya) (Ahuja 2005, 2009a). Sequoia is an endemic restricted in its natural

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habitat to the fog belt of California and southern Oregon, USA. It grows from sea level to 900 m altitude, but prefers altitudes lower than 750 m, in a frost-free period ranging from 6-11 months in a year (Olson et al. 1990). Because of its polyploid nature, meiosis is irregular and seed set is low in Sequoia, varying from 1-10%, and only 5-10% of the seeds are viable (Olson et al. 1990; Ahuja and Neale 2002; Ahuja 2005). Although older Sequoia trees (genotype dependent) are relatively less susceptible to frost, seedlings in the Pacific Northwest region, where Sequoia is an endemic, are vulnerable to infection by a fungus Botrytis and frost damage (Olson et al. 1990). For this reason, clonal forestry offers prospects for propagating selected genotypes by tissue culture.

Sequoia has been grown out of its endemic region with varying degrees of success in different parts of the world, including France, Germany, Spain, United Kingdom, Turkey, Chile, South Africa, and New Zealand (Kuser et al. 1995; Meason et al. 2016).

Micropropagation has been achieved in Sequoia by using meristems, nodal stem explants, and needles of juvenile explants, as well tissues from stump shoots of mature trees that were 90 years old (Boulay 1978; Ball et al. 1978; Arnaud et al. 1993; Bon et al. 1994; Sul and Korban 2005; Korban and Sul 2007). Somatic embryogenesis and organogenesis was also reported by tissue culture of needles of Sequoia (Liu et al. 2006). In general, stump shoots from the base of mature Sequoia trees are more responsive to *in vitro* regeneration compared to shoots from the crown of the same mature trees.

During the winter of 1985-1986, a Sequoia plantation near Cologne, Germany was badly hit by the frost. A number of trees survived the winter. The owner of the plantation sent branches from three (two 25 years old, and one aged 8) frost-tolerant Sequoia trees, to the Institute of Forest Genetics, Grosshansdorf, for clonal propagation and to test their ability to tolerate frost in Germany. We cultured bud meristems from the three trees on a modified woody plant medium fortified with low concentrations of BAP (0.2 to 0.5 mg/l) and NAA (0.02 mg/l), and rooted microshoots (**Fig. 3**) in a hormone-free medium, and *ex vitro* in a peat-perlite potting mixture (Ahuja 1996, 2011a). More than 1000 plants were produced from the three trees by tissue culture, and several hundred clones were tested under field conditions for testing their overwintering capacity. Field trials from the clones derived from three frost-tolerant trees were conducted for a number of years at Grosshansdorf and Trenthost, in northern Germany. Trees in Grosshansdorf arboretum seem to be frost-tolerant and have survived more than 25 winters in Germany (**Fig. 3**). However, under field conditions at Trenthorst, only those clones survived that were sheltered by a tree canopy towards the edge of the field. Therefore, sheltering of Sequoia clones derived from frost-tolerant trees were necessary during several years of their early growth period for their later survival in harsh winters in temperate climate zones (Ahuja, 1996, 2011a, 2017).

Based on these regeneration studies on forest trees, it is suggested that an ideal method for micropropagation should have the following criteria (modified from Ahuja, 1987):

- It is relatively simple,
- Gives high frequency microshoot multiplication rate,
- Has a high degree of reproducibility,
- Applicable to juvenile and mature tissue,
- Applicable to different genotypes
- Induces direct differentiation of microshoots, or somatic embryos, with minimum callus formation,
- Gives high frequency of plantlet formation *in vitro*, or *ex vitro*,
- Gives high survivable of plantlets or somatic embryos after potting and transfer to greenhouse,
- Easily adaptable to greenhouse and nursery conditions for acclimatization,
- Ensures, to a large extent, genetic stability of propagules under *in vitro* environment, *ex vitro* greenhouse, nursery and field conditions, and in progeny tests,

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- Most importantly, it is cost effective, that is, costs of micropropagated plantlets or somatic embryos are comparable to zygotic seedlings.

Although such an ideal method of micropropagation is not yet available for organogenesis or somatic embryogenesis for forest trees, at least some aspects of such a procedure have been accomplished in *Populus* and some other tree species. And major biotech efforts are progressing rapidly for making clonal forestry by micropropagation commercially feasible.
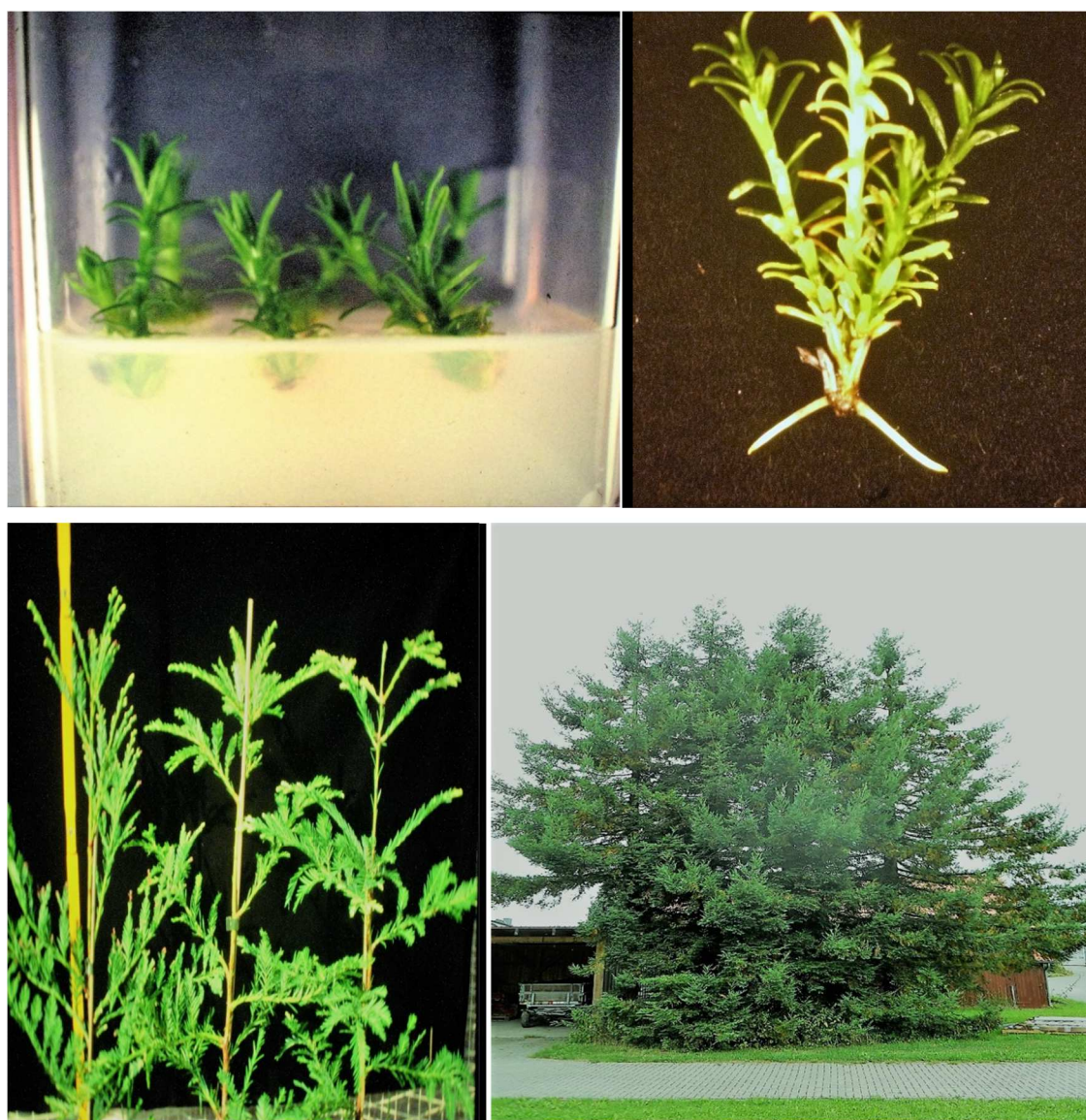
Figure 3. Micropropagation of *Sequoia sempervirens* showing microshoots, rooted plantlet (top row), greenhouse grown plants, and more than 25 years old frost-tolerant Sequoia trees (photograph by Dr. Georg von Wühlisch) in the arboretum (bottom row) of Institute of Forest Genetics, Grosshansdorf, Germany.

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Regeneration systems and genetic engineering in trees

Although genetic improvement of forest trees has mainly progressed by selection and breeding methods (Zobel and Talbert 1884; White et al. 2007; Neale and Kremer 2011; Eriksson et al. 2013), these approaches have limited applicability in long-lived forest trees, with extended vegetative phases ranging from one to many decades. Genetic engineering (GE), on the other hand, offers prospects of transfer of desirable genes into selected genotypes at a comparatively faster rate by bypassing the prolonged breeding process. Thus, transfer of genes with desirable traits by traditional approaches, involving breeding and recurrent selection that would take decades to centuries in forest trees, can be accomplished by genetic engineering in a single generation. Furthermore, GE overrides incompatibility barriers and allows gene transfer not only in unrelated tree species, but also between widely divergent taxa. At the same time GE also removes potentially undesirable effects of linked alleles to transferred genes which could be inadvertently introduced to the progeny by conventional breeding programs (Ahuja 2009b, 2011b).

After establishing well defined regeneration systems, both by organogenesis and somatic embryogenesis, it became possible to transfer recombinant genes in forest trees by GE. Ever since the first report on the *Agrobacterium*-mediated transfer of herbicide tolerant gene to poplars (Fillatti et al. 1987), there has been considerable interest in the application of GE to forest trees by *Agrobacterium*-mediated or biolistic methods. During the last three decades, a number of forest trees have been genetically engineered with a variety of different recombinant genes, using tissues derived from organogenesis (leaf discs, microshoots, different tissues/ organs) and somatic embryogenesis (embryonal cells/mass) for the production of transgenic plants (Charest and Michel 1991; Ahuja 2000; Trontin et al. 2002, 2007; Tang and Newton 2003, Charity et al. 2005; Klimaszewska et al. 2007; Lee et al. 2014; Uddenberg et al. 2016). The first wave of GE involved the transfer of foreign genes (mostly bacterial genes) used as reporter genes to monitor the integration and expression of transgenes in forest trees. This was followed by a second wave which included transfer of economically important genes for herbicide tolerance, pest resistance, lignin modification, yield traits, and reproduction manipulation (both bacterial and plant genes) to the forest tree genome (Ahuja, 2001, 2009b, 2011b, 2014a; Boerjan 2005; Brunner et al. 2007 van Acker et al. 2014; Klocko et al. 2016). However, there were a number of technical problems and limitations to *in vitro* regeneration of selected genotypes in forest trees. Some of these problems have been addressed and progress has been made and genetically modified forest trees are undergoing confined field testing of their growth performance and biosafety (Häggman et al. 2013; Porth and El-Kassaby 2014; Ahuja 2014a, 2014b; Vettori et al. 2016). The future of genetically modified forest trees still remains controversial and uncertain, as a number of biological and regulatory issues relevant to transgenic trees need to be addressed and resolved before their commercial deployment (Ahuja, 2009b, 2011b, 2014a, Bradford et al. 2005; Finstad et al. 2007; Sederoff 2007). However, pest resistant poplars have been commercialized in China in 1990's, and frost-tolerant transgenic eucalypts and genetically modified loblolly pine engineered for increased wood density are expected to be released in the US market in the near future.

Transgene stability

Forest trees have long generational cycles with vegetative phases extending from one to several decades. Genetic and phenotypic stability of genetically modified trees are important considerations for subsequent large scale plantations of GE trees (Ahuja 1988b, 1997, 2000; Hawkins et al. 2003; Brunner et al. 2007; Porth and El-Kassaby 2014). In addition, the potential risk concerning escape of transgenes from GE trees and feral tree populations acquiring the transgenes needs to be addressed before commercial deployment of GE trees (Brunner et al. 2007; Farnum et al. 2007; Smouse et al. 2007; Ahuja 2009b, 2011b, 2014a). Stability of transgenes in the vegetative propagules and across reproductive generations, and transgene containment are important considerations for long-lived forest trees that will ultimately determine their future in forestry (Ahuja, 2009b, 2011b; Ahuja and Fladung 2014). Stability of transgene expression and

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functional utility of a transgene in time and space in the forest tree genome are also important considerations for transgenic research (Ahuja 2000). As trees grow, they increase in size and complexity with their increasing age, and changes in gene expression are likely to occur during seasonal cycles and in a changing environment. Therefore, promoter fidelity and genetic stability of transgene expression have to be viewed at several different levels in the long-lived forest trees. In this context, the activity of some transgenes (for example, herbicide tolerance) may be required for a short duration during early growth, while others to remain active throughout the life (for example, pest resistance, lignin reduction, yield traits), and still others would be required at a specific time (for example, reproductive sterility) of the forest tree growth and development (Ahuja, 2009b, 2011b, 2014a).

Variation in transgene expression has been widely reported in transgenic herbaceous plants and forest trees. Variation in gene expression may be caused by somaclonal variation (Larkin and Scowcroft 19981; Ahuja 1987, 1998), integration patterns and copy number of a transgene, and inactivation/silencing of the transgenes in the host genome (Finnegan and McElroy 1994; Ahuja and Fladung 1996; Fladung et al. 1997; Ahuja 1997; Kumar and Fladung 2002; Butaye et al. 2005; Wagner et al. 2005). Detailed studies have been carried out in *Populus* and several other forest tree species for determining the stability of transgenes on a short-term and long-term basis.

One study used the *rolC* gene from *Agrobacterium rhizogenes*, which is a dominant gene that affects the phenotype, particularly the leaf size and color when introduced in a plant. Since *rolC* gene expression can be monitored at the phenotypic level, it can be used as a selectable marker to assess its phenotypic and molecular expression, as well as genetic stability in the transgenic plants. A morphological marker has a distinct advantage, when compared to the time-consuming analysis of biochemical markers (such as GUS) at specific intervals, because it can be easily observed at all times during the life cycle of a transgenic plant. Four selected clones from aspen (*P. tremula*) and hybrid aspen (*P. tremula x P. tremuloides*) were genetically transformed by using the leaf-disc *Agrobacterium* co-cultivation method. Two *rolC* reporter gene constructs with different promoters (35S::*rolC* and *rbcS::rolC*) were employed in this study, showing different leaf sizes (**Fig. 4**) (Ahuja and Fladung 1996; Fladung et al. 1996, 1997). Transgenic aspens carried 1-3 copies of the *rolC* transgene (**Fig. 4**). Transgenic lines carrying the *rolC* transgene were grown in the greenhouse for more than 19 years (Fladung et al. 2013), and in a confined field trial for 5 years Fladung et al. 2004; Fladung and Hoenicka 2012; Pilate et al. 2016). Variation in phenotypic expression was observed in a number of transgenic plants both in the greenhouse (**Fig. 4**) and field conditions (Ahuja 2000; Fladung et al. 2004). Results from this study have revealed that most of the transgenic plants carrying a single copy of the transgenes were relatively more stable than those carrying more than one copy of the transgene (Ahuja 2000; Fladung and Kumar 2002; Kumar and Fladung 2002; Faldung et al. 2013).

Detailed confined field studies, ranging from 3 to 8 years have also been carried out in genetically modified poplars and white spruce. Transgenic poplars showed relatively stable expression of the herbicide tolerant gene up to 8 years under confined field trials (Li et al. 2008). Genetically modified spruce (*Picea glauca*) carrying the *Bt* gene also showed a continued insecticidal activity in confined field trials for 5 years (Lachance et al. 2007). Generally, stable expression of transgenes was detected with one to fewer copies of transgenes in these studies (Brunner et al. 2007; Li et al. 2008). However, there were exceptions in another study, where one or fewer copies of a transgene were not always associated with stable expression of the transgene, as was reported in the antifungal transgene (pinosylvin synthase) activity in poplars (Seppänen et al. 2004).

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Figure 4. Transgenic hybrid aspen clone carrying 35S::rolC and rbsc::rolC transgenes, along with a control plant (left to right plants) in the top row; next southern blot showing 1-3 copies of the transgenes in transgenic aspens. Bottom row shows chimeras in transgenic hybrid aspens, one with Hernán Mattes Fernández from Argentina while visiting my lab in Germany.

Because of the randomness of transgene integration and instability in the *Agrobacterium*-mediated and biolistically transformed plants, a site-specific, and a highly efficient and   precise gene integration strategy, involving clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein9 (Cas9) genome editing system (CRISPR-Cas9) has been used for targeted mutagenesis in forest trees (Tsai and Xue 2015; Fan et al. 2015; Zhou et al. 2015). The power of the CRISPR-Cas9 platform lies not only in its ability to precisely engineer foreign genes at specific locations in the genome, but also native genes for the production of transgenic plants. Another utility of this gene targeting system lies in the potential for

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activating/editing native plant genes for drought tolerance, frost tolerance, disease resistance and other qualitative and possibly quantitative traits, rather than introducing exogenous genes for these traits. Such innovations will pave the way for next generation biotech crops/trees to be less regulated or not regulated by federal oversights, as these novel genotypes will be, more or less, substantially equivalent to genetically unmodified plants (Ahuja 2014b). Although still in infancy, the CRISPR-Cas9 genome targeting system is evolving rapidly and seems to accomplish both gene insertion and gene mutation in plants/trees (Li et al. 2015; Ma et al. 2016; Tycko et al. 2016; Puchta 2017). The CRISPR-Cas9 gene targeting system also offers prospects for altering the genome in somatic embryogenesis or organogenesis cultures of forest tree species for genetic improvement and perhaps scaled-up, cost effective clonal forestry.

Transgene containment

Several approaches have been proposed to impede the escape and containment of transgenes (Brunner et al. 2007; Ahuja 2011b). These include: (1) reproductive sterility (Strauss et al. 1995; Brunner et al. 2007; Klocko et al. 2016), (2) site-specific excision of transgene mediated by recombinase systems, where the functional transgene is removed from the gametes before flowering (Luo et al. 2007; Gidoni et al. 2008; Fladung et al. 2010), and (3) introducing transgenes into the chloroplasts genome to reduced transgene escape via pollen (Bock 2006; Verma and Daneill 2007). Transgene escape from transgenic populations is a virtual reality, and none of these approaches would likely achieve 100 percent transgene containment in the forest trees in a real world. Although, studies on containment of transgenes are being actively pursued, their successful application to forest trees, with extended vegetative phases, still remains a challenging problem. One alternative non-GE approach would be by isolating the transgenic populations to delimited areas, or using incompatible genotypes of wild tree populations neighboring the genetically modified trees (Ahuja 2011b).

Clonal forestry and transgenic forestry facing climate change

Global climate has been changing in the past and will continue to change in the future. What is new is that according to current estimates by meteorologists global warming has been occurring at an accelerated rate. The Intergovernmental Panel on Climate Change (IPCC) has made projections on increase of global mean temperatures by the end of the current century. The Fourth Assessment Report (AR4) by IPCC (2007) predicted a global temperature increase between 2.4°C to 6.4°C, accompanied by significant changes in precipitation (Allan and Sodan 2008; O'Gorman and Schneider 2009; Trentberth 2011), while the AR5 report by IPCC (2014) predicted a temperature rise between 2.6°C and 4.8°C, depending on the locations with low or high emission of carbon dioxide (CO_2). These changes in global warming are likely caused by anthropogenic greenhouse gases, mainly CO_2, resulting from the industrial combustion of fossil fuel in the atmosphere, and have contributed to about 78% of the total greenhouse gas emissions increase from 1970 to 2010 (IPCC 2014).

Rapid climate change may impact plant /tree species in a number of different ways, including: (1) range shifts, migration, and geographical redistribution (Parmesan and Yohe 2003; Aitken et al. 2008; Iverson et al. 2005, 2008; Kelly and Gouldon 2008; Thuiller et al. 2008; Iverson and McKenzie 2013), (2) shifts in phenology (e.g. flowering time) (Franks et al. 2007; Craufurd and Wheeler 2009; Anderson et al. 2012; Gaira et al. 2014), (3) threat to biodiversity (Botkin et al. 2007; Bellard et al. 2012; Javeline et al. 2013), (4) ecosystem disturbance (Littell et al. 2010; Stuadet et al. 2013; Bellard et al. 2014), (5) forest growth, drought and mortality (Battles et al;. 2008; Allen et al. 2010, 2015; Quirk et al. 2013), (6) changes in genetic and evolutionary response (Davis et al. 2005; Hoffmann and Willi 2008; Hoffmann and Sgro 2010; Rehfeldt et al. 2014), and (7) extinction risks (Thomas et al. 2004; Parmesan 2006; Foden et al. 2008; Pearson et al. 2014; Urban 2015). In addition to the above mentioned impacts on plants, climate change is

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also presumable causing changes in the environment, including increased drought conditions, changes in precipitation, and increase in sea levels. Clonal forestry and transgenic forestry will have to cope with rapidly changing climate and global warming.

There are at least four broad options for adaptation of forest trees to rapid climate change: (1) strategies for mitigation of climate change, (2) translocation of clonal material by managed relocation (one type of assisted migration) to new locations to pursue biological diversity, ecosystem functioning as an adaptation strategy for coping with climate change, in the event that the current habitat is to become unsuitable because of climate change (Richardson et al. 2009; Schwartz et al. 2012), (3) selection of genotypes from the germplasm in the forest tree species of interest that are tolerant to environmental stresses, including drought, frost, and pests, and are amenable for *in vitro* clonal propagation, and (4) employment of biotechnological approaches to produce novel genotypes that are resilient and tolerant to climate change effects. I shall discuss climate change mitigation, and biotechnological approaches to cope with climate change in the following sections.

Mitigation of climate change

Different strategies present themselves to combat climate change. These include: (1) mitigation of CO_2 in the factories, (2) nuclear power plants (with little or no CO_2 emission) for energy, rather than fossil fuels, and (3) geo-engineering, which is deliberate large scale manipulation of the planetary environment to counteract anthropogenic climate change (Marchetti 1977). In fact, geo-engineering does both, it removes CO_2 from the atmosphere and manages solar radiation reaching the surface of the earth.

There are at least four geo-engineering approaches that are currently employed to mitigate CO_2 and global warning (Zhang et al. 2015). One is land-based for CO_2 sequestration by large scale reforestation and afforestation programs. Second approach is *atmosphere-based* and involves injecting aerosols (e.g. sulfur dioxide) into the stratosphere by aircrafts for solar radiation management. The sulfur dioxide is oxidized in the atmosphere to sulfuric acid, which has a low enough vapor pressure to form cloud droplets to block solar radiation (Robock 2014). The third approach is *ocean-based*, and involves ocean fertilization by adding iron (iron sulfate), urea (source of nitrogen), and phosphorus to stimulate phytoplankton growth and population. Phytoplanktons absorb CO_2 through photosynthesis and form a food chain in the ocean. When phytoplanktons die, they sink taking the photosynthate with them to ocean floor, thus sequestering atmospheric CO_2 (Williamson et al. 2012). The fourth approach is *albedo-based* that involves reflection of incoming short-wave radiation from sunlight by the leaf surface by plants and tree canopy (Davies-Barnard 2014). It might be possible by genetic engineering to produce transgenic trees that have broad and waxy leaf surface facing sunlight to reflect heat back into space.

Biotechnological approaches to cope with climate change

I shall present two biotechnological approaches that can be employed to generate new genotypes that are able to cope with climate change. Here I shall focus on two stress-related traits that will be important for the adaptation of trees to climate change. These include drought tolerance and frost-tolerance. One approach involves use of *somaclonal variation* in tissues of selected clones *in vitro* to induce epigenetic or genetic changes for stress tolerant trait. Towards this goal, I suggest, induction of callus formation in both organogenesis and somatic embryogenesis cultures of forest tree species by using appropriate levels of PEG and/or 2,4-D, or temperature ranges (25-30ºC), and select for plantlets or somatic seedlings tolerant to drought and/or frost.

The second strategy calls for transfer of stress tolerant genes (drought tolerant or frost tolerant) into selected clones of a forest tree species by *genetic engineering*. Although both drought tolerance and frost tolerance

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may be controlled by a number of genes, it should be possible to induce tolerance in both cases by introduction of a single gene, as has been shown in modification of lignin (Vanholme et al. 2008; van Acker et al. 2014), which is also a polygenic trait. Candidate genes have been reported for drought tolerance (Du et al. 2013; Blum 2014; Gao et al. 2013; Gartland and Gartland 2016) and frost tolerance (Feng et al. 2012; Wisniewski et al. 2014; Wang et al. 2016), and some have been have been used for genetic engineering.

There are two options for stress tolerance by gene transfer: (1) transfer of stress tolerant gene (drought tolerance or frost tolerance), along with a pest tolerant (BT) gene in a selected forest tree clone, and/or (2) genetically engineer drought tolerance or frost tolerance gene into a transgenic genotype that already carries a transgene for an economic trait (for example, pest resistance). As it turns out, both pest resistance and drought tolerance traits would be useful, as with increasing temperatures and humidity, pest population will also increase (Battisti and Larsson 2015; Sangle et al. 2015). Test these genotypes in greenhouse with drier or colder atmospheres. Alternatively, move transgenic genotypes by managed relocation (a type of assisted migration) to pole-ward locations to test for frost-tolerance, or in drier southward locations for testing drought tolerance. It is suggested that tissues/organs from these transgenic genotypes should be stored in gene banks using cryopreservation techniques (Keller and Senula 2010; Gonzalez-Arnao et al. 2014) for future exploitation to cope with the rapid climate change.

Concluding statement

Clonal forestry by organogenesis and somatic embryogenesis has been making progress. However, there are still some limitations to the commercial deployment of clonal material. Among them are the technical problems to clonally propagate mature selected genotypes *in vitro*, and costs of propagule production. These problems may be addressed by innovative techniques in biotechnology for cost-effective production of forest tree clones by *in vitro*. Clonal forestry and transgenic forestry will have to cope with rapid climate change. CO_2 sequestration by large-scale reforestation and afforestation programs, and geo-engineering offers prospects for combating climate change. Managed relocation offers opportunities to test the capacity of the forest clonal material to adapt to the new environment in the new locations in the face of climate change. Biotechnological approaches using somaclonal variation and genetic engineering would be necessary to produce clonal transgenic materials that are tolerant to climate change stresses.

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Vegetatively propagating forest trees

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Abstract

Propagation by seeds gives rise to individuals which are all genetically different from each other. By contrast, asexual or vegetative propagation consists in duplicating, theoretically unlimitedly, genotypes while preserving through mitotic divisions their original genetic make-up, and consequently all their individual characteristics. This is essential to ensure the transfer of economically important traits which are under non-additive control. Vegetative propagation can be applied to any individual that does not produce fertile seeds, either because it has not entered the mature reproductive stage yet, or due to unfavorable environmental conditions. Its usefulness is obvious for research as well as for operational activities, depending on the ultimate objectives and on the most suitable strategies to meet the goals. Conventional nursery techniques and *in vitro* culture can be used for vegetatively propagating forest tree species. The respective pros and cons of these various vegetative propagation methods, which can synergistically complement each other, are considered, mainly from an operational viewpoint. Species characteristics and cost effectiveness must be taken into account for applications while pondering the real advantages and limitations of vegetative versus seed-based propagation strategies in the general context of forest tree plantations.

Keywords: axillary budding, clone, cuttings, grafting, *in vitro* culture, organogenesis, self-rooted plants, somatic embryogenesis.

Foreword

The 4th conference of the IUFRO working unit 2.09.02 has recently given us the opportunity to discuss the last advances of somatic embryogenesis (SE) and other vegetative propagation (VP) technologies applied to forest tree species. SE was given preponderant consideration during the past conferences of the IUFRO working unit 2.09.02 consistently with its affiliation to IUFRO unit 2.09.00 dealing with "Tree seed, physiology and biotechnology". There is, however, a need to broaden the scope to other VP methods, formerly regrouped within the IUFRO working unit 2.01.17 "Physiology of vegetative reproduction" before its fusion with 2.09.2 in 2010. This will give the opportunity to widen the range of forest tree species amenable to different VP techniques, with special mention for those planted in tropical countries due to their worldwide importance. Organizing the venue of the conference in La Plata, which was attended by a great number of participants from the Northern but also from the Southern hemispheres, supports this view. Consistently, it seems relevant to reconsider the particularities and usefulness of VP as a whole, as well as the various ways it can be applied for managing and using sustainably forest tree resources in view of the forthcoming challenges of the 21th century. Improving, the sooner the better, the productivity and the quality of wood production in plantations, in order to lessen the pressure on the irreplaceable natural forest ecosystems that are more and more endangered, is a major issue. The purpose of this paper is to examine, from realistic and practical viewpoints, what can be expected from VP for meeting this goal with the shortest delays, emphasizing concrete outcomes rather than longer-term and more uncertain expectations.

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Vegetative propagation: main features and current importance for forest tree plantations

Seed-derived individuals are all genetically different from one another. By contrast, asexual or vegetative propagation (VP) consists in duplicating, theoretically unlimitedly, genotypes while preserving, through mitotic divisions, their original genetic make-up, and consequently all their individual characteristics. For several tree species, VP is a natural propagation procedure, be it by suckers (*Populus spp, Robinia pseudoacacia*), layers (*Thuya spp, Argania spinosa*) or even apomixis (*Citrus spp, Cupressus dupreziana*). Usually, the separation from the mother plant takes place once the offspring can rely on functional roots. VP can also be induced artificially for more specific purposes as described in this paper. It can be applied to any individual that does not produce fertile seeds, either because it has not yet entered the mature stage or, as is mostly the case for exotic species, due to unfavorable environmental conditions. By producing genetically identical offspring, VP is a way to improve wood population uniformity in yield and quality especially for plantations established in monoclonal blocks (Zobel and Talbert 1984, Ahuja and Libby 1993a, Lindgren 2002). Conversely VP is associated with a substantial impoverishment in genetic diversity, depending on the number and the genetic relatedness of the clones deployed. Contrary to propagation by seeds, VP cannot create any new genotypes. It must therefore be considered as a dead-end from a genetic improvement viewpoint, except when the clones produced are used for breeding through suitably established clonal seed orchards (CSOs) or for genetic engineering (White et al. 2007, Ahuja 2011, Harfouche et al. 2011).

Possible usefulness of VP for forest tree species

VP can be used in forestry for different purposes (Libby 1974, Zobel and Talbert 1984, Ahuja and Libby 1993a) such as:

- *ex-situ* conservation of genepools which are endangered *in situ,* like in the case of *Cupressus dupreziana* which is restricted to very few individuals in its native Thassili mountains in Algeria. Thirty two genotypes were successfully grafted, physiologically rejuvenated and clonally propagated by rooted cuttings at the French R and D institute AFOCEL (Franclet 1977, Monteuuis, unpublished results). The rooted clones were then planted in different clonal test locations in France, which can be converted into CSOs for producing new genotypes and enriching thereby the narrow genetic diversity of this species in peril (Fauconnier 2012).
- Genetic enrichment or improvement through seeds produced from clones selected and then planted for this purpose within wisely designed CSOs.
- Assessing the influence of the environment (E) on field behavior of the clone which is liable to vary according to genotype (G), certain clones being more adaptable or plastic than others, as reflected by G X E interactions.
- Evaluation of various genetic parameters such as broad sense heritabilities, genetic correlations… (Burdon and Shelbourne 1974).
- Physiological studies, including phase change, resistance or tolerance to environmental constraints like frost, drought…
- Genetic engineering using somatic embryogenesis (SE), preferably when derived from single DNA-transformed cells, for regenerating with the higher chances of genetic and phenotypic stability completely transgenic trees (Trontin et al. 2007, Ahuja 2011, Hazubska-Przybył and Bojarczuk 2016). Nonetheless, it should be kept in mind that the few transgenes available to date are restricted to traits under monogenic control whereas most of the economically important forest tree features are assumed to be polygenically determined (Ahuja 2011, Harfouche et al. 2011). The risks of modifying host plant characteristics by genetic transformation should not be ignored. Society's reluctance and strict jurisdictions towards transgenic plants, trees included, are also increasingly interfering with genetic engineering activities.

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- From an operational standpoint, the mass production of superior and uniform planting material in order to generate with the shortest delay a high yield of outstanding quality wood.

VP techniques

Trees can be vegetatively propagated in bulk or as separate clones (Talbert et al. 1993, Ritchie 1994, Monteuuis 2016), by grafting or on their own roots, self-rooted, in nursery or *in vitro* culture, distinguishing between axillary budding, organogenesis and SE origins (Ahuja and Libby 1993a, Hartmann et al. 1997).

Grafting

Grafting is a helpful alternative for cloning individuals that are reluctant to *de novo* organogenesis, *i.e.,* adventitious rooting, budding and somatic embryogenesis. It consists in combining a root system, the rootstock, to an aerial part, the scion, to give rise to a bi-genotypic new individual. Grafted plants differ in this respect from self-rooted vegetative offspring, usually produced by rooted cuttings under suitable nursery conditions profitably equipped with a mist or fog-system (Hartmann et al. 1997). These are not required for grafting. Seedlings or clones can be utilized as rootstocks. The term homograft is employed when rootstock and scion are from the same species and heterograft when the two symbionts are from different species. Pros and cons of grafting have already been extensively described (Hartmann et al. 1997). It is used for instance in fruit tree orchards for increasing tree density and hence overall productivity by grafting on specific rootstock clones. The vigorous tap roots produced by seed-issued rootstocks can overcome the limitations of clones that are difficult to root or produce a deficient adventitious root system that is non-adapted to the planting site conditions. Operationally, grafting has had a tremendous impact on the industrial development of rubber tree clonal plantations which are all grafted (Masson and Monteuuis 2017). It is also widely used on a smaller scale for reproducing special ornamental growth habits, like witches' broom, 'compactum', 'nana' or 'pendulum' cultivars that could have resulted from an auto-maintained disease or from maturation-associated physiological deficiencies. Thus the weeping variety "pendulum" of *Sequoiadendron giganteum* could be maintained by heterografting onto *Sequoia sempervirens*, which was less vigorous than homografting (Monteuuis 1985), but disappeared after cloning by microcuttings (Franclet 1981). Grafting is also a means of producing clonal plants that do not delay the onset of flowering and seed or fruit production in seed orchards, as physiologically mature material can be propagated by grafting but not self-rooted (Borchert 1976, Hackett 1985, Bonga 1987). Its main usefulness in forestry is for breeding or for operational production of genetically improved seeds from judiciously set up CSOs, as well as for establishing *ex-situ* genepool conservation stands (Zobel and Talbert 1984, Ahuja and Libby 1993a, White et al. 2007). Grafting has also been used under certain circumstances for physiologically rejuvenating mature selected genotypes as a prerequisite to the true-to-type production of self-rooted clonal offspring (Franclet 1977, Cauvin and Marien 1979, Monteuuis 1985). The possibilities to graft miniaturized scions onto juvenile rootstocks *in vitro,* but also the most suitable "physiological window" for collecting the scion from the donor plant, have been assumed to play a key role in this regard (Monteuuis 2012). However, due to its particularities and constraints (Hartmann et al. 1997, Zobel and Talbert 1984), grafting is not compatible with large scale clonal forestry which consists of self-rooted clones exclusively.

Self-rooted shoots produced by axillary budding

Cuttings, minicuttings and microcuttings differ from one another basically in the length of the shoots used to be rooted. They all result from the elongation of preexisting shoot meristems. Propagation by axillary

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budding is hence the most natural and reliable artificial VP method, whose efficiency has already been proven in nursery as well as in *in vitro* conditions.

Macropropagation

Propagation by rooted cuttings or minicuttings in nursery conditions, also referred to as macropropagation (Rauter 1983), is the most widely used technique for vegetatively mass producing forest trees. It has been proven to be more effective and efficient than tissue culture for a wider range of genotypes of several species, especially *E. urophylla X E. grandis* (Saya et al. 2008) and *Hevea brasiliensis* (Masson and Monteuuis in these proceedings). The main and first requisite is to make sure that the shoot (ramet) removed from the donor tree *in situ* (ortet) or stock plant remains alive long enough for proper rooting. Adventitious root formation is determined by endogenous and exogenous factors notwithstanding their likely interactions (Rauter 1983, Davis et al. 1988). Certain cells, assumedly in shoot perivascular tissues, must have the capacity to dedifferentiate and give rise to *de novo* produced root tips which are different in many respects, anatomically in particular, from the shoot structures from which they are derived (Davis and Haissig 1994). The basic requirements for mass propagating the genotypes selected by rooted cuttings are *i)* adapted nursery facilities, resorting to a reliable automatic mist or fog system for maintaining the cuttings alive during the length of time needed for roots to develop and to become functional and *ii)* have the proper know-how for inducing and sustaining a sufficiently high capacity for true-to-type cloning by rooted cuttings of the plant material (Rauter 1983, Hackett 1985, Hartmann et al. 1997). Starting in the late 70's, AFOCEL has had a pioneering influence in the development of efficient techniques for mass clonally propagating by rooted cuttings in nursery conditions mature ortets of various tropical and temperate tree species (Franclet 1977, 1981). The importance of shortening the distance within the donor plant between the shoots to be used as cuttings, and the roots, especially the tips from where the hypothetical rejuvenating hormones the cytokinins originate (George 1993), accounted for intensive and repeated shoot hedging and pruning practices (Franclet 1977, Hackett 1985, Monteuuis 1989). These methods were combined with serial rooting for regenerating new root systems with root tips closer to the shoots (Monteuuis 1993). This gave rise to the minicutting system: the nth plant generation is serially produced from short axillary bottom shoots collected from the previous $n-1^{th}$ generation of plants raised in small volume Melfert plugs before field planting (Monteuuis et al. 1987). Another advantage of this system is to save nursery or greenhouse space. Initially developed by AFOCEL for *Eucalyptus gunnii*, *E. globulus* and related interspecific eucalypt hybrids (Cauvin 1982, Chaperon et al. 1984) as well as for other temperate species like *Sequoia sempervirens* (Monteuuis et al. 1987), the minicutting propagation system has subsequently been applied with great success in warmer countries for (sub) tropical tree species. These encompass eucalypt clones and interspecific hybrids (Wendling and Xavier 2003, Titon et al. 2006, Saya et al. 2008), teak (Ugalde Arias 2013), *Gmelina arborea* and others of more local interest (Monteuuis 1993). Propagation by minicuttings is nowadays more and more used for reducing the cost of plants produced by advanced *in vitro* methods, especially SE (Thompson 2014, Bonga 2015, Georget et al. 2017). The benefits of advanced and sophisticated nursery equipment like aero and hydroponics seem however questionable, the priority being to produce at lower cost the quantity of good quality clonal offspring required. Moreover, macropropagation methods, no matter how elaborate, will always face limitations that micropropagation techniques can overcome (Monteuuis 2016).

Micropropagation

Its main interest lies in the possibility to use *in vitro* culture for initiating the mass production by axillary budding from miniaturized ramets, the microcuttings, which are too tiny to survive in natural conditions. A microcutting or microshoot is a shoot portion that has at least one apical or axillary meristem. Its initial size

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usually ranges between 1 and 2 cm but can be as small as 0.1mm when it is restricted to the shoot apical meristem (SAM). The pros and cons as well as the successive steps of micropropagation by axillary budding have been developed recently, emphasizing the benefits of using miniaturized ramets for mass clonally propagating true-to-type selected genotypes with greater efficiency than by more conventional methods (Bonga 1987, Monteuuis 1989, 2016). The microshoots can be maintained in the absence of any root and serially subcultured *in vitro* on proper culture media long enough to ensure their mass multiplication and their physiological rejuvenation needed for efficient adventitious rooting, preferably carried out in nursery conditions (Bonga 1982, Durzan 1984, Hackett 1985). As an illustration, a SAM-issued rejuvenated *Sequoiadendron giganteum* line and an *Acacia mangium* mature selected genotype introduced *in vitro* in 1986 and 1995 respectively have been maintained through serial subcultures up to now (Monteuuis et al. 2008, Monteuuis and Bon 2000). This prolonged subculture procedure had no noticeable effect on the growth and organogenic capacities but could be associated to a significant increase of DNA methylation compared to the same material grown outdoors (Monteuuis et al. 2008, 2009). The use of simple *in vitro* protocols and the possibility to mass micropropagate in a restricted space, year around, regardless of the local outdoor conditions can also reduce production costs (Monteuuis 2000). A comparative study made for teak within the same company in Sabah, East Malaysia showed that for more than 100 000 clonal offspring produced annually, micropropagation was more cost effective than nursery techniques (Monteuuis 2000). This was mainly due to the savings made on intensive and time consuming management of stock plants which are not needed in tissue culture. Lastly, being contamination-free, tissue-culture remains to date the only way to dispatch vegetative plant material for research as well as for operational and commercial purposes to any international destination. This is of determining importance for the rapid diffusion of the YSG BIOTECH TG1-8 teak clones from East Malaysia to various tropical countries worldwide (Goh and Monteuuis 2012, 2016, Monteuuis and Goh 2017).

Self-rooted shoots produced by in vitro *organogenesis*

Unlike propagation by axillary budding, organogenesis or *de novo* propagation requires first that particular cells from superficial tissues are able to dedifferentiate and then to reinitiate a new organogenic program leading to the formation of adventitious meristems that can subsequently develop into shoots (Durzan 1984, Thorpe and Patel 1986, Bonga and von Aderkas 1992). Because ontogenetic ageing is localized in the SAMs (Fortanier and Jonkers 1976), organogenesis-issued shoots can thus be assumed to be completely ontogenetically rejuvenated. *De novo* shoot formation can occur naturally, for instance from sphaeroblasts in certain tree species like beech or eucalypt. It can also be induced artificially in tissue culture from various organs which are usually associated with the first stages of the ontogeny like cotyledons, hypocotyls, epicotyls or primary needles for *Pinus spp*. (Dunstan and Thorpe 1986, Thorpe et al. 1991). Therefore, except for a few cases of morphological rejuvenation (Fouret et al. 1989, Bonga and Pond 1991, Dumas and Monteuuis 1991), very juvenile genotypes, too young to be reliably selected on their real genetic value, are mostly used for micropropagation by organogenesis. These primary explants are inoculated on suitable *in vitro* culture media enriched with high concentrations of growth regulators for inducing, directly or indirectly from a possible transitory callus, the formation of adventitious meristems, meristemoids or meristematic nodules (Dunstan and Thorpe 1986, Bonga and von Aderkas 1992). Only a part of these newly formed meristems will eventually elongate into shoots that can then be further micropropagated by axillary budding to be ultimately rooted and acclimated. Initially viewed as a highly efficient micropropagation technique (Thorpe and Biondi 1984, Dunstan and Thorpe 1986, Thorpe et al. 1991), it has become obvious that the expectations were far greater than the actual outcomes (Thompson 2014, Bonga 2015). This can be due to several reasons: *i)* only very juvenile genotypes can be *de novo* micropropagated, with noticeable variations of responsiveness between and within species at the family or individual levels (Durzan 1984, Thorpe and Biondi 1984, Hargreaves et al. 2005); *ii)* adventitiously produced juvenile clones can hardly be maintained in micropropagation the time needed for reliable field testing and cryopreservation of this

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material has limitations (Bonga 2015); *iii)* the field behavior of the adventitiously-produced plants of different species was disappointing, showing growth, vigor and conformity abnormalities as well as symptoms of early physiological maturation (Bonga 1991, Gupta et al. 1991, Hargreaves et al. 2005), despite the organogenesis-induced ontogenetic rejuvenation; *iv)* the successive *in vitro* transfers and manipulations required are constraining and result in prohibitive production costs (Bonga 2015). For *Pinus radiata*, the species for which *de novo* micropropagation has been the more utilized so far, adventitiously-derived plants were reported to cost 7 times more than open-pollinated seedlings (Menzies and Aimers-Halliday 1997). This situation has warranted an increasing interest in the use of SE for mass clonal propagation (Hazubska-Przybył and Bojarczuk 2016).

Somatic embryogenesis

In vitro propagation by SE has already been largely documented (Thorpe 1995, Germania and Lambardi 2016, Hazubska-Przybył and Bojarczuk 2016). Briefly, it consists in producing embryos from somatic cells by mitotic divisions, hence preserving their original genetic make-up. This is the only and fundamental difference with zygotic embryos to which somatic embryos are identical in many other respects. SE is therefore a cloning technique. Except for rare occurrences of direct embryogenesis, including genotype-dependent cleavage polyembryogenesis (Durzan and Gupta 1987; Sharma and Thorpe 1995; Durzan 2008), the somatic embryos are formed indirectly usually after an intermediate callus stage artificially induced by the application of strong growth regulators which could cause somaclonal variations (Bairu et al. 2011). In the most favorable situations, undifferentiated cells of these calli can gradually evolve into somatic embryos characterized, similarly to zygotic embryos, by a shoot–root bipolar structure prefiguring the future plant (Thorpe 1995). This basically distinguishes somatic embryos from adventitious and axillary budding-derived microcuttings consisting of shoots from which adventitious roots must develop subsequently. When originating from a single cell, SE is the most striking and concrete illustration of cell totipotency (Durzan 1984, Thorpe 1995). SE remains the supreme and only way of achieving complete ontogenetic rejuvenation for the whole plant by virtue of its power to reset ontogenetic ageing to zero through the formation of embryonic structures that characterize the very first stages of ontogeny. The older and more developed the mother plant, the greater the magnitude of this ontogenetic rejuvenation. In this respect, *Hevea brasiliensis* (Carron and Enjalric 1985), *Quercus robur* (Toribio et al. 2004, San–José et al. 2010; Ballester and Vieitez 2012), *Quercus ilex* (Barra-Jiménez et al. 2014), *Eucalyptus globulus* and *E. saligna* × *E. maidenii* (Corredoira et al. 2015) deserve special consideration as SE could be obtained from sporophytic tissues of mature genotypes whereas in most cases, and especially in conifers, only certain immature or mature zygotic embryos respond positively (Thorpe 1995, Germania and Lambardi 2016, Hazubska-Przybył and Bojarczuk 2016). It can logically be assumed that ontogenetic and physiological rejuvenation are positively related (Borchert 1976, Fortanier and Jonkers 1976). This could explain that embling-derived *H. brasiliensis* trees showed a higher capacity for SE than their grafted same age, same size and same clone homologs (Lardet et al. 2009). The physiological rejuvenation associated with the SE-induced ontogenetical rejuvenation has also been proven helpful for further mass clonally propagating by rooted cuttings the few SE-derived industrial genotypes of *H. brasiliensis* that have been obtained (Masson et al. 2013). In *Abies nordmanniana*, SE has been adopted as the solution for overcoming the poor rooting rates and ageing-related plagiotropic effects associated with VP by rooted cuttings (Find 2016). Occasionally, emblings can also demonstrate a higher capacity for SE than usually expected for seedlings of the same age as observed for *Picea abies* (Ruaud et al. 1992, Harvengt et al. 2001) and *Picea glauca* (Klimaszewska et al. 2011). It has been hypothesized that this could be due to a possible SE-induced delay of physiological ageing (Klimaszewska et al. 2011, Bonga 2016). However, the fact that only particular embling-derived genotypes responded positively also suggest the influence of a genetic predisposition for SE.

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For a long time considered to be the most promising and efficient cloning technology after the disillusion encountered with organogenesis (Hazubska-Przybył and Bojarczuk 2016), SE is still facing major scale-up hindrances. According to Thompson (2014), these are: *i)* a too limited effectiveness due to a strong genotype-dependent control of capacity for SE at the genus, species, provenances, family and individual levels; *ii)* lack of efficiency of the protocols used starting with the initiation and conversion rates, then affecting the quality of the emblings produced (Timmis 1998, Bonga 2016); *iii)* excessive production cost that cannot be offset by a high enough field superiority compared to much cheaper good quality seedlings. Emblings, at least in their first stages of development, have often been observed to grow slower than seedlings and to be prone to within sample variations according to species and procedures (Aronen 2016, Högberg 2016, Trontin et al. 2016a). Moreover, although completely ontogenetically rejuvenated by SE, emblings can exhibit several symptoms of early maturation such as premature flowering (Colas and Lamhamedi 2010, Breton, personal communication), lower ability for adventitious rooting (Högberg 2016)… This could be caused by non-optimal *in vitro* culture conditions liable to affect prematurely the sensitive and permeable cells from which SE and adventitious buds are derived (Meiland 1997, von Aderkas and Bonga 2000, Högberg et al. 2001). For instance, culture media are empirically made with a restricted list of components that are liable to interact and vary during the course of time, independent of the physiological requirements of the more or less organized cells or group of cells involved at different stages of the SE process. This will always be a constraint, even though SE efficiency can very likely be increased by improving the current protocols (Park and Bonga 2011), notwithstanding possible genotype x culture medium interactions. There is still a need to fine tune the first steps of the SE procedure for higher effectiveness, efficiency and cheaper costs in very realistic conditions with big enough and replicated representative samples before focusing on automation with the hope of reducing costs (Timmis 1998, Thompson 2014). Prohibitive production cost for SE-derived materials of insufficient quality remains the major deterrent to industrial use of SE for large scale plantation programs. Mass multiplying by rooted cuttings the insufficient numbers of expensive emblings managed as responsive stock plants has been viewed as an option for producing more planting material at cheaper cost (Lelu-Walter et al. 2013, Thompson 2014, Bonga 2015). The possibility to cryopreserve the SE-derived clones and a higher number of representatives per clone are the main advantages of such a procedure, compared to direct and more effective as less genotype-dependent bulk propagation by rooted cuttings of more genotypes from the same genetic background (Talbert et al. 1993, Ritchie 1994). Similarly to organogenesis, SE scaling-up may have been anticipated with excessive optimism based on findings drawn from limited scale research experiments too much disconnected from operational conditions (Thompson 2014). The fact that SE of *Picea abies* benefitted from thirty years of heavy research investments that did not result in any industrial application (Lelu et al. 2013) is enlightening in this respect. In addition to economic considerations and except for rare species, it should be emphasized that only very juvenile genotypes that are predisposed to SE and too young to be reliably selected on their field value, are liable to respond successfully to SE (Dunstan and Thorpe 1986, Bonga 2016, Hazubska-Przybył and Bojarczuk 2016). These aspects should be seriously considered when selecting material to be propagated by SE or other means.

Material to be vegetatively propagated

The material to be vegetatively propagated must:

i. thrive under planting site conditions, untested exotic origins being more prone to inadaptability to local environment than native ones. In this respect, it is symptomatic to note the preference given to clones of exotic species, with special mention for eucalypts, to start new industrial forest plantations in places where these materials have never been introduced before, without even considering the potential of native species to meet plantation objectives, at least partly. Planting local species

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contributes to the preservation of the natural biodiversity, bearing in mind that the genetic erosion associated with the use of clones compared to seedlings should not be minimized.

ii.   be well known and prized by the end-users. As an illustration, AFOCEL has during the late 70's-80's strongly and dynamically promoted clonal plantations of exotic species to be intensively managed in order to feed the increasing needs of pulp and paper mills (Afocel 1982). *Sequoia sempervirens* for instance was chosen for its specific properties and more particularly its high growth rate, low resin content and long fibers that are valuable assets for papermaking. Efficient and innovative propagation techniques were developed for mass producing by rooted cuttings in nursery conditions clones from mature selected Plus trees of 100 yr-old and older with average rooting rates of more than 80% (Monteuuis et al. 1987). Experimental plots were set up and 40 years of field observations have demonstrated the remarkable vigor of this species (Harvengt et al. 2013). In spite of such success at the experimental level, *Sequoia sempervirens* has never been and is still not planted at an industrial scale to be used by the pulp and paper industry, at least in Europe.

iii. generate the greatest genetic gain, the magnitude of which depends on the range of variability for traits of commercial interest within the seedling population. The wider this variability, the more room for selection at higher intensity by focusing on the few best genotypes which are far distant from the average for the most prized criteria. The stronger the selection pressure, the greater the expected returns (Zobel and Talbert 1984, Ahuja and Libby 1993a, White et al. 2007).

iv.   have a sufficient capacity to be cost-efficiently mass clonally propagated true-to-type: the quality-price ratio of the planting material has a determining impact on return on investment for forest plantations in accordance with the market value of the end product.

High value vs pulpwood species

High value timber species will definitely fetch higher market prices than pulpwood species at the end of a growing period or rotation, with the objective of producing with the shortest delay the highest volume of the best quality wood, in accordance with the relevant business plans. Promoting fast growth is also a concern of prime importance for high value timber because it reduces weeding and maintenance costs, it shortens the rotation and thus assures sooner delivery of valuable logs, the bigger the more prized. But volume is not the only criterion: log quality matters also and to a far greater extent than it does for pulp or fuelwood tree species. Clear bole length, shape, deformities like knots, buttresses, forks and branches have a big impact on log processing into sawn timber, veneer peeling and even splicing, by reducing waste and by enhancing the quality of the final product. For species like teak for instance, wood characteristics and aesthetic features, especially for refined furniture or yacht deck end-uses, have also a great market value influence (FAO 2009, Kollert and Cherubini 2012, Ugalde Arias 2013). The most highly valued products combine the most prized log and wood traits and it is very unlikely that these can be captured altogether by seed propagation. For such materials, and when possible, cloning seems therefore greatly justified, the superior cost of the clonal offspring compared to seedlings being offset by the much higher value of the final product. The situation is quite different for tree species planted mainly for pulp or fuelwood production. These are mainly propagated by seeds despite overly optimistic speculations that could have been drawn from the particularities of other species in different contexts. In *Abies nordmanniana* and *Picea abies*, true-to-type cloning by SE has been limited to the production of nicely shaped Christmas and ornamental tree clones respectively as these end-uses generate much higher added value than wood production (Lelu et al. 2013, Find 2016, Högberg and Varis 2016).

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Mature vs juvenile genotypes

The foregoing arguments plead strongly for the selection of candidate plus trees (CPT) for cloning (**Fig. 1**). A CPT is individually or mass selected based on phenotypic traits irrespective of its breeding value which characterizes an elite tree (Zobel and Talbert 1984). For reliable selection, a CPT requires to be developed hence old enough to express its superiority for as many traits of high commercial value as possible (Bonga 1982, Zobel and Talbert 1984, Ahuja and Libby 1993a). The rationale of preferring to clone mature selected genotypes rather than juvenile ones has already been largely argued (Zobel 1981, Bonga 1982, 1987), but is less and less acknowledged when not simply ignored (Hazubska-Przybył and Bojarczuk 2016), except for a few persevering research teams (Ballester et al. 2016, Klimaszewska and Rutledge 2016, Trontin et al. 2016b). Several traits of major economic importance like volume, branchiness, clear bole length and shape, wood characters especially for high value timber species are the priority criteria used for CPT selection. These criteria can be combined under the terminology of multi-trait selection for upgrading the superiority of the candidate clone (Zobel and Talbert 1984, White et al. 2007).

In teak more specifically, the initial multi-trait-based phenotypic selection of the CPTs is upgraded by taking into consideration wood value indications obtained by non-destructive core sampling as well as genotypic information drawn from DNA molecular markers (Goh et al. 2007, **Fig. 2**). Such multi-trait mass selection is immediate, very practical and efficient regardless of information on the additive vs non additive control, in other words on the heritability of the traits desired or on the genetic pedigree and relatedness of the CPTs, notwithstanding the importance of such indications for safe clonal deployment (Zobel 1981, Zobel and Talbert 1984, Ahuja and Libby 1993a). This clonal forestry strategy, starting from mature selected CPTs, is quite rational and attractive in many respects, at least theoretically (Bonga 1982, Libby and Rauter 1984, Ahuja and Libby 1993a). Practically, it can be implemented very quickly, providing suitable true-to-type and cost effective mass cloning methods are available, as demonstrated recently for teak (Goh et al. 2005, 2007, Goh and Monteuuis 2016, **Fig. 3**). This is the only but unavoidable limitation, the capacity for true-to-type cloning of most tree species by *de novo* organogenesis, *i.e.,* adventitious rooting, budding and somatic embryogenesis being severely antagonized by ageing (Bonga 1982, Hackett 1985, Bonga and von Aderkas 1992). The possibilities of physiologically rejuvenating mature selected genotypes for overcoming this cloning reluctance have been investigated during many years, but not enough, too superficially and without the discernment required (Franclet 1981, Monteuuis 1989, Bonga and von Aderkas 1993). For instance reinvigoration and rejuvenation have been for a long time confounded (Pierik 1990, Monteuuis et al. 2011a, Wendling et al. 2014). Likewise, investigations on physiological ageing should have given more consideration to likely interactions between variations in physiological state and growth activity, distinguishing between resting, bud break and active elongation periods in relation to phenology, seasonal variations and endogenous rhythms (Monteuuis 1989, Monteuuis et al. 1995, Mankessi et al. 2009). Practically, rejuvenation successes have been too anecdotal and limited in scope to warrant their application to the true-to-type mass clonal propagation of mature CPTs, especially for tree species of major economic importance (Bonga et al. 2010). These activities on physiological rejuvenation of mature selected genotypes, with the possibility to resorting to SE for complete rejuvenation, as a requisite for efficient mass clonal propagation by rooted cuttings have progressively been abandoned, although maybe too soon with reference to *Hevea brasiliensis* (Masson et al. 2013, Masson and Monteuuis in these proceedings).

Another option is to start the clonal selection from a large population of clones initially derived from young selected genotypes and serially propagated by rooted cuttings to be established within properly designed clonal tests (Kleinschmit 1974, Menzies and Aimers-Halliday 1997). The inferior clones are rogued progressively as they express their field characteristics while developing in the course of time. The superior genotypes remaining can then be mass cloned, provided that the rooting ability of the cuttings can be maintained over the years (St. Clair et al. 1985, Mason et al. 2002). Attempted initially on a large scale with Norway spruce clones and later with other coniferous species, this strategy has proven to be too time, space

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Figure 1. Candidate Plus Trees (CPTs) for cloning in even-aged family planted stands: *Eucalyptus spp*: 6 monh-old in Pointe-Noire, Congo (1a) and 2-year old in Budkinon, Mindanao, Philippines (1b); *Tectona grandis*, 22 year-old, Tchorogo, Togo (2); unexpected occurrence of a natural *Acacia auriculiformis X A. mangium* hybrid within a 2 year-old *A. mangium* family plot, Lai Uyên, Binh Duong, Vietnam (3); 10 year-old *Acacia mangium* in Luasong Forestry Center, Sabah, East Malaysia (4).

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and money consuming to be sustained (Kleinschmit 1974, Kleinschmit and Schmidt 1977, Högberg and Varis 2016). Therefore, and because of an increasing interest in advanced biotechnology rather than for outdoor experimentation, research on forest tree cloning has been focused on the development of protocols for mass clonally propagating by SE very young *i.e.* mature or even immature genotypes (Thorpe et al. 1991, Bonga 2015, Masson and Monteuuis in these proceedings). The relevant prevailing argument is that efficient breeding programs, supported by molecular marker-assisted selection and cryopreservation, do not warrant any more work on true-to-type cloning of mature selected CPTs (Park and Gupta 2012, personal conversations during the 2nd IUFRO 2.09.02 conference in Brno, Park et al. 2016).
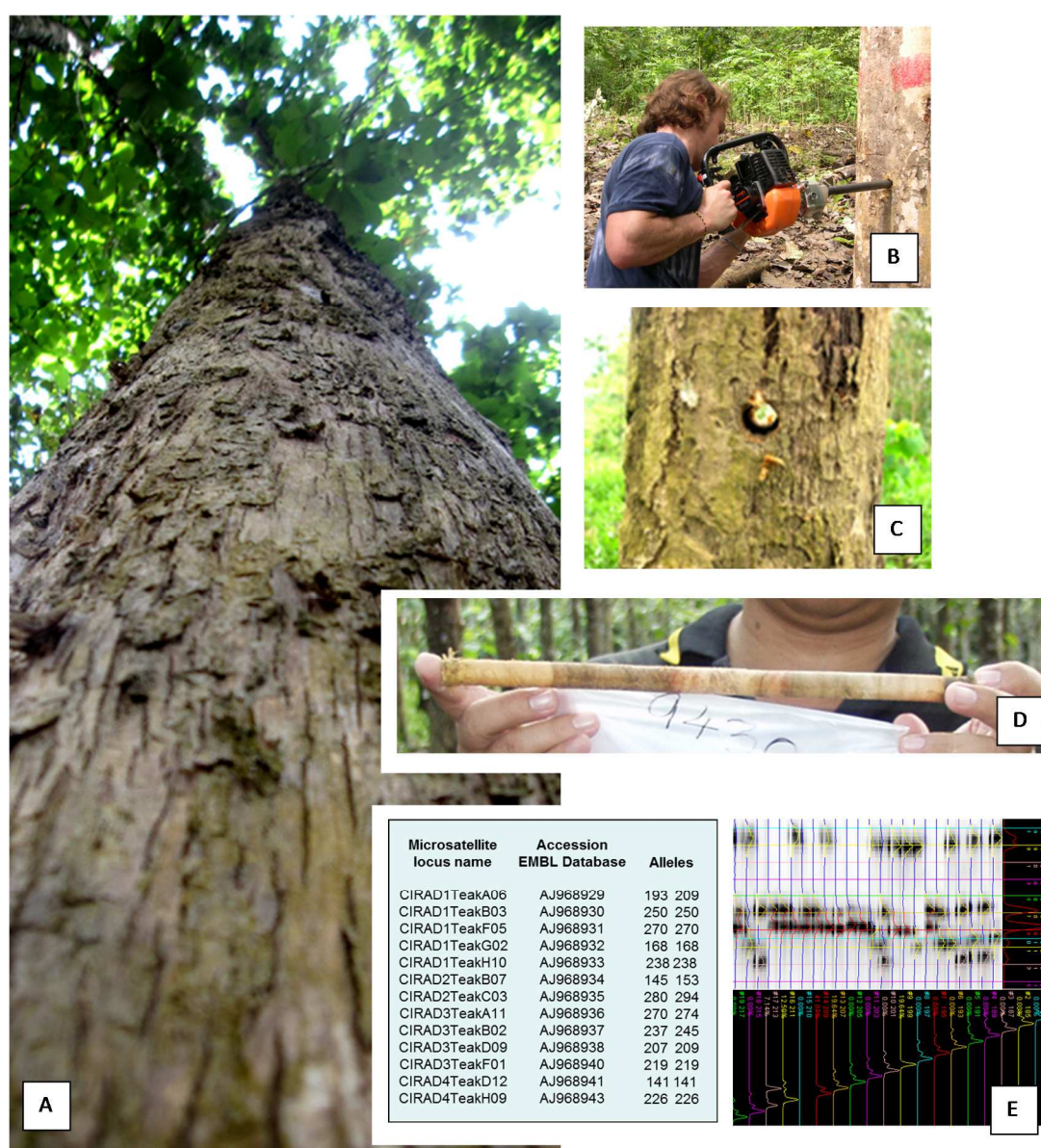
Figure 2: Multitrait mass selection of mature teak CPTs (A) upgraded by the use of non-destructive core sampling wood analysis procedures (B, C, D) and microsatellite molecular markers (E).

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Figure 3: The mature selected teak genotypes are rejuvenated, then mass clonally propagated *by in vitro* micropropagation (A) or by macropropagation, with the possibility for the microshoots only to be dispatched worldwide under proper conditioning (B, C). The microcuttings are rooted and acclimatized (D) under similar nursery conditions as for macropropagation by rooted cuttings (E). After proper raising (F, G), the clonal offspring are ready to be field planted.

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Although interesting, this view seems in some respects to be overly speculative and optimistic. First of all, each seed-issued genotype is unique by virtue of the DNA recombinations resulting from the crossing overs occurring during meiosis, over which breeders have no control. Therefore, the new genotypes derived from advanced breeding programs will be different from any other genotype and hopefully better than a particular outstanding CPT. However there will always be a risk that this will not happen and that the time, energy, land and cost invested may not pay off ultimately, especially for selections based on a combination of several traits which are not necessarily genetically linked and are assumed to be under non additive control (Zobel and Talbert 1984, Cornelius 1994, White et al. 2007). What can be realistically expected from marker assisted selection in such particular cases, as well as the relevant cost and time frame needed remain after 20 years of investment still questionable (Muranty et al. 2014). Another major concern is the strong additive genetic control of SE initiation capacity (Park et al. 1998, Klimaszewska et al. 2007), at least for certain species. This tends to promote the selection in the first place of SE responsive families, with the possibility to further select clones within these families resorting to cryopreservation technology (MacKay et al. 2006, Park and Bonga 2011, Bonga 2016). The preponderant importance given to SE initiation capacity, even for orienting breeding programs (Park et al. 1998, Klimaszewska et al. 2007) favors the selection of heritable characters at the expense of traits under non additive control, which could have a great economic impact (Zobel and Talbert 1984, Timmis 1985, Timmis et al. 1987). Growth and form for instance have been observed to vary substantially within progenies between genetically related individuals (Zobel and Talbert 1984, Chaix et al. 2011, Monteuuis et al. 2011b, **Fig. 1**). The comparison between container-grown white spruce seedlings and embling clones for various traits, at the between and within family levels, are quite enlightening, within family differences of mean values being greater among embling clones than the mean value among the seedlings (Lamhamedi et al. 2000). CPT-derived clones differ also from the average of the population, but primarily in their superiority in economically important traits. Initially selecting genotypes on their capacity for SE initiation may result in the elimination of clones with superior characteristics if these and SE responsiveness are not positively correlated. Furthermore, there is the risk of adverse selection in case of negative correlations (Haines and Woolaston 1991, Adams et al. 2016, Högberg and Varis 2016). In addition, there are still some uncertainties as regards possible risks of genetic instability associated with the cryopreservation of the SE-derived clones during the time required for reliable field testing (Park and Bonga 2011, Bonga 2016, Hazubska-Przybyl and Bojarczuk 2016). Such problems do not exist for clonal forestry programs based on individual selection of mature CPT, as has long ago been demonstrated for a large number of planted tree species and more recently for teak (Ahuja and Libby 1993b, Lindgren 2002, Monteuuis and Goh 2017). In any case, priority for selecting CPT must be given to economically important traits over ease of clonal techniques that need as much as possible to be adapted to the particularities of the CPT rather than the reverse.

Deployment of VP-issued wood populations

The rationale of propagating trees vegetatively has been to plant populations that can generate, with the shortest delays, the highest volume of premium and uniform wood quality to best meet end-user expectations. Several success stories have clearly demonstrated during the past decades the practical advantages for some forest tree species of preferring clones to seedlings for producing wood (Ahuja and Libby 1993b, Talbert et al. 1993). Mature selected CPTs were mass clonally propagated true-to-type by rooted cuttings to be field-planted. The number of clones as well as the size and the design of the monoclonal blocks planted at the same time must be adapted to the particularities of each clone and to between clone genetic relatedness. All this has been abundantly documented (Ahuja and Libby 1993a and b, Lindgren 2002). Production of self-rooted clones remains however strongly influenced by the genetic origin of the ortet and the negative effect of physiological ageing on adventitious rooting capacity. This has warranted, for certain species, the mass propagation by rooted cuttings of seedlings in their early stage of development. These, presumably being of high genetic value but available in insufficient numbers, are too

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young to be individually selected on their field characteristics. They are, therefore, propagated in mixture, as a bulk without keeping the individual genotypic identity during the successive cycles of propagation (Talbert et al. 1993, Ritchie 1994). This will ultimately result in a drastic and uncontrolled reduction of the initial genetic diversity of the bulk population (Monteuuis 2016). Besides and as previously argued, there are always risks of propagating genotypes that are too young to be field tested due to uncertainty about correlations between economically important traits and VP capacity (Haines and Woolaston 1991). Therefore, bulk propagation differs basically from the clonal option, the latter offering the possibility of deploying clones in a mixture, as a polyclonal variety consisting of a well-known number of representatives of each clone. All these reasons could explain why polyclonal varieties, especially when derived from mature selected CPTs, have gradually supplanted bulk propagated VP populations. This strategy has so far mainly prevailed for teak clonal forestry, which is still in its infancy and hence facing time limitations for properly testing the clones, as it should be done, notwithstanding the time, space, manpower and money constraints associated with the process (Zobel and Talbert 1984, Lindgren 2002). The most widely used teak polyclonal variety consists of 8 mature selected clones (Goh and Monteuuis 2012, Monteuuis and Goh 2017, **Fig. 4**). These are mostly planted in a mixture with the purpose of buffering possible risks of clone inadaptability to the planting site conditions, as compared to monoclonal blocks which are more uniform, for better or worse.

Independent of the intensification of clonal forestry activities with broadleaf tree species mainly, the strong interest devoted to SE during the past decades for coniferous species of the northern hemisphere has logically given rise to the emergence of new deployment strategies with special emphasis on Multi Varietal Forestry (MVF) (Park 2002). Being SE-based, MVF faces the previously argued limitations, in particular a too low initiation and plant conversion rates, and risks of genetic instability in long-term cryopreserved embryogenic lines (Park 2002, Klimaszewska et al. 2007). Primarily driven by SE capacity, MVF may also miss traits of great economic impact that are under additive control but not positively correlated with the ability for SE initiation, in addition to all the valuable characters which are non-additively controlled and as such will be excluded. This constitutes a major drawback compared to the returns expected from clonal forestry programs based on the multi-trait mass selection of mature CPTs. Resorting to molecular markers for refining the selections (Park et al. 2016), mixing emblings and seedlings in plantations (Park 2002, Thompson 2014, Adams et al. 2016), and preferring to use the name varieties instead of clones (Klimaszewska et al. 2007, Park and Bonga 2011, Park et al. 2016), have been proposed for wider acceptance and promotion. This makes the field situation quite confusing and heterogeneous, especially as regards to the genetic composition of the relevant tree populations, which matters the most *in fine* (Burdon and Aimers-Halliday 2006). All this contrasts with the specificities of clonal forestry, whose field benefits, when wisely implemented, have already been clearly established (Zobel 1981, Ahuja and Libby 1993a,b).

Concluding remarks

The decision to vegetatively propagate forest trees must be pondered due to the practical consequences in several domains. Species characteristics as well as the returns expected and the best ways to meet plantation objectives deserve major consideration. Attractiveness for the latest biotechnologies supported by too far reaching and overly optimistic speculation, should not be given excessive importance at the expense of more concrete and adapted options whose efficiency have already been demonstrated. Owing to its particularities and higher cost, VP seems to be more suitable for the true-to type mass clonal propagation of mature CPTs selected for their outstanding superiority in traits of great economic value, the more the better. Preference must be devoted to the more efficient but not necessarily the more advanced VP methods, or combinations thereof, for reaching this goal. A likely prerequisite for that is the physiological rejuvenation of the mature selected genotypes. Again, SE due to its capacity to achieve complete rejuvenation deserves special consideration. Within such a context and due to all these VP limitations, the

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advantages associated with the propagation of forest tree species by seeds should be kept in mind. In addition to cost efficiency, propagation by seed is the more natural and powerful way of creating the genetic diversity so much needed for the environment and for tree genetic improvement.

Figure 4: Four-year-old monoclonal blocks of YSG Biotech teak clones produced from mature selected CPT on steep slopes of southern Java, Indonesia. Maintenance was limited to weeding the first year, in absence of any pruning operation. The trees display the YSG Biotech TG1-8 characteristic features i.e. excellent straightness, reduced lateral branching and high leaf density accounting for increased photosynthesis and impressive growth rate.

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Contributions of somatic embryogenesis and other *in vitro* propagation techniques to the genetic improvement of tropical woody species: *Coffee arabica*, *Tectona grandis* and *Gmelina arborea*

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Abstract

Somatic embryogenesis is a technique that has been used in Central America for the multiplication of *Coffea arabica* F1 hybrids since its creation. The coffee breeding program for Central America - PROMECAFE (1992- 2006) - was implemented with the participation of CATIE, CIRAD and the coffee institutes in the region. Of the 98 hybrids obtained, three met the objectives of the selection satisfactorily, i.e., the new varieties are more vigorous than the traditional ones with higher value in productivity, precocity, tolerance to leaf rust and cup quality. Although somatic embryogenesis is the ideal technique for the multiplication of these varieties; the process is intensive and of long duration, so the final cost per plant is very high in comparison with that of plants obtained from seeds. These factors and others have limited the transfer of these hybrids to the producers who need to renew their plantations. To facilitate this process, CATIE established a two-stage multiplication strategy: 1-The regeneration of juvenile mother plants by somatic embryogenesis; and 2-The establishment of clonal gardens of these mother plants in the greenhouse for horticultural multiplication. This would allow the rapid multiplication of the hybrids and reduce the cost per plant produced in the greenhouse. Starting in 2017, the alliance with a horticultural company specializing in commercial scale vegetative propagation, will enable the multiplication of hybrids, and the transfer of plants to producers, at lower cost than if the plants had come directly from the laboratory.

The sylviculture of exotic, rapid growth species such as *Tectona grandis* and *Gmelina arborea* has gained recognition in Latin America for good yields in commercial plantations, producing high-value timber for international markets. Although these species are easily propagated using rooted cuttings, it is increasingly more common for forestry companies to request technical services from laboratories for rapid multiplication, disease cleansing or reinvigoration of their mother plants. CATIE, in collaboration with the National Institute of Forest Science (NIFoS) of the Republic of Korea, is working on improving the micropropagation protocols for these species and promoting the use of high-quality clonal material in the region. The excellent *in vitro* response of teak has allowed some companies to rely on the laboratory for production of mother plants from superior clones and their subsequent use in the establishment of clonal gardens for shoot production. Therefore, these *in vitro* techniques constitute a support tool to clonal sylviculture programs.

Keywords: *Coffee*, tropical forest species, micropropagation, temporary immersion culture - RITA, horticultural multiplication, mass propagation.

Introduction

Major biotechnological advances have been made mainly with agricultural crops that have been widely domesticated while forest trees have received little attention, even though biotechnology could potentially have a major impact on sylviculture and forest products (Merkle and Nairn 2005). Arabica coffee is an

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example of domestication in Central America where it has been cultivated since the 18th century. Coffee has changed from an exotic beverage to a very important product for export to Europe and North America in the 19th century, even becoming a fundamental component of the economies and societies of the isthmus (Samper 1999). Although *Coffea arabica* in America has, since its introduction, been characterized as having a narrow genetic base, advances in biotechnology techniques have enabled breeders to create new high-yielding varieties that can be propagated by high-efficiency *in vitro* techniques such as somatic embryogenesis (Etienne et al. 2002). In Central America, the PROMECAFE regional breeding program was implemented from 1992 to 2006 with the technical participation of CATIE (Tropical Agricultural Research and Higher Education Center), CIRAD (French Agricultural Research Centre for International Development) and the coffee institutes of the region (Etienne et al. 1999; Bertrand et al. 2005). The main objective of this program was to increase the genetic base of *C. arabica* in the region and develop F1 hybrids that are more vigorous than traditional varieties with optimal agronomic traits such as resistance to diseases and cup quality (Etienne et al. 2002). Due to their heterozygous structure, these hybrids can only be propagated vegetatively. In the context of collaboration, the pilot-scale multiplication of these hybrids by somatic embryogenesis (SE) was developed at CATIE (Van Boxtel and Berthouly 1996; Etienne et al. 1999; Etienne-Barry et al. 1999). Today, SE is still the ideal technique for the multiplication of these materials; the process is intensive and time consuming, so the final cost per plant is very high compared to the cost of plants obtained traditionally from seeds. These technical factors, added to the lack of promotion and financing policies, have limited the distribution of these hybrids in Central America for more than 10 years. More recently, CATIE developed a two phase strategy for the multiplication of hybrids: *1- regeneration of mother plants by SE*; and *2- multiplication of mother plants from mini-cuttings rooted in the greenhouse* (Mesén and Jiménez 2016). This procedure allows rapid hybrid multiplication at low cost, taking advantage of the juvenile trait of the mother plants produced by SE.

The focus of conventional tree breeding and, more recently, biotechnology research, has been mainly limited to conifers due to their economic importance in developed countries (Merkle and Nairn 2005). In spite of this, the value of sylviculture of exotic species in tropical America has gained recognition and thanks to the unique traits of species like teak, commercial plantations are raising expectations in the tropics, particularly in America in recent decades (Ugalde 2013). However, according to Merkle and Nairn (2005), little progress has been reported on the *in vitro* propagation of high-value tropical species, which is usually restricted to the multiplication of axillary buds as in the case of teak (Gupta et al. 1980; Sunitibala et al. 1994; Monteuuis et al. 1998; Daquinta et al. 2001; Mendoza et al. 2007; Quiala et al. 2011; Rojas and Abdelnour-Esquivel 2012), and *Gmelina arborea,* which is widely recommended for reforestation in the tropics (Kannan and Jasrai 1996; Sukartiningsih et al. 1999; Valverde-Cerdas et al. 2004). High microbial contamination, oxidation, hyperhydricity and the low *in vitro* morphogenetic response observed in explants of adult tropical trees explains why the results of many studies have not been published (Pérez 2006). However, it is more and more common for forest companies to request propagation services from laboratories for the multiplication, disease cleansing, and the reinvigoration of mother plants from selected individuals of these species.

The general objective of this publication is to show how *in vitro* propagation techniques can be integrated into breeding programs and make important contributions toward developing strategies for the multiplication and distribution of new varieties and selections that were produced.

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Somatic embryogenesis used in the multiplication and distribution of F1 coffee hybrids in Central America.

Coffea arabica breeding program in Central America

Coffea arabica represents 70% of the world's coffee production. In Latin America, traditional Arabica coffee varieties are characterized by having a very narrow genetic base since they are derived from only a few plants introduced in the 18th century (Bertrand et al. 2005). In the 1990s, a *C. arabica* breeding program was initiated by the coffee institutes of Central America (PROMECAFÉ), CIRAD and CATIE, in order to increase genetic variability in Central America. This program consisted of making directed crosses of traditional cultivated varieties with recognized cup quality such as 'Caturra' and 'Catuaí', with introgressed varieties such as 'Catimores' and 'Sarchimores', and wild individuals from Ethiopia, Sudan, Kenya and Tanzania as sources of genetic variability that are preserved in the CATIE germplasm collection (PROMECAFE 2003; Bertrand et al. 2005). As an outcome of this program and after evaluations and pre-selections in field tests in different agroecological areas of Costa Rica, only three hybrids were recommended and named for Central America ('Centroamericano'- L13A44, 'Milenio'- L12A28, and 'Casiopea'- L4A34) based on the plant's architecture, precocity, yield, cup quality and pest tolerance (Bertrand et al. 2005). The L4A5 hybrid was recently named 'Esperanza´ for having shown very good growth in some moist lowland zones of Costa Rica. Although *C. arabica* is autogamous, it shows high heterosis (Srinivasan and Vishveshvara 1978); consequently the F1 hybrids can only be propagated vegetatively. Therefore, SE has so far been the best means for the initial propagation of these F1 hybrids (Berthouly and Michaux-Ferriére 1996; Etienne and Bertrand 2001).

Background of coffee somatic embryogenesis in Central America

Early investigations on SE for coffee during the last 40 years include the works of Staritsky (1970); Sharp et al. (1973); Sondahl and Sharp (1977); Dublin (1981, 1984) and Yasuda et al. (1985), who reported that coffee SE is obtained from the culture of leaf fragments using two well-defined strategies: - *direct or low frequency SE* – which occurs in a single phase, in a single culture medium to produce somatic embryos rapidly but in limited quantities; and - *indirect or high frequency SE* – which consists of two phases; the induction of primary callogenesis in an initial medium and the regeneration of friable embryogenic callus masses in a second medium that enables the regeneration of thousands of somatic embryos (Berthouly and Michaux-Ferriere 1996). Indirect embryogenesis is the most used method because of its high yields in embryo production, which is why it has been adopted by numerous work teams for the multiplication of different coffee species and their varieties (Zamarripa et al. 1991; Van Boxtel and Berthouly 1996; Berthouly and Michaux-Ferriére 1996; Ducos et al. 2007). In Central America, CATIE in conjunction with CIRAD carried out research on *C. arabica* and its selected hybrids (Etienne and Bertrand 2001). In the context of scientific collaboration between 1996 and 2001, a pilot-scale production unit was established at CATIE with the objective of optimizing the process and developing a change of scale in the propagation of the new coffee materials (Etienne et al. 1999; Etienne et al. 2012). Taking as a reference the advantages of high frequency SE in the mass production of plants (Van Boxtel and Berthouly 1996), an original procedure was developed consisting of four culture phases - 1. a*cquisition of embryogenic calluses (EC)*; 2. *multiplication of EC in liquid medium (ECS)*; 3. *germination* and; 4. c*onversion of somatic embryos into plants – in liquid medium with temporary immersion, using RITA® containers* (Etienne et al. 1999). However, the most remarkable technical innovation was the development of two original culture systems - 1. *the production of somatic embryos in simplified RITA® bioreactors* (Berthouly et al. 1995; Etienne et al. 1997a); and - 2. *the direct planting of the somatic embryos obtained from bioreactors in the greenhouse for conversion into plants* (Etienne-Barry et al. 1999; Barry-Etienne et al. 2002; Etienne et al. 2013). This pilot production facilitated an evaluation of the risks and limitations of the process and determination of

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production costs with a view toward industrial-scale development (Etienne et al. 1999; Etienne et al. 2012). Training of technical personnel and technology transfer was done in the laboratories of the coffee institutes of the region; however, some of these laboratories failed to develop SE, while others only achieved this on a small scale.

At the beginning of the 2000s, CIRAD transferred the technology to a private company which established laboratories in Nicaragua and Mexico for commercial hybrid production (Etienne et al. 2012, 2013). Since then, this company has produced several million plants; however, despite high production volumes, the authors mention that the cost–benefit ratio of the process is not yet satisfactory, and that this can only be increased by an improvement in SE production and optimization. According to Etienne et al. (2012, 2013), conversion into plants is the main bottleneck in the implementation of this technology. Evaluation of 11 genotypes indicated that only 50% of the embryos regenerated plants and indicated a strong genotypic effect among the 11 genotypes.

The genetic conformation of the plants obtained by SE has also been the subject of research by a French team. They revealed that the frequency of abnormal, variant plants in the greenhouse is very low (0.1 to 0.2%), and other variants are only detectable at the field level (Menéndez-Yuffá et al. 2010). Other studies show that genetic and epigenetic alterations are limited, indicating that plants derived from ECS and secondary embryogenesis have high stability (Bobadilla et al. 2013; Bobadilla et al. 2015).

CATIE continued working on small-scale hybrid multiplication mainly to meet the Center's research and teaching needs and to supply small quantities of plants to local producers. Despite all the efforts made on different fronts, there are still technical and logistical factors that have limited the distribution and dissemination of the F1 hybrids in Central America for more than 10 years. Among these factors, SE is a methodology of long duration with many different phases, some more complex than others and difficult to carry out in laboratories dedicated to simpler micropropagation techniques. On the other hand, there is the challenge of changes of scale, impossible to carry out when one does not have control of the SE process. These drawbacks have meant that plant production has been limited and therefore the cost of the final product is very high. At the same time, the strategies used to promote, disseminate and market these materials in Central America might not have been the most appropriate.

More recently (2012-2013), during the last outbreak of coffee rust that affected 55% of the total area under cultivation in Central America, the regional institutions linked to coffee research and production activated their alert systems. About 300,000 farmers would need to renovate their plantations, which revived interest in F1 hybrids due to their good performance against leaf rust. CATIE began a new period of capacity-building to increase the multiplication of these materials and offer them at a better price to coffee producers. A two-phase multiplication strategy was developed: 1- *the regeneration of F1 hybrids by SE*; and 2- *the multiplication of the mother plants of the F1 hybrids from mini-cuttings rooted in the greenhouse* (Mesén and Jiménez 2016). This innovation allows the rapid and low cost multiplication of hybrids taking advantage of the juvenile trait of the mother plants produced by SE. The recent partnership of CATIE with the FJA HOLDING S.A. firm, which specializes in vegetative propagation on a commercial scale, allows the multiplication of hybrids on a large scale and the transfer of plants to the producers at a lower cost than if the plants came directly from the laboratory.

Multiplication of F1 hybrids by somatic embryogenesis.

From a practical point of view, this document divides coffee somatic embryogenesis into the five phases detailed below:

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Establishment of embryogenic cultures

The induction and proliferation of embryogenic calluses *(*EC) from immature coffee leaves is done according to the improved procedure of Van Boxtel and Berthouly (1996). During disinfection the leaves are washed with running water and soap, then placed in 50% (v/v) sodium hypochlorite 3% for 30 minutes followed by three washes with sterile distilled water. The leaves are sectioned into explants of about 1 cm which are inoculated with the adaxial surface in contact with the callus induction medium. After 30 days in the dark, explants with scar calluses are transferred to embryo induction medium to initiate the second stage under indirect light conditions; this medium shows significant variation in auxin and cytokinin concentrations, causing a hormonal shock that allows the evolution of a non-embryogenic primary callus into the EC (Etienne et al. 1999). This process varies among the different hybrids, with 'Centroamericano' as the most recalcitrant since its EC develops only in very small amounts after 8 to 10 months of culture, while in the other genotypes the occurrence of EC can occur in large quantities after 4 or 6 months of culture (**Fig. 1a**). One of the limitations of this EC is that it loses its optimal condition in a short time, so cryopreservation becomes necessary to conserve calluses or ECS of good quality or difficult to obtain, to allow routine management of these cultures and reduce losses due to deterioration.

Multiplication of embryogenic cell cultures.

EC multiplication is done in a liquid culture medium with the establishment of ECS (Van Boxtel and Berthouly 1996), to make better use of EC mainly in the case of the most recalcitrant hybrids ('Centroamericano'). ECSs are initiated by inoculating small amounts of EC into the six cavities of multi-well dishes containing 7 ml of liquid callus proliferation medium, or 50 ml Erlenmeyer flasks are used with 12.5 ml of the same medium. As cell volume increases, the culture is transferred to larger volume Erlenmeyer flasks to finally establish ECS in 250 ml containers with a cell volume of 1.5 ml PCV (Packed Cell Volume) in 50 ml of liquid proliferation medium. Throughout the entire process, the culture medium is renewed every 15 days; and at 30 days the finest aggregate filtrates are transferred to a larger Erlenmeyer flask. The cultures are kept on rotary shakers at a speed of 100 rpm under indirect light.

Regeneration of somatic embryos

Several alternatives have been used to regenerate coffee embryogenic cultures. Etienne et al. (1999) mention that during large-scale micropropagation, the recommended method for initiating ECS regeneration uses liquid medium in 100 to 250-ml Erlenmeyer flasks according to Van Boxtel and Berthouly (1996). However, with the development of the RITA® container (Alvard et al. 1993), the system was innovated with the direct transfer of 200 mg of ECS to the bioreactor with 200 ml of regeneration medium. The complete development of the embryo is achieved after 4 months of culture on development medium, with subcultures every two months and using an immersion frequency of two times per day for one minute. This allows regeneration, germination and conversion into plants in the same receptacle, simplifying the process and avoiding the use of a gel medium (Berthouly et al. 1995; Etienne et al. 1999). Using the same methodology at CATIE, we have observed high rates of hyperhydricity, asynchrony and malformations of the embryos mainly in the 'Centroamericano' hybrid. In order to reduce these drawbacks, we have alternatively implemented the regeneration phase in semi-solid medium. Small portions of EC are transferred to YASUDA medium (Yasuda et al. 1985), in Petri dishes and kept under indirect light. With many of the EC, hundreds of thousands of very good quality embryos are regenerated after 2 to 2.5 months of culture (**Fig. 1c-d**). Some hybrids such as 'Milenio' or 'Esperanza' produce large masses of EC in the embryo induction medium, so we consider the establishment of ECS unnecessary; this reduces a possible source of variability because of the reduction in time the culture spends in suspension.

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Germination of somatic embryos and conversion into plants.

At CATIE, germination of coffee somatic embryos and their conversion into plants is normally carried out in RITA® bioreactors after regeneration in semi-solid medium according to the aforementioned procedure. A density of 1.25 g of regenerated embryos per container is used with 200 ml of development medium with two one-minute immersions per day. However, despite the initial control of culture density, some tissue masses must be divided after 2 or 3 months to reduce density and favor embryo development. Many advantages are attributed to the use of RITA® containers during coffee SE, since they facilitate handling during different phases, large numbers of embryos are accommodated in a single container, and the use of liquid medium is less labor intensive and reduces production costs (Berthouly et al. 1995; Etienne et al. 1999). However, in practical terms, certain limitations are often observed in the system, mainly the vulnerability of the cultures to hyperhydricity causing the loss of numerous embryos. In order to reduce this drawback, we made changes to immersion times and frequencies. For the 'Milenio' hybrid, we compared the control condition of 2 immersions per day for 1 minute (every 12 hours) with the use of one daily immersion; and 1 and 2 immersions per day for 1 minute every two days. At 5 months of culture, we observed the highest number of plants in good condition in cultures subjected to 1 or 2 immersions every two days. Cultures with 1 or 2 daily immersions had the highest number of malformed, hyperhydric and dead embryos. According to these results, the two best treatments were evaluated in the 'Centroamericano', 'Milenio' and 'Esperanza' hybrids using three RITA® containers per treatment (**Tab. 1**).

Table 1. Number of individuals of 'Centroamericano', 'Milenio' and 'Esperanza' hybrids that achieved conversion into plants and number of hyperhydric individuals following culture at different temporary immersion frequencies in RITA®.

Treatments*	Sample (gr)	Centroamericano'	Milenio'	Esperanza'
		Plants**		
2 Imm/Day/1Min	10.3	12.0 c	35.0 b	40.0 b
1 Imm/2 Day/1Min	10.0	62.5 a	30.0 b	35.0 b
2 Imm/2 Day/1Min	9.6	73.5 a	37.5 b	45.0 b
		Hyperhidricity**		
2 Imm/Day/1Min	10.3	259.0 a	169.5 b	138.0 b
1 Imm/2 Day/1Min	10.0	146.5 b	163.0 b	122.0 b
2 Imm/2 Day/1Min	9.6	140.0 b	144.5 b	55.5 c

*Number of immersions daily /or every two days
**Treatments with letters in common are not significantly different (p > 0.05)

The 'Centroamericano' hybrid produced significantly more uniform plants and lower hyperhydricity by using immersions every two days (**Fig. 1f-g**). The 'Milenio' and 'Esperanza' hybrids did not show significant differences between treatments, however the greatest number of plants was achieved with 2 daily immersions every two days. It was observed that when immersion frequency is reduced, plant growth is slower but hyperhydricity is less and secondary embryogenesis is reduced. Therefore, the increase in biomass is visually inferior, favoring synchronization in development and the conversion of embryos into plants. These results indicate that two daily immersions may be excessive since it appears to cause the high

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hyperhydricity observed (**Fig. 1e**). The observed disadvantages coupled with the high price of RITA® units (≥ 57 €) and the cost of installation of the system may be limiting factors for many laboratories in the region.

In order to reduce the impact of these limiting factors, an alternative method of conversion into plants was put into practice in semi-solid medium to obtain plants rapidly without excessive cost. In this case, 1.25g of regenerated embryos were pre-germinated in a 250-ml Erlenmeyer flask, containing 50 ml of liquid development medium and cultured in the dark on a rotary shaker (100 rpm) for 15 days. As a result, elongated embryos were obtained with evident cotyledons, some roots and a strong pink coloration (**Fig. 1h**). Subsequently, pre-germinated embryos were rapidly transferred by spatula to culture dishes containing 75 ml of semi-solid development medium and kept under a 12 hr light/12 hr dark photoperiod for 8 or 10 weeks. This process has allowed us to generate 50 good quality plants with root per dish on average in only two steps without excessive handling (**Fig. 1i-j**).

Acclimatization of plants in the greenhouse.

The direct planting in horticultural substrate of embryos germinated in RITA® bioreactors was developed (Etienne-Barry et al. 1999) and evaluated (Barry-Etienne et al. 2002) to simplify the process and reduce production costs associated with the plant conversion phase under *in vitro* conditions. At CATIE, the acclimatization of germinated embryos as well as plants with at least one pair of true leaves was put into practice. In the case of germinated embryos, many remained in this state and never developed ('Centroamericano'), or they showed mortality above 40% for all the hybrids, similar to what was found by Etienne-Barry et al. (1999). The selection of plants with at least one pair of leaves (**Fig. 1k**), and improvement of the acclimatization protocol enabled survival to rise from 45-50% to 75-85% or more, depending on the hybrid. Acclimatization is carried out in plastic trays with a peat moss substrate (Canadian sphagnum 65%, horticultural perlite, horticultural vermiculite) mixed with Osmocote slow release fertilizer (14-14-14). The trays are placed in the greenhouse inside plastic tunnels with a relative humidity close to 100%. An irrigation regimen of 15 seconds, 3 or 4 times per day is used, depending on weather conditions. Fertilizers and pesticides for pest control are applied regularly. After 4 months in the greenhouse the plants are acclimated and ready for the horticultural multiplication stage by rooting of cuttings (**Fig. 1l-m**).

Mass multiplication of F1 hybrids by rooting of mini-cuttings in the greenhouse.

In order to facilitate the transfer of F1 hybrids to coffee producers, the Forest Seed Bank at CATIE developed an alternative process for rapid hybrid multiplication by rooting mini-cuttings (Mesén and Jiménez 2016). The plants produced by SE (**Fig. 2a**) are used as mother plants for the establishment of clonal gardens in greenhouses to generate shoots by successive pruning (**Fig. 2b**). However, success in the continuous production of shoots is due to the juvenile trait of the mother plants produced by SE in the laboratory, which can be used as cutting producers for more than two years (**Fig. 2c**). This complementary method allows rapid hybrid multiplication and considerably reduces the unit cost per plant produced under horticultural conditions (**Fig. 2d-g**). If both techniques are properly implemented, they can be used to generate beds of plantlets for plantations (Mesén and Jiménez 2016). The partnership with the Costa Rican company FJA HOLDING S.A., which specializes in vegetative propagation on an industrial scale, allows the mass production of F1 hybrids by rooted cuttings and the marketing of plants to producers at a lower cost (≤ $0.75) than if the plants come directly from the laboratory. This company has developed a three-year pilot production plan that began in 2016 with the multiplication of 240,000 plants; 640,000 for 2017, and a projected production of 1,000,000 plants for 2018 (Altmann 2016, personal communication).

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Figure 1. Somatic embryogenesis in F1 hybrids of *Coffea arabica*. a. Embryogenic callus. b. Embryogenic cell suspension. c. Regeneration of ECS in RITA®. d. Regeneration of EC in semi-solid medium. e. RITA® culture with 2 immersions per day for 1 minute. f. RITA® culture with 2 immersions per day for 1 minute every two days. g. RITA® culture with 1 immersions per day for 1 minute every two days. h. Pregermination of regenerated embryos. i. Embryo germination and plant conversion in semi-solid medium. j. Roots of plants in semisolid medium. k-l. Acclimatization of plants from somatic embryogenesis. m. Plants 4 months after acclimatization.

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Figure 2. Coffee F1 hybrids from the laboratory to the field. a. Plants from SE were used as mother plants. b. Clonal garden of mother plants that produce cuttings. c. Rooting of mini-cuttings. d. Development of plants for distribution to farmers. Photos FJA HOLDING S.A.

Mother plant production from *T. grandis* and *G. arborea* superior clones by micropropagation

Micropropagation of T. grandis (teak)

For teak, large-scale production *in vitro* has been based on the culture of axillary buds due to the high sustainability of the culture and genotypic fidelity compared to *de novo* procedures; to date, millions of plants have been propagated this way (Goh and Monteuuis 2016). We have established and improved a protocol using different publications on the culture of axillary buds and apices as references (Gupta et al. 1980; Sunitibala et al. 1994; Monteuuis et al. 1998; Daquinta et al. 2001; Rojas and Abdelnour-Esquivel 2012; Mendoza et al. 2007; Quiala et al. 2011). Our work is based on clones from the Hojancha Canton Agricultural Center in the province of Guanacaste, Costa Rica.

Initial culture

For the introduction *in vitro* we use apices harvested from mother plants in the greenhouse, which are subjected to a double disinfection with calcium hypochlorite at 10% and at 8% for 20 and 15 minutes respectively, followed by three rinses with sterile distilled water. Initially, the complete MS culture medium

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is used with 30 g of sucrose and supplemented with 0.2 mg/l of Kinetin and 0.1 mg/l of Indoleacetic acid (IAA). Agar (SIGMA) is used as a gelling agent and the pH is adjusted to 5.7.

Multiplication in semi-solid medium

In the multiplication phase the MS medium was supplemented with 0.5 mg/l of N6 benzyladenine (BA) and 0.5 mg/l of Kinetin. This medium allowed the development of numerous shoots, but with excessive callus formation at the base and in some cases much hyperhydricity, mainly in liquid culture with temporary immersion. In order to improve these conditions, the reduction of the cytokinin source led us to use 0.05 mg/l BA during the multiplication phase, obtaining a lower number of shoots but of better quality than those previously obtained. Although most publications on teak (Daquinta et al. 2001; Mendoza et al. 2007; Rojas and Abdelnour-Esquivel 2012; Singh and Mishra 2016) mention the use of high concentrations of BA (0.5 to 2.5 mg/l), alone or in combination with other regulators, they do not indicate the formation of hyperhydric shoots. In order to make better use of the initial explant, it was reused several times to produce several crops of shoots, according to the methodology used by Lardet et al. (1998) in *Hevea brasiliensis*. The base of the primary nodal explant was transferred to fresh medium (subculture of the primary nodal explant) to produce a new generation of shoots, taking advantage of the axillary buds at the base. In addition, shoots from the primary nodal explant were used as secondary nodal explants to develop a new cycle of multiplication, or they were used in rooting and acclimatization. This method has allowed the development of a simplified protocol that enables plant production in a simple and efficient way, avoiding contamination from new introductions of explants and facilitating handling by having an *in vitro* stock of mother plants for producing shoots (**Fig. 3**).
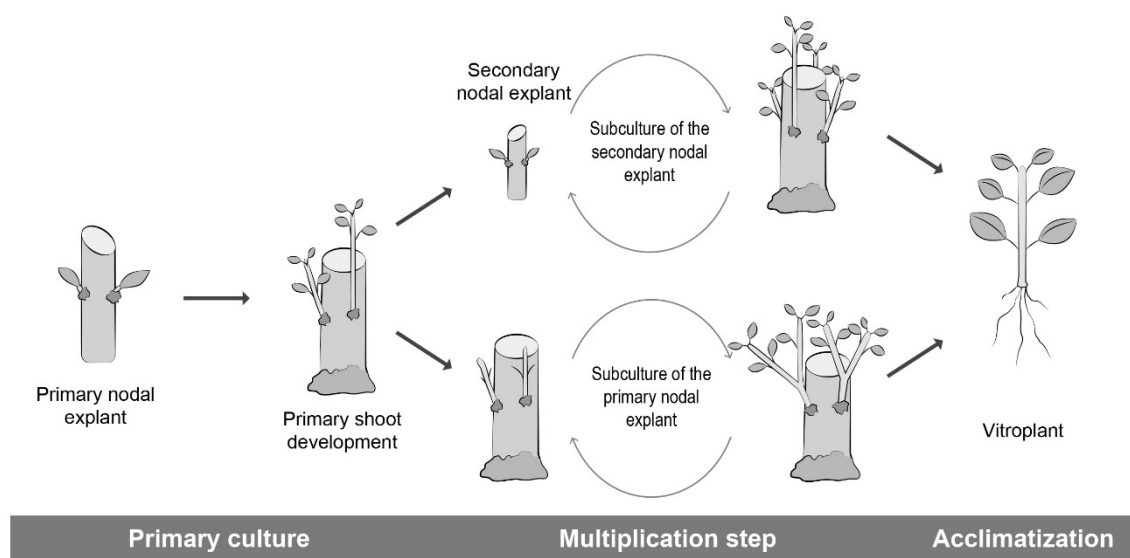
Figure 3. Multiplication of teak including reuse of the primary explant and the subculture of primary and secondary nodal explants

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The primary nodal explant was cultured for three cycles of 30 days each in jars containing 3 explants in 20 ml of semi-solid multiplication medium supplemented with 0.05 mg/l of BA. Cultures were kept in a photoperiod of 12 hours light and 12 hours dark at a temperature of $27 \pm 2°$ C. The number of shoots increased with each culture cycle, being significant in the third cycle where they tripled (4.5) with respect to the first cycle (1.5). However, shoot length was lower in the third subculture as the number of shoots produced increased. This assay was replicated using a 0.5-liter vessel with 100 ml of semi-solid multiplication medium. The number of explants per recipient was evaluated for R3= 3 explants, R4= 4 explants and R5= 5 explants during three cultures lasting 30-days (**Fig. 4**).
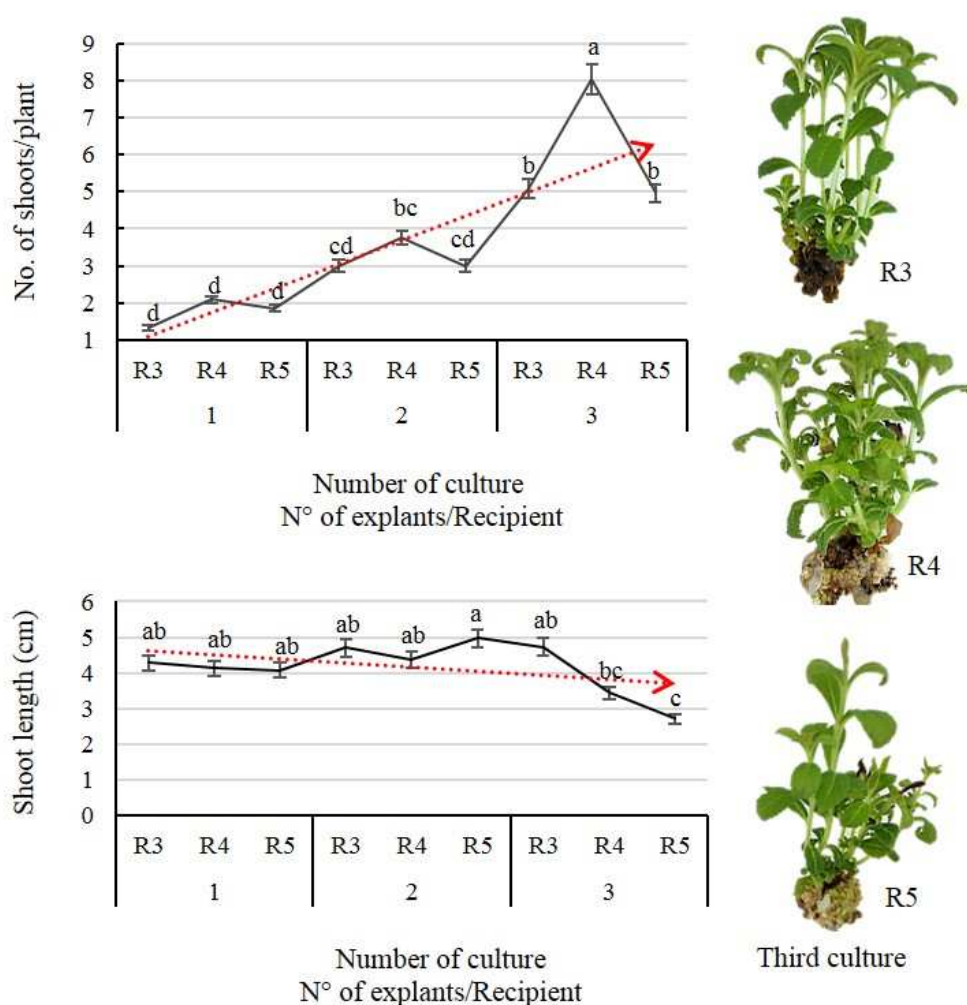
Figure 4 Number and average length of shoots produced during three subcultures from the primary nodal explant cultured in semi-solid medium. R3, R4 and R5 correspond to the number of primary nodal explants for each recipient. Means with the same letter are not significantly different (LSD Fisher; p≤ 0.05)

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The production of shoots per explant showed exponential growth in each subculture; however, the highest number of shoots was observed in R4 in the three cultures, being highly significant in the third culture (8.1); the R3 and R5 treatments did not show significant differences. Monteuuis et al. (1998) mentioned average coefficients of 3 in the multiplication of teak working with thousands of microcuttings of different geographic and genetic origins. We observed that shoot length is fairly homogeneous in all treatments, except for the third subculture where R4 and R5 showed the highest numbers of shoots, but of shorter length. The best condition in terms of shoot production yield and shoot quality is undoubtedly the R4 condition with the highest number of shoots larger than 3 cm. Possibly, the larger size of the containers improved culture atmosphere and the development of shoots when adequate density was used. Some R5 shoots showed growth in rosettes and scorched leaves, possibly due to overcrowding inside the recipient given the number of explants and their respective shoots. We also observed strong callus formation at the base of the explant, mainly in the third subculture. A negative correlation between teak shoot length and basal callus formation was observed by Mendoza et al. (2007); other authors also mention callus formation associated with high concentrations of BA in semi-solid medium (Rojas and Abdelnour-Esquivel 2012; Singh and Mishra 2016).

Multiplication in RITA® temporary immersion bioreactors

Liquid medium has been recommended as ideal for automated culture and cost reduction during micropropagation (Aitken-Christie et al. 1995). However, the disadvantages associated with liquid media such as plant asphyxiation and hyperhydricity under these conditions can be avoided by using temporary immersion (Etienne and Berthouly 2002; Escalona et al. 2003). Few forest species, including *Pinus radiata* (Aitken-Christie and Jones 1987), *Hevea brasiliensis* (Etienne et al. 1997b), *Crescentia cujete* (Murch et al. 2004) and *Eucalyptus* (McAlister et al. 2005) have been propagated in temporary immersion culture systems. The only work on culture by temporary immersion of teak reported to date (Quiala et al. 2011) was done using the TIS system described by Escalona (1999), but using 2-liter glass vessels. In teak, the unpublished results obtained in our laboratory with RITA® containers using 0.5 mg/l BA and Kinetin determined the suitable number of explants and the high multiplication rate; however, this was accompanied by high rates of hyperhydricity. Quiala et al. (2011) obtained similar results in a culture container at high concentrations of BA. Using histochemical analysis of the shoots, they found that high concentrations of BA (6.66 μM) contributed to a lower accumulation of phenolic compounds and lignin deposition in shoot vascular tissue; in addition, the leaves of these shoots showed deformed stomata and broken epidermal layers in guard cells, typical of hyperhydric leaves. The duration and frequency of immersion is the most decisive parameter for system efficiency, in addition to optimization of nutrient medium volume, especially during shoot proliferation (Etienne and Berthouly 2002). In this regard, we have reduced the concentration of BA to 0.05 mg/l; and we have also evaluated 1 and 2 daily immersions, or 1 and 2 immersions every two days for 1 minute. In each RITA®, 200 ml of liquid medium was used with three replicas of 30 nodal explants per container. An evaluation was made after three weeks of culture. Significant differences were observed between the immersion frequencies used (**Tab. 2**).

The highest average number of shoots per explant was achieved when 1 (3.3) and 2 immersions per day (3.2) were used; however, the highest shoot hyperhydricity (26 and 29%) was also observed with these cycles (**Fig. 5a-b**). With immersions every two days, the number of shoots was somewhat lowered (2.3 and 1.7) and hyperhydricity was significantly reduced to 7% (**Fig. 5c-d**). These results are difficult to compare with those obtained by Quiala et al. (2011) because they used a TIS culture system other than RITA® containers, in addition to other culture conditions and parameters that differed from ours. In *Crescentia cujete* (Murch et al. 2004) and *Eucalyptus* (McAlister et al. 2005), although the investigators used very frequent immersions and prolonged immersion times of 3 minutes every 3 hours, and 30 seconds every 10 minutes respectively, they did not report hyperhydricity in the shoots.

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Table 2. Effect of the immersion frequency on the production and the quality of
the teak shoots in temporal immersion culture in RITA© containers.

Treatment	Budbreak (%)	No. of shoots	Shoot length (cm)	Hyperhydricity (%)
1 imm/day/1 min	100.0 a	3.3 a	2.2 b	29.0 a
2 imm/day/1 min	96.0 b	3.2 a	3.4 a	26.0 a
1 imm/2 day/1 min	100.0 a	2.3 b	3.7 a	7.0 b
2 imm/2 day/1 min	100.0 a	1.7 c	2.2 b	7.0 b

Means with the same letter are not significantly different (LSD Fisher; $p \leq 0.05$).

Figure 5. Teak shoots produced in RITA® with different immersion frequencies and their acclimatization. a. Shoots produced at 2 daily immersions 1 minute (Control). b. Hyperhydric shoots (Control). c-d. Shoots produced at 1 immersion every two days for 1 minute. e. Shoot used for *ex vitro* rooting. f-g. Rooting of shoots. h. Acclimated shoots.

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In our case, by using a low concentration of BA (0.05 mg/l) and a single one-minute immersion every 2 days, we reduced hyperhydricity and produced more than 60 shoots per RITA® container that were approximately 4 cm in length with good morphology and synchronized development (**Fig. 5c-d**).

Rooting and acclimatization

Initially rooting was done *in vitro* using 2 cm shoots cultured in MS 1/2 medium, with 30 g of sucrose, 1 mg/l Indolebutyric acid (IBA), at a pH of 5.7, and 2 g/l of Phytagel (SIGMA) as the gelling agent. Subsequently shoots approximately 2 cm in length from semi-solid culture medium or liquid in temporary immersion (RITA®) were taken to the greenhouse and planted in trays containing peat moss as a substrate. As a rooting agent, the commercial hormone MAGIC ROOT (EVER GREEN) powder was used, containing 0.3% IBA (w/w). High relative humidity and regular fertilizations were applied during acclimatization and most shoots from semi-solid medium developed some type of root and survived acclimatization (79%). For shoots from liquid medium in RITA® container, the best survival was 71.6% in shoots exposed to one immersion every 2 days, which clearly corresponds to the treatment that gave rise to better quality shoots during multiplication. Acclimated plants showed good adventitious root systems (**Fig. 5g**). Daquinta et al. (2001) achieved 92.5% *ex vitro* rooting, using rooting powder with 1000 mg/l IBA and NAA, while Monteuuis et al. (1998) and Goh and Monteuuis (2016) reported 90% acclimatization of plants with or without *in vitro* rooting, indicating that the physiologically rejuvenated material does not require hormone application if environmental conditions are adequate during acclimatization. Quiala et al. (2011) observed that a high concentration of BA affected the survival of teak plants *ex vitro* during acclimatization, possibly associated with the decrease in rooting rates at increased BA doses. For *Eucalyptus* clones the *ex vitro* rooting of plantlets grown in RITA® containers was much better than in plants grown on solid media (McAlister et al. 2005). We consider that there is a direct impact between the quality of the shoots produced in RITA® containers and success during acclimatization. However, once the plants survived acclimatization, their development was very similar regardless of the previous treatments. Goh and Monteuuis (2016) mention that protocol simplicity reduces plant production costs and ensures reasonable selling prices for quality plantation renovation material.

Micropropagation of Gmelina

G.arborea is a commercially important exotic species that is widely cultivated in Costa Rica (Valverde-Cerdas et al. 2004). Although this species can be propagated by seeds, cuttings and grafting, these techniques have not been sufficiently effective to meet the demand for clonal material in industrial plantations (Kannan and Jasrai 1996; Sukartiningsih et al. 1999; Romero 2004). Micropropagation can greatly reduce the time for the multiplication of clones necessary for the establishment of clonal gardens or field trials (Romero 2004). Most publications about this species refer to the use of seeds (Sukartiningsih et al. 1999; Gamboa and Abdelnour 1999; Valverde-Cerdas et al. 2004) and very few publications are related to explants from older trees (Kannan and Jasrai 1996; Suarez et al. 2013).

The genetic improvement program of CATIE's Forest Seed Bank has developed *Gmelina* materials with high genetic quality, which are certified by the National Seed Office of Costa Rica; these have performed well in plantations at the international level (www.catie.ac.cr/productos-y-servicios/banco-de-semillas-forestales). Clones of plus trees selected in a 26-month old plantation grown from superior CATIE seeds were used for this work. Although this species responds well to micropropagation, we have observed marked clonal differences and to date, of the nine clones used only four performed well. Different genotypic responses of *Gmelina* during micropropagation were also reported by Valverde-Cerdas et al. (2004) and Suárez et al. (2013).

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Initial culture

We start with the culture of apices from mother plants in the greenhouse. The disinfection method used is the same as the one described for teak. Initial culture was done for 30 days in the complete MS medium with 0.2 mg/l of BA, 30 g of sucrose and 0.7% agar as a gelling agent, and the pH was adjusted to 5.7. The photoperiod consisted of 12 hours light and 12 hours dark at a temperature of $27 \pm 2°$ C in the culture room.

Multiplication in semi-solid medium

Multiplication was done by culturing nodal explants in the same MS medium as mentioned above, but enriched with 0.5 mg/l BA. We observed that when using BA concentrations of 0.5 - 0.25 - 0.125 mg/l during the multiplication of clone G5, a large number of shoots with high percentages of hyperhydricity were produced and large masses of callus formed at the base of the explants, which limited the use of the shoots. Other studies have shown the positive effect of the use of similar BA concentrations on the formation of *Gmelina* shoots (Kannan and Jasrai 1996; Gamboa and Abdelnour 1999; Suárez et al. 2013). However, Kannan and Jasrai (1996); and Sukartiningsih et al. (1999) also reported pronounced callus formation at the base of the shoots at high concentrations of BA to the detriment of shoot quality. Gamboa and Abdelnour (1999) also produced 29% hyperhydric shoots using 1 mg/l BA, similar to the 30% obtained when we used 0.5 mg/l BA. By using proportionally lower BA concentrations (0 - 0.03 - 0.06 mg/l) with the same G5 clone, we were able to reduce this problem without significantly affecting shoot production. As for teak, we sub-cultured the primary explant to generate new shoots from axillary buds at the explant base. In both primary and secondary cultures (**Tab. 3**), the average number of shoots increased proportionally as the BA concentration increased, at the expense of shoot length, which was shorter but without any loss of quality; shoots larger than 3 cm in height enable the continuation of a new cycle of multiplication or they can be subjected to acclimatization. Large callus masses (** / ***) were formed proportionally as a function of BA concentration and the absence of BA showed the lowest formation of callus at the base of the explants. **Fig. 6a-c** show the shoots produced from secondary explants that were cultured in a second multiplication cycle.

Table 3. Production of shoots in primary and secondary nodal explants of *Gmelina* cultured in different concentrations of BA.

BAP (mg/l)	No. of shoots / explant	Shoot length (cm)	Callus formation (%)		
			(*)	(**)	(***)
Primary culture					
0	2.4 b	3.52 a	20.0 a	0.0 b	0.0 b
0.03	3.17 a	4.74 a	10.0 ab	5.0 b	11.7 a
0.06	3.0 ab	4.49 a	5.0 b	20.0 a	5.0 ab
Secondary culture					
0	2.2 b	3.1 a	13.0 a	8.0 a	0.0 c
0.03	2.8 ab	3.3 a	5.0 ab	17.0 b	5.0 b
0.06	4.0 a	3.4 a	1.7 b	10.0 a	16.7 a

Means with the same letter are not significantly different (LSD Fisher; p≤ 0.05).

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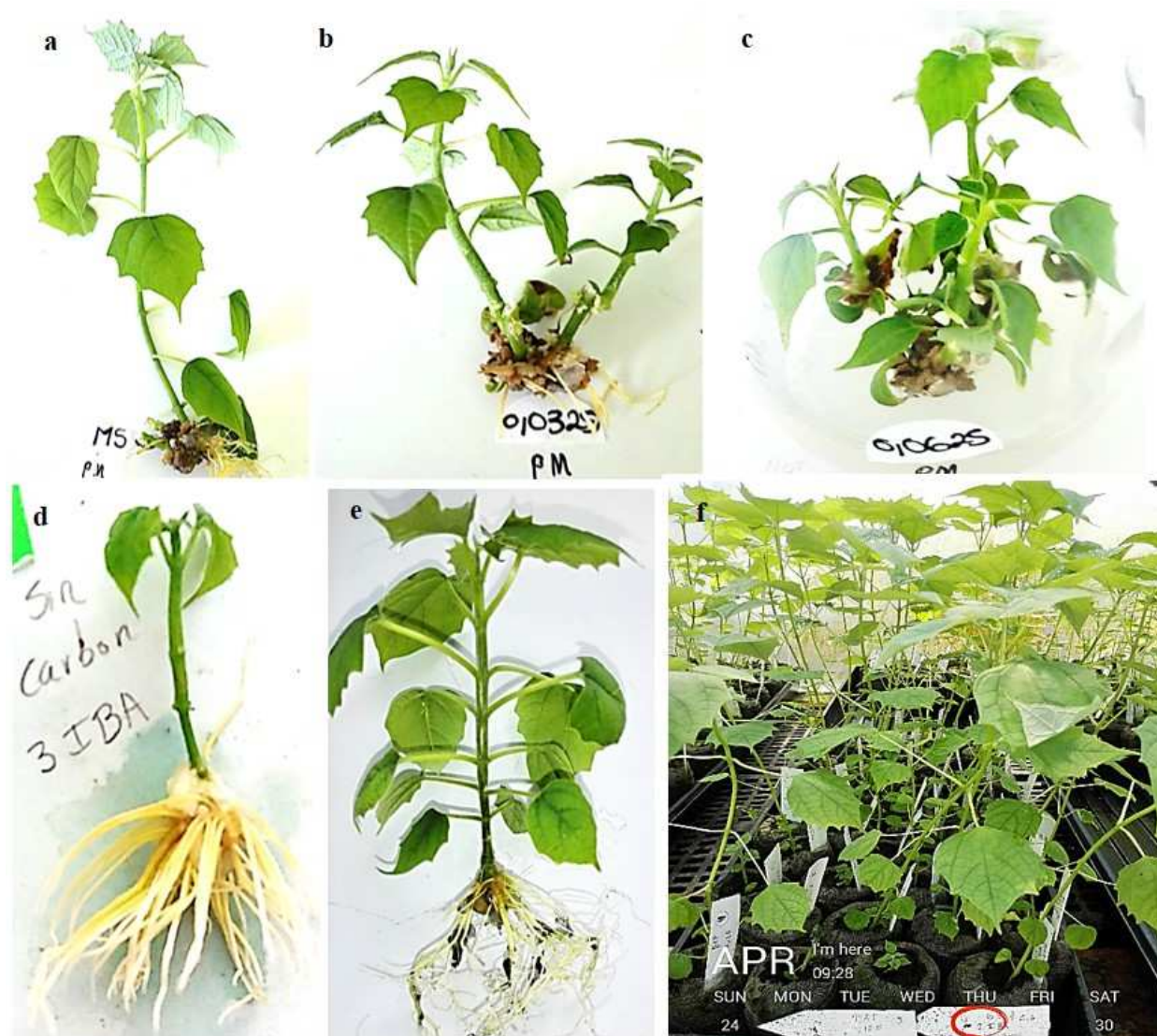
Figure 6. Multiplication, rooting and acclimatization of secondary shoots of *Gmelina*. a. Shoots without BA. b. Shoots with 0.03 mg/l BA. c. Shoots with 0.06 mg/l BA. d. Rooting with 3 mg/l IBA, without activated charcoal (AC). e. Rooting with 1mg/l IBA, with AC. f. Shoots acclimatization.

In vitro *rooting*

For rooting, shoots 2 cm in length were inoculated into semi-solid MS 1/2 medium containing 1 and 3 mg/l IBA, 20 g sucrose and 7 g agar, as recommended by Valverde-Cerdas et al. (2004). In our case, after 6 days in the root induction medium, some of the shoots were transferred to another medium with 1 g of activated charcoal (AC) and no IBA, other shoots remained in the induction medium until root formation, and a third group was transferred to pellets (Jiffy) placed on plastic trays and covered with a layer of plastic to maintain high relative humidity. Each treatment consisted of three replicas of 25 shoots each. All treatments

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remained in the culture room for two weeks and were then transferred to the greenhouse; after 8 days the plastic layer was removed and the fertilization and irrigation cycle started. After 6 days of induction all the shoots showed scar callus formation at the base. By 45 days after transplant, almost 100% of the shoots had formed roots (**Tab. 4**). The lowest and highest numbers of roots were observed with 1 and 3 mg/l of IBA without activated charcoal (the induction medium); however, these roots were very thick, brittle, and had large masses of callus at the base of the explant (**Fig. 6d**). In contrast, the best root quality was observed in shoots transferred to the medium with activated charcoal and without IBA (**Fig. 6e**). Possibly the activated charcoal absorbed remnant auxin present at the base of the microcutting. The shoots kept in the induction medium with IBA probably were exposed to toxicity leading to abnormal callus development, root malformation and premature leaf drop, conditions that were more intense when 3 mg/l IBA was used. Valverde-Cerdas et al. (2004) observed that different exposure times of stems to auxin affected rooting ability whether solid or liquid medium was used.

Table 4 Rooting percentage, average number and length of roots in *Gmelina* shoots subject to different rooting treatments. AC: 1 g activated charcoal.

IBA (mg/l)	Rooting (%)	No. of roots	Root length (cm)
1	96.0 b	3.1 c	2.8 b
1 + AC	100.0 a	4.4 bc	6.2 a
3	100.0 a	10.7 a	2.9 b
3 + AC	100.0 a	5.7 b	5.9 a

Means with the same letter are not significantly different (LSD Fisher; $p \leq 0.05$).

Acclimatization

During acclimatization, the impact of the rooting treatments used was evident. Survival during acclimatization (**Fig. 6f**) was higher when 1 mg/l IBA was used during induction with activated charcoal (83%) in the subculture medium (IBA-1 /AC). Similarly, the survival of shoots transferred to pellets (IBA-1 /P; IBA-3 /P) was higher (67%) than those rooted in the same induction medium (38%). These results indicate that the rooting of *Gmelina* shoots can be induced *in vitro* at low auxin concentration in a few days, followed by planting in pellets or substrate in the greenhouse. Sukartiningsih et al. (1999) observed that high (1-2 mg/l) and low concentrations of IBA (0.02-0.2 mg/l), alone or in combination with NAA (0.02 mg/l) in the culture medium favored root development. Other studies found 80% rooting at similar concentrations of IBA (4.9 μM) in semi-solid MS ½ medium (Sen et al. 1992; Kannan and Jasrai 1996), and 22 to 50% in the absence of auxin respectively (Suárez et al. 2013; Gamboa and Abdelnour 1999). Kannan and Jasrai (1996) also observed that the induction of roots *in vitro* with a high concentration of auxin (9.8 μM) caused senescence of the shoots and leaf fall. Valverde-Cerdas et al. (2004) mentioned that a combined system of controlled *in vitro* conditions and sterile pellets during rooting ensures the survival of the plants during acclimatization. Recently we evaluated the harvest of shoots *in vitro* and direct rooting in the greenhouse according to the protocol described for teak. More than 90% of the shoots survived acclimatization.

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Conclusions

Although coffee somatic embryogenesis under our conditions still presents important technical limitations in different phases of the process and further study is required, the strategy presented here offers good expectations for increasing the scale of multiplication and producing plants at low cost to meet the demand for quality genetic material needed for the renovation of Central America's coffee plantations.

Although teak and Gmelina are species that respond well to micropropagation, they also present limitations when propagating different genotypes or increasing the scale of multiplication. However, a simplified protocol combining *in vitro* multiplication with rooting and simultaneous acclimatization in the greenhouse could be highly useful in genetic improvement programs for the reinvigoration, disease cleansing, and renovation of mother plants in clonal gardens.

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Induction of somatic embryogenesis in explants derived from axillary shoot cultures established from adult holm oak tree

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Abstract

The successful induction of somatic embryogenesis in shoot apex and leaf explants derived from axillary shoot cultures established from two mature *Quercus ilex* L. trees is, for the first time, reported. The embryogenic response was strongly influenced by explant type, auxin and genotype. Shoot apex explants displayed a higher embryogenic capacity than leaf explants. In the latter, somatic embryo initiation was only achieved on Murashige and Skoog (1962) medium (MS) without plant growth regulators or on MS medium supplemented with 4 mg/l naphthaleneacetic acid (NAA) plus 0.5 mg/l benzyladenine (BA). In apex explants, the effect of the type of auxin was closely related to the genotype. The highest induction rate was achieved with apex explants of genotype Q3 and medium containing 4 mg/l NAA and 0.5 mg/l BA, in which 11% of the shoot apices yielded somatic embryos or embryogenic structures. Conversely, in genotype Q10 the highest induction rate (8%) was obtained in apex explants cultured on 4 mg/l indoleacetic acid in combination with 0.5 mg/l BA, although an embryogenic response was observed in all treatments evaluated in this genotype. The embryogenic capacity was maintained by repetitive somatic embryogenesis through subculture of proembryogenic masses, at 6-week intervals, on Schenk and Hildebrandt medium (1972) without plant growth regulators. For plantlet conversion, somatic embryos were stored for 2 months at 4ºC before being transferred to germination medium consisting of Gresshoff and Doy medium (1972) supplemented with 0.1 mg/l BA and 20 μM silver thiosulphate.

Keywords: adult trees, apex explants, IAA, leaf explants, NAA, *Quercus ilex*

Introduction

Quercus ilex L. (holm oak) is one of the most important *Quercus* species in the Mediterranean area as regards the area of land occupied, economic interest, ecological and landscape value, and rusticity. *Quercus ilex* together with *Q. suber* are the main constituents of the Spanish "dehesas", considered the most widespread agroforestry system in Europe, covering a total area of 3.1 million hectares (Moreno and Pulido 2009). Holm oak acorns provide a rich source of nutrients for foraging animals, especially Iberian pigs (whose meat is the basis of a high-quality food industry) as well as sheep, goats, cattle, horses and fighting bulls (Cañellas et al. 2007). The ectomycorrhizal fungus *Tuber melanosporum* develops in a symbiotic relationship with holm oak roots, producing the black truffle, one of the most expensive edible mushrooms in the world due to its use in international *haute cuisine* (Reyna-Domenech and García-Barreda 2009). In addition to these traditional uses, holm oak woodland (*dehesa*) provides ecosystem services of increasing interest, such as recreational opportunities, carbon storage and wildlife habitat.

Over the last few decades, millions of oak trees have been affected by a complex disease known as oak decline, oak dieback or oak mortality. In the "dehesas", this disease is called "la seca" and refers to the

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decline and death of individual specimens of *Quercus suber* and *Quercus ilex*. Studies carried out by Portuguese and Spanish research groups have identified *Phytophthora cinnamomi* Rands as the main biotic agent of the disease (Pérez-Sierra et al. 2013).

Holm oak is considered very recalcitrant to traditional propagation methods. Difficulties in propagation are caused by the irregular acorn production (ranging from 79.3-469.6 kg acorns/ha), the difficulty in germinating, dispersing and conserving seeds and the absence of effective conventional vegetative propagation methods, mainly in adult trees. Although *in vitro* culture may represent an alternative solution to conventional propagation, micropropagation by using axillary or adventitious buds has not yet been reported in mature holm oak (Vieitez et al. 2012). Somatic embryogenesis (SE) may offer many advantages over organogenesis as it provides a more achievable means of clonal propagation, cryopreservation, genetic transformation or large-scale production in bioreactors (Corredoira et al. 2006; Guan et al. 2016; Bonga 2017). SE has been induced from immature zygotic embryos of holm oak (Mauri and Manzanera 2003), from catkins collected from adult holm oak trees (Blasco et al. 2013), and from developing ovules (Barra-Jiménez et al. 2014). However, robust SE protocols are required not only to micropropagate selected genotypes that have good attributes such as high fruit production, quality of wood or natural resistance to "la seca" and that can also be applied in genetic transformation studies for the introduction of genes conferring resistance to the disease. There is an evident need to carry out further research aimed at the successful micropropagation of this species, particularly to develop regeneration protocols involving the induction of somatic embryos from non-embryogenic material. Future holm oak improvement programmes should consider the introduction of genes conferring resistance to "la seca". However, appropriate embryogenic regeneration systems are first required as SE is considered the most efficient means of regeneration and application of genetic transformation.

For all of the above-mentioned reasons, the main objective of the present study was to develop a reliable procedure for inducing SE in holm oak adult trees. We specifically investigated the potential use, for this purpose, of leaf and shoot apex explants excised from *in vitro* axillary shoot proliferation cultures established from mature trees.

Material and methods

Plant material

Axillary shoot cultures were established *in vitro* from shoot tips and nodal explants excised from forced shoots originating from branches of two centenary *Quercus ilex* trees (denominated Q3 and Q10). Shoot cultures were initiated and clonal axillary shoots were proliferated following previously described procedures (Martínez et al. 2017). Briefly, forced shoots were stripped of all leaves and surface sterilized by immersion in a 0.3% (w/v) solution of free chlorine (Millipore® chlorine tablets) for 3 min. The shoots were then rinsed three times in sterile water for 10 min. Shoot tips and nodal explants (1 cm long) were cut from the disinfected shoots and used to initiate *in vitro* cultures on shoot initiation medium consisting of Woody Plant Medium (WPM, Lloyd and McCown 1981) supplemented with 0.5 mg/l benzyladenine (BA), 3% (w/v) sucrose and 0.65% (w/v) Vitroagar (Pronadisa, Spain). The explants were maintained in the same medium with transfer to fresh medium every 2 weeks during the 6 week culture period. The explants were then transferred to initiation medium in which the concentration of BA was reduced to 0.2 mg/l.

After 8 weeks from the start of culture, new shoots longer than 0.5 cm were excised from the original explants and cultured vertically in 500 ml glass jars containing 70 ml of shoot proliferation medium. This medium consisted of WPM supplemented with 3% (w/v) sucrose, 0.8% (w/v) Sigma agar (A-1296, Sigma-Aldrich, St. Louis, Mo, USA) and different cytokinins. Shoots were transferred every 2 weeks to fresh proliferation medium, in the following sequence: 0.1 mg/l BA for the first 2 weeks; 0.05 mg/l BA for the

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next 2 weeks; and 0.01 mg/l BA plus 0.1 mg/l zeatin for the last 2 weeks in a 6-week multiplication cycle. At the end of the 6-week cycle, vigorous shoots longer than 0.5-1.0 cm that had developed from axillary buds were isolated and used for subsequent culture. Shoot subculture on the proliferation medium was successively repeated until a sufficient number of shoots was obtained as a source of explants for SE induction. All culture media (pH 5.7) were autoclaved at 115 ºC for 20 min prior to use. Unless otherwise indicated, all cultures were incubated in a growth chamber with a 16-h photoperiod, provided by cool-white fluorescent lamps (photon flux density of 50-60 µmol m^{-2} s^{-1}) at 25ºC light/20ºC dark (standard culture conditions).

Induction of somatic embryogenesis

For SE initiation, shoot apex explants (2–2.5 mm long, comprising the apical meristem and several pairs of leaf primordia) and leaf explants (the most apical expanding-leaf below the shoot apex) of genotypes Q3 and Q10 were cultured on embryo induction medium consisting of Murashige and Skoog (1962) basal medium (MS) supplemented with 500 mg/l casein hydrolysate, 3% (w/v) sucrose and 0.6% (w/v) Vitroagar. Induction treatments consisted of induction medium without plant growth regulators (PGRs) or induction medium supplemented with 0.5 mg/l BA in combination with 1, 2, 4 mg/l indoleacetic acid (IAA) or 1, 2, 4 mg/l naphthaleneacetic acid (NAA). The explants were cultured for 8 weeks in darkness at 25 ºC, before being transferred to induction medium with lower concentrations of auxin and BA, of 0.1 mg/l and 0.1 mg/l respectively (M2 medium), and were finally cultured under standard conditions for a further 4 weeks. The explants were transferred to a third medium consisting of M2 medium without PGRs (expression medium M3) and were maintained under standard conditions for at least 32 weeks (i.e., overall for 44 weeks after the start of culture). Ten shoot apices and ten leaf explants were placed in 90 mm Petri dishes containing 25 ml of medium. At least 50 explants were used per treatment, and the experiments were repeated at least two times. The embryogenic response was determined after successive culture of explants in M1, M2 and M3 media, by recording the percentage of explants forming callus and the percentage of explants forming somatic embryos in each genotype, explant type and treatment.

Maintenance of embryogenic cultures

Somatic embryos or embryogenic structures were isolated from initial explants and cultured in Petri dishes containing 25 ml of embryo proliferation medium consisting of Schenk and Hildebrandt (1972) basal medium (SH), 3% (w/v) sucrose and 0.6% (w/v) Vitroagar. The new proembryogenic masses (PEMs), considered subcultured explants, were isolated and were cultured on embryo proliferation medium with sequential subculture at 6-week intervals under standard conditions to maintain the embryogenic capacity.

Germination and plantlet conversion

Cotyledonary-stage embryos (≥ 5 mm) developed on embryo proliferation medium were placed in empty Petri dishes and stored at 4ºC for two or three months prior to culture in germination medium. The somatic embryos were then transferred to 500 ml glass jars containing 70 ml of germination medium consisting of Gresshoff and Doy (1972) basal medium (GD) supplemented with 3% (w/v) sucrose, 0.6% (w/v) Vitroagar, 0.1 mg/l BA and 20 µM silver thiosulphate (STS). The effect of each of the two cold storage durations was evaluated using four replicate jars, each containing six mature somatic embryos (24 embryos per treatment). The germination response was determined after 8 weeks by recording the number of embryos with roots only and the number of embryos that converted into plants (both shoot and root development ≥ 5 mm).

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Results

All treatments, except the treatment without PGRs, yielded acceptable rates of callus formation in both genotypes and explant types after culture of the explants for 8 weeks on induction medium (data not shown). However, the amount of callus tissue formed differed greatly depending on the type of explant. In leaf explants, the first response was swelling and this was reflected in a watery appearance during the culture on induction medium. After transfer to M2 medium only small callus proliferation, mainly developed in the petiole region, was observed. By contrast, apex explants generated larger amounts of callus tissue that began to form in the cut area and finally covered all of the initial explant, making it almost unrecognisable.

SE was induced in the shoot apex and in leaf explants of both genotypes evaluated, although the rate of SE induction depended on the genotype, auxin and explant type (**Fig. 1**). In both clones, shoot apex explants (**Fig. 2A, B**) were significantly more responsive to somatic embryogenesis than leaf explants (**Fig. 1**). In leaf explants, somatic embryos were only initiated at a low frequency (1-3%) and induction was only successful in two of the eight treatments evaluated (induction medium without PGRs or supplemented with NAA 4 mg/l plus BA 0.5 mg/l) **Fig. 1**). By contrast, in shoot apex explants an embryogenic response was observed in all treatments tested in the Q10 genotype and in seven out of the eight treatments evaluated in the Q3 genotype (**Fig. 1**). The addition of auxin improved the rate of induction, especially in apex explants, although the effect of auxin type on this explant type varied with the genotype (**Fig. 1**). Thus, in genotype Q3 the highest induction rate (11%) was achieved with medium containing 4 mg/l NAA plus 0.5 mg/l BA, whereas in genotype Q10 the highest rate (8%) was obtained with apex cultured on medium supplemented with 4 mg/l IAA plus 0.5 mg/l BA.

In the treatments with auxin, somatic embryos or embryogenic structures only began to appear after culture of the explants on medium M3 for at least two months (approximately 20 weeks after the start of the culture period). However, in explants cultured on embryo induction medium without PGRs, the embryos/embryonic structures appeared very quickly, generally within 8-12 weeks after the start of culture. Only one somatic embryo or one embryogenic structure was generated per embryogenic explant, which made later establishment of the different embryogenic lines more difficult.

Holm oak somatic embryos or embryogenic structures were isolated from initial explants and cultured on SH medium to maintain embryogenic competence (**Fig. 2C**). However, maintenance of embryogenic competence proved difficult and more than half of the lines initially obtained were lost after few subcultures. The embryogenic capacity was only maintained by culturing PEMs on SH medium without PGRs. Low embryo multiplication rates were obtained when torpedo or early cotyledonary-stage somatic embryos were used as the initial explant for embryo proliferation. In order to establish embryogenic lines, it is generally essential to isolate the embryos from the initial explant at very early stages of embryogenic development and always before the cotyledonary stage is reached.

Cold stratification was a necessary step for plantlet conversion. The best results were obtained by placing cotyledonary-stage embryos in empty Petri dishes and storing them at 4°C for two months. After 8 weeks on germination medium, at least 60% of the somatic embryos regenerated into plantlets (**Fig. 2D**). The plants that developed were vigorous and of good quality in terms of root and shoot length. Nonetheless, the acclimatization capacity of these plants was very low and only 10% of plants survived after transplanting.

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Genotype Q3

Somatic embryogenesis (%)

	Zero	NAA1	NAA2	NAA4	IAA 0.5	IAA1	IAA2	IAA4
Leaf	0		0	1	0	0	0	0
Apex	1		10	11	3	2	4	0

Genotype Q10

Somatic embryogenesis (%)

	Zero	NAA1	NAA2	NAA4	IAA 0.5	IAA 1	IAA 2	IAA 4
Leaf	2	0	0	3	0	0	0	0
Apex	3	2	5	4	3	3	3	8

Figure 1. Influence of genotype, explant type and auxin treatment on the rate of induction of somatic embryogenesis in two holm oak genotypes. Auxin concentration is represented in mg/l. In genotype Q3, treatment with 1 mg/l of NAA was not performed.

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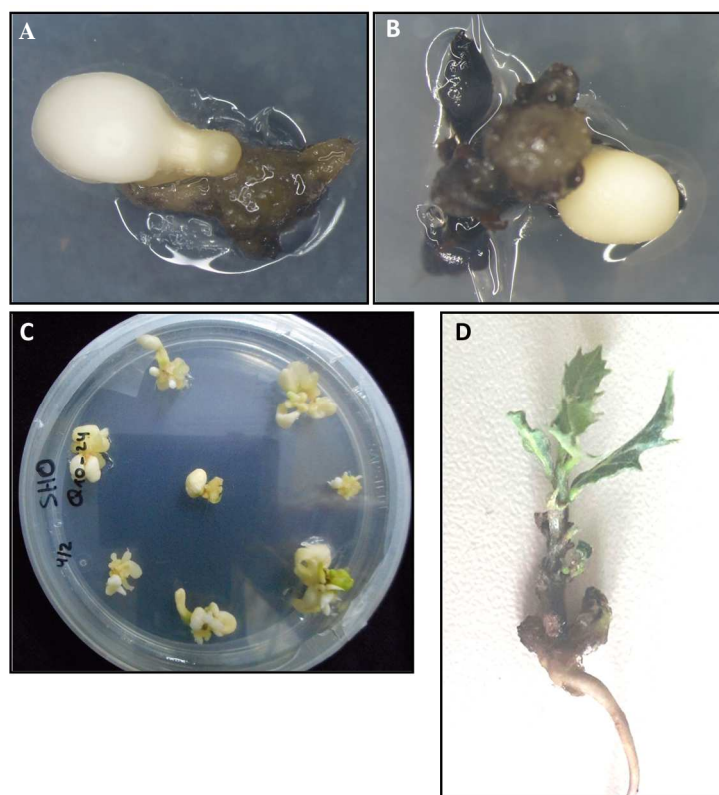
Figure 2. Somatic embryogenesis in leaf and shoot apex explants of holm oak. A, B. Somatic embryos initiated in shoot apex explants of genotypes Q3 (A) and Q10 (B). C. Morphological aspect of somatic embryo cultures after 6 weeks on proliferation medium. D. Plant regeneration from a somatic embryo after two months of cold storage and 8 weeks of culture in germination medium.

Discussion

Several researchers have investigated SE induction in *Q. ilex* with varying degrees of success. In an early study, induction of SE in leaf explants derived from mature holm oak trees was achieved, but plants were not successfully regenerated from somatic embryos (Féraud-Keller and Espagnac 1989). Similarly, an embryogenic response, but not plant regeneration, was achieved in two out of six tested genotypes (induction rates 3.3%) when isolated male flowers and catkins were cultured in a three-step system on medium supplemented with NAA and BA (Blasco et al. 2013). More recently, both embryogenic response and plant regeneration were attained (induction rate ranging from 1.2-3.2%) by culturing teguments of developing ovules isolated from immature acorns on medium without PGRs (Barra-Jiménez et al. 2014). However, several aspects associated with the use of floral explants for inducing SE, including seasonal limitation in the availability of the material and the need to sterilize the plant tissues, must be taken into consideration. To circumvent these aspects we have defined a protocol for inducing somatic embryos from leaves and apices excised from *in vitro* shoot cultures established by using material selected mature holm oak trees. The use of axillary shoot cultures as a source of initial explants enables better control of the

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growth conditions for the source material. As in holm oak, shoot apex and leaf explants excised from axillary shoot proliferation cultures established from material originating from mature field-grown trees has also proved suitable for initiating SE in other *Quercus* species (San-José et al. 2010; Corredoira et al. 2012; Mallón et al. 2013; Martínez et al. 2015).

The SE induction capacity of holm oak was significantly influenced by the explant type. The source of initial explants and their developmental stage are known to be key elements in SE initiation (Fehér 2006). In holm oak, in contrast to most species of the genus *Quercus*, the embryogenic response of apex explants was much higher than that of leaf explants (Corredoira et al. 2014; Martínez et al. 2015). Similarly, apex explants of *Eucalyptus globulus* have also been found to yield a higher embryogenic response as compared to leaf explants (Corredoira et al. 2015). One possible explanation for these findings may be the high degree of necrosis that holm oak leaves undergo, as observed after only a few weeks of culture in induction medium.

Auxins are considered the main signalling molecules involved in induction of somatic embryogenesis (Jiménez 2005; Fehér 2006). They appear to play a dual role during induction, one related to auxin signalling and the other to a stress component that also changes the endogenous content of auxins. In the present study, we found that addition of an auxin (NAA or IAA) to the induction medium improved the embryogenic response in holm oak, especially when apex explants were used as the starting material. The auxin NAA has been the plant growth regulator of choice for inducing somatic embryos in most previous studies in adult somatic tissues of *Quercus* species (Corredoira et al. 2014) and in other woody species such as chestnut (Corredoira et al. 2016). However, use of IAA for inducing somatic embryogenesis in *Quercus* species has not been reported.

In addition to the type of explant and auxin used, we also observed that the genotype had a strong effect on the embryogenic response, particularly in relation to the auxin treatment. The effect of genotype is currently considered a crucial factor in micropropagation and particularly in SE induction in *Quercus* species (Ballester et al. 2016).

We can conclude that the SE induction was not the most critical step in the embryogenic process in holm oak. In most woody species, once embryos are initiated, although at a low rate, large numbers of somatic embryos can be obtained by secondary embryogenesis. However, in holm oak, loss of the embryogenic capacity of many of the embryos/embryogenic structures has been observed after only few subcultures. This appears to be characteristic of this species and this problem has also been described in relation to other embryogenic systems also developed from adult somatic tissues of holm oak (Blasco et al. 2013; Barra-Jiménez 2014). By contrast, plant regeneration was successful and high plant conversion rates were obtained after two-months of cold storage of the somatic embryos.

Conclusions

Although holm oak is usually considered recalcitrant to vegetative propagation, important progress was made in this respect in the present study. We have developed a protocol for somatic embryogenesis that utilizes leaf and apex explants derived from axillary shoot cultures. This procedure represents a useful tool for mass propagation of holm oak trees tolerant to *P. cinnamomi*. However, further research on maintenance of embryogenic capacity and plant acclimatization is required.

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Pinus radiata and *Pinus halepensis* somatic embryogenesis: can we modulate the success of the process by provoking abiotic stress at the initial stages?

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Abstract

The development of somatic cells to somatic plantlets comprises three stages: induction of embryonal masses, maturation of embryogenic tissues and conversion into somatic plants. We focused our study on the effect of environmental conditions, such as water availability and temperature, at the initial stages of somatic embryogenesis in two different species, *Pinus radiata* and *Pinus halepensis*. The modification of environmental conditions affected the success rate of the phase in which these conditions were applied; however, only the modification of radiata pine environmental conditions during initiation led to significant changes in the number of somatic embryos obtained. The global objective of our project is the development of somatic plants with different adaptability to abiotic stress situations taking into account the epigenetic changes acquired by tissues during the embryogenesis process. Specifically, we wanted to evaluate if embryogenic tissue initiation or proliferation stage was critical in improving drought tolerance of the plantlets produced. Furthermore, we wanted to analyse if the differences observed could be related to the protein profile of somatic embryos and the physiological parameters of somatic emblings.

Keywords: Aleppo pine, drought stress, embryogenic cell lines, radiata pine, somatic embryo, water use efficiency.

Abbreviations: ECLs: Embryogenic Cell Lines; SE: Somatic Embryogenesis; Se: Somatic embryos

Introduction

The current context of climate change will lead to environmental variation; mainly higher temperatures and lower water availability. Drought stress is one of the main problems in survival and forestry plant productivity in many countries. For this reason, many research projects are focused on the application of biotechnological tools to improve the tolerance of trees to low water availability. It has been demonstrated for species such as *Picea abies* that temperature during maternal reproduction affects adaptive traits (Johnsen et al. 2005). Somatic embryogenesis (SE) brings great advantages as a model to study this fact and has the additional benefit of being a large-scale propagation system for ''elite'' clones (Isah 2016).

In previous studies we optimized the SE process in *Pinus radiata* and *P. halepensis* (Montalbán et al. 2016) and we chose these species for our experiments due to their different ecological characteristics. Radiata pine has a commercial potential worldwide and it has become one of the most widely planted exotic pine species in rainfall environments of the Southern hemisphere (Yan et al. 2006). On the other hand, Aleppo pine is of a great importance due to its adaptability to dry, calcareous and poor soils. In light of predictions of global warming, there is some interest about the physiological ability of *P. halepensis* to survive in the

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drought conditions expected to occur in large afforestations in the future (Oliveras et al. 2003; Maestre and Cortina 2004).

Our general aim was to produce somatic plantlets tolerant to abiotic stress through the modification of environmental conditions at the initial stages of SE. This objective was divided in the following tasks:

> ➢ -To analyse if the modification of physical and chemical conditions at initial stages of radiata and Aleppo pine SE had an impact in the SE stage in which they were applied or in the subsequent phases.

> ➢ -To elucidate whether the differences observed during the SE process persist over time in the proteomic profile of somatic embryos (Se) obtained under different initiation conditions.

> ➢ -To analyse if plants obtained from somatic embryos coming from embryogenic tissue initiated at different environmental conditions showed different behaviour *ex vitro* under stressful conditions.

Material and methods

Plant material and culture media

Immature cones from open pollinated radiata and Aleppo pine trees (four mother trees for radiata pine and five mother trees for Aleppo pine) were collected when zygotic embryos exhibited a dominant embryo (Montalbán et al. 2012; Montalbán et al. 2013). The seeds were sterilized and the megagametophytes extracted and laid on culture medium following methods developed by Montalbán et al. (2016). The basal medium was EDM (Walter et al. 1998) for *P. radiata* and DCR (Gupta and Durzan 1985) for *P. halepensis*. For initiation and proliferation 4.5 µM 2,4-dichlorophenoxyacetic acid and 2.7 µM benzyladenine were added to radiata pine medium and 4.5 µM 2,4- dichlorophenoxyacetic acid and 2.7 µM kinetin were added to Aleppo pine medium. Embryogenic cell lines (ECLs) of radiata and Aleppo pine were subjected to maturation on the same basal medium for each species following Montalbán et al. (2010 and 2013), using abscisic acid at 60 µM and 75 µM, respectively. Somatic embryos from both species were germinated on half strength LP [Quoirin and Lepoivre 1977, modified by Aitken-Christie et al. (1988)].

Initiation and proliferation experiments

The environmental conditions were modified at two different stages of the SE process: initiation (Experiment 1) for both species, and proliferation (Experiment 2) for *P. radiata*, a scheme of the experiments is shown in **Fig. 1**.

In each of the experiments, the amount of agar (Gelrite®) was increased or reduced by one gram with respect to the standard conditions (3 gL^{-1} for initiation and 4.5 gL^{-1} for proliferation), in order to increase or reduce the water availability of the medium (**Fig. 1**). Also in each experiment the cultures were kept under different temperatures, 5ºC above and below the standard (23ºC, **Fig. 1**). Thus, in each experiment nine different treatments were tested, carrying out the other SE steps under standard conditions.

The success of the SE process at different stages was calculated as follows: percentage of initiation (cell lines initiated of the total number of megagametophytes cultured) for Experiment 1; percentage of proliferation (ECLs that proliferated of the total number of initiated cell lines) for Experiment 1 and 2; percentage of maturation (ECLs that produced Se of the total number of ECLs subjected to maturation) and number of Se per gram of embryogenic tissue for those ECLs producing Se (Experiment 1 and 2).

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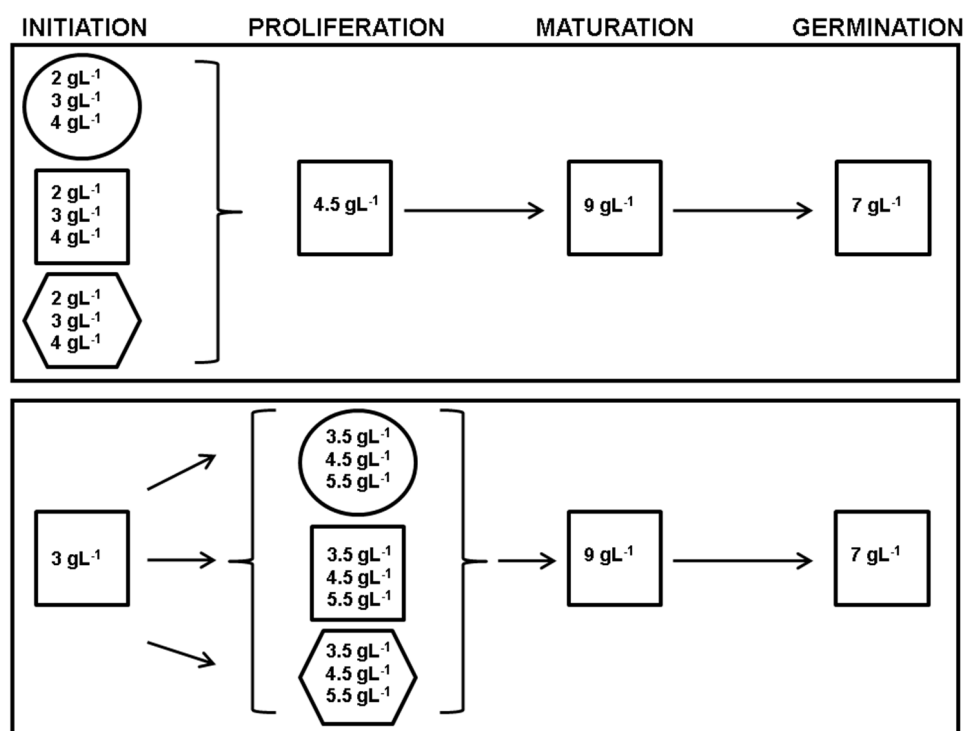
Figure 1. Scheme of the experimental design of experiments 1 and 2 (upper and lower rectangles, respectively). Cultures were stored at three different temperatures: 18ºC (circle), 23ºC (square) or 28ºC (hexagon) and at three different agar concentrations (inside circles, squares and hexagons).

A two-way analysis of variance was performed to assess the effect of temperature and agar concentration on the different stages of the SE process. When significant differences between the levels of the two factor variables were found, Tukey HSD and Benjamini-Yekutieli post hoc tests were carried out with the Aleppo and radiata pine data, respectively, to find out which levels were statistically different.

Protein determination

Radiata pine Se grown under conditions that led to high initiation percentages (from ECLs initiated at 18ºC or 23ºC and at 4gL^{-1} agar) and Se cultured under conditions that led to low initiation percentages (from ECLs initiated at 28ºC and 2gL^{-1} or 3gL^{-1} agar) were analyzed (García-Mendiguren et al. 2016b).

Protein extraction, 2-D Electrophoresis, Image Analysis and protein identification by MALDI-TOF mass spectrometry were carried out following Correia et al. (2012).

Drought experiment

After germination we established the plantlets *ex vitro* in a controlled greenhouse, and then an experiment was carried out to assess if the plants had acquired a tolerance to drought stress. Radiata pine somatic plants were selected from the different initiation temperatures experiment. Half of them were randomly selected for drought stress treatment (no irrigation), and the remaining plants were maintained with a water supply.

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Different hydric parameters, such as leaf water potential and transpiration were analyzed. Furthermore, gas exchange parameters like net photosynthesis, stomatal conductance, etc. were determined with a LICOR 6400 chamber. All mentioned parameters were measured at time zero (the first day) and when plants from each group tested showed epinasty symptoms (**Fig. 2**).

Figure 2. *Pinus radiata* somatic plant with needle epinasty and apical curvature after 4 weeks under drought conditions.

Results

Experiment 1

The modification of environmental conditions (temperature and agar concentration) at the initiation stage had a significant effect on both radiata (**Fig. 3a**) (García-Mendiguren et al. 2016a) and Aleppo pine (Pereira et al. 2016). However the interaction of the factors tested was not statistically significant. Initiation percentages were significantly higher when megagametophytes from radiata and Aleppo pine were cultured at the highest agar concentration (16 and 49%, respectively) (**Fig. 4**). The standard initiation temperature (23ºC) led to significantly better initiation percentages (53%) than the other temperatures tested for *P. halepensis* (below 40%, Pereira et al. 2016); in the case of *P. radiata* the results at this temperature were not statistically different from those obtained at 18ºC (18% and 14%, respectively, García-Mendiguren et al. 2016a) (**Fig. 5**).

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At proliferation stage in Experiment 1 only initiation temperature had a significant effect on proliferation rates. For *P. radiata* initiation at 28ºC led to significantly higher proliferation percentages (66%) than the other temperatures tested (36%, García-Mendiguren et al. 2016a). On the contrary, *P. halepensis* initiation at 23ºC produced significantly better proliferation rates (79%, **Fig. 3b**) than initiation at 18ºC (60%, Pereira et al. 2016, **Fig. 6**).
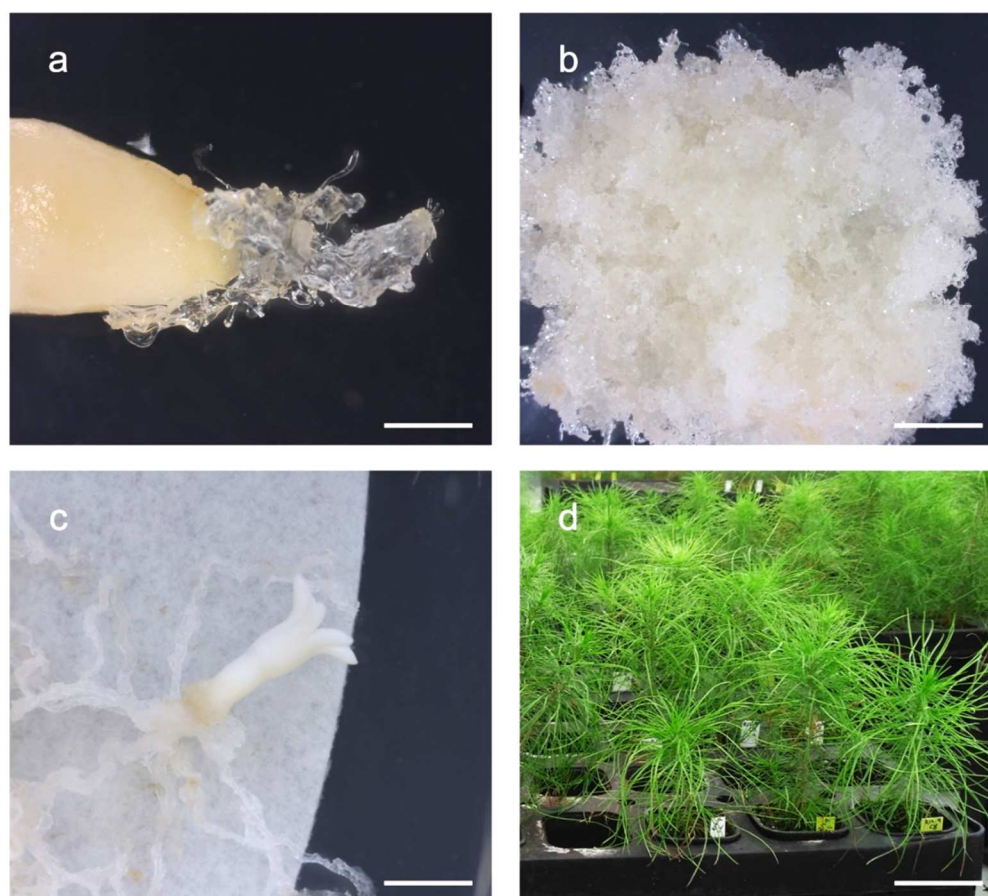
Figure 3. Somatic embryogenesis, a) tissue extrusion in *Pinus radiata* at 23ºC and 4gL^{-1} agar (bar=2mm); b) embryogenic tissue proliferation in *P. halepensis* at 23ºC and 4.5 gL^{-1} agar (bar=5mm); c) *P. radiata* somatic embryo from an embryogenic cell line initiated at 23ºC and 4gL^{-1} agar (bar=3 mm); d) *P. radiata* somatic plantlets from an embryogenic cell line initiated at 23ºC and 4gL^{-1} (bar=50mm).

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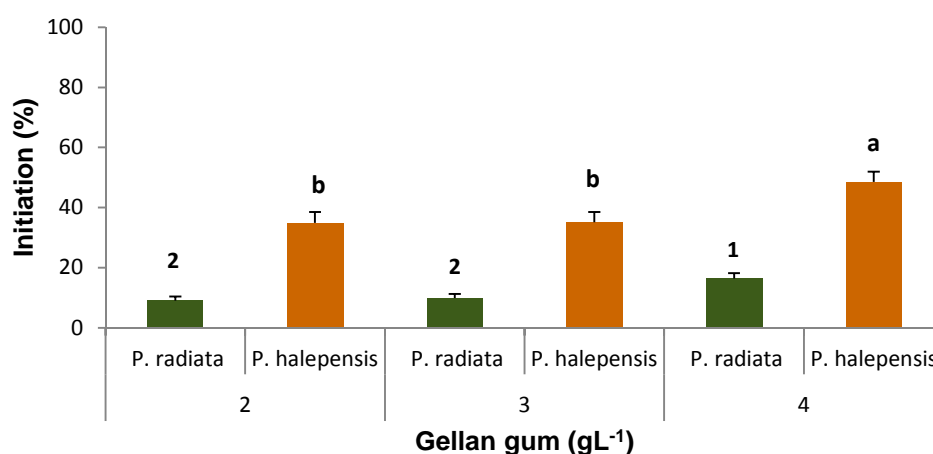
Figure 4. Embryonal mass tissue initiation (%) in *P. radiata* and *P. halepensis* megagametophytes initiated on EDM at different gellan gum concentrations (2, 3 and 4 g L^{-1}). Values represent mean ± S.E. Different letters and numbers indicate significance at p = 0.05.
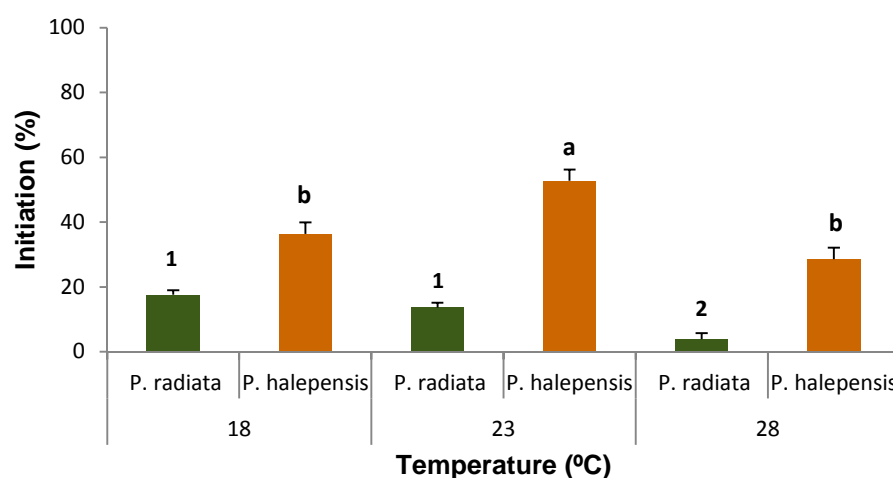
Figure 5. Embryonal mass tissue initiation (%) in *P. radiata* and *P. halepensis* megagametophytes initiated on EDM at different temperatures (18, 23 and 28 g L^{-1}); Values represent mean ± S.E. Different letters and numbers indicate significance at p = 0.05.

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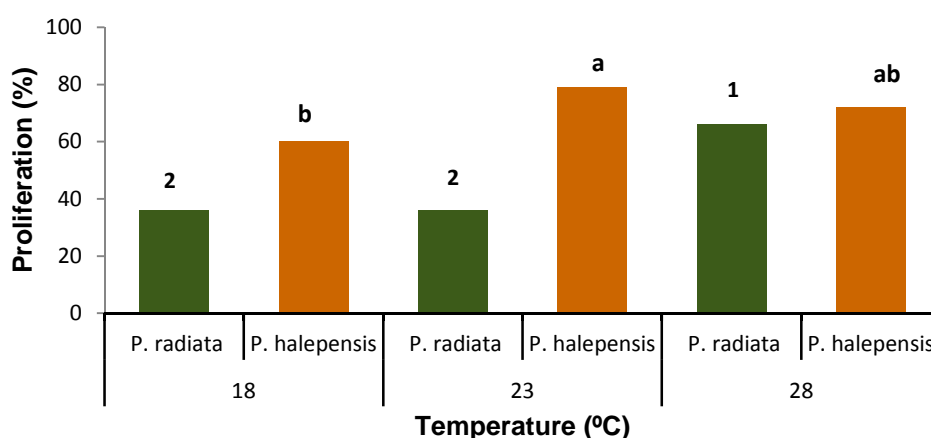
Figure 6. Embryonal mass tissue proliferation (%) in *P. radiata* and *P. halepensis* megagametophytes proliferated on EDM at different temperatures (18, 23 and 28 ℃); values represent mean. Different letters and numbers indicate significance at p= 0.05.

Changing environmental conditions at initiation did not affect significantly the maturation percentages, these percentages were above 89% for radiata pine and above 95% for Aleppo pine.

When the number of Se was analyzed (**Fig. 3c**), in radiata pine, significantly better results were obtained in embryonal masses initiated at 28℃ (García-Mendiguren et al. 2016a) (**Fig. 7**). No differences were found in Aleppo pine when modifying temperature or agar at initial stage of SE (Pereira et al. 2016).
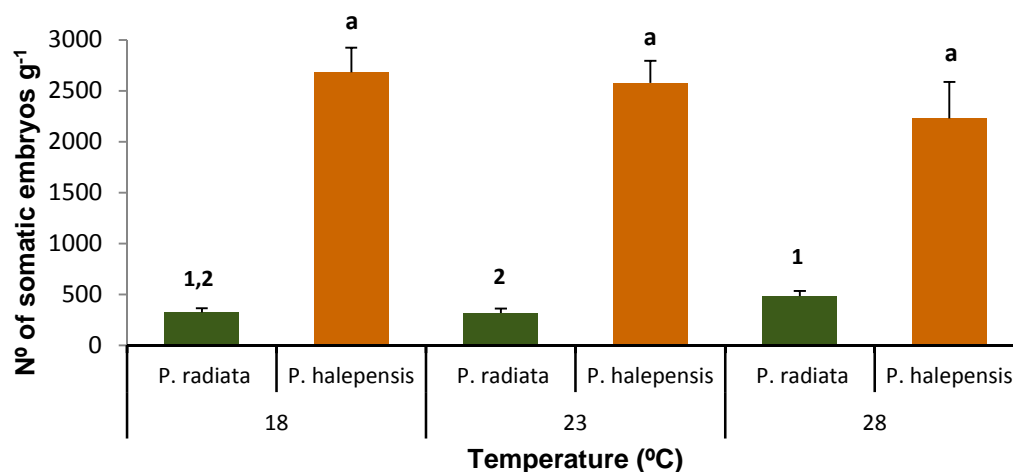
Figure 7. Number of somatic embryos produced per gram of embryonal mass (fresh weight) of *P. radiata* and *P. halepensis* initiated at different temperatures (18, 23 or 28℃). Values represent mean ± S.E. Different letters indicate significance at p = 0.05

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Protein determination

Eleven differentially expressed spots were identified in Se initiated under conditions that led to low initiation rates. Based on the Swiss-Prot database, identified proteins were organized into different functional categories. Most of the proteins were involved in defense responses, such as the osmotically inducible protein OsmC, chaperon protein and vicilins. Proteins associated with other functional groups, such as proteins related to response to reactive oxygen species and carbohydrate metabolic process, such as enolase or phosphoglycerate kinase or proteins related to gene expression, were also found in lower proportions (García-Mendiguren et a. 2016b).

Drought experiment

When radiata pine somatic plantlets from Experiment 1 were subjected to drought conditions (**Fig. 3d**), the first individual to show epinasty belonged to initiation temperature 23ºC (**Fig. 2**). As observed in previous studies carried out in *Pinus radiata* in our research group (De Diego et al. 2012), the turgor loss point was at -2 MPa. After 14 days the results were according to our hypothesis, showing that plants exposed to initiation temperature 28ºC preserved their turgor (-1.25 MPa). Nevertheless, after 22 days plants from embryonal masses initiated at 18ºC showed the lowest loss of turgor (-1.55 MPa). The Net photosynthesis results corroborate the results from leaf water potential measurements, and the best results in stressed plants were obtained with embryonal masses initiated at 18ºC (**Fig. 4**, Montalbán et al. 2014). Water-use efficiency, calculated as the ratio of photosynthesis to stomatal conductance, was 50 times higher in plants coming from embryonal masses initiated at 18ºC when compared with stressed plants exposed to other initiation temperatures.

Discussion

As reviewed by Stasolla and Yeung (2003), most studies on the effect of environmental conditions during conifer SE are carried out focusing on the maturation stage; however, from our experiments one general conclusion is clear, the early stages of SE have a great impact on the overall SE process. Whereas standard initiation temperature produced the best results in *P. halepensis* (Pereira et al. 2016), no significant differences were found between initiation temperature 18ºC and 23ºC for *P. radiata* (García-Mendiguren et al. 2016a).

The water availability of the medium plays a major role at different phases of the process as has been found by several authors (Choudhury et al. 2008). In both species the best initiation results were obtained in the medium with the lowest water availability. , this is in accordance with the results obtained in different *Pinus* species were initiation has been achieved at this agar concentration (Lelu-Walter et al. 2008, Humánez et al. 2012). In contrast other authors have reported better initiation results when agar concentration is lowered or even in liquid cultures (Pullman and Skryabina 2007).

In radiata pine, the highest initiation temperature seems to produce a selective pressure as pointed by Fehér in 2015, so that the ECLs initiated at this temperature were more successful in terms of proliferation rates and number of Se obtained. In this regard, Bonga et al. (2010) suggested that reducing or increasing temperature regimes may improve initiation and proliferation since temperature stress may promote cellular reprogramming. This prolonged effect was not observed for Aleppo pine where the temperatures that gave the lowest initiation percentages (18ºC and 28ºC) did not lead to better results for proliferation or maturation stages (Pereira et al. 2016).The lack of this selective pressure could be due to the high adaptability of this species to different environments, a characteristic already observed in the field (Botella et al. 2010).

In the proliferation experiment, the highest temperature produced the lowest results but did not exert a beneficial selective pressure on subsequent phases as observed for the initiation experiment; these results

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are in disagreement with those by Kvaalen and Johnsen (2008) in *Picea abies*, who reported that proliferation at 28ºC led to a larger number of Se than cultures maintained at lower temperatures. Regarding the proteomic analysis, the presence in Se from ECLs initiated at high temperature of proteins involved in defense responses such as Chaperones is interesting as these proteins are known as heat shock proteins. Among their functions is the stabilization of proteins and membranes, and can assist in protein refolding under stress conditions (Wang et al. 2004). Heat shock proteins are known to be induced not only in response to short-term stress, but their production is a necessary step in plant heat acclimation (Timperio et al. 2008). At the protein level, a signature of desiccation tolerance is the accumulation of these proteins that function in the protection of cellular components during seed desiccation (Timperio et al. 2008).

The *ex vitro* drought experiment showed that our plantlets behaved differently regarding the initiation temperature. However, the selective pressure we exerted had a beneficial effect in ECLs exposed to initiation temperature 28ºC but did not have a positive effect in the *ex vitro* experiments, where somatic plantlets exposed to the lowest initiation temperature showed the best performance against drought stress.

Future experiments will try to clarify if the effects can be more drastic if more extreme temperatures are used at the initial stages of SE.

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Somatic Embryogenesis in *Musa* clones

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Abstract

Banana is a major crop in Venezuela as well as in most tropical countries, thanks to its rich content in vitamins and minerals. Due to the high demand for healthy plants, and for genetically improved materials with resistance to diseases, biotechnology is a useful alternative to produce these plants. The objective of the present work is to induce somatic embryogenesis from apical shoots as a plant regeneration process of three *in vitro* clones of *Musa*: Titiaro (AA), Giant Pineo (AAA) and Tetraploide (AAAA), economically important cultivars in Venezuela. In order to induce somatic embryogenesis, MS culture medium, supplemented with Morel vitamins (10 ml/l), sucrose (30 g/l), cysteine (60 mg/l), 2,4-D (0, 2 and 4 mg l^{-1})and Dicamba(0, 2 and 4 mg l^{-1}) and gelrite (2 g/l) were used as solidifying agent. Somatic embryogenesis was carried out with vitro-plants that had originated from two groups: a) vitro-plants after 3 subcultures, with BA (5 mg/l) and b) vitro-plants after 3 subcultures, two with 5 mg/l and one with 10 mg/l of BA. The induction of embryogenic callus was achieved in three clones in both experimental groups when 4 mg/l of 2,4-D was used. The multiplication of embryogenic callus is obtained with the same concentration used in the induction phase. The induction and multiplication of embryogenic callus were developed in dark conditions. For differentiation of embryos, zeatin (1.50 mg/l) was added to the same basic medium. The acclimatization of plants obtained by somatic embryogenesis was successful in the two selected clones for this research.

Keywords: somatic embryogenesis, *Musa*, clone, vitro-plant

Introduction

The genus *Musa* has a wide coverage in the tropical areas of the world, where it represents a basic element of the daily diet, because it is an excellent source of carbohydrates (35%), fiber (6-7%), macroelements such as potassium, magnesium, phosphorus, calcium, iron and vitamins such as A and C (20%), and their fruits are widely accepted. In addition, it represents one of the essential bases in the typical food usage in some countries, where about 90% of its production is used for domestic consumption. On the other hand, it represents an agent of vital importance in the economy of many Latin American and Caribbean countries, due to the industrial export trade of this fruit. Clones of Musaceae are very important for human consumption. It has been demonstrated that it is the fourth most commercialized plant in the world, making its production useful at an economic level (Vázquez et al. 2008). It should be noted that there are references to the nutritional properties of bananas, which can be useful for feeding programs. In addition, components used in the therapeutic area have also been found. Banana is an important crop in Venezuela, as well as in most tropical countries, thanks to its rich content in vitamins and minerals. Most cultivated clones are: Giant Pineo (AAA), Titiaro (AA), Topocho (AAAB), Plátano Hartón (ABB) and Cambur manzano (AAB).

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Musaceae are plants that have a great value as food, which allows them to be used by people who require a balanced diet in order to reduce nutritional problems. In many regions of the world, food availability is truly problematic and it is necessary to use bananas to solve this situation. (Devatkal et al. 2014).

Bananas can be classified according to the taxonomic logic in: Order: Zingeberales, Family: Musaceae, Genus: *Musa*. Most of the edible cultivars are the product of the hybridization of *M. acuminata* and M. *balbisiana*, (Haddad et al. 2005). The genus *Musa* consists of 4 sections: Australimusa, Callimusa, Rhodoclamys and Eumusa. The crossing of *M. acuminata* and *M. balbisiana* varieties gave rise to edible bananas. There are clones with *M. acuminata* and *balbisiana* genomes: diploids (AB), triploids with *M. acuminata* dominance (AAB), triploids with *M. balbisiana* dominance (ABB) and tetraploids with dominance (ABBB) (Haddad et al. 2005; Vásquez et al. 2008).

The processing of bananas has granted them diverse uses in the preparation of commercial products that are very important in the ingestion of food, because they contain nutrients that are vital for the organism's functionality. It is also important to note that bananas are widely used to treat various health conditions, such as: gastrointestinal problems, infections, among others (Albornoz 1997; Martínez et al. 1999). For this reason, several biotechnological alternatives are proposed for the genetic improvement of bananas, in order to maintain these species over time, with less susceptibility to pest and disease attack. In some locations, the frequency of plant usage is determined by its availability in the study area; therefore, its use varies in relation to cultivated species (Perez, 2012). Due to the importance of Musaceae at the nutritional and medicinal levels, it is fundamental to optimize their production by implementing viable conservation and propagation strategies that allow maintaining a production of pathogen free cultivars with great adaptability to the environment.

One of the alternatives to increase banana production is the implementation of *in vitro* cultures, in order to obtain plants in good sanitary condition, free of pathogens and versatile to deal with biotic and abiotic stress. As for the *in vitro* culture, the increase of the cytokinin levels in the culture media can be implemented, in order to induce variability and at the same time ensure a massive propagation of explants. Through *in vitro* cultivation, plant species can be conserved in a germplasm bank, and thus ensure future availability. Plant biotechnology is a widely used tool for plant breeding, which consists of the following techniques: tissue culture, genetic engineering and the use of molecular markers (Vallejo and Estrada, 2002). The plant genetic material obtained by propagation techniques guarantees the preservation of genetic diversity. Therefore, the *in vitro* cultivation of *Musa* clones is widely performed in order to guarantee the availability of this plant, and the safety and food sovereignty (Albarrán et al. 2011).

The propagation of plants *in vitro*, is usually a viable alternative for the production of large-scale, pathogen-free plants. Currently, the lack of healthy and competent corms for the establishment of new cropping areas and the potential danger of pathogen-infected seeds represent a serious disadvantage in in vivo breeding. In this sense, the plant tissue culture is a tool that allows to move healthy material to the field, to have plants available at any time of the year, to multiply desirable genotypes and to transport the propagules easily. Some of the "*in vitro*" techniques used in *Musa* culture are based on the isolation of the apex, clonal propagation (Trujillo and García, 1996) and the use of vegetative tissue to obtain embryogenic callus, with the aim of inducing somatic embryogenesis (Novak et al, 1989; Escalant and Teisson, 1989; Escalant et al, 1989; Gerth and Gomez, 1991; Trujillo and García, 1996).

Somatic embryogenesis has been suggested as a method to improve genetic traits in *Musa*. For the initiation of embryogenic cultures, various parts of the plant have been used as an explant: inflorescence, leaves and young corm tissues (Perea-Dallos and Novak, 1988). The regeneration of whole plants from cultures of somatic cells has been one of the problems while using somatic embryogenesis in the improvement of *Musa*. Research on somatic embryogenesis in *Musa* species from vegetative tissues has been identified as an atypical process, with embryos that do not easily develop into complete plants (Cronauer and Krikorian, 1983; Banerjee et al, 1987;

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Novak et al, 1989). However, some authors have developed procedures for embryogenesis that resulted in normal, complete embryos (Novak et al, 1989; Gerth and Gomez, 1991).

The objective of this investigation is to induce somatic embryogenesis for the regeneration of plants from apical buds of three clones of *Musa in vitro*: Titiaro (AA), Giant Pineo (AAA) and Tetraploid (AAAA) cultivars economically important in Venezuela. Due to the high demand for healthy plants that are genetically enhanced and disease resistant, biotechnology is a useful alternative to produce these plants.

Material and methods

Vegetative material

In this research, three varieties of Musaceae were used with the *acuminata* genome: Titiaro (AA); Giant Pineo (AAA) and Tetraploide (AAAA).

To induce somatic embryogenesis we used an MS culture medium (1962) supplemented with vitamins of Morel (10 ml/l), sucrose (30 g/l), cysteine (60 mg/l), and as growth regulators 2,4-D (0, 2 and 4 mg/l), Dicamba (0, 2 and 4 mg/l) and as solidification agent gelrite (2 g/l). Somatic embryogenesis was carried out from apical buds from plantlets obtained from two groups:

A. Vitro-plants after 3 subcultures, two of which contained 5 mg/l and one with 10 mg/l BA.
B. Vitro-plants after 3 subcultures with 5 mg/l BA.

Induction of the embryogenic callus

Callus induction was obtained from the base of the leaves of the vitro-plants, including the meristematic primordium. Somatic embryogenesis was induced in two groups: vitro-plants: group A, with three subcultures *in vitro* (two with 5 mg/l BA and one using 10 mg/l) and group B, also with 3 subcultures with 5 mg/l BA. The average size of the explants was 1-1.5 cm long. For the induction of the somatic embryogenesis process different means were tested. All media prepared were based on Murashige and Skoog (1962) salts supplemented with Morel vitamins (10 ml/l), cysteine (60 mg/l), sucrose (40 g/l) and gelrite (2.5 g/l) as the gelling agent and various concentrations of growth substances (**Tab. 1**).

Table 1. Concentrations of growth substances in various media used for initiation of somatic embryogenesis in *Musa* clones.

Media	2,4-D (mg/l)	Dicamba (mg/l)
1	0	-
2	2	-
3	4	-
4	-	0
5	-	2
6	-	4

The C medium was used in the callus induction process; 2.4 D (dichlorophenoxyacetic acid) was added as auxin at a concentration of 4 mg/l (this concentration was selected as a result of previous experiments),

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sucrose (40 g/l) and gelrite (2 mg/l) was used as the gelling agent. The pH of the medium was adjusted to 5.8. At this stage, the explants were incubated at a temperature of 26 °C for 40 days under absolute darkness.

Morphological observations were performed periodically using a Wild Heerebrugg Type 376788 stereoscopic microscope, to determine the appearance of callus on the explants and to estimate the amount of callus in each explant. Additionally, qualitative evaluations were carried out on explants that formed callus, and characteristics concerning color and texture of the explants were determined.

Multiplication of embryogenic callus

The process of multiplication of the embryogenic callus was carried out using the method described above, where only the sucrose concentration was modified from 40 g/l to 30 g/l, and the 2,4-D as the auxin source (4 mg/l). The callus was incubated for 30 days in total darkness at 26°C.

Induction of embryo differentiation

The differentiation of embryos from the callus was obtained using the medium initially described, with a reduced concentration of sucrose (20 g/l) and the cytokinin zeatin (1.50 mg/l) was added. The evolution of the embryos was monitored periodically through photographs using a Stereoscopic microscope brand Wild Heerbrugg with a camera, Pentax (35 millimeters) with a Pentax M lens of 121.50 mm.

Germination of embryos

Germination of the embryos was obtained in the same medium as used for differentiation and under continuous lighting (50 µE. m^{-2}.sec^{-1}).

Adaptation of plants to natural conditions

The plants obtained from the somatic embryos were then transplanted into a mixture of soil and peat (3:1), previously sterilized in an autoclave at 121 °C for 15 minutes. The seedlings were placed in propagation boxes for a period of three weeks and then transferred to the greenhouse. These plants were fertilized every 15 days using a diluted liquid vermicompost.

Histological Methods

Several stages of the embryogenic process were analyzed through a histological analysis. The tissue samples were fixed in a FAA solution (5 parts 37% v/v formaldehyde, 5 parts glacial acetic acid and 90 parts 70% ethanol) where they remained until processing time. Subsequently, they were stained with a mixture of safranin-toluidine blue at a 3:1 ratio, and embeded in paraffin and sectioned. The treatment of the samples for the histological study was divided into the following steps: fixation and dehydration, inclusion in paraplast, sectioning and observation. The method used for this process was the one described by Johansen (1970) and Perez-Cortez et al. (2003).

Results and discussion

Induction of somatic embryogenesis in *Musa* sp. was obtained from leaves of banana clones with different ploidy, i.e., genome *acuminata:* Titiaro (AA), Giant Pineo (AAA) and Tetraploide (AAAA).

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Embryogenic callus induction was achieved in the three clones tested with both experimental groups, using 2.4-D (4 mg/l) as growth regulator. Multiplication of embryogenic callus was obtained with the same concentration used in the induction phase, using 2.4-D (4 mg/l). Induction and multiplication of embryogenic callus occurred in the dark. For embryo differentiation, zeatin was added (1.5 mg/l) to the same basic medium.

Induction of somatic embryogenesis was obtained in *Musa* by using similar explants as used by Santos et al. (2002). Somatic embryogenesis in all the clones studied began with the formation of a white callus which appeared after about 40 days which is a shorter time span than was described in previous studies (**Fig. 1**). This phase was performed under total darkness to avoid accelerated oxidation of the explants, although oxidation in Musaceae did not appear to be a problem for the development of somatic embryogenesis in this investigation.

However, one of the main problems presented in the induction of somatic embryogenesis in *Musa*, is the oxidation of explants and calli due to the high amount of phenols present in this tissue. To solve this problem, the addition of antioxidants is recommended (Novak et al, 1989). In our case, the addition of cysteine to the culture medium, eliminated oxidation as a limiting factor of this process.

Somatic embryogenesis depends basically on three essential factors: the tissue to be used, the mother plant from which the tissue is obtained and the culture medium. Generally, the explant type is a determining factor for initiation of somatic embryogenesis. The specific differences of each tissue in achieving the competence that allows inducing and expressing the regenerative capacity, suggests that a specific, essential factor is needed for somatic embryogenesis to occur (Novak et al, 1989; Gerth and Gomez, 1991). Factors such as physiological status and age of the mother plant are also considered to be of great importance, so the use of young plants is highly recommended (Escalant and Teisson, 1989; García and Martinez, 1995).
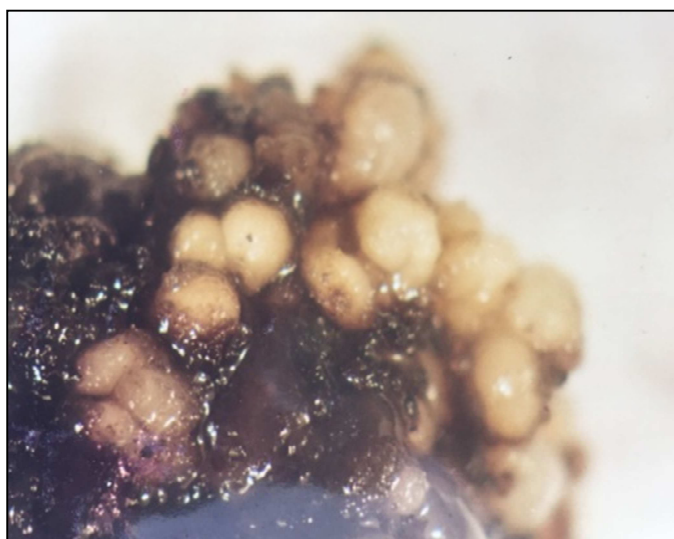
Figure 1. Embryogenic callus in *Musa* spp. (clone Giant Pineo).

Somatic embryogenesis can occur directly if competent cells already exist in the tissue used as an explant, or indirectly, if carried out through a preliminary phase where the formation of new cells is observed (Ammirato, 1987) as in this investigation.

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A higher percentage of calli induction was observed for the Pineo Giant clone than for the Titiaro and Tetraploide clones. The use of Dicamba as growth substance in the initiation of somatic embryogenesis of *Musa* clones was not successful. **Tab. 2** shows the results of the induction of this process, using 2,4-D (4 mg/l) as the source of auxin.

Table 2. Calli formation during the initiation phase of somatic embryogenesis using 2,4-D.

Clone	Group A	Group B
Pineo Gigante (AAA)	+++	+++
Titiaro (AA)	++	++
Tetraploide (AAAA)	++	++

The use of 2.4-D or other auxin is generally sufficient to initiate somatic embryogenesis. Different interpretations have been proposed for this process. It is thought that there are several cells in the explants that are preconditioned for the morphogenetic events that lead to the formation of somatic embryos (Thorpe, 1983). Growth regulators, generally 2.4-D, not only initiate the development of somatic embryos, but also stimulate clonal multiplication of predetermined cells.

The multiplication of the embryogenic callus was obtained in the medium C, where the concentration of the sucrose was reduced while maintaining the concentration of 2.4-D. This phase lasted approximately 30 days. Differentiation of somatic embryos occurred when 2.4-D was removed from the medium. It has been established that the presence of auxins is necessary for the formation of embryogenic cell aggregates. However, the use of auxins in subsequent phases of somatic embryogenesis inhibits the evolution of this process. Embryo differentiation was detected by the appearance of rounded, cream and white colored structures, approximately forty days after the beginning of the culture, where globular, globular embryos with invagination, and elongated embryo forms were observed (**Fig. 2**). For the differentiation of the embryos, zeatin was added (1.5 mg/l) to the same basic medium. When the cytokine (Z) was added, some embryos remained in the globular stage with some sign of enlargement but with no later development of invagination; other embryos reached the invagination stage; and some reached the enlargement stage and became actively photosynthetic. At this stage, the embryos were placed under light conditions (50 µE.m^{-2}.sec^{-1}) and exposed to 27°C.

Asynchrony often occurs in somatic embryogenesis, including in many genera of *Musa*. It has been established that this asynchrony is due to the production of new somatic embryos on the originally formed ones and the formation of new embryogenic callus with the subsequent differentiation of new embryos (Ammirato, 1987; Novak et al, 1989; Escalant and Teisson, 1989; Escalant et al, 1994; Garcia and Martinez, 1995; Grapin et al, 1996; Deo et al, 2010). The results obtained during three months of asynchrony indicated different percentages in the production of embryos; where the highest percentages were for clones Giant Pineo and Titiaro. Embryos at different stages of development were isolated and subcultured for further differentiation. Germination of the embryos "*in vitro*" begins with the development of the plumula, followed by the formation of the complete plant with the formation of adventitious roots, which occurs after 2 months of cultivation (**Fig. 2**). Not all somatic embryos regenerated a complete plant, however, the rate of regeneration was high. The development of complete plants was observed for the Giant Pineo and Titiaro clones, but not for the Tetraploide clone. In relation to the percentage of regenerated plants, Giant Pineo exhibited the highest values while the Titiaro clone had the lowest percentage.

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Figure 2. *In vitro* germination of the embryos and regeneration of a complete plant

Of the groups of vitro-plants used for the induction of somatic embryogenesis, group B showed high percentages of regenerated plants for Giant Pineo that Titiaro, the group A showed a better percentage of embryo survival than the ones in group B. This can be related to the endogenous hormonal concentration in the explants obtained from the vitro-plants used in this experiment since two different treatments were used during their "*in vitro*" multiplication phase.

The regeneration of a complete plant from embryos has limitations in the use of this biotechnology for crop improvement. The genus *Musa* is an excellent example of this since the species of this genus are extremely recalcitrant, and success with this genus has been limited (Novak et al, 1989). However, the regeneration of plants from somatic embryos has been obtained in several investigations (Novak et al, 1989; Escalant and Teisson, 1994; Gerth and Gomez, 1991; Lopez-Gomez et al, 2016).

Histological studies of somatic embryogenesis are very important to understand and improve this morphogenetic process. The embryogenic callus showed isodiametrical small cells with thick walls and small vacuoles (**Fig 3**). In studies of histological sections, groups of embryogenic cells surrounded by a thick wall could be located. These cell aggregates have been termed pro-embryonic masses, which appeared after two months of culture.
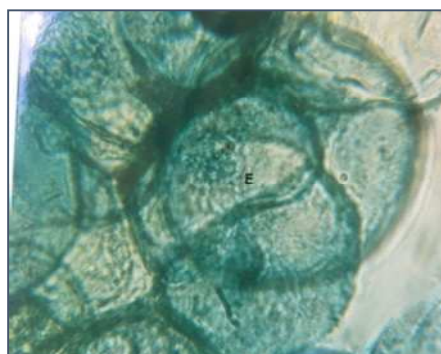
Figure 3. Embryogenic callus cells in the Giant Pineo clone (AAA)

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The origin of somatic embryos obtained from meristematic apexes of the selected clones of *Musa* was analyzed by histological and morphological studies during the various stages of process development. Histological sections of globular embryos showed the radial arrangement of cells and the existence of an epidermal layer surrounding the entire embryo.

Histological analysis of somatic embryogenesis in *Musa* sp. in the clones studied, indicate that the transparent, non-embryogenic callus, is made up of elongated, large, thin-walled cells. On the other hand, the white or cream colored embryogenic callus is composed of cells that have a dense cytoplasm, a prominent nucleus and a large amount of stored proteins. The histochemical characteristics of these cells suggest an intense synthesis of RNA and a high metabolic activity, characteristics that have been pointed out in other research as being characteristic of embryogenic cells (Williams and Maheswaran, 1986; Escalant et al, 1989 (a); Escalant et al, 1989(b); Escalant and Teisson, 1989; Garcia and Martinez, 1995; Grapin et al, 1996; Garcia and Martinez, 1995; Grapin et al, 1996).

The acclimatization of plantlets obtained by somatic embryogenesis was successful in the two clones selected for this research (Giant Pineo (AAA) and Titiaro (AA) (**Fig. 4**).

Figure 4. *Musa* clones plantlets obtained from somatic embryogenesis

Conclusions

The induction of somatic embryogenesis was successful when using the bases of leaves of vitro-plants including the meristematic primordium. This induced a high frequency of somatic embryogenesis and resulted in the regeneration of complete plants of the genus *Musa*.

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The importance of somatic embryogenesis is that it allows multiplication of genotypes on a large scale. It facilitates the cultivation in liquid media and favors the automation of the culture system. Somatic embryogenesis in *Musa* is an excellent alternative for rapid propagation of elite materials and may eventually lead to the production of *Musa* commercially by *in vitro* means.

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Histological analysis of somatic embryogenesis of *Melia azedarach* L. and *Prosopis alpataco* Phil.

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Abstract

Histological methods contribute significantly to the understanding of *in vitro* tissue culture systems, since they provide information that enable us to make the right decisions to optimize *in vitro* propagation protocols. *Melia azedarach* (chinaberry) and *Prosopis alpataco* (alpataco) are two multipurpose woody species. They provide wood, food and various bio-active compounds. Cotyledons placed in the induction medium were used to induce somatic embryogenesis (Sharry et al. 2006; Boeri et al. 2016). Explants of both species differentiated either morphogenic or non-morphogenic callus. In chinaberry there were no noticeable differences in appearance between the embryogenic and organogenic calli, since both embryogenesis and organogenesis took place in the same type of callus. This callus originated both organs and embryos. Microscopic analysis showed the coexistence of meristemoids, shoots, somatic embryos and vascular tissue, as well as still dedifferentiated cells. *Prosopis* cotyledons produced only somatic embryos. In both, pro-embryogenic (ce) and non-embryogenic (cne) cells were observed. A re-differentiation process took place in different types of cells with intense cell divisions that were located in regions distributed randomly. The cne were rounded or elongated, had thin walls and little cytoplasm. The ce were rounded, small and relatively thick-walled and had a dense cytoplasm and a prominent, deep red nucleus which are typical characters of meristematic and pro embryogenic cells. Embryogenesis was initiated in individual cells located in the periphery of the callus and from superficial cells from existing ce cells. Divisions observed in these surface cells affirm the unicellular origin of embryos obtained in alpataco and chinaberry cultures. The different stages of embryogenic development observed in both species were similar to embryogenesis *in vivo*; suggesting the genetic potential of the plant is being used. The formation of somatic embryos was a continuous process during the period of incubation of the explants. Given that not all cells differentiate into somatic embryos at the same time, it was possible to observe all the stages of development of the embryo in the same callus. Finally, somatic embryos germinated normally. Somatic embryogenesis was highly similar for both species even though no systematic relationship existed between the species studied. The explants and environmental conditions used for both species were similar.

Keywords: somatic embryo, chinaberry, alpataco, histology

Introduction

Somatic embryogenesis has been a focal point of research in plant development. This process relies on somatic cell totipotency (i.e., the capacity to regenerate the entire plant from single somatic cells), and it has long been used as an efficient system for regenerating plants on a large-scale basis. Also, because it is

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a unique system which includes a large number of events such as physiological reprogramming of explants as well as changes in gene expression and cell division patterns, and in cell fate (Fehér 2008; Rose et al. 2010), somatic embryogenesis has also become an appropriate method for studying the morphophysiological and molecular aspects of cell differentiation. Understanding the developmental events during the induction and development of somatic embryos is essential if one intends to regulate each stage of the somatic embryogenesis developmental program efficiently. Additionally, such knowledge may be useful for the development of efficient protocols for somatic embryogenesis induction and for validation of genetic transformation systems (Fehér et al. 2003; Yang and Zhang 2010; Rocha and Dornelas 2013; Mahdavi-Darvari 2015). Anatomical and ultrastructural studies have contributed to a better understanding of the basic cellular mechanisms involved in the acquisition of competence and histodifferentiation of somatic embryos (Canhoto et al. 1996; Verdeil et al. 2001; Moura et al. 2008; 2010; de Almeida et al. 2012; Rocha et al. 2012, 2013, 2016). In other words, the dynamic and fate of cells committed to somatic embryogenesis can be investigated by microscopy techniques (Rocha et al. 2016). The formation of an embryogenic callus and the subsequent differentiation of somatic embryos can be analyzed over time, and studies of the cytological changes that have occurred during these processes can also be of great value. *In vitro* development of cells and tissues depends on different factors such as: genotype, type of plant, age and developmental stage of an explant, physiological state of an explant-donor plant, and the external environment, which includes composition of media and physical culture conditions. Histological methods contribute significantly to the understanding of *in vitro* tissue culture systems in woody perennials, since they provide information that enables us to make the right decisions to optimize *in vitro* propagation protocols. Here, we report studies of the histological changes that characterize the somatic embryogenesis developmental pathway in two woody perennial species. *Melia azedarach* L. (chinaberry) and *Prosopis alpataco* Phil. (alpataco) are two multipurpose trees. They provide wood, food and various bio-active compounds. Cotyledons placed in the induction medium were used to induce somatic embryogenesis (Sharry et al. 2006; Boeri et al. 2016). Both species will differentiate embryogenic calli in induction culture medium and these were analyzed microscopically.

Material and methods

***Prosopis alpataco* Phil.**

Embryogenic calli of alpataco, previously fixed in FAA, were treated by two different ways, one as a squash technique and the other by microtome sectioning. In the first case, the calli were immersed in a 1:1 solution of 5% NaOH and 5% NaOCl for 8 hours (during that time the material turns translucent). They were then rinsed in distilled water and immersed in cloral hydrate (5%) for 24 hours and finally embedded in gelatin-glycerin with safranin. For the microtome sectioning, the methodology used was the one proposed by D'Ambrogio de Argueso's (1986), where the times of each treatment were adjusted as recommended by Arambarri (2015, personal communication). Finally, staining with Cresyl violet was performed and gelatin –glycerine was used for assembly. A Schönfeld's binocular loupe and a Motic BA310 microscope were used to evaluate the samples and photographs were taken with a Motic Image Plus 2.0 camera.

***Melia azedarach* L.**

The 4-year-old embryogenic calli were fixed and embedded in paraffin and cross-sectional and longitudinal cross-sections were made. Infiltration and paraffin inclusion were performed according to the technique of Jeff Long and Kathy Bartons (Arabidopsis'98 CSHL Course Manual). Schiffs staining technique was used for total carbohydrate and protein determination that stained with a fuchsia or strong pink color. In order to obtain serial cuts of 5 μm, a rotating microtome was used in radial and longitudinal planes. Samples were

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placed on slides, labeled and collected for further observation. For this samples a GEMALUX binocular loupe, IROSCOPE optical microscope and Olympus BX40 microscope were used; and photographs were taken with a SEAGULL DF-300X or RGB CCD cameras.

In both species, the following characteristics were observed: friability, compaction, differentiated structures (conduction system, somatic embryos, and organs), color, size, cellular types and presence of starch granules.

Results and discussion

Unlike *Prosopis alpataco* (alpataco), for which only embryos were produced, *Melia azedarach* (chinaberry) formed callus of a mixed type (organogenic and embryogenic). In both species, the calli derived from cotyledons, presented a greenish coloration due to the presence of chloroplasts, because they were induced in the presence of a photoperiod of 16 hours of light. Histological analysis showed that embryogenic calli of chinaberry originated from cotyledon parenchyma cells, whereas in alpataco the embryogenic response was initiated in tissues associated with the embryonic axis close to the cotyledons. The cells which are more competent for somatic embryogenesis are generally those of young tissues, immature zygotic embryos among them.

In both species, typical callus contained pro-embryogenic (ce) and non-embryogenic (cne) cells (**Fig. 1** and **Fig**. **2**). Non-embryogenic cells were elongated and thin-walled while containing less-dense cytoplasm. They were individualized, poorly differentiated and very little colored. The embryogenic ones were rounded and had relatively thick walls, dense cytoplasm and a prominent nucleus. All these traits are typical of meristematic and proembryogenic cells. These characteristics have also been described in other cultures (Dussert et al. 1995).
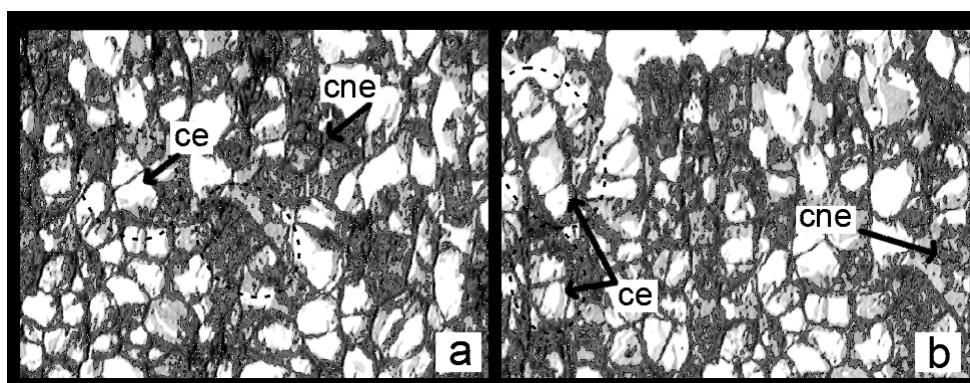
Figure 1. Somatic embryos originated from little isodiametric parenchymal cells covered by a mono-stratified epidermis which by cell division formed a bicellular pro-embryo *(a, Chinaberry)*. Continued division of cells limited by a relatively thick wall resulted in the development of pro-somatic embryos *(b, Chinaberry)*. ce: embryogenic cells; cne: non-embryogenic cells.

The process covered different stages. In Chinaberry, it began by induction, that is, the conversion of a somatic cell into a pro-embryogenic cell (**Fig. 3a,b**) and then continued by histodifferentiation (**Fig. 3c-e**), in which pro-embryogenic cells differentiated into somatic embryos (**Fig. 3f,g**), with both division and differentiation occurring simultaneously.

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Figure 2. Embryogenic callus of *P. alpataco* obtained from cotyledons with embryonic axis on SE induction medium. Differentiation of embryogenic cells (ce) and non-embryogenic cells (cne). Scale: 100 µm.
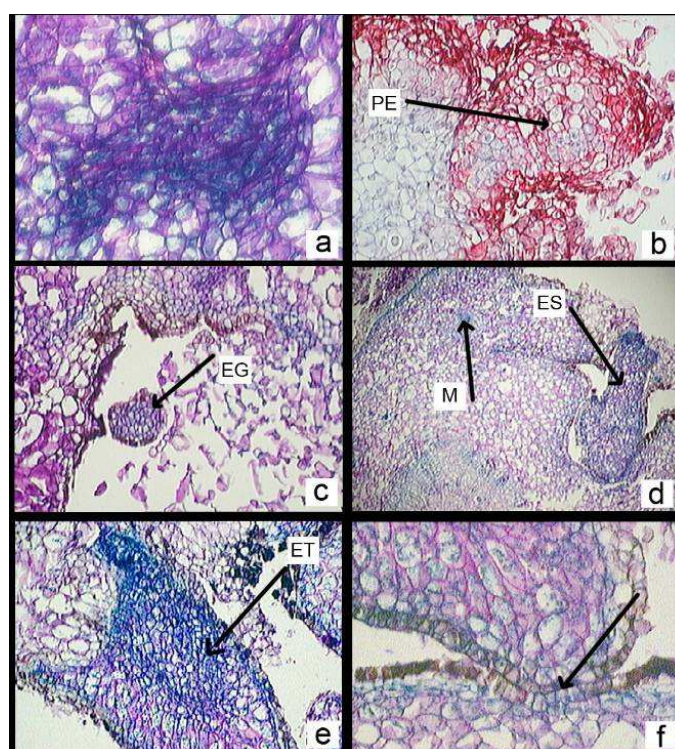
Figure 3. Somatic embryogenesis in Chinaberry. Initiation of indirect somatic embryogenesis inside little, isodiametric parenchymatic cells, covered by a mono-stratified epidermis (a-b). The pro-embryogenic cells differentiated into embryos that passed through a series of intermediate stages: globular (c), heart (d) and torpedo (e). There is no connection between somatic embryos and the callus tissue (f). PE: pro-embryos; ES: somatic embryos; M: meristemoid; EG: globular somatic embryo: ET: torpedo somatic embryo.

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The divisions observed in these superficial cells (initially periclinal and then, periclinal, anticlinal and oblique) would support the assertion of the unicellular origin of the embryos obtained in *P. alpataco* and *M. azedarach*. The proembryos were observed initially growing in the embryogenic mass, being individualized later as spherical or proembryonarial structures, covered by a protodermis. This histogenesis process occurred along with the emergence of a fundamental meristem and procambial tissue. Other processes observed were the formation of isolated xylem vessels, and areas where meristematic cells were found (**Fig. 4**). These meristematic regions continued to divide actively and, accompanied by a rearrangement in the length of the vessel cells, gave rise to the formation of embryos at different stages of development (globular, heart, torpedo and cotyledon) with conduction tissue (**Fig. 5** and **6**).
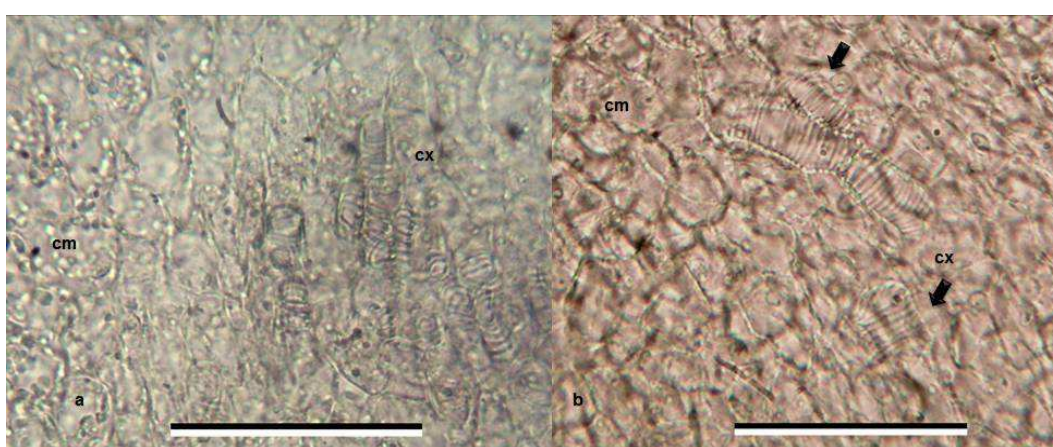
Figure 4. Differentiation of individual xylematic vessels (cx) and areas with meristematic cells (cm) in the embryogenic callus of *P. alpataco*. Scale: 50 µm.
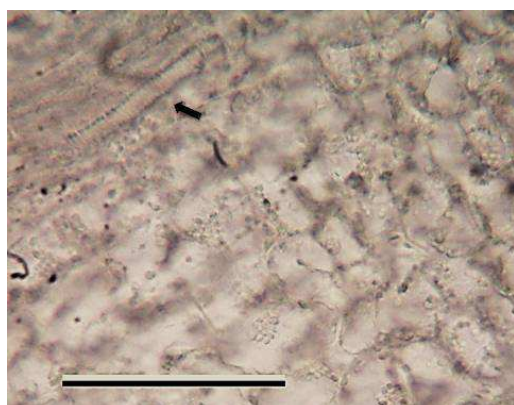
Figure 5. Length reordering of vessel cells in the embryogenic callus of *P. alpataco* and formation of conduction tissue as indicated by an arrow. Scale: 50 µm.
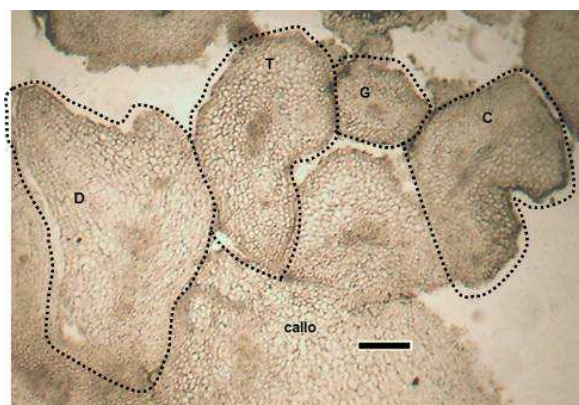
Figure 6. Somatic embryos of *P. alpataco* at different stages of development: a globular stage embryo individualized on the surface of the embryogenic callus (G); embryo at heart stage (C), torpedo embryo (T) clearly showing the well-differentiated caulinar and root apices; early stage of dicotyledonous embryo (D). Scale: 100 µm.

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These structures, originated from individual cells of the surface of the embryogenic callus. They were variable in shape and size and gave rise to the initial stages of somatic embryos (globular and heart states) (**Fig. 7a,b**). During these embryogenic stages, the formation of conduction tissue was observed in the center of the somatic embryos. After the appearance of the first embryonic stages, the elongation of the hypocotyl-radicle axis resulted in the torpedo shape, clearly showing a distinct root and shoot apex. This elongation was accompanied by a rearrangement of vascular tissue and adjacent parenchymal cells. The expansion and separation of the cotyledons marked the cotyledonary phase with two cotyledons and an apical meristem in which mitotic activity clearly occurred. Torpedo and cotyledonary embryos were obtained that were comparable to zygotic embryos (**Fig. 7c,d**).
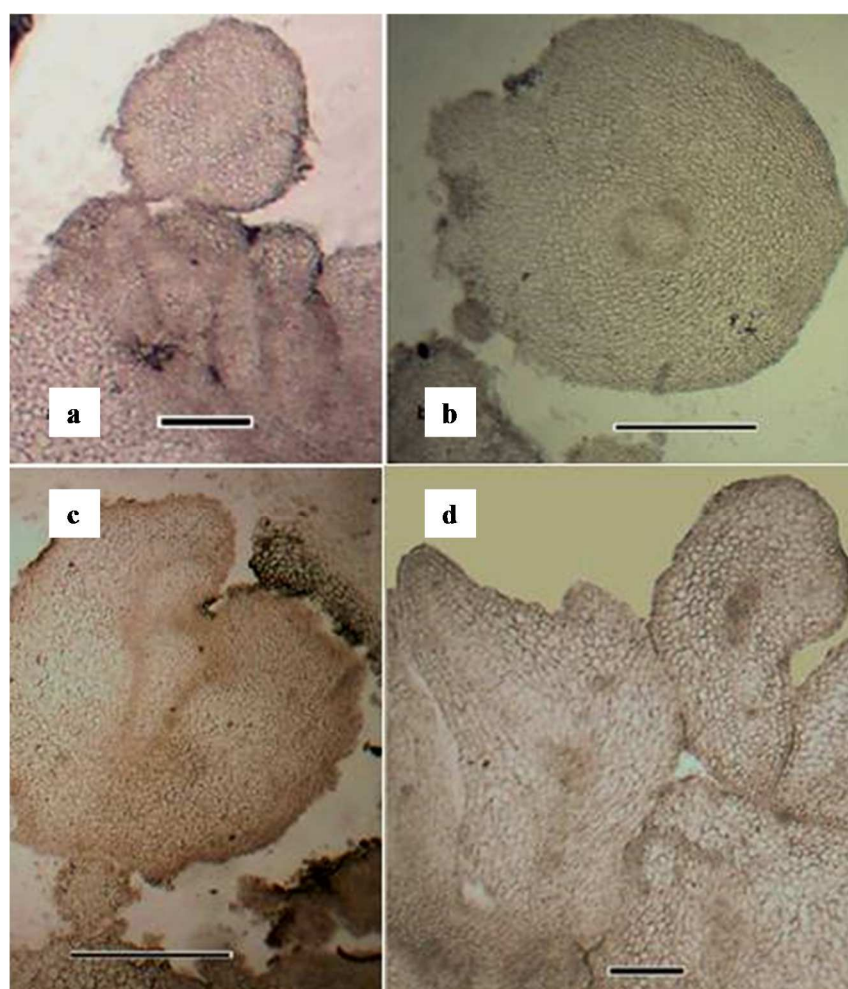
Figure 7. Initial stages of somatic embryos of *P. alpataco*: incipient globular stage (a); globular somatic embryo with a developed conduction tissue in the center of the structure (b); somatic embryo of *P. alpataco* at the heart stage (c). In the center a well-developed conduction tissue and the lack of connexion with the subjacent tissue are shown; somatic embryos of *P. alpataco* in torpedo stage and early phase of the embryo in cotyledonary stage (d). Scale: 100 µm.

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The initiation of polarity in embryos is often regarded as the first step in embryogenesis (Warren and Warren 1993). In carrot it appears that the first division has to be asymmetric, producing two cells of different sizes, in order to confer embryogenic competence to the individual cells (Dudits et al. 1991). In the case of carrot, the first division of single suspension cells capable of forming embryogenic cells is unequal (Komamine et al. 1992), and only the smaller daughter cell will ultimately develop into an embryo (de Jong et al. 1993). A polarized DNA synthesis in these cells was reported by Komamine et al. (1992), Tsukahara and Komamine (1997) and Sato-Nara and Fukuda (2000). In most species showing embryogenic capacity, the asymmetric division does not form an embryo directly, but forms a pro-embryogenic mass (PEM), in which only one or a few cells can subsequently develop into an embryo (Komamine et al. 1992; Ronchi and Giorgetti 1995). Filonova et al. (2000) observed in Norway spruce that the rest of the PEM cells are probably eliminated through a cycle of programmed cell death. In a close relationship with *in vitro* embryogenesis, the orientation of the first cell division has also been considered to be essential to the establishment of the basic polarity of the zygotic embryo (Jimenez et al. 2001).
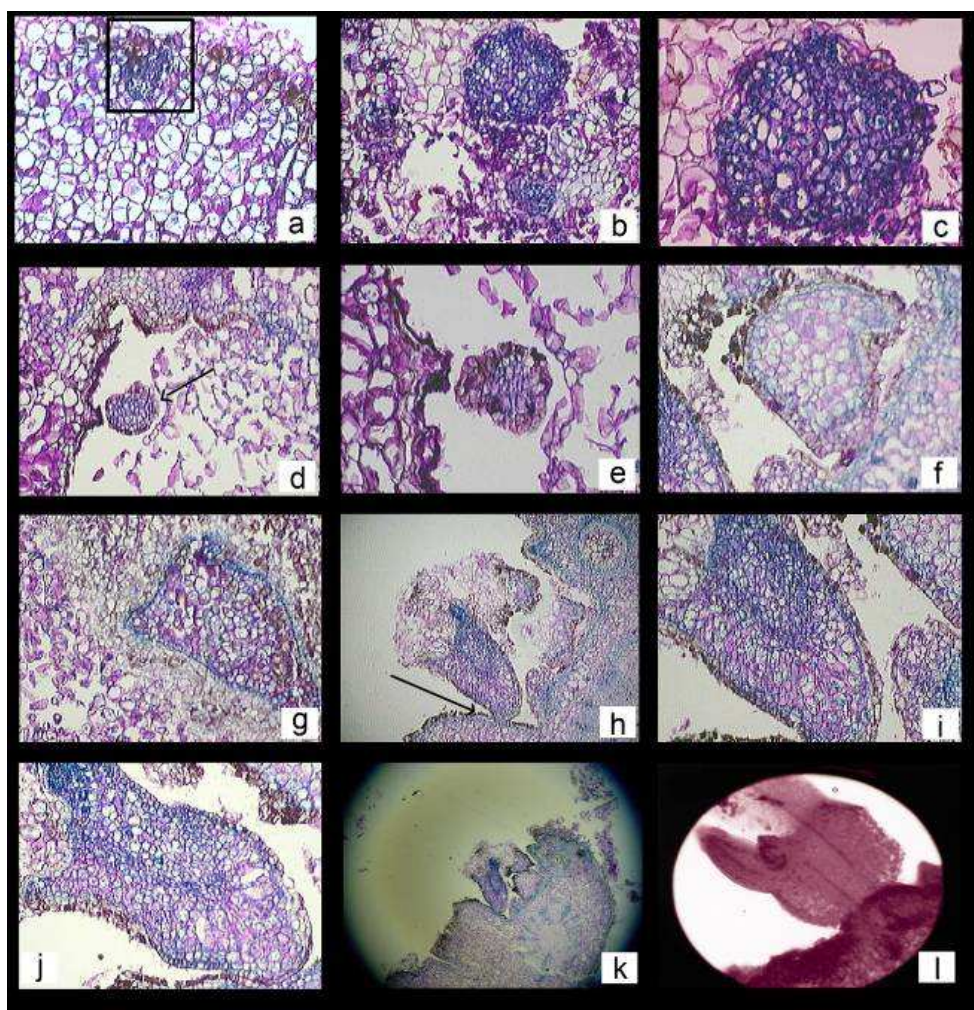
Figure 8. Somatic embryogenesis of *Melia azedarach* from the globular stage (a-d) to the heart (e,f) and cotyledonary stages (g-l).

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In the case of *Melia*, the embryos passed through a series of intermediate stages. It was observed that the transition from the globular stage (**Fig. 8d**) to the heart stage (**Fig. 8e**,**f**) is a sign of bilateral symmetry, characterized by the cessation of uniform growth and the initiation of the two cotyledons (**Fig. 8g**). At this point, cell division occurred predominantly in the cotyledonary portions, while the cells present between the cotyledons ceased their division (**Fig. 8h,i**). The elongation of the hypocotyl-radicle axis defined the torpedo shape, clearly establishing the difference between the root apex and the shoot apex (**Fig. 8j,k**). Also, the expansion and separation of the cotyledons marked the mature embryo phase (**Fig. 8j,k**), where the two cotyledons and the apical meristem zone showed great mitotic activity and vascular tissue. The apical meristem developed between the two cotyledon primordia in the apical zone of the somatic embryo (**Fig. 8l**).

The squash technique applied to the embryogenic calli of *P. alpataco and M. azedarach* allowed us to confirm the formation of somatic embryos as it was possible to observe the lack of vascular connections between the embryonic tissues and the parenchyma tissues of the callus (**Fig. 9**). Finally, the somatic embryos obtained constitute bipolar structures with a radicle and a plumule.
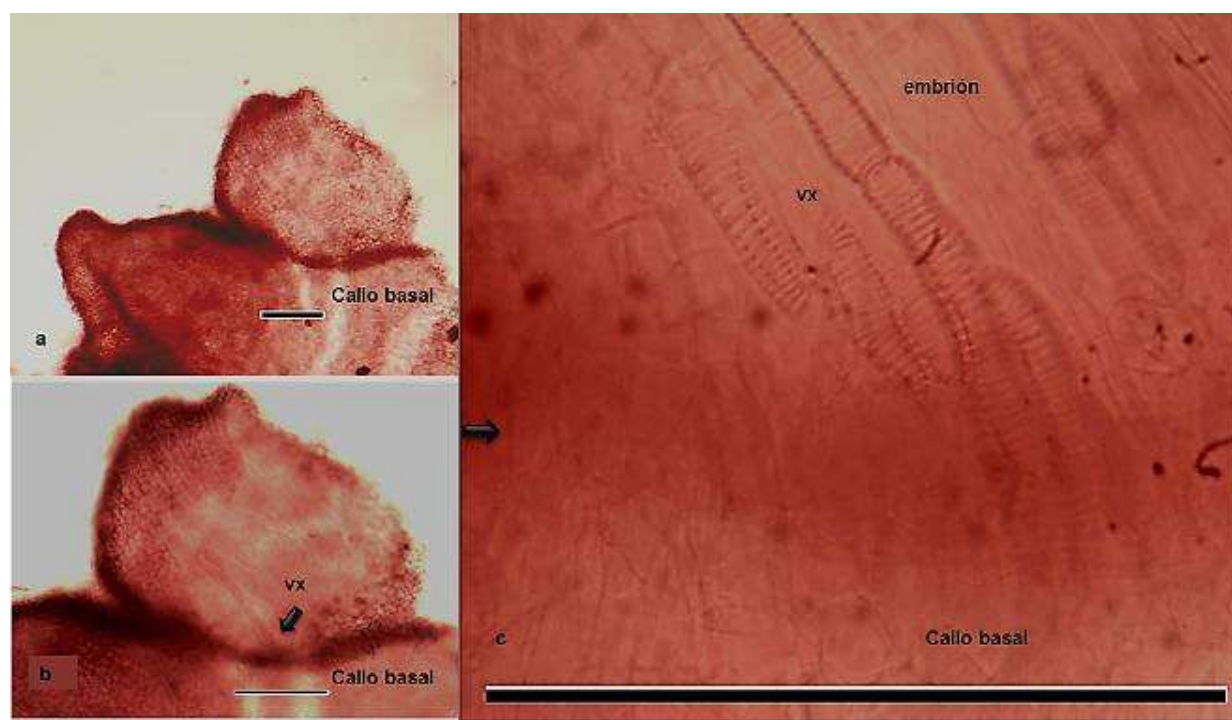
Figure 9. Squash of embryogenic callus of *P. alpataco*. Somatic embryo of *P. alpataco* on parenchymatic tissue of the calli (a); union zone from the embryo to the explant, indicated by an arrow (b); union zone between the embryo and the callus (c). Xylem vessels (VX). Scale: 100 μm.

Conclusions

For two woody perennial species similarities and differences of somatic embryogenesis at microscopy level were studied. For both species somatic embryogenesis was similar despite the fact that no systematic relationship between the species exists. However, the explants and environmental conditions were similar. This study is important because of the need to identify the cells associated with the induction process and

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the formation of structures capable of organized growth and eventual development into seedlings. For plant regeneration from somatic embryos, histological analyses must be carried out to determine the route of regeneration and to discover whether the plant originated from somatic embryogenesis or organogenesis.

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Proliferation and rooting of chestnut under photoautotrophic conditions

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Abstract

In this study we used chestnut clones selected for their resistance to ink disease and propagated them under photoautotrophic conditions during both proliferation and adventitious rooting stages. For successful proliferation, shoots were first exposed to decreasing sugar levels for some months, to enable their survival in sucrose-free medium. *In vitro* rooting in photoautotrophic conditions was applied to 15 chestnut genotypes. More than 6000 shoots were tested, and average values for rooting and acclimation were better than in conventional micropropagation.

Keywords: bioreactors, CO_2, liquid culture, PPF, sucrose

Introduction

European chestnut (*Castanea sativa* Mill.) is of major ecological and economic importance in Europe. However, this tree species has been threatened for more than a century by the spread of ink disease caused by the fungi *Phytophthora cinnamomi* and *P. cambivora* (González et al. 2011). Chestnut trees showing different degrees of resistance to the disease have been obtained by hybridizing European and Asian chestnut species (*C. crenata* and *C. mollissima*, respectively). Both species display natural resistance/tolerance to the pathogens.

Interspecific hybrids should be propagated vegetatively in order to maintain their resistance. As chestnut is highly recalcitrant to macropropagation, agar-based micropropagation protocols have been developed (Vieitez et al. 2007). With the aim of reducing production costs and improving plant quality for acclimatization, we have previously cultured chestnut shoots in liquid medium by using temporary (Vidal et al. 2015) and continuous immersion systems (Cuenca et al. 2015), always with sugar added as a carbon source.

Photoautotrophic micropropagation (PAM) involves the elimination of exogenous sugar from nutritive media to promote photosynthetic activity in the cultures. Shoots cultured by PAM are considered to be physiologically healthier and better adapted to acclimation than shoots cultured in the presence of sugar under photomixotrophic conditions (Xiao et al. 2011).

In the present study, we used PAM to propagate selected chestnut clones (displaying resistance to ink disease) during proliferation and adventitious rooting stages.

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Materials and methods

Continuous immersion system

Chestnut shoots were cultured in a continuous immersion system by using bioreactors in-house adapted from 10-16 L polypropylene food storage containers (Lock&Lock$^{©}$). The bioreactors were equipped with 0.2 µm filters to receive forced ventilation with CO_2-enriched air and were placed in an experimental unit, as previously described (Cuenca et al. 2015), under a 16-h photoperiod provided by white light-emitting diodes at photosynthetic photon flux densities (PPF) ranging from 50 to 150 µmol m^{-2} s^{-1}, and a 25°C light/ 20°C dark temperature regime. Standard conditions were established as ambient CO_2 (~ 400 ppm) and low PPF (~ 50 µmol m^{-2} s^{-1}) and photoautotrophic conditions consisted in elevated CO_2 levels (~ 2000 ppm) and high PPF (~ 150 µmol m^{-2} s^{-1}).

Proliferation

Shoots from four chestnut genotypes (C042, C053, P042 and P043) were cultured in 10 L bioreactors with 1 L of Murashige and Skoog medium (Murashige and Skoog 1962) containing half strength nitrates (MS^{N½}) and supplemented with 0.05 mg/L N^{6}-benzyladenine (BA). Rockwool cubes (1 cm^{3}) were used as support material. Shoot quality and proliferation rates were evaluated, 5 weeks after culture initiation, in relation to explant type (apical sections or basal sections attached to the basal callus), explant size (1.5 or 3 cm), amount of sucrose added to the medium (0, 1 or 3% (w/v)), PPF density (50 and 150 µmol m^{-2} s^{-1}) and CO_2 level (400 and 2000 ppm).

The following variables were determined: 1) number of shoots/explant, 2) multiplication coefficient, calculated as the number of new 1.5 cm segments obtained per initial explant, 3) the length of the longest shoot per explant (SL), 4) the number of rootable shoots per explant (shoots longer than 3-4 cm with an actively growing apex), 5) pigment levels (chlorophyll a, b and carotenoids, extracted with dimethylformamide and quantified following the method described by Wellburn 1994).

Rooting

Shoots from fifteen chestnut genotypes were dipped in 1 g L^{-1} IBA (indole-3-butyric acid) for 2 min before being cultured in 16 L bioreactors containing 2.5 L of Gresshoff and Doy medium (Gresshoff and Doy, 1972) with 1/3 strength macronutrients, but without plant growth regulators or sucrose, under white LEDs (PPF of 150 µmol m^{-2} s^{-1}). Rooting rates were evaluated 6 weeks after culture initiation in relation to parameters such as the shape and size of rockwool cubes (Kiemplugs or AO [Grodan$^{®}$] blocks) and CO_2 level (400 to 2000 ppm). Rooted plantlets were transferred to polystyrene trays in a peat:perlite (3:1) mixture. Acclimation success was assessed two months after rooting evaluation.

Results and discussion

Proliferation

Initial experiments performed without sucrose and using explants previously grown in standard conditions (low PPF and ambient CO_2 levels) with 3% sucrose were not successful, and the shoots did not proliferate. In order to modify the physiological state of the chestnut shoots towards photoautotrophy, the shoots were

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subcultured in media in which the sucrose levels were lowered over at least three subcultures (to 1% and then 0.5%). Simultaneously, the effect of manipulating light intensity and CO_2 levels was evaluated.

During the first experiments, apical sections showed a poor ability to grow, which led us to adjust the size of the explants to cope with the new conditions of sugar limitation. The effect of increasing explant size on the proliferation of apical sections of clone P042 cultured with sucrose 3% under low light intensity and without CO_2-enriched air (400 ppm) is shown in **Fig. 1a**. Even under these photomixotrophic conditions, 1.5 cm sections (with one or two leaves) showed limited proliferation; however, in 3 cm sections with four leaves, the number of segments obtained was almost triplicated. The role of explant size in conventional micropropagation has been highlighted by Pierik (1987), who also pointed out that larger explants may be less dependent on the addition of nutrients, including sugars. Miyashita et al. (1996) reported that potato explants with a large leaf area responded better to PAM than those with smaller leaves or without leaves. Mingozzi et al. (2009) was not successful in proliferating *Populus deltoides* in a PAM system when they used 1-cm-long stem sections devoid of leaves as explants. For these reasons, we used only 3-4 cm apical or basal sections, with at least 4 expanded leaves, in subsequent experiments with chestnut.
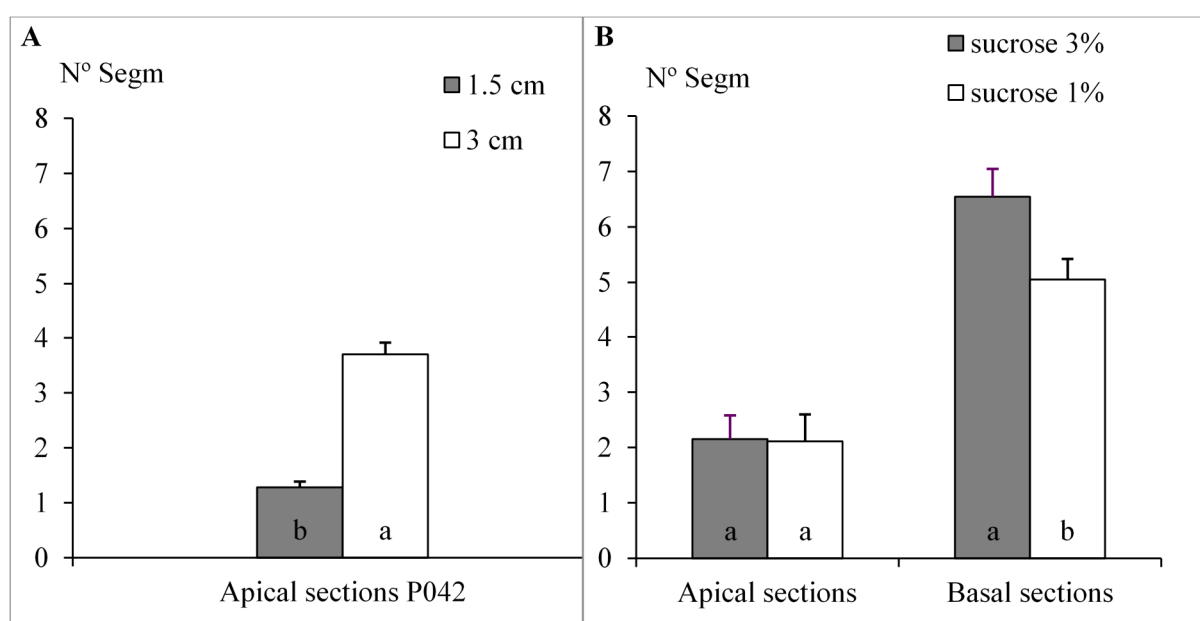
Figure 1. Effect of explant size (A) and sucrose (B) on rates of proliferation of chestnut shoots cultured with 400 ppm CO_2. A) Clone P042: Apical sections (1.5 and 3 cm) cultured under 50 µmol m^{-2} s^{-1} PPF and with sucrose 3%. B) Clone P043: Apical and basal sections of 3 cm cultured under 150 µmol m^{-2} s^{-1} PPF and with two levels of sucrose (1% and 3%). For each clone and explant type, different letters indicate significant differences at p < 0.05.

To study the effect of sucrose content on shoots cultured without CO_2-enrichment (400 ppm), 3-cm apical and basal sections of clone P043 were grown under high light intensity in media containing 1% and 3% sucrose. High proliferation rates were obtained under both conditions, although basal sections treated with 3% sucrose produced significantly more segments than those cultured with lower levels of sucrose (**Fig. 1b**). Similar results have been reported for *Phalaenopsis* (Yoon et al. 2009) and *Pfaffia glomerata*

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(Saldanha et al. 2013), which grew better when sucrose 3% was added to the medium in ambient CO_2 conditions.

In order to increase the rate of proliferation of shoots cultured with low amounts of sugar, the effect of applying CO_2-enriched air was studied in basal sections of clone C053 cultured with 1% sucrose and under high PPF (150 µmol m^{-2} s^{-1}). A greater number of longer shoots were produced with CO_2-enriched air, which lead to significantly higher proliferation rates (**Fig. 2a**). However, photosynthetic pigments (chlorophylls a, b, and total carotenoids) were not affected by CO_2 levels (**Fig. 2b**). The presence of CO_2 has been found to promote growth of apple cultured with or without sugar (Morini and Melai 2003/4), kiwi cultured with 1-2% sucrose (Arigita et al. 2010) and also Brazilian ginseng (Saldanha et al. 2013) and *Paulownia fortunei* (Sha Valli Khan et al. 2003), both cultured without sugar.
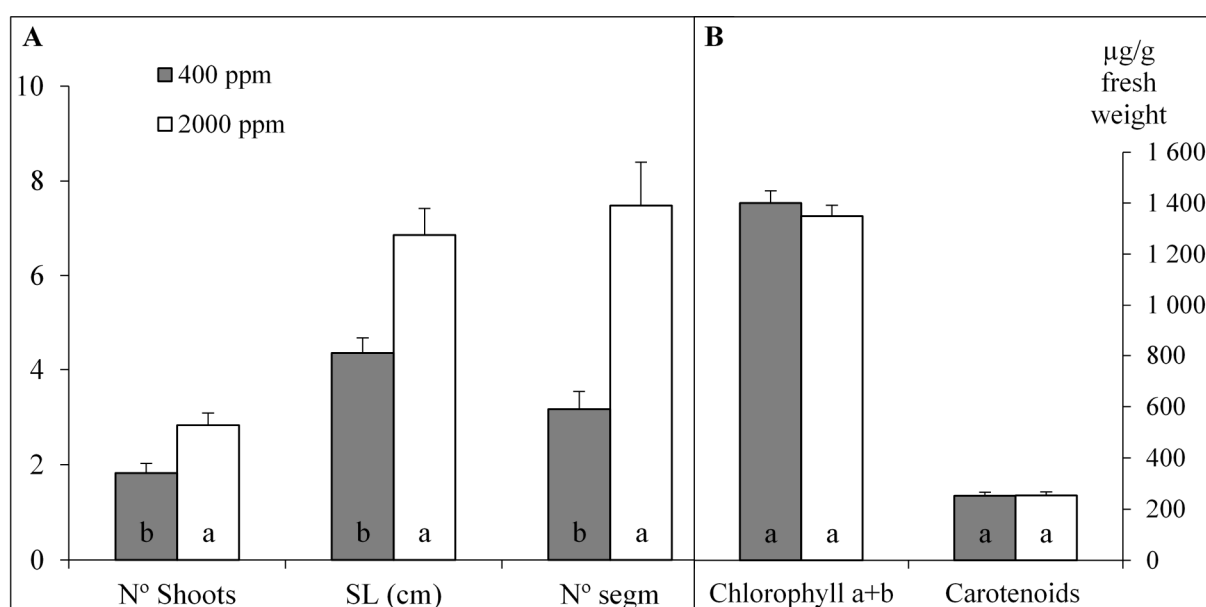
Figure. 2. Effect of CO_2 levels (400 and 2000 ppm) on proliferation rates (A) and photosynthetic pigments (B) in basal sections of clone C053 cultured with 1% sucrose and 150 µmol m^{-2} s^{-1} PPF. For each variable, different letters indicate significant differences at $p < 0.05$. SL: Shoot length.

The effect of light intensity (PPF, 50 and 150 µmol m^{-2} s^{-1}) was investigated in another experiment with the same chestnut genotype. In this case, both basal and apical sections were included, and CO_2-enriched air (2000 ppm) was applied to all the vessels. The results of the two-way ANOVA (**Fig. 3**) indicate that basal explants cultured under high light intensity performed best. Light intensity did not significantly affect growth parameters, as p values of 0.434, 0.197 and 0.239 were obtained for respectively number of shoots, number of segments and number of rootable shoots. However, explant type strongly influenced the proliferation rate, with p values of less than 0.001 obtained for all the variables. Similar findings regarding light intensity have been reported for *Doritaenopsis* (Shin et al. 2013) and for *Eucalyptus uro-grandis* propagated without sucrose under a relatively low PPF (45 µmol m^{-2} s^{-1}), but elevated CO_2 levels (Tanaka et al. 2005).

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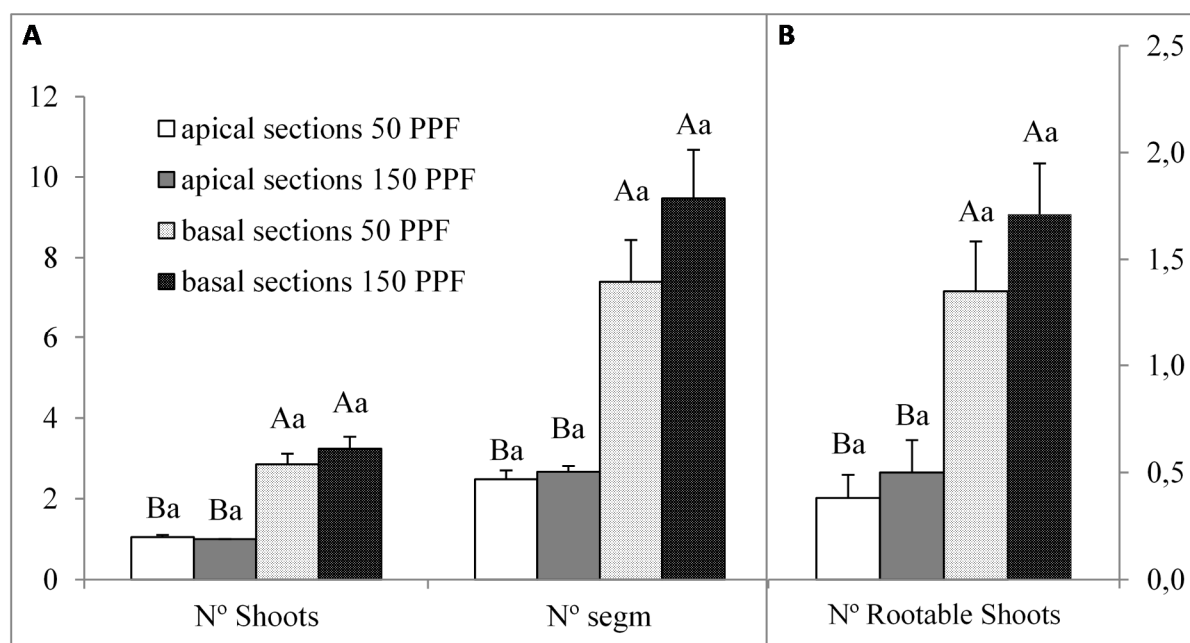
Figure 3. Effect of light intensity (50 and 150 µmol m^{-2} s^{-1} PPF) and explant type (apical and basal sections) on proliferation rates (A) and number of rootable shoots (B) in clone C053 cultured with 1% sucrose and 2000 ppm CO_2. For each variable, different capital letters indicate significant differences regarding explant type, and different small letters indicate significant differences regarding light intensity (p < 0.05).

Once acceptable proliferation rates were obtained using shoots cultured with 1% sucrose, the conditions that supported this growth (2000 ppm CO_2 and 150 µmol m^{-2} s^{-1} PPF) were applied to shoots cultured with 0.5% sucrose for one month, followed by subsequent culture in medium devoid of sugar. Proliferation rates of apical and basal sections of clone P042 after three subcultures under PAM without sucrose are summarized in **Fig. 4**, and the quality of shoots of clones P043, C053 and C042, also cultured without exogenous sugar, is shown in **Fig. 5a-c**.

Chestnut shoots were able to grow under photoautotrophic conditions without sucrose (**Fig. 4** and **5**). Healthy shoots and high proliferation rates (average multiplication coefficients of 3.0 and 4.9 for apical and basal sections respectively) were obtained, although in some genotypes these values were not as high as those obtained when sugar was added to the culture medium (data not shown). A similar response to PAM has been obtained with *Paulownia fortunei* (Sha Valli Khan et al. 2003), while in other plants, such as *Samanea saman* (Mosaleeyanon et al. 2004), *Oplopanax elatum* (Park et al. 2011) and *Billbergia zebrina* (Martins et al. 2015), the addition of sugar hampered growth or diminished shoot quality.

Sugar has also been found to promote growth under photoautotrophic conditions, as reported for *Alocasia amazonica* (Jo et al. 2009), *Populus deltoides* (Mingozzi et al. 2009) and *Juglans regia* (Hassankhah et al. 2014). Interestingly, Iarema et al. (2012) reported that *Pfaffia glomerata* cultured without sugar produced taller shoots, whereas Saldanha et al. (2013) observed the opposite. In the latter case, the initial explants were devoid of leaves, which may have hampered their development in the PAM system (Miyashita et al. 1996). Sáez et al. (2016) was not successful in achieving proliferation of *Castanea sativa* shoots cultured under 50 or 150 PPF with 0.5% sucrose. However, the lowered sucrose content was not accompanied by an increase in CO_2 levels, as recommended by Kozai and Kubota (2001) for photoautotrophic development.

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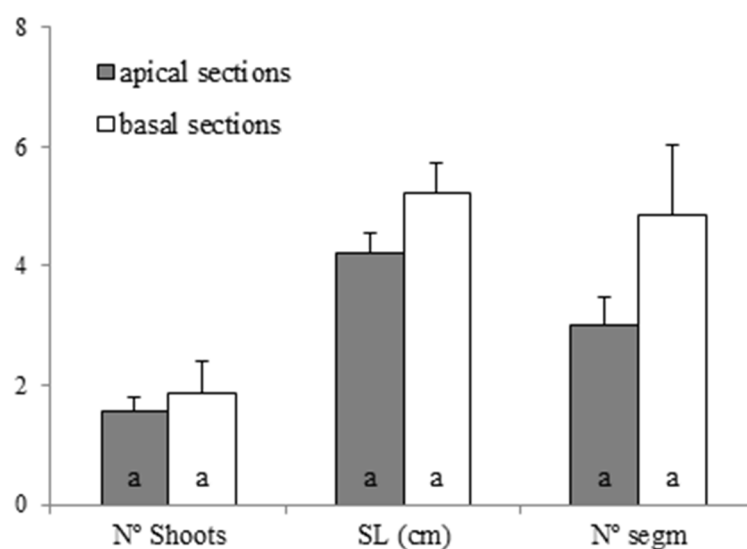
Figure 4. Effect of explant type on proliferation rates in shoots of clone P042 cultured without sucrose under 150 μmol m^{-2} s^{-1} PPF and with 2000 ppm CO_2. For each variable, different letters indicate significant differences at $p < 0.05$. SL: Shoot length.
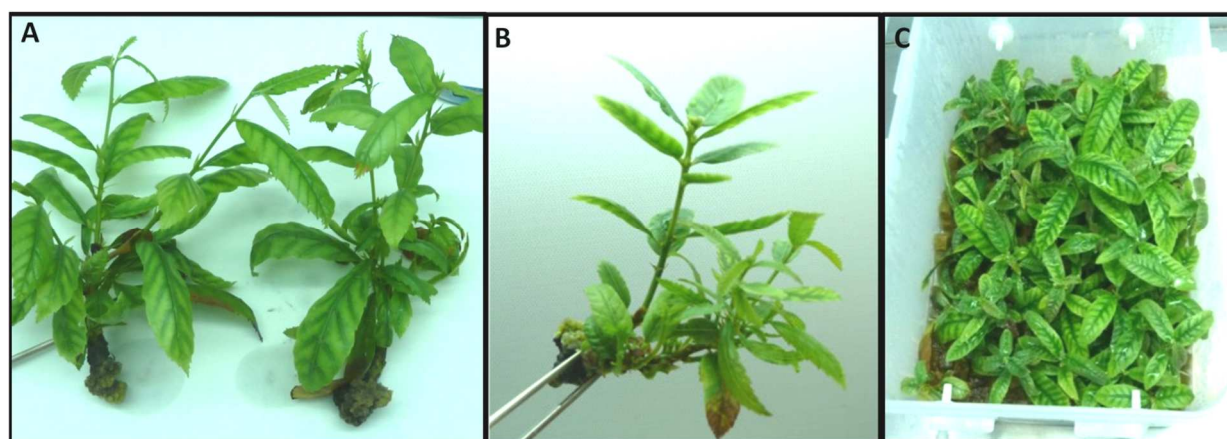
Figure 5. Chestnut shoots cultured without sucrose under 150 μmol m^{-2} s^{-1} PPF and with 2000 ppm CO_2. A) Clone P043. B) Clone C053. C) Clone C042.

Rooting

More than 6000 vigorous chestnut shoots belonging to 15 genotypes were used in rooting experiments. The shoots were dipped in 1 g L^{-1} IBA and placed 16 L bioreactors in sucrose-free medium, with rockwool cubes as inert substrate. The effect of CO_2 level on the rooting rates is shown in **Fig. 6a**. Although the effects were not significant, CO_2 levels of 1200-1500 ppm were the most suitable for root development and

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shoot growth. Rooting success was not significantly affected by the shape of the rockwool cubes used to maintain the shoots in a vertical position. The AO blocks yielded a rooting rate of 71%, and Kiemplugs a rooting rate of 51% (**Fig. 6b**). High survival rates and shoot regrowth were observed after transplantation. All shoots rooted in the PAM system were subsequently successfully acclimated (**Fig. 7a-b**). An average rooting plus acclimation rate of 52% was obtained, indicating the feasibility of applying photoautotrophic conditions for rooting and acclimating chestnut shoots. Similar results have been reported for other species such as *Eucalyptus calmadulensis* (Zobayed et al. 2001), *Epidendrum* (Teixeira da Silva et al. 2005), *Myrtus communis* (Lucchesini et al. 2006), *Populus deltoides* (Mingozzi et al. 2009), *Macadamia tetraphylla* (Cha-um et al. 2011), *Oplopanax elatum* (Park et al. 2011) and *Billbergia zebrina* (Martins et al. 2015).
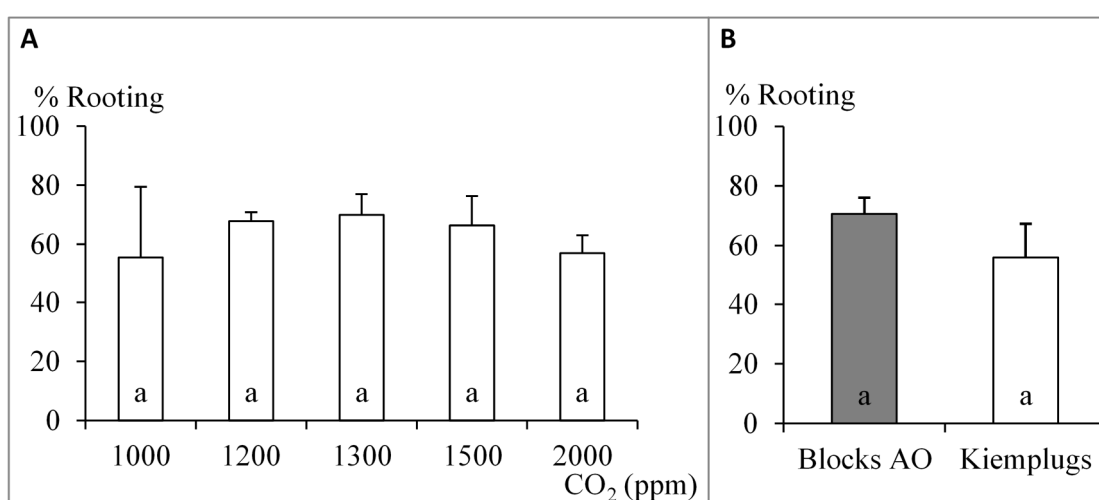
Figure 6. Effect of CO_2 (A) and substrate type (B) on rooting success in chestnut shoots. Explants derived from 15 clones (6300 shoots) were dipped in 1g/L IBA for 2 min before being transferred to rockwool plugs (AO blocks or Kiemplugs) in 16 L bioreactors with different levels of CO_2. Mean values indicated by the same letter were not significantly different at $p < 0.05$.

Figure 7. A) Shoots of clone 90025 rooted in different types of rockwool plugs. B) Acclimated plants of Prêcoce Migoule 14 weeks after rooting induction in the PAM system.

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Conclusions

The present findings showed for the first time the feasibility of proliferating and rooting axillary chestnut shoots under photoautotrophic conditions. For successful proliferation, shoots were first exposed to decreasing sugar levels for some months to enable their survival in sucrose-free medium. They were then easily rooted and acclimated, without sugar. Biochemical studies comparing the physiological state of shoots cultured in different conditions are in progress.

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Expression of a *GH3* gene during adventitious rooting in chestnut

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Abstract

Induction of adventitious roots (ARs) is an auxin-regulated developmental process which is negatively affected by the maturity of the plant. Analysis of the sequence encoded by the *CsGH3-1* gene isolated from chestnut revealed that the putative protein belongs to group II of the GH3 family and contains the enzyme domains involved in conjugating indole-3-acetic acid (IAA) to amino acids. Here, we characterized the gene expression induced in chestnut microshoots treated with indole-3 butyric acid (IBA) to promote AR formation. Expression of *CsGH3-1* was strongly induced in IBA-treated microshoots, whereas the transcripts were barely detected in control (untreated) microshoots. Comparison of the expression patterns in the rooting-competent and rooting-incompetent shoots confirmed that expression of the gene was correlated with the ontogenetic stage of the microshoots. The *CsGH3-1* mRNA levels were higher in mature shoots than in rooting-competent shoots during the period of cell dedifferentiation prior to cell division, which suggests a putative role for the gene in this process. Furthermore, accumulation of the transcripts in the cambium cells, only detected in root-forming shoots, was highly correlated with the ability of these cells to generate ARs. Overall, our findings indicate that the *CsGH3-1* gene is involved in auxin homeostasis and has a tissue-specific function in generating the auxin gradient required for transition of cambium cells into root initials during induction of ARs.

Keywords: *Castanea sativa,* homeostasis, maturation, ontogenetic stage, root induction, shoot cultures

Introduction

The formation of adventitious roots (ARs) in forest species is crucial for the massive propagation of selected genotypes. Characterization of economically relevant traits in trees is only possible after maturation, which exerts a negative effect on the competence of tissues to respond to root-inducing signals in many forest species and thus causes tissues from mature trees to lose the capacity to form ARs. Maturation, therefore, limits the successful large-scale propagation of elite trees for commercial purposes.

An experimental chestnut (*Castanea sativa* Mill.) microshoot system was developed from the same 80-year old field-grown tree previously used to study phase change and maturation (Sánchez and Vieitez 1991). Basal sprouts (BS) and crown branches (C) were used as the source of material to establish two shoot culture lines sharing the same genetic background, but exhibiting different *in vitro* behaviour (reviewed in Ballester et al. 2009; Covelo et al. 2009). In particular, a high proportion (90%) of juvenile-like BS shoots form roots in response to exogenous auxin treatment, whereas adult C shoots only form callus-like structures (Ballester et al. 1999, 2009). The different rooting ability has been maintained, along with other morphological features, for more than 20 years in the *in vitro* lines. *In vitro* culture techniques provide controlled environmental conditions that help minimize perturbing signals. As both lines are maintained under the same culture conditions, this system is suitable for analyzing the loss of plasticity associated with the ontogenetic stage of tissues and thus for studying the decline in adventitious rooting during maturation.

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Auxin is a key player in most aspects of plant development and is an essential regulator of AR formation (reviewed in Geiss et al. 2009; Legué et al. 2014). Auxin levels increase during activation/induction (dedifferentiation), then decrease through the initiation process, finally increasing again during expression (emergence) of new roots (Kevers et al. 1997; De Klerk et al. 1999). Indeed, auxin is required for the dedifferentiation and acquisition of meristematic identity of differentiated cells that initiate a new developmental programme, thus producing changes at the molecular level (Abarca and Díaz-Sala 2009). In forest species, rooting competent cells are generally located in or close to the vascular cambium, which represents a secondary meristem where signals are integrated to adapt plant development to the current conditions (Risopatron et al. 2010). In rooting-competent chestnut shoots, derived from BS, auxin-induced cellular reprogramming takes place in cambial cells and their derivatives (where mitotic figures are seen 24 h after hormone application), thus initiating new divisions that eventually lead to the formation of ARs (Ballester et al. 1999). However, the activity of auxin depends on how the hormone is distributed within and between tissues, as well as on the sensitivity of the cells to the hormone. Therefore, both the amount and specific distribution of active auxin in the tissues strongly influence the physiological responses of the plant.

The active auxin content is directly influenced by the action of proteins in the Gretchen Hagen 3 (GH3) family of early auxin-responsive genes. The GH3 genes encode proteins related to the firefly-luciferase superfamily of enzymes (Chang et al. 1997). The family is divided into three subfamilies. Members of subfamily II are capable of conjugating auxin to amino acids, with GH3.5 also conjugating salicylic acid; some members in subfamily III activate jasmonic acid (JA) through conjugation with isoleucine, and the biochemical activity of members in subfamily I remains uncharacterized, except for GH3.12 in *Arabidopsis*, which is active on benzoates (Staswick et al. 2005; Westfall et al. 2012, 2013, 2016). The GH3 family is widely conserved and exclusive to the plant kingdom, although the number of genes varies between species (from 2 members in *Physcomitrella patens* to 19 in Arabidopsis). To date, the family has been characterized in only a few forest species, including model species such as aspen (Okrent and Wildermuth 2011) and fruit species such as apple (Yuan et al. 2013).

In Arabidopsis, the activity of specific members of the GH3 family has been linked to the formation of ARs. AtGH3.3, AtGH3.5 and AtGH3.6 have been shown to be involved in the interaction between auxin and JA signalling routes during the initiation of ARs (Gutierrez et al. 2012). In woody species, a GH3 gene from pine has been implicated in the molecular cross-talk between tree roots and two fungal species during establishment of a symbiotic mycorrhizal system (Reddy et al. 2006). However, the capacity of the GH3 proteins to modulate levels of active auxin suggests that these proteins may have a strong influence on the developmental process.

The aims of this study were 1) to characterize the structure of the CsGH3-1 protein and to predict the interactions with three auxin substrates and 2) to analyze expression of the gene in auxin-treated chestnut microshoots during the adventitious rooting process, by using different techniques such as northern blotting, quantitative PCR and in situ localization.

Material and methods

Plant material and root induction

Two stock lines of chestnut (*Castanea sativa* Mill) shoot cultures (clone P2), established *in vitro* from basal sprouts (BS) and crown branches (C) of the same mature tree (Sánchez and Vieitez 1991), were used in this study. ARs were induced by dipping the base of the shoots in a 5 mM IBA solution for 1 min and then placing them in 1/3 strength GD medium (Gresshoff and Doy 1972), as previously described (Sánchez et al. 2007). Microshoots were divided into basal and apical parts and harvested separately at 6, 12, 24 and

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48 h (Vielba et al. 2011) for RNA extraction or in situ hybridization. Control shoots were not treated with IBA. Microshoots were also harvested at the end of the proliferation cycle, designated as "time 0".

Phylogenetic analysis

We used GH3 sequences described for *Populus trichocarpa* (Okrent and Wildermuth 2011) and from *Castanea mollissima* to construct the phylogenetic tree. Data on the latter species were retrieved from the hardwood genomics database (www.hardwoodgenomics.org). Although 11 members of the GH3 family were identified in the *C. mollissima* genome database, two of these were not considered further as they did not contain all the functional domains. To build the phylogenetic tree, protein sequences were aligned with Muscle (Edgar 2004) and a maximum likelihood approach was used. Analysis was conducted with MEGA version 6 (Tamura et al. 2013).

Homology modelling

The model for the structure of CsGH3-1 was constructed using Swiss-Model, an automated protein structure homology–modelling program server (www.swissmodel.expasy.org), with a homologous protein structure as template (Arnold et al. 2006; Guex et al. 2009; Kiefer et al. 2009; Biasini et al. 2014). The 3-D structures of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and 1-naphthalene-acetic acid (NAA) were obtained using Chem3D pro 14.0 (www.cambridgesoft.com) and the UCSF Chimera package (Pettersen et al. 2004). Auxins were docked into the active site of CsGH3-1 by using the Autodock 4.2.6 molecular docking software package (Morris et al. 1998, 2009). We selected poses with lowest binding affinity to yield the three complexes: CsGH3-1–IAA, CsGH3-1–IBA and CsGH3-1–NAA. All molecular graphics were performed using Discovery Studio 4.1 Visualizer (Accelrys Software Inc., California, USA).

Analysis of CsGH3-1 expression

Total RNA was extracted from apical and basal parts of the shoots as previously described (Sánchez et al. 2007). For northern blotting analysis, the RNA was electrophoretically separated on agarose gels and transferred to a Zeta-Probe® blotting membrane as described by Gil et al. (2003). The filters were hybridized with a specific radio-labelled probe (400 nucleotide) generated from the 3´UTR region of the *CsGH3-1* sequence.

Quantitative PCR analysis was carried out as described by Sánchez et al. (2007). *CsGH3-1* specific primers were designed using the Primers Express software (*CsGH3-1 Fwd* 5´-GTGAAGGATCCAGGCAATTCA-3´ and *CsGH3-1 Rev* 5´-CCACTCGGCCTTGTCTGTAAA-3´). A ubiquitin gene from chestnut (*CsUBQ Fwd 5´-*AGGAATCAACCCTTCACCTTGTC-3´ and *CsUBQ Rev* 5´-GAACTCTCCACCTCCAAAGTGATG-3´) was used as a control.

In situ localization was carried out as previously described (Vielba et al. 2011; Valladares et al. 2013). Sense- and antisense-specific probes were generated from a small fragment (300-nucleotide) corresponding to the 3´UTR of *CsGH3-1* cloned into the PCRII ® vector containing the SP6 and T7 promoters. The fragment was amplified by PCR and used as template for synthesis of DIG-labelled probes with the SP6/T7 RNA labelling kit (Roche).

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Results and discussion

The cDNA and deduced protein sequences of *CsGH3-1* were deposited in the European Molecular Biology laboratory (EMBL), under accession numbers JN900481.1 and AFC364431.1. Several sequences were selected to construct a phylogenetic tree and thus explore the evolutionary relationship between CsGH3-1 and other members of the GH3 family (**Fig. 1**). Analysis of the first draft of the Chinese chestnut (*C. mollissima*) genome enabled us to identify nine putative active genes plus two partial sequences that did not contain certain domains (results not shown) and that were thus excluded. Three sequences from *C. sativa* (including CsGH3-1) were also included in the analysis, as well as the complete GH3 family from *Populus trichocarpa*, a model woody species in which the GH3 family has been characterized (Okrent and Wildermuth 2011). The results indicated that CsGH3-1 belongs to subfamily II, whose members are capable of catalyzing the formation of auxin conjugates. CsGH3-2 also belongs to group II, as well as four proteins of Chinese chestnut (**Fig. 1**). The sequence CmGH3.12 could not be assigned to any of the pre-established sub-families. This aspect requires further research.
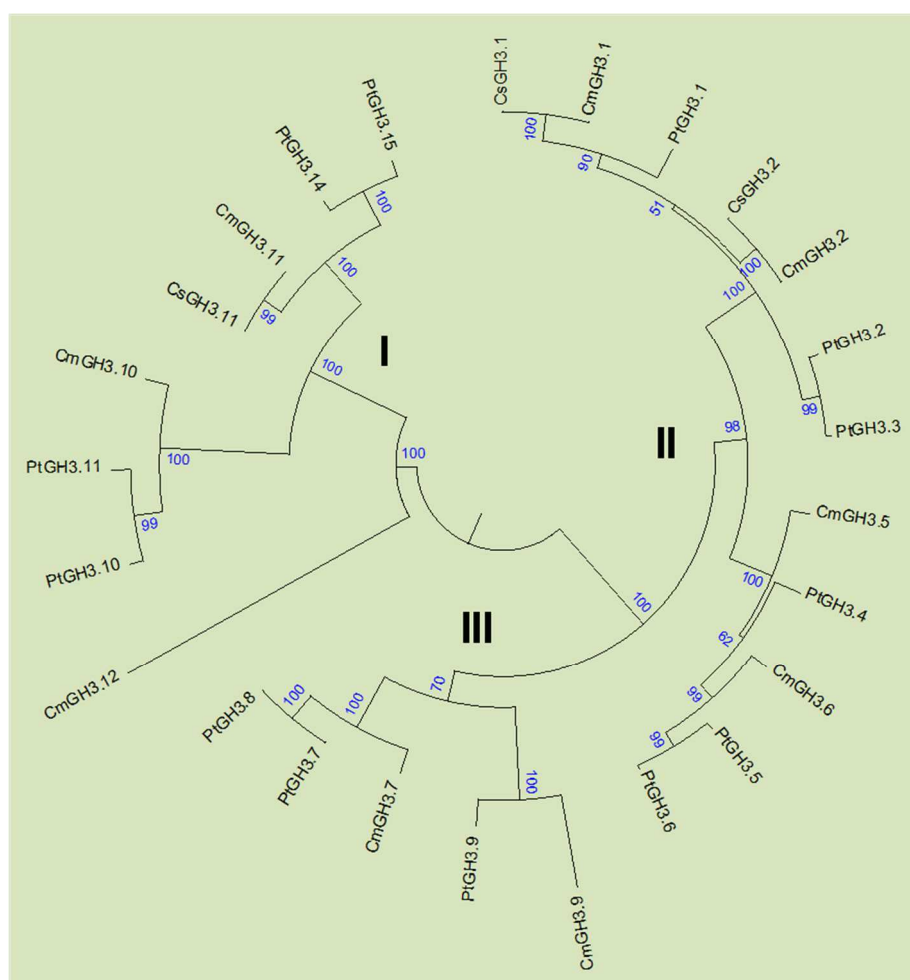
Figure 1. Phylogenetic tree of the GH3 proteins from *Castanea sativa* (Cs), *Castanea mollissima* (Cm) and from *Populus trichocarpa* (Pt). I, II and III represent the three sub-families of the GH3 family. Support values for each branch are shown in blue.

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The crystal structure and detailed analysis of the grapevine (*Vitis vinifera*) IAA-amidosynthetase (VvGH3-1) has previously been reported (Peat et al. 2012; Böttcher et al. 2012). Due to the high sequence similarity of CsGH3-1 and VvGH3-1 proteins, the latter was used as a template to generate the three-dimensional structure of CsGH3-1 (**Fig. 2**). The similarity between both proteins suggests that these enzymes catalyze the same biochemical reactions. Data from the structural models of both proteins also showed a high degree of conservation of critical residues responsible for their biochemical activities. In the GH3-1 protein, we identified a large N-terminal domain (first 442 amino acids) and a smaller C-domain (residues 443 to 602), with an active site located at the interface of the two domains (**Fig. 2a**). The presence of conserved domains and specific residues confirmed the ability of this putative protein to catalyze the formation of auxin conjugates. The deduced CsGH3-1 protein contains identical motifs (^{112}SSGTSAGERK^{121}, ^{336}YASSE^{340} and ^{417}YRVGD^{421}) to those identified in other members of this family (Terol et al. 2006). These essential motifs critically help to define the active site of the protein where the auxin indole ring will fit. VvGH3-1 has been shown to catalyze, *in vitro*, the conjugation of IAA with glutamic acid and, at a lower rate, with aspartic acid (Böttcher et al. 2010). Comparison of VvGH3-1 and CsGH3-1 sequences enabled us to identify several conserved residues involved in the acyl group and nucleotide-binding site, which are located in the same position throughout the protein sequences. Moreover, in CsGH3-1 the Lys^{153} and Arg^{120} residues, which are located in almost the same positions in other GH3 proteins, indicate a preference for acidic amino acids as a substrate when Lys^{153} is conserved, or a greater affinity for aspartic than for glutamic acid, in the case of the Arg^{120} (Westfall et al. 2012; Peat et al. 2012). The fate of auxin conjugates differs depending on the amino acid involved in the reaction and may involve catabolism, storage or signalling (Westfall et al. 2013). The expected conjugates with glutamic or aspartic acid are believed to be involved in catabolism. Together, the data on the structures and similarities of chestnut and grapevine GH3 proteins indicate that the putative CsGH3-1 enzyme is involved in the conjugation of amino acids to IAA.

A molecular docking approach was used to analyze the binding sites of different substrates with the CsGH3-1 protein (**Fig. 2b**). The results showed that the auxins tested (IAA, IBA and NAA) fit into the active site of the protein without significant steric hindrance. The findings also suggest that CsGH3-1 is capable of conjugating amino acids to at least two different naturally occurring auxins (and a synthetic one), thus expanding the number of possible products from the enzymatic activity. Auxin works as a chemical transducer of exogenous and endogenous cues (Simon and Petrásek 2011). The common strategy of conjugating this and other signalling molecules to regulate their activity (Westfall et al. 2012) is a valuable mechanism that allows fast fine-tuning of hormone action in plants and the consequent avoidance of an energetically expensive degradation/recycling process (Sztein et al. 2000). In addition, the putative diverse biological functions of the resulting conjugates also increase the possible outcomes for the plant and may provide significant adaptive advantages. The ability of the putative CsGH3-1 enzyme to accept different substrates is consistent with previous findings related to the *in vitro* enzymatic activity of GH3 proteins. Proteins showing a specific preference for a particular substrate (amino acid or auxin) often also display lower rates of activity on other substrates (Staswick et al. 2005; Böttcher et al. 2010, Westfall et al. 2016).

Analysis of *CsGH3-1* expression was performed during the auxin-induction of ARs in two different chestnut shoot lines, to investigate the putative role of the gene in regulating IAA levels in similar tissues at different ontogenetic stage. Maturation (i.e., the transition from the juvenile to the adult state) imposes several changes in physiological and adaptive responses of plants, and these changes are more pronounced in long-lived woody species. In the case of chestnut, several traits, such as growth rate and polyphenol content, are affected by maturation (reviewed in Ballester et al. 2009). As mentioned above, the loss of rooting ability of adult chestnut microshoots is of particular relevance for the clonal propagation of selected genotypes. Therefore, our rooting-competent (BS) and rooting-incompetent (C) shoot system enables detailed analysis of the genetic and molecular differences underlying the different behaviour of the two lines.

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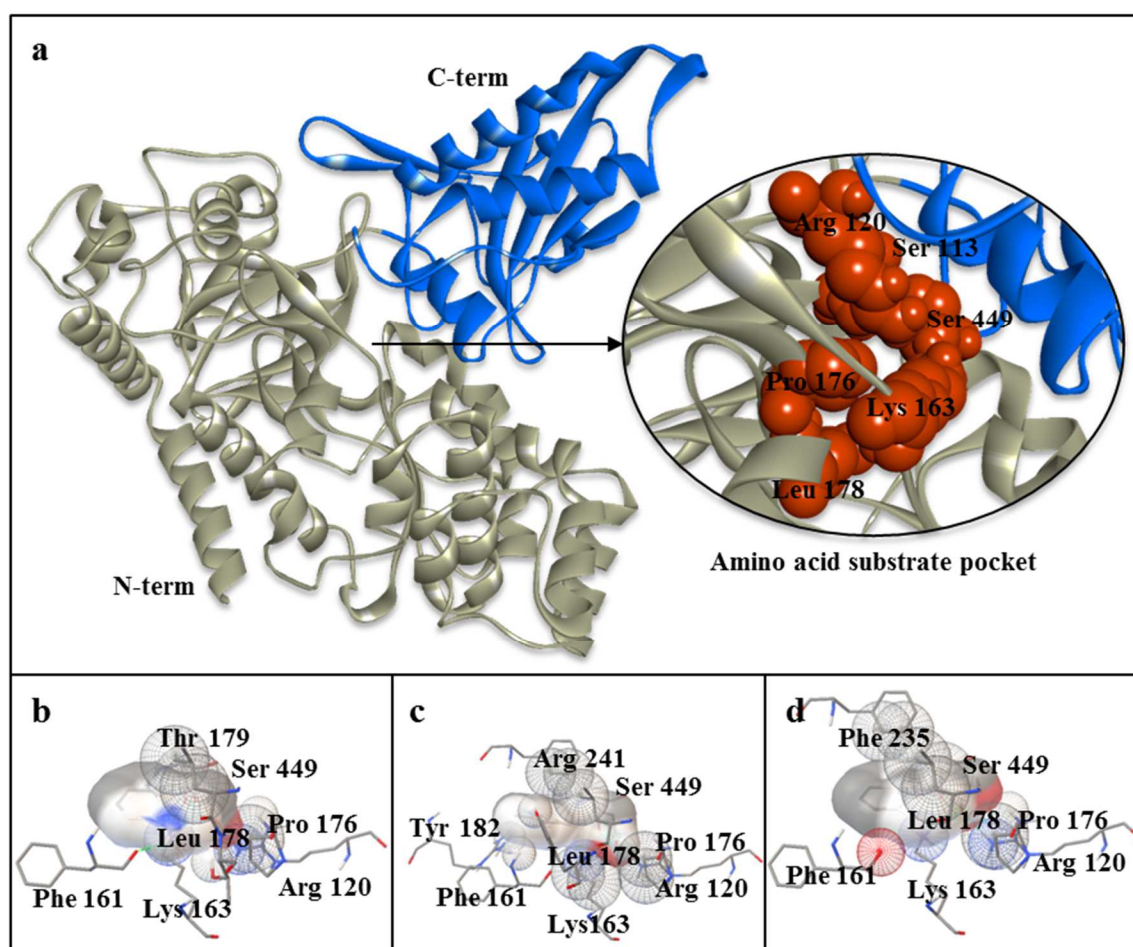
Figure 2. Overall structure of the *CsGH3-1* protein and binding modes of IAA, IBA and NAA into de active site of the protein predicted by molecular docking. **a**) Model built for CsGH3-1 colored from the N-terminal domain (grey) to the C-terminal domain (blue). Residues forming the amino acid substrate pocket are shown in red in the close-up. **b)** CsGH3-1–IAA complex. **c)** CsGH3-1–IBA complex. **d)** CsGH3-1–NAA complex. Residues interacting with each substrate are shown in b, c and d.

Due to the auxin responsiveness of members of group II of the GH3 family, analysis of the expression of these genes could be used to trace auxin activity and distribution in the different tissues (Ahkami et al. 2013) and could help us to understand the putative role of *CsGH3-1* during formation of ARs. Northern blot analysis revealed strong induction of auxin by *CsGH3-1* in the apical part of the shoots between 12 and 24 h after IBA treatment, indicating that the auxin stimulus reached the whole shoot (**Fig. 3**). Moreover, the level of induction was greater in C shoots, particularly in the samples harvested after 24 h of IBA treatment (**Fig. 3b**), yielding a different expression of *CsGH3-1* in both types of shoots. ARs are formed in the lower part of the stem of BS-derived shoots, suggesting that although all tissues are stimulated by auxin, the particular distribution of the hormone is important for triggering the process of AR formation. Indeed, these new roots are mainly formed in zones close to the wound site.

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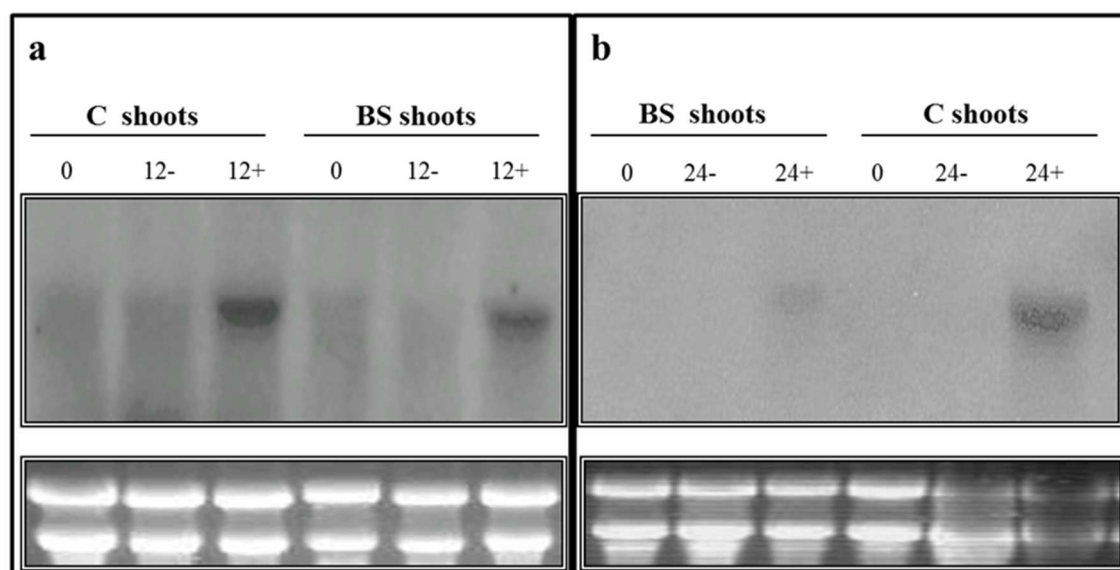
Figure 3. Northern blot analysis of *CsGH3-1* mRNA in the apical parts of juvenile-like (BS) and mature (C) shoots of the P2 clone. Numbers indicate time (h) after the shoots were treated (+) or not treated (-) with auxin. Time 0 samples were harvested from shoots at the end of the 4-week multiplication cycle. Ribosomal RNA bands stained with ethidium bromide are shown (bottom panels) as loading controls.

qPCR analysis was performed in the basal part of the BS and C shoots to investigate the dynamics of gene expression during induction of AR. Relative mRNA levels in *CsGH3-1* were higher and lasted longer in C-derived than in BS-derived shoots after IBA treatment (**Fig. 4**). These data confirmed that *CsGH3-1* expression is positively and differentially regulated by auxin in both types of shoots. The differential gene expression is associated with the ontogenetic stage of tissues, and levels are higher in mature shoots, as also observed in the apical parts of shoots (**Fig. 3**). By 6-12 h after the IBA treatment, expression profiles were similar in BS- and C-derived shoots; however, the mRNA levels decreased sharply in BS shoots after 24 h, but remained stable in C-derived shoots. Such a decrease in the level of expression was only detected in rooting-competent shoots and may be related to their rooting ability. The *CsGH3-1* expression appeared to shift in BS-derived shoots when cell differentiation occurred after IBA induction, as observed at a histological level during the first 24 h of root initiation (Ballester et al. 1999). On the contrary, high mRNA levels were detected after 24 h in IBA-treated C-derived shoots, in which cell dedifferentiation was observed, but further cell divisions led to formation of callus rather than root primordia. Expression of GH3 genes was also elevated in *Arabidopsis* mutants with a reduced ability to form lateral roots (Seo and Park 2009). By 48 h after the IBA treatment, *CsGH3-1* mRNA levels in C-derived shoots were still similar to those detected at 24 h in the BS-derived counterparts. Although endogenous auxin plays an important role in the process of reprogramming cell fate and is essential for inducing ARs, the auxin content of the tissues does not seem to be the main factor limiting AR formation in adult tissues (Ballester et al. 1999; Vidal et al. 2003). Ballester et al. (1999) showed that the IAA content of IBA-treated C-derived shoots in same chestnut microshoot system as used here was several times higher than in BS-derived shoots, while IBA and IAA-Aspartic Acid (IAA-Asp) contents were similar in both types of shoots. These auxin levels do not seem to be consistent with the level of *CsGH3-1* expression detected. We believe that these differences may be a consequence of differences in the activity of auxin conjugating enzymes, or to differences in the auxin transport machinery. Indeed, they clearly suggest that auxin-conjugating enzymes act differently depending on the ontogenetic state. The levels of expression attained (in the order of hundreds fold-change)

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do not indicate that all of these transcripts will generate a functional protein. Several post-transcriptional mechanisms actively control the amount of protein generated, and it is generally considered that the mRNA content and the derived protein are weakly correlated (Maier et al. 2009).
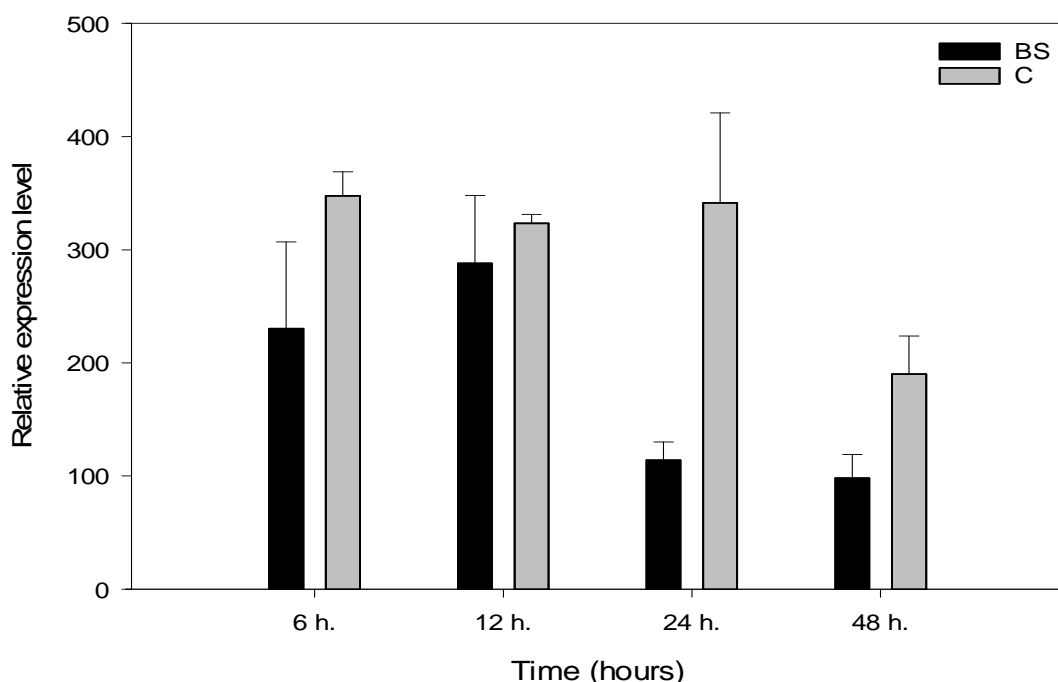
Figure 4. Relative mRNA levels of *CsGH3-1* in the basal part of chestnut microshoots treated with 5 mM of IBA for 1 min and subsequently transferred to IBA-free medium for the indicated times. Bars represent standard errors. BS, basal microshoots. C, crown-derived microshoots.

We performed in situ localization of transcripts to test whether the different levels of *CsGH3-1* expression detected by qPCR are a consequence of a broad/general response to the exogenous IBA of all tissues in each type of shoot or whether they represent a cell- and shoot-type specific response. The analysis was performed in the basal part of the IBA-treated shoots near the wounded area. Sections from BS-derived control shoots harvested at time 0 were also included.

The *CsGH3-1* transcripts accumulated throughout the different tissues of the C-derived shoot samples (**Fig. 5**), suggesting a rather uniform distribution of auxin, which consequently mediates a similar gene response in these tissues. On the other hand, *CsGH3-1* mRNA levels were low in control BS-derived shoots harvested at time 0 (**Fig. 6a**). However, IBA treatment increased the expression of the gene in BS-derived shoots during the first 24h of root induction, and transcripts were mainly localized within the vascular cambium area, where the divisions that lead to the formation of ARs begin (**Fig. 6b, c**, and **d**; Ballester et al. 1999), as well as in the root primordia developed from these shoots (**Fig. 6f**).

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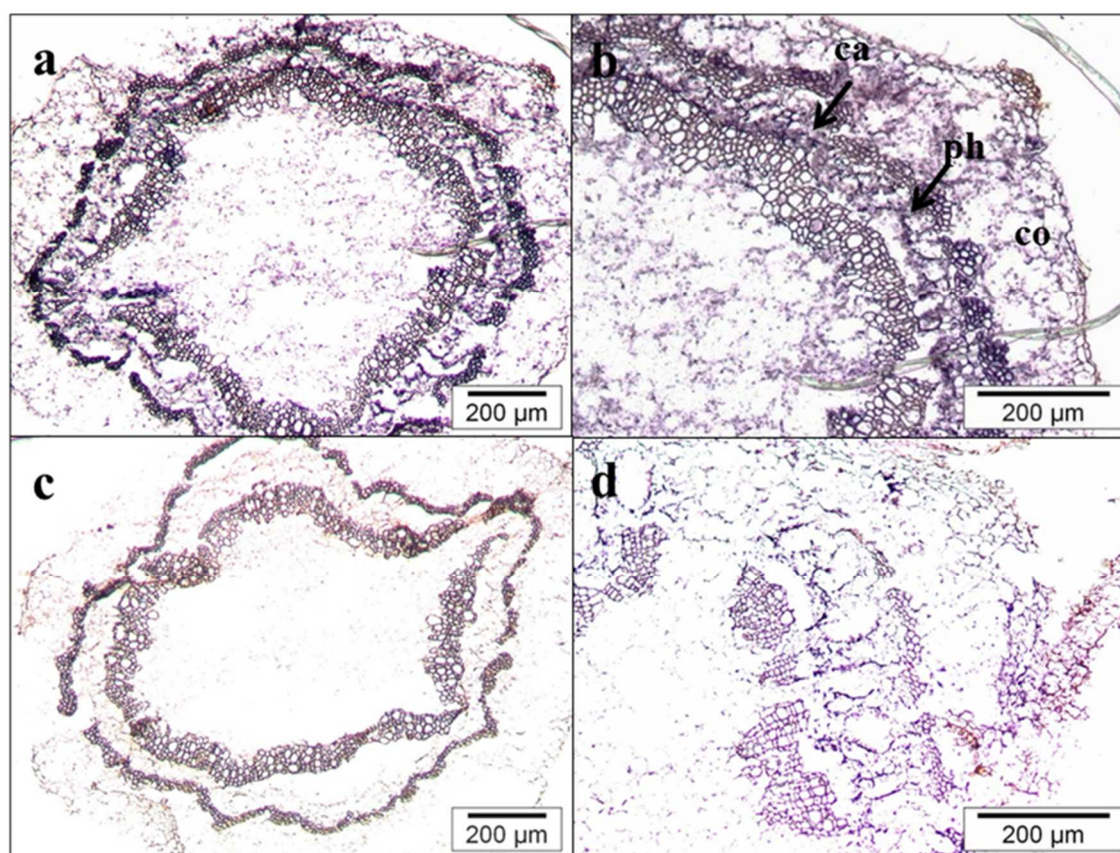
Figure 5. *In situ* analysis of *CsGH3-1* mRNA in transverse sections of the basal part of microshoots derived from crown branches (C) treated with IBA (5 mM for 1 min) and harvested 24 h after treatment. (a,b) Hybridization signal was extended to the phloem (ph) and cortex (co). (c, d) No hybridization signal was detected in sections hybridized with the sense probe. (ca) cambium

These findings indicate that an auxin gradient was created after application of both stimuli (wounding and addition of exogenous auxin), leading to the AR formation. This gradient may directly influence the physiological responses in the tissues. It has been suggested that auxin-driven responses are not a consequence of the total hormone content but represent a specific response to the hormone gradients in the tissues or to changes in hormone concentrations (Benková et al. 2003; Nilsson et al. 2008). In this context, the role played by specific auxin transporters is crucial, as their activity will help create the gradients. Although strongly suggested by our findings, it is not known whether auxin transporters, like the PIN family or a member of the ABC superfamily (Zažímalová et al. 2010), are also influenced by the ontogenetic state. Nonetheless, the gradient is important because it is created around the vascular cambium, where GH3 genes may be involved in generating adaptive responses (Teichmann et al. 2008). Moreover, in many forest species, including chestnut, ARs originate from the cambium and their derivatives (Ballester et al. 1999). The activity of the CsGH3-1 protein in cambial cells should reduce the amount of free auxin. However, CsGH3-1 may help to maintain the auxin level required for the fate transition of a small number of competent cells into root initial cells. Indeed, the levels of IAA-Asp conjugates were not significantly different in BS-derived and C-derived shoots (Ballester et al. 1999). As discussed above, although other conjugates are probably a product of the activity of the protein, the gene response seems to be tissue-specific

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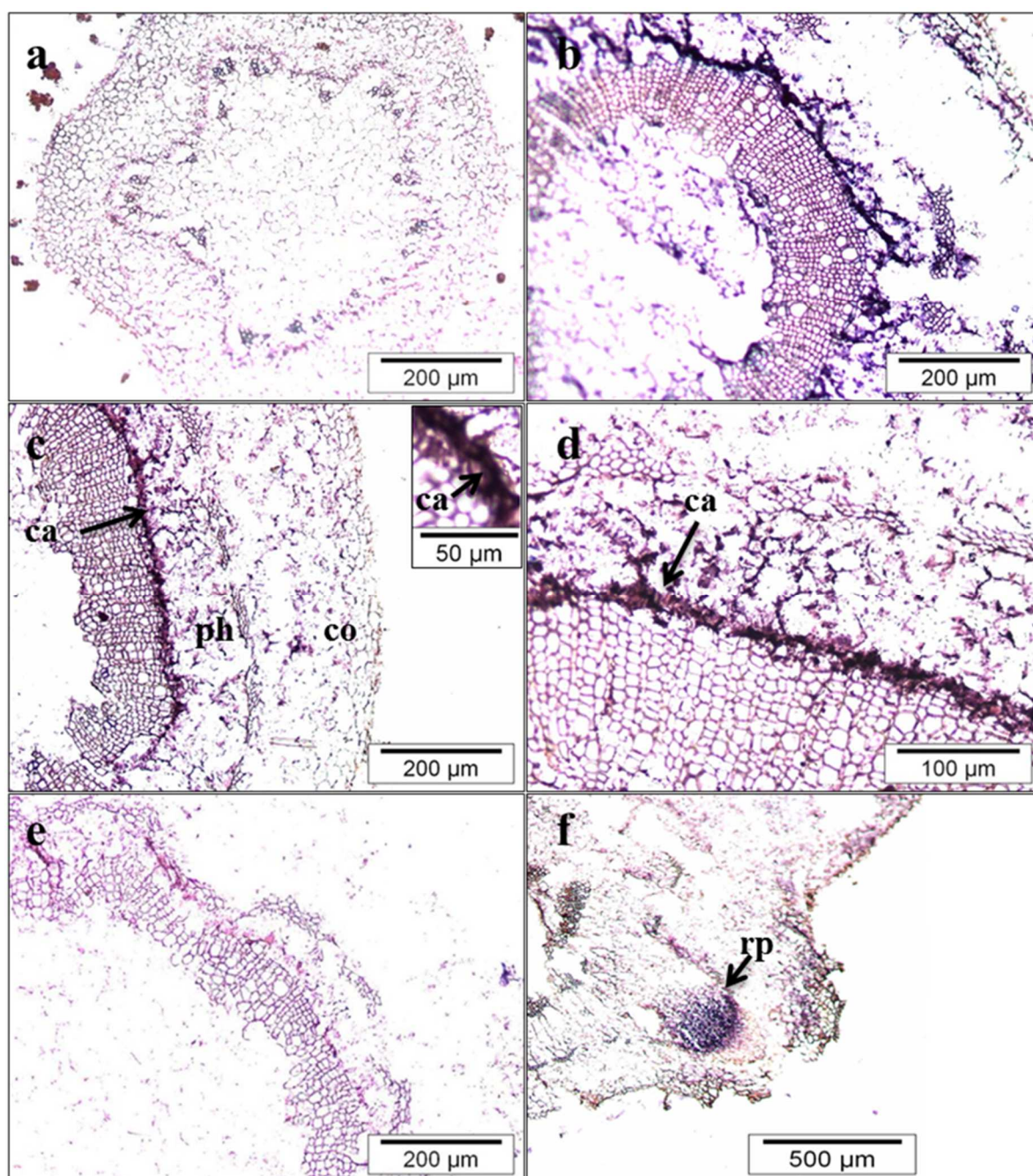
Figure 6. *In situ* analysis of *CsGH3-1* mRNA in transverse sections of the basal part of microshoots derived from basal shoots (BS) harvested at the end of the multiplication period (a) or from shoots treated with IBA (5mM) for 1 min and harvested after 24 h (b, c, d, e) and 10 days (f) after IBA treatment. (a) Expression of *CsGH3-1* was low with a diffuse signal extended throughout the section tissues. (b, c, d) The signal was localized in the cambium (ca).(e) No hybridization signal was detected in IBA-treated shoots hybridized with the sense probe. (f) Strong CsGH3-1 expression was observed in the root primordia (rp) developed in IBA-treated microshoots. co: cortex; ph: phloem

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in relation to the ontogenetic state of the shoots. Maturation therefore exerts a significant effect on auxinhomeostasis. The role of CsGH3-1 in the cambial cells may be related to the withdrawal of excessive auxin and/or the need to maintain auxin gradients. Moreover, the putative destiny of auxin conjugates and their specific nature adds another layer of complexity to the process. A similar expression pattern (regarding quantity and tissue-specificity) was detected during analysis of the expression of the putative transcription factor *CsSCL1*, performed in the same *in vitro* system of chestnut microshoots that was used in the present study (Vielba et al. 2011). Accumulation of *CsSCL1* was also confined to the vascular cambium of rooting-competent shoots and to the root primordia, and it was suggested to play an important role in the very early stages of root initiation and also in root meristem maintenance. Expression of the GH3 promoter was reported during the lateral root development in rice (Sreevidya et al. 2010), associated with the pericycle areas of the root, where lateral root primordia would be initiated from a few founder cells (located at the xylem pole) that retain the ability to undergo asymmetric cell division (Dubrowsky et al. 2000; De Smet 2011).

Conclusions

The findings of this research reveal that the putative CsGH3-1 enzyme may be capable of catalyzing the synthesis of different auxin conjugates. Relative levels and tissue localization of *CsGH3-1* transcripts differed in rooting-competent and rooting-incompetent shoots after 24 h of IBA treatment, indicating that the gene is regulated in an ontogenetic stage- and tissue type-dependent manner. The lack of rooting ability in adult-like material appears to be directly linked to a shift in gene regulation and auxin homeostasis during maturation. The findings thus suggest the involvement of the *CsGH3-1* gene in generating the auxin gradient required for induction of ARs, as well as in other physiological responses.

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Agrobacterium-mediated DNA delivery into somatic embryogenic tissue of *Pinus pinea*

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Abstract

A standardized protocol for *Agrobacterium*-mediated transformation of *Pinus pinea* (stone pine) embryogenic callus is presented after testing the effect of factors such as amount of initial tissue, infection period, bacterial dilution, acetosyringone concentration, plasmid constructions, and *Agrobacterium* strains. Transient GUS (beta-glucuronidase) gene expression was used to monitor T-DNA delivery into targeted cells. The most efficient concentrations of the selective agents (kanamycin and phosphinothricin, PPT) have also been determined. Significant genotypic variation in response to transformation was observed between the two embryogenic lines tested. Co-culturing of 12 g calli with *Agrobacterium* for 5 min, including 1 minute in vacuum, followed by co-cultivation with 200µM acetosyringone for 3 days was found to be optimum for maximum transformation efficiency. Among constructions, the AGL1 strain harboring the pTAB16 plasmid at an OD of 0.8 significantly increased DNA delivery into the cells. Selection of transformed tissue required 1 mg PPT or 5 mg/l kanamycin. The optimized protocol would be useful for *Agrobacterium*-mediated genetic transformation of stone pine for genetic and agronomical studies.

Keywords: *Agrobacterium,* bacterial strains, *Pinus pinea,* plasmids, transient expression.

Key message: We describe an enhanced protocol for transformation and selection of transformants of *Pinus pinea* embryogenic callus.

Abbreviations: AS, acetosyringone; Kan, Kanamycin; OD, optical density at 600 nm; PPT, phosphinotricin.

Introduction

Pinus pinea L. (stone pine) is an economically important forest species of the Mediterranean region, where it has been widely cultivated due mainly to the high economic value of its seed crops (Calama et al. 2007). Besides its agronomical value, the species is also used for ecological and ornamental purposes. Stone pine is characterized by very low genetic variation and a high adaptative plasticity (Sáez-Laguna et al. 2014). Thus, the species is well adapted to the high temperatures and drought characteristics of Mediterranean climates; also it is less sensitive to diseases and pests than other Mediterranean pines, particularly to the pine wilt nematode *Bursaphelenchus xylophilus* (Santos et al. 2012). In spite of these environmental adaptations, seed production varies annually mainly due to water shortage (Mutke et al. 2005). Recently, several studies on gene expression and epigenetic variability under drought stress have been described (Sáez-Laguna et al. 2014; Perdiguero et al. 2015). Further research on assessment of gene function requires

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the generation of mutants or transgenic plants with altered gene expression (Uddenberg et al. 2015). Protocols for *Agrobacterium*-mediated transformation from stone pine isolated cotyledons have been described (Humara et al. 1999). Furthermore, the advantages of somatic embryogenesis (SE), particularly maintenance of regeneration potential by cryopreservation while the testing of clones is in progress (Park, 2002). This prompted the development of SE protocols for this species as first described by Carneros et al. (2009). In the present work the conditions for an efficient DNA delivery into stone pine embryogenic lines are reported.

Materials and methods

Plant material and tissue culture

Pinus pinea embryogenic lines (1F11 and 7F11) used in this study were generated as described inCarneros et al. (2009) and provided by Dr. Mariano Toribio (InstitutoMadrileño de Investigación y Desarrollo Rural, Agrario y Alimentario, IMIDRA, Spain). Embryogenic lines were maintained by subcultures to fresh mLV medium (Lelu-Walter et al. 2006) every two weeks. Embryogenic calli, grown for 4 days after their transfer to fresh medium, were used in all experiments.

Bacterial strains and plasmids

Agrobacterium tumefaciens AGL1 (Schroeder et al. 1993), EHA105 (Hood et al. 1993), C58 (Koncz and Schell 1986), and GV3101 (Holsters et al. 1980) strains, and plasmid constructions pABC, pBIN35SGUSINT, pBINUbiGUSINT and pTAB16 were used in the experiments. To facilitate transient expression assays, all constructions included the *gus*A gene driven by the CaMV 35S promoter, unless otherwise stated. This gene is not expressed in *A. tumefaciens* due to the insertion of an intron plant in the protein-coding region. The pABC plasmid (Mentewab and Steward 2005), carries the *Atwbc*19 gene that confers tolerance to kanamycin (Kan), driven by the CaMV35S promoter (gift of Dr. Neal Stewart, University of Tennessee; USA). The pBIN35SGUSINT plasmid contains the *npt*II gene, controlled by the *nos* promoter (nopaline synthase). This gene confers kan resistance (Vancanneyt 1990). The pBINUbiGUSINT plasmid contains the *npt*IIgene driven by the *nos* promoter, but the *gus*A gene is under the control of the *ubi*I maize polyubiquitin promoter (Humara et al. 1999). The pTAB16 plasmid includes the *bar* gene, which confers phosphinothricin (PPT) tolerance, driven by the CaMV 35S promoter (Schroeder et al. 1993).

Bacterial strains were cultured in LB (Luria Broth) medium for 16 hours at 28°C in a horizontal orbital shaker (200 rpm), with appropriate antibiotics to reach the desired optical density (OD). LB for AGL1 strain cultures also contained 0.4 g/l of $MgSO_4$. The antibiotics used were rifampicin 50 µg/ml for selection of AGL1 strain, rifampicin 50 µg/ml and tetracycline 5 µg/ml for the C58 strain, nalidixic acid 30 µg/ml for the EHA105 strain, gentamicin 25 µg/ml and tetracycline 5 µg/ml for the GV3101 strain, tetracycline 5 µg/ml for the pTAB16 construction and kanamycin (Kan) 50 µg/ml for the pBIN35SGUSINT, pBINUbiGUSINT and pABC constructions.

Transformation experiments

Unless otherwise stated, the bacterial suspensions were centrifuged (3000 rpm, 20 min) and the precipitate re-suspended in MS liquid medium (Murashige and Skoog 1962) to reach the desired OD_{600} and 100 µM acetosyringone (AS) was added. For infection, equal volumes of bacterial suspension and tissue (6 g/50 ml mLV) were mixed in a 125 ml kitasato flask and a 1 min vacuum infiltration period was applied. Ten

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minutes later, the mix was recovered on 55 mm Ø Whatman no. 2 filter paper and placed on absorbent paper to drain excess liquid. The filter paper was placed on semi-solid mLV medium without casein hydrolysate for a 3 days co-culture period at 25 ± 2ºC in darkness.

To optimize *Pinus pinea* transformation conditions, the effect of different parameters on DNA delivery were sequentially assayed. These included initial amount of calli (6 or 12 g/50 ml of mLV); infection period (5 or 10 min); bacterial dilution (OD_{600} 0.3 or 0.8); AS concentration (100 vs. 200µM); plasmids (pABC, pBIN35SGUSINT, pBINUbiGUSINT and pTAB16) and *Agrobacterium* strains (AGL1, EHA105, GV3101 and C58). Four replications were prepared for each experiment and transient *gus*A (*GUS*) expression was determined after 3 days co-culture.

GUS histological assay

A histological assay for *GUS* transient expression was performed 3 days after co-culture. Filter paper with infected calli was deposited into 60 mm Ø empty plates, with 2 mL of the reagent mix described by Jefferson et al. (1987) containing 2 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), and incubated at 37ºC for 16 h in the dark. Then, each plate was examined microscopically and the number of foci (areas of blue cell staining) was recorded.

Sensitivity to selection agents

The concentrations of two selective agents (Kan and PPT) that inhibited growth of untransformed tissue were determined. To this end, 0.5 g of each embryogenic line was suspended in 5 ml mLV liquid medium. The mix was softly shaken and then poured on a Buchner funnel with a 70 mm Ø Whatman filter paper. After draining, aided by a final vacuum pulse, the filter paper with embryogenic cells was transferred to a semisolid mLV medium with PPT (0, 2, 4 or 6 mg/l) or Kan (0, 5, 10, 20 or 30 mg/l). Glutamine and casein hydrolysate were excluded from PTT-supplemented media. Three replicates were performed per treatment and calli fresh weight was determined after two weeks in culture.

Statistical analysis

Significance of the different treatment effects on transient *GUS* expression was determined using analysis of variance (ANOVA). Percentage data were subjected to arcsine transformation prior to statistical analysis. Variation among treatments means was analyzed using Tukey´s procedure (1953). All statistical analyses were performed using SPSS Statistics 20.0.0.

Results and discussion

Effect of initial tissue amount

Six or 12 g of the 1F11 embryogenic line were infected with AGL1-pTAB16 (OD_{600} 0.8). The infection of 12 g tissue produced ten times more transient GUS expressions than 6 g of tissue (291.0 vs. 27.3 blue foci per plate, respectively p≤0.05). Similar rates (10 mg/50mL of embryogenic cells) were successfully used to transform other pine species (Charity et al. 2005; Levée et al. 1999; Trontin et al. 2007). Based on these results, 12 g/50 ml mLV of callus was selected for subsequent experiments.

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Effect of infection period

Twelve g of 1F11 and 7F11 embryogenic lines were infected with AGL1-pTAB16 (OD_{600nm} 0.8) for 5 or 10 min. No significant differences were observed between the embryogenic lines, but a 5 minutes infection period was significantly more effective ($p \leq 0.05$) than 10 minutes (232.0 blue foci versus 60.0, respectively). Five min infection also produced the highest transformation rates from stone pine cotyledons (Humara et al. 1999). However, shorter periods of infection have been used on *Pinus radiate* (Charity et al. 2005) and *Pinus pinaster* (Trontin et al. 2002). The 5-min infection period was selected for subsequent experiments.

Effect of bacterial dilution

The effect of two AGL1-pTAB16 bacterial dilutions (OD_{600} 0.3 and 0.8) was tested on 1F11 and 7F11 lines. An OD_{600} of 0.8 increased GUS expression on both embryogenic lines (average of 173.1 blue foci vs. 25.1, for an OD of 0.8 and 0.3, respectively; $p \leq 0.05$). Similar results were previously reported for cotyledon transformation of the species (Humara et al. 1999). On the contrary, bacterial dilution did not affect *GUS* transient expression of *Pinus pinaster* embryogenic lines (Trontin et al. 2002). An OD of 0.8 was selected for subsequent studies.

Effect of AS concentration

Three different AS concentrations (0, 100 and 200 µM) in the bacterial dilution medium used for infection of 1F11 and 7F11 embryogenic lines were tested. Irrespective of the embryogenic line, the highest AS concentration favored significantly *GUS* transient expression (average of 462.1, 178.2 and 38.6 blue foci for 200, 100 and 0 µM AS, respectively; $p \leq 0.05$). The interaction between AS concentration and the embryogenic line was also significant, bests results being obtained on 1F11 line and AS 200 µM, where a mean of 647.7 blue foci were observed (**Fig. 1**). In some conifers, adding AS to the co-culture medium increases transient expression (Levée et al. 1999; Wenck et al. 1999; Le-Feuvre et al. 2013). On the contrary, in *P. pinaster* AS did not increase *GUS* expression (Trontin et al. 2002; Tereso et al. 2006). A concentration of 200 µM AS was selected for further studies.
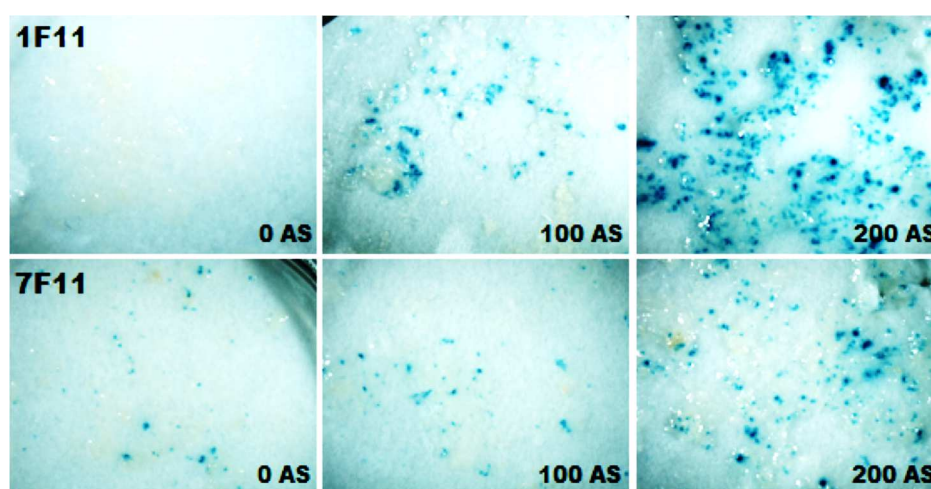
Figure 1. Effect of AS concentration (0, 100 and 200 µM) on *GUS* expression in two stone pine embryogenic lines, 1F11 (up) and 7F11 (bottom).

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Effect of plasmid constructions

Plasmids pABC, pBIN35SGUSINT, pBINUbiGUSINT and pTAB16 integrated into AGL1 strain were assayed. Regardless of the embryogenic line, pTAB16 plasmid was more effective in terms of transient GUS expression especially in line 1F11 (data not shown). The pTAB16 plasmid had not been previously assayed to transform *Pinus* species although the *bar* gene was already used for maritime pine transformation (Trontin et al. 2007).

Effect of bacterial strains

Lines 1F11 and 7F11 were infected with AGL1, EHA105, GV3101 or C58 strains harboring the pBIN35SGUSINT plasmid. Irrespective of the embryogenic line, AGL1 induced the highest levels of GUS expression (average of 323.1 blue foci/plate vs. 6.8 and 0.0 for EHA105 and each of the GV3101 and C58 bacterial strains, respectively; **Tab. 1**). The low susceptibility of stone pine embryogenic tissue to EHA105 and C58 infection was not found in other pine species. Thus, although the C58 strain was also ineffective in transforming stone pine cotyledons, EHA 105 successfully did it (Humara et al. 1999), and C58 is now being used routinely to transform maritime pine (Trontin et al. 2002). AGL1 was also effective in the transformation of radiata pine cotyledons (Grant et al. 2015); nevertheless, its effect on embryogenic lines from maritime pine seems to be genotype-dependent (Trontin et al. 2002; Álvarez et al. 2013).

Table 1. Effect of bacterial strains on *GUS* expression in 1F11 and 7F11 embryogenic lines of *Pinus pinea* (mean number of GUS foci per plate). Data are the mean of at least four replications. For each column, values with different letters are significantly different according to Tukey´s test ($p \leq 0.05$).

Line	Bacterial strains			
	AGL1	EHA105	C58	GV3101
1F11	332.5	11.5	0.0	0.0
7F11	313.7	2.2	0.0	0.0
Mean	323.1a	6.8b	0.0b	0.0b

Selective agents' sensitivity

In both embryogenic lines, callus growth was significantly reduced by all Kan and PPT concentrations tested. Growth reduction percentages ranged from 40 to 90% (data no shown). Thus, 5-10 mg/l Kan or 1-2 mg/l PPT can be used to select transformed stone pine tissue. Similar concentrations have been used to select other pine species (Trontin et al. 2007; Grant et al. 2015).

Our results do not differ substantially from those previously reported for other *Pinus* species except for the requirement of a higher AS concentration, and for the high infection capability of AGL1 which can be explained by the presence of extra virulent factors in this strain (Guy et al. 2016). The variability detected in the mean number of GUS foci among different series of similar experiments, even when the same infection protocol was used, might be due to the metabolic state of the tissue, including its degree of phenolization, which may vary within the time that the two trials were performed, affecting differently to the interaction between *A. tumefaciens* and plant cells (Levée et al. 1999; Malabadi and Nataraja, 2007).

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Conclusions

Here we present for the first time a protocol for DNA transfer into *Pinus pinea* embryogenic lines that includes: a) The use of AGL1 strain harboring the pTAB16 plasmid grown at a bacterial OD_{600} of 0.8; b) the centrifugation and resuspension in MS liquid medium with 200 μM AS; c) five minutes infection with an initial one minute vacuum; d) the regeneration of transgenic lines, after 3 days co-culture, on selection medium with 1 mg/l PPT. In plasmids with *npt*II gene, the selection medium must include 5 mg/l Kan. This protocol opens up significant possibilities for genetic improvement of *Pinus pinea* selected lines.

Author contributions: JS and IA conceived and designed the experiments. MB and JM performed the experiments. MB and IA analyzed the data. MB, JS and IA wrote the paper.

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Transformation of *Quercus suber* and *Quercus ilex* somatic embryos with a gene encoding a thaumatin-like protein

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Abstract

An efficient protocol for the genetic transformation of cork oak and holm oak somatic embryos (SEs) with the *CsTL1* gene that encodes a thaumatin-like protein is described. Cork oak SEs at the globular or early torpedo stages, isolated from three embryogenic lines, were pre-cultured for one day and co-cultured for 5 days with *Agrobacterium tumefaciens* strain EHA105 harbouring the pK7WG2D-tau plasmid. Genetic transformation was successfully achieved in all three embryogenic lines studied. Transformation efficiency was clearly genotype-dependent, and the highest rate (17%) was obtained with SEs of line TGR3. Successful transformation of holm oak SEs was not achieved under the above-described conditions; transgenic SEs were only obtained, and at a lower frequency (2%), when the original SEs were pre-cultured for 1-2 weeks with the *Agrobacterium* strain. At present, 30 *GFP*-positive cork oak embryogenic lines and 10 *GFP*-positive holm oak embryogenic lines are being maintained in our laboratory by secondary embryogenesis. The presence of different transgenes in the SEs was confirmed by PCR analysis.

Keywords: *Agrobacterium*, cork oak, holm oak, embryogenic cultures, genetic transformation

Abbreviations: *GFP*, Green Fluorescent Protein; kan, kanamycin; SEs, Somatic Embryos

Introduction

Cork oak (*Quercus suber* L.) and holm oak (*Quercus ilex* L.) are widely distributed in the Mediterranean ecosystem. The economic importance of these tree species in the region is mainly due to production of cork and acorns, respectively. However, populations of both species have been decimated over the last few decades by a syndrome denominated as oak decline, which is mainly caused by *Phytophthora cinnamomi*, *Diplodia mutila* and *Biscogniauxia mediterranea* (Muñoz López et al. 1992; Vannini et al. 1996; Sánchez et al. 2002).

Phytophthora cinnamomi Rands. is an oomycete belonging to the family Pythiaceae (European and Mediterranean Plant Protection Organization 2004). This pathogen infects more than 3,000 potential hosts (Hardham 2005). It invades the root by the spread of primary hyphae from the cortex to parenchymal tissues via apoplast and symplast pathways (Ruiz-Gómez et al. 2012). *Quercus ilex* is more susceptible to *P. cinnamomi* than *Q. suber*, although both trees undergo a decrease in stomatal conductance when infected, due to root loss and root lesions (Robin et al. 2001). Other symptoms caused by *Phytophthora* include bleeding cankers on trunks, crown symptoms, chronic decline and death (Robin et al. 1992). Although in nature some trees show tolerance to oak decline, their distribution and breeding by traditional means are constrained by a long juvenile period and reproductive cycle, complex reproductive characteristics (such

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as self-incompatibility and a high degree of heterozygosis) and recalcitrance to conventional vegetative propagation methods.

Genetic transformation can be used to produce individual trees that are tolerant to oak decline. Over-expression of genes conferring resistance is not yet possible, because specific genes for resistance to oak decline have not been identified. Tolerance to fungal pathogens can occur via expression of heterologous genes whose products, including pathogenesis-related (PR) proteins, display *in vitro* antifungal activity (Van Loon 1997; Lorito et al. 1998). Such proteins are expressed in response to pathogen attack, abiotic stress and developmental signals (Veluthakkal and Dasgupta 2010). Among these, the PR-5 family of proteins (thaumatin-like proteins) are generally of low molecular weight (below 35 kDa) and may produce transmembrane pores on fungal plasma membranes, thus promoting osmotic rupture and inhibition of hyphal growth and spore germination.

The isolation of PR-proteins from chestnut seeds provides an opportunity to produce transgenic cork oak and holm oak trees with genes encoding these proteins isolated from a species in the same family (Fagaceae). The *CsTL1* gene, which encodes a thaumatin-like protein, has been isolated from mature European chestnut cotyledons (García-Casado et al. 2000). Moreover, this protein exerts *in vitro* antifungal activity against *Trichoderma viride* and *Fusarium oxysporum* (García-Casado et al. 2000).

The objective of this study was to develop a protocol for the genetic transformation of cork oak and holm oak somatic embryos (SEs) with the *CsTL1* gene, in a first step towards producing cork and holm oak trees tolerant to oak decline.

Material and methods

Plant material

Three different embryogenic lines of *Quercus suber* (ALM6, ALM80 and TGR3) were used as sources of target explants. All were initiated from leaves of adult trees selected for the high quality and yield of cork produced (Toribio et al. 2005). One embryogenic line of *Quercus ilex*, named Q8, was used as the source of explants. In this case, SEs were induced from adult trees selected for their high fruit yield (Barra-Jiménez et al. 2014).

The embryogenic cultures were maintained by secondary embryogenesis with sequential subculture at 6-week intervals on proliferation medium consisting of SH medium (Schenk and Hildebrant 1972) supplemented with 3% (w/v) sucrose and 0.6% (w/v) Vitro-agar (Pronadisa, Spain). The pH of the medium was adjusted to 5.6 before sterilization by autoclaving at 120ºC for 20 min. The embryogenic cultures were subjected to a 16 h 25ºC light/8h 20ºC dark photoperiod (provided by cool-white fluorescent lamps at a photon flux density of 50-60 mmol m^{-2} s^{-1}).

Agrobacterium strain and plasmid

The chestnut gene encoding the thaumatin-like protein *CsTL1* was cloned using the Gateway cloning system (Invitrogen, USA) into plasmid pK7WG2D (VIB, Ghent University, Belgium) under control of the CaMV35S promoter.

The *CsTL1* coding region was mobilized into the pENTRY/D-TOPO intermediate vector, and the resulting pENTRY/D-TOPO-*CsTL1* was transferred into the Gateway-compatible plasmid pK7WG2D by the LR clonase reaction (Invitrogen, USA). The vector, pK7WG2D-TAU (**Fig. 1**), was introduced into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) by the freeze-thaw method (Xu and Li 2008).

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This plasmid also includes the green fluorescence protein (*EGFP*) as a reporter gene, driven by the rol root loci D (rolD) promoter, and neomycin phosphotransferase (*NPTII*) as selectable marker gene, driven by the nopaline synthase (nos) promoter.
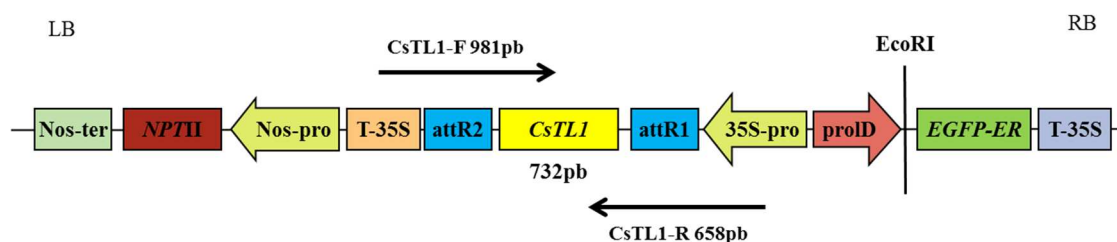
Figure 1. Representation of the T-DNA region of plasmid pK7WG2D-TAU used for the genetic transformation of cork oak and holm oak somatic embryos.

Transformation procedures

Explants consisting of small groups of two-three SEs at globular and early torpedo stages were used for the cork oak transformation experiments, whereas in holm oak the target explants consisted of one-two isolated pro-embryogenic masses (PEMs) (**Fig. 2**). The cork oak explants were pre-cultured for one day, whereas holm oak explants were pre-cultured for different times (one day, one week and two weeks) prior to transformation (**Fig. 2**).

Cultures of *Agrobacterium* were initiated from a glycerol stock and grown overnight in liquid Luria-Bertani medium (LB: 1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0, Sambrook et al. 1989) containing kan (10 mg/l) at 28°C with shaking (200 rpm). A single colony of the bacterium was inoculated into 2 ml of LB medium containing 10 mg/l kan, and the culture was incubated overnight at 28°C at 200 rpm in darkness. One ml of the bacterial suspension was inoculated into 600 ml of LB liquid medium with the appropriate antibiotics. The bacterial suspension was then incubated at 28°C at 90 rpm until an OD_{600} of 0.6-0.7 was achieved. The bacterial culture was then centrifuged at 6500 rpm for 10 min at 10°C and re-suspended in 200 ml of Murashige and Skoog (1962) liquid medium containing 5% sucrose (pH 5.6-5.7).

Pre-cultured SEs were transformed by immersion in bacterial suspension for 30 min, before being blotted dry on sterile paper and transferred to proliferation medium. The SEs were co-cultured for 5 days in darkness at 25°C. The SEs were then washed for 30 min with sterile water containing 300 mg/l carbenicillin.

The explants were transferred to Petri dishes with selective medium consisting of proliferation medium containing 300 mg/l carbenicillin plus 100 mg/l kan (for cork oak) or 75 mg/l kan (for holm oak). The explants were incubated under standard conditions, with transfer to fresh medium every two weeks. After 10 weeks, kan-resistant embryos were transferred to fresh proliferation medium containing 300 mg/l carbenicillin plus 150 mg/l kan (for cork oak) or 125 mg/l kan (for holm oak). Somatic embryos were maintained for a further four weeks in this medium (14 weeks in total) and putative transformants identified by growth on selection medium were then evaluated by the *GFP*-specific fluorescence technique (*GFP+*).

Genetic transformation was indicated by *GFP*-specific fluorescence observed under a Leica M205 FA epi-fluorescence stereomicroscope equipped with a light source consisting of a 100-W mercury bulb and a filter set including a 470/40x nm excitation filter and a 525/50m nm long-pass emission filter. Images were taken

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with a Leica DSC7000T camera. The transformation efficiency was defined as the percentage of initial explants that are *GFP*-positive after culture for 14 weeks in selection medium.

For each cork oak embryogenic line, duplicate experimental set-ups comprising ten explants placed in each of 10 Petri dishes were established. For holm oak, ten explants were placed in each of 10 Petri dishes for each pre-culture treatment, and the experiment was duplicated. In each transformation experiment, 20 non-inoculated (wild type, WT) embryogenic explants were cultured on proliferation medium with or without antibiotics (negative and positive controls).
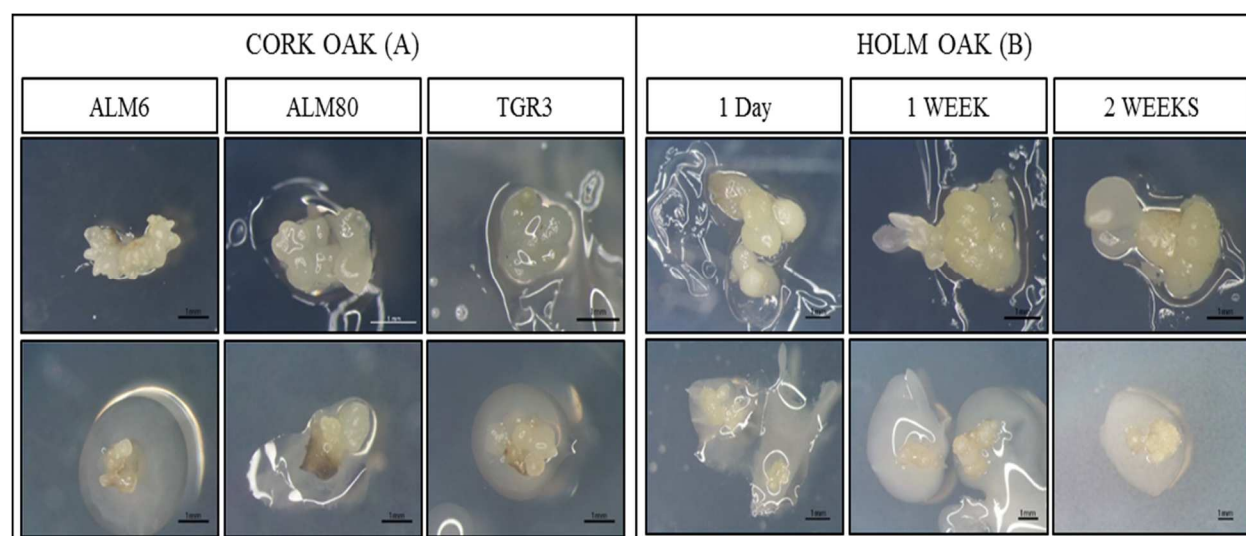
Figure 2. Morphological appearance of individual cork oak (A) and holm oak (B) somatic embryos after the pre-culture period (upper row) and after the *Agrobacterium* cultivation period (bottom row). Scale bar 1 mm.

PCR analysis

We extracted genomic DNA from cork oak and holm oak SEs derived from untransformed and putative transgenic embryo lines, by using the Real Plant and Fungi Extraction Kit according to the manufacturer's instructions (Durviz, Spain). The presence of the *CsTL1* (in both transcriptional senses), *NPTII* and *EGFP* transgenes was confirmed by PCR analysis. Reactions were carried out in a 50 μL volume containing 1× supplied Taq buffer, 2.5 mM MgCl$_2$, 200 μM dNTPs, 0.6 μM each primer, 1 U Taq DNA polymerase (Qiagen, Germany) and 100-200 ng of genomic DNA. PCR analysis was conducted with gene-specific primers: *CsTL1-F* (forward: 5´-AGGTCACTGGATTTTGGT-3´; reverse: 5'-CACCATGATGAAAACCCTG-3'; including T-35S region (**Fig. 1**)), *CsTL1-R* (forward: 5´-GGTAAGGCCGTAGAGT-3´; reverse: 5´-GATCTAACAGAACTCGCC-3´; including p35S region (**Fig. 1**)), *NPTII* (forward: 5'-GTCATCTCACCTTGCTCCTGCC-3'; reverse: 5'-AAGAAGGCGATAGAAGGCGA-3') and *EGFP* (forward: 5'-CACCGGGGTGGTGCCCAT-3'; reverse: 5'-CTAGTGGATCCCCCGGGC-3'). The expected sizes of PCR fragments were 981 bp for *CsTL1-F*, 658 bp for *CsTL1-R*, 472 bp for *NPTII*, and 740 bp for *EGFP*. Amplifications were carried out in a MJ Mini™ thermal cycler (Bio-Rad, Hercules, CA) by applying the following programs after initial polymerase activation: 40 cycles at 94ºC for 15 s, 60ºC for 30 s and 72ºC for 1 min for *CsTL1* gene, 35 cycles at 94ºC for 50 s, 60ºC for 50 s and 72ºC for 70 s for the *NPTII* gene,

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and 40 cycles at 94ºC for 15 s, 56ºC for 30 s and 72ºC for 1 min for *EGFP* gene. The amplified products were resolved on 1.2% (w/v) agarose gel and confirmed by the presence of a single band of expected size.

Maturation and germination

Cotyledonary-stage somatic embryos (≥ 5 mm) were isolated from transgenic lines of both species. Cork oak somatic embryos were transferred to baby food jars with 30 ml of SH medium and stored at 4ºC in total darkness for two months according to Toribio et al. (2005). Somatic embryos were then transferred to jars with 70 ml of SH medium supplemented with 0.025 mg/l 6-benzyladenine (BA) plus 0.05 mg/l indole-3-butyric acid. Holm oak somatic embryos were transferred to empty Petri dishes and stored at 4ºC in semi-darkness for two months. The explants were then transferred to jars containing 70 ml of Gresshoff and Doy (1972) medium with 0.1 mg/l BA. In both cases, SEs were cultured under standard conditions for 6 weeks.

Results

Evaluation of GFP fluorescence and development of transformants

Following the co-cultivation period, the explants gradually turned brown and many showed signs of necrosis. Newly emerging somatic embryos or embryogenic structures were observed in necrotic explants after culture for 6 (cork oak) or 8 weeks (holm oak). Kan-resistant explants were observed in all four embryogenic lines after 10 weeks of culture on selective medium, although the highest rate of production was achieved with line TGR3 (**Fig. 3A, B**). Only kan-resistant explants were isolated after 10 weeks of culture on selective medium. In both species, all explants cultured in the absence of the *Agrobacterium* suspension but in the presence of antibiotics (selective medium) became necrotic and did not survive (negative control). In both species, the transformation efficiency was determined on the basis of the fluorescence of surviving explants (**Fig. 4**). In cork oak, this parameter was clearly genotype-dependent, with rates of 17%, 4.5% and 2% obtained for lines TGR-3, ALM80 and ALM6, respectively (**Fig. 3A**).

In holm oak, no response was obtained when the SEs were pre-cultured for only one day; however, a transformation frequency of 2% was obtained when the explants were pre-cultured for one or two weeks (**Fig. 3B**). Although larger explants were obtained after two weeks and were easier to manipulate (**Fig. 2B**), several of them were contaminated with *Agrobacterium*, probably due to the complex shapes of the embryogenic forms, which may have impeded exposure of the surfaces to the bactericidal agent used during the washing step. A total of 30 *GFP*-positive embryogenic lines of cork oak were obtained and 10 *GFP*-positive lines of holm oak.

After GFP evaluation, new embryogenic transgenic lines of each genotype were established. One cotyledonary-stage embryo was isolated from each GFP-positive line and subcultured on selective medium for proliferation and establishment of different embryogenic transgenic lines. These lines were then successfully maintained by secondary embryogenesis on proliferation medium.

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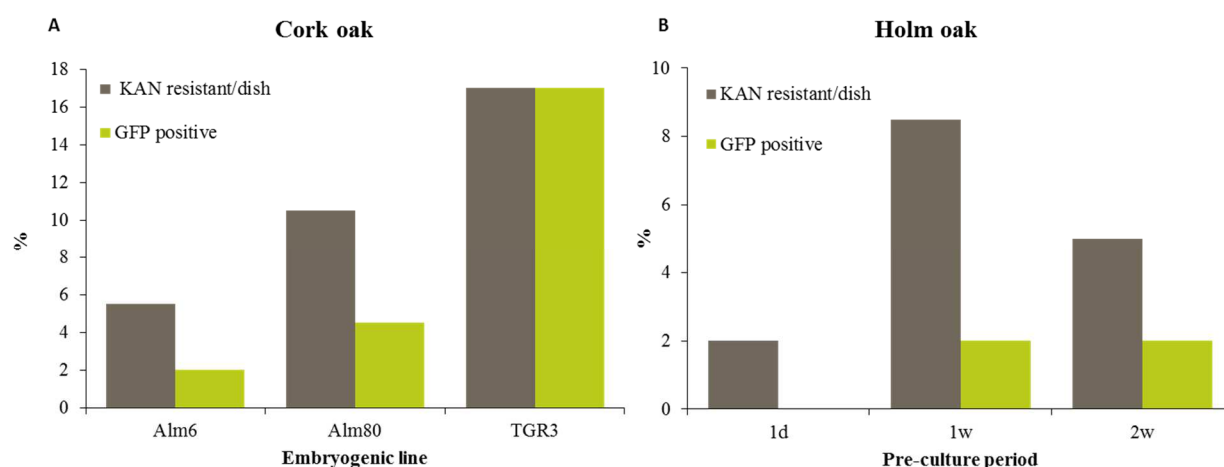
Figure 3. Kanamycin resistance frequency and transformation efficiency obtained for somatic embryos of cork oak (A) and holm oak (B). In cork oak the three different embryogenic lines are specified. In holm oak are shown the three pre-culture times.
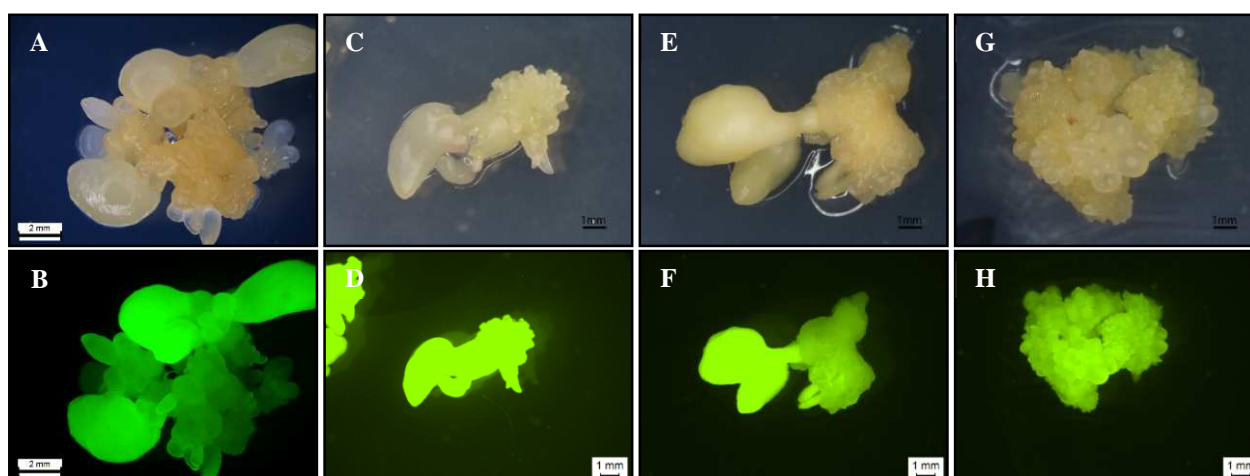
Figure 4. Kanamycin resistant explants of holm oak (A) and cork oak (C, E, G) observed under white light after culture for 8 weeks in selection medium. B, D, F, H: The same somatic embryos as shown in A, C, E, G observed under blue light and showing green fluorescence.

PCR analysis

The presence of the three transgenes was confirmed by PCR analysis in all putative transgenic lines analysed, but not in untransformed somatic embryos. A single band of the expected size was obtained in each embryogenic line. The presence of a thaumatin-like gene was confirmed in both transcriptional senses (**Fig. 5**).

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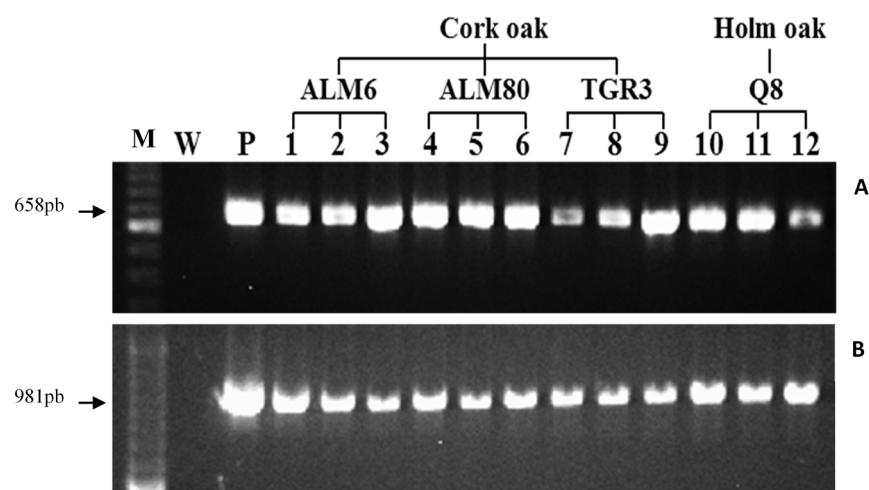
Figure 5. PCR amplification obtained with primers specific for production of a 658-bp (A) and 981-bp (B) *CsTL-F* and *CsTL-R* fragments, respectively. M: DNA ladder; W: non-transgenic somatic embryos (negative control); P corresponds to plasmid DNA (positive control); Lanes 1-12: cork oak transgenic lines (lanes 1-9) and holm oak transgenic lines (lanes 10-12).
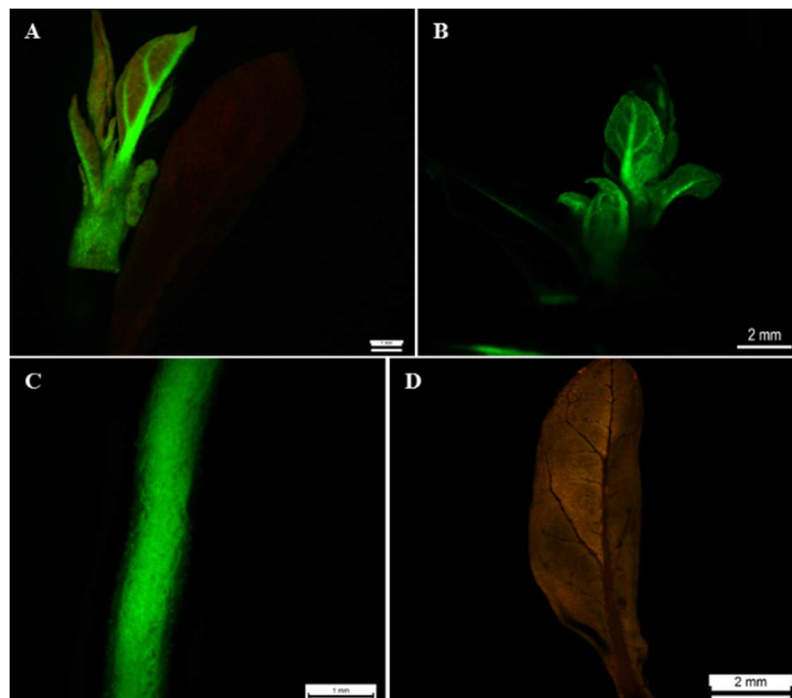
Figure 6. *GFP* expression on apex (A, B) and root (C) derived from transgenic plants visualized in an epi-fluorescence stereomicroscope. Untransformed leaf without *GFP* expression (D) visualized in an epi-fluorescence stereomicroscope.

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Evaluation of GFP in plants

Transgenic plants were obtained after germination of SEs derived from cork oak and holm oak transgenic lines. No phenotypic differences were found relative to control plants, suggesting no potential cytotoxic effects of the *GFP*.

GFP expression was also verified in shoots, leaves and roots derived from plantlets obtained after somatic embryo germination (**Fig. 6A, B, C**). Fluorescence was also more intense in young, small leaves than in older, larger leaves, and it was easier to detect in veins, midrib and petioles than in blades. *GFP* expression was not detected in roots, leaves (**Fig. 6D**) or shoots obtained from the non-transformed plants used as negative control.

Discussion

This is the first report of the genetic transformation of cork oak and holm oak with a gene from the Fagaceae family that describes attempts to improve the tolerance of the species to oak decline. Genes that encode thaumatin-like protein have been tested in other species, such as European chestnut (Corredoira et al. 2012), *Citrus* (Fagoaga et al. 2001; Zanek et al. 2008), wheat (Mackintosh et al. 2007) and pedunculate oak (Mallón et al. 2014).

Genetic transformation has been achieved in several oak species, including cork oak. In *Quercus robur*, successful transformation of SEs initiated from mature and juvenile pedunculate oak trees has been reported with the *Agrobacterium* strain EHA 105 harbouring the p35SGUSINT plasmid containing a *uidA* reporter gene and *ntptII* marker gene. Transformation efficiencies of 6% and 2% were achieved in juvenile material and in the mature genotype, respectively (Vidal et al. 2010; Mallón et al. 2013). Different strategies for genetic transformation have been proposed for cork oak. Álvarez et al. (2004) reported a transformation frequency of 4% when embryogenic clusters and isolated embryos derived from a single embryogenic line were used in transformation experiments. These explants, without pre-culture, were infected with different *Agrobacterium* strains harbouring pBINUbiGUSint, co-cultured for 2 days in darkness, washed with a solution of cefotaxime (600 mg/l) for 2 hours, and cultured on proliferation medium supplemented with 500 mg/l cefotaxime and 100 mg/l kan. In a subsequent study using the same protocol, Álvarez and Ordás (2007) achieved successful transformation of only 3 of the 6 embryogenic lines evaluated using the *Agrobacterium* strain AGL1 transformed with the plasmid pBINUbiGUSint. Later on, somatic embryos isolated from an embryogenic line with a higher transformation capacity were subsequently transformed with *Agrobacterium* strain AGL1 harbouring the plasmid pBINUbiBar, with the objective of producing adult transgenic cork oak trees resistant to the herbicide phosphinothricin (Álvarez et al. 2009).

The protocol defined in this study differs in several aspects from those previously applied to cork oak. For example, we used carbenicillin (300 mg/l) as the bactericidal agent, after earlier attempts using cefotaxime proved unsuccessful (the embryos tended to acquire a dark yellowish colour and did not grow). Moreover, as cefotaxime is thermolabile and photolabile, it is advisable to maintain the cultures in darkness (Álvarez and Ordás 2007). We also used *GFP* rather than *uidA* (GUS) as a selectable marker. The *GFP* assay greatly simplified and improved evaluation of the transformation events in real time, relative to the GUS assay. The presence of necrotic tissues or green spots may interfere in the GUS assay, thus requiring longer periods of incubation or maintenance of the embryos in darkness to avoid greening (Álvarez and Ordás 2007); however, these difficulties were precluded by the use of *GFP*. Detection of *GFP* fluorescence is easy and non-destructive, whereas large numbers of explants must be sacrificed in the GUS assay (Wu et al. 2015). As each putative transformed line is established from a single transformation event, *GFP* helps to isolate only transformed embryogenic masses. In our study, only one fluorescent embryo at its cotyledonary-stage was isolated in order to establish the putative transgenic lines.

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In all previous reports related with genetic transformation of cork oak, the genotype was indicated to have a strong influence. Álvarez and Ordás (2007) reported higher transformation efficiency with the M10 line (43%) than with lines ALM6 and ALM80, which died after 4 months of culture in the presence of kan. In the present study, genetic transformation of all three cork oak lines was achieved (17% in TGR3, 4.5% in ALM80 and 2% in ALM6), including the lines ALM6 and ALM80 which were not transformed in previous studies.

To our knowledge, this is the first report of the successful genetic transformation of holm oak. A similar protocol was also applied to cork oak, although the procedure had to be modified as pre-culturing the explants for only one day did not produce successful results. When the explants were pre-cultured for one or two weeks, a comparable transformation efficiency (~2%) was achieved. Although low, this rate was sufficient to establish ten transgenic embryogenic lines in holm oak. The same pre-culture time was necessary for the genetic transformation of pedunculate oak somatic embryos (Vidal et al. 2010).

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Elicitation of holm oak somatic embryos and dual culture with *Phytophthora cinnamomi*

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Abstract

Holm oak (*Quercus ilex* L.) is the main tree species of the "dehesa" and "montado", typical Mediterranean agroforestry systems of Spain and Portugal. Acorns produced by this species are an important component of the Iberian pig's diet, whose products are the basis of a high-quality gastronomy industry. As a consequence of a decline syndrome called "seca" there has been a high mortality among trees of this species in the last years. One of the agents causing this syndrome is the oomycete *Phytophthora cinnamomi* Rands. One of the possibilities to address the mortality problem is the production of plants tolerant to the infection. Priming plants by the induction of epigenetic changes to elicit a defense response has long been used for producing tolerant plants. In addition, the induction of a transgenerational defense in offspring occurs by maternal signals at the time of seed formation. The formation of somatic embryos offers an opportunity to give "somatic seeds" suitable cues to prime regenerated plants for tolerance. Embryo clumps from three holm oak embryogenic lines were treated with BABA, BTH, Salicylic Acid and Methyl-Jasmonate. They were also treated with filtrates of a cinnamomin-inducing culture medium in which the oomycete was cultured. Treatments to somatic embryos consisted in the culture on semisolid SH medium supplemented with 50µM of each of the compounds separately or with filtrates at 10 or 30% for 60 days (long-term elicitation), or immersion in the same liquid medium for 3 days (short-term elicitation) followed by culture on medium without elicitor for up to 60 days. Data on the number of somatic embryos produced at the end of each treatment and their germination ability were recorded. Single somatic embryos were challenged with the oomycete in a dual culture. Data of differential growth of mycelium and of necrosis of somatic embryos were daily collected from day 2 to day 10 from the beginning. Treatments with different elicitors did not significantly affect the multiplication rate of the embryogenic lines, but long-term elicitation with BTH strongly reduced the embryo differentiation ability. Conversion of somatic embryos into plants was not significantly affected. When somatic embryos were challenged with *Phytophthora cinnamomi* in a dual culture they markedly enhanced the growth of mycelium. However this chemotactic attraction was altered in some cases when somatic embryos were treated with elicitors. Some significant differences were also recorded among treatments for necrosis of somatic embryos. Therefore elicitation induced changes in the holm oak somatic embryos that could be detected in dual culture with the oomycete.

Keywords: epigenetic changes, forest biotechnology, disease tolerance, *Quercus ilex*, somatic embryogenesis, transgenerational resistance.

Key message: holm oak somatic embryos of the same genotypes treated with elicitors of defense response produced different epitypes that behaved differently when they were challenged with *Phytophthora cinnamomi* in dual culture.

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Introduction

Holm oak (*Quercus ilex* L.) is a main tree species of the "dehesa" and "montado", typical Mediterranean agroforestry systems of Spain and Portugal. Acorns produced by these species are an important component of the diet of the Iberian pig, a local race that is the basis of a high quality gastronomic industry (García-Valverde et al. 2010). In the past decades there has been a high mortality among trees of this species, at a rate that has increased lately. This is the consequence of a decline syndrome called "seca" one of whose causative agents is a root infection by the oomycete *Phytophthora cinnamomi* Rands (De Sampaio e Paiva Camilo-Alves et al. 2013).

Genetic improvement techniques such as the capturing of tolerance traits that are part of the natural genetic variability, or the induction of new variability by the direct transfer of pathogenesis-related genes, could be used to produce tolerant varieties (Dungey et al. 2014; Corredoira et al. 2016). The induction of epigenetic changes to induce a defense response is another possibility to produce tolerant plants (Zhu et al. 2016). Transgenerational defense induction is a well-known phenomenon (Holeski et al. 2012). This induction occurs in the parental generation and is transferred to the offspring through maternal signals at the time of seed formation. In addition, an epigenetic memory that is set during seed formation and that is a long-lasting condition that affects the behavior of plants produced from those seeds, has been described for several conifers (Yakovlev et al. 2012). Therefore, the formation of somatic embryos in a controlled environment offers an opportunity to give "somatic seeds" suitable cues to prime regenerated plants for tolerance.

Several substances have been used for treating seeds that confers to germinants an improved resistance or tolerance against different kinds of stresses, both abiotic and biotic. Plants derived from seeds of several species treated with salicylic acid showed enhanced vigor and defense responses (Rajjou et al. 2006). Other substances used for priming seeds to induce resistance/tolerance to several pathogens are methyl jasmonate (Król et al. 2015), β-aminobutyric acid (Cohen et al. 2016) and benzothiadiazole (Ramasamy et al. 2015). In addition, substances secreted by several microorganisms also may induce an elicitation response in treated seeds that improves the defense responses of the seedlings (Ahmed et al. 2000). In the case of *Phytophthora cinnamomi* these elicitors are called cinnamomins (Ebadzad et al. 2015).

Responses of plant tissues of different species, challenged with fungal pathogens in dual cultures to study pathogenicity and host tolerance, have been reported (Nawrot-Chorabik 2013). This technique has been applied both to callus (Kvaalen and Solheim 2000; Nagy et al. 2005) and embryogenic cultures of forest species (Nawrot-Chorabik 2014; Nawrot-Chorabik et al. 2016). Recently published studies suggest that transgenerational, induced resistance against *Phytophthora cinnamomi* occurs in holm oak (Solla et al. 2013). Therefore the elicitation of induced tolerance could be possible in somatic embryos of this species. The objective of this study was to test whether changes in somatic embryos could be induced by elicitors of the defense response, and to assess whether these changes could be detected by a dual-culture bioassay. Also the effect of the application of elicitors on the multiplication and conversion ability of holm oak somatic embryos was evaluated.

Material and Methods

Dual culture

Holm oak embryogenic lines were obtained by induction of somatic embryogenesis in teguments of developing ovules isolated from acorns collected from a tree standing in Quintos de Mora (line Q8; Toledo, Spain), and two trees growing in El Encín (lines E00 and E2; Madrid, Spain). They were maintained by recurrent embryogenesis on semisolid SH medium lacking plant growth regulators (PGR) with monthly subculture (Barra-Jiménez et al. 2014). A virulent strain of *Phytophthora cinnamomi,* donated by the

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University of Extremadura (Spain), was used in the dual culture experiments. This strain (UEX1) was isolated in a stand of diseased, declining holm oak trees (Corcobado et al. 2014), and was maintained on PDA-agar medium in darkness at 4 °C with monthly subcultures. To obtain cultures with active mycelial growth, 10 sq mm of the cold-conserved cultures were cultured in 90 mm diameter Petri dishes with the same medium and placed in darkness at 23 °C for one week.

Effect of elicitation

To study the effect of elicitation, embryo clumps from the three embryogenic lines were treated with each elicitor separately. They were β-aminobutyric acid (BABA), benzothiadiazole (BTH), salicylic acid (SA), and methyl jasmonate (MJA), all of them at 50μM, and a filtrate (FILT) at 10 and 30% (v/v) of a cinnamomin-inducing liquid medium (ESM) in which the strain of *Phytophthora cinnamomi* was cultured (Horta et al. 2008). These elicitors were applied in two ways. Treatments named "long term elicitation" consisted in culturing the embryo clumps on semisolid SH medium without PGR supplemented with each elicitor for 60 days, with a subculture to the same medium after the first month. In the treatments termed "short term elicitation" the embryo clumps were cultured in liquid SH medium lacking PGR and supplemented with each elicitor for 3 days under shaking conditions, and then they were transferred to semi-solid basal SH medium without elicitors and cultured up to a period of 60 days with an intermediate subculture. All cultures were performed under a 16-h photoperiod at 25 °C. At the beginning of each treatment five vessels, inoculated with the same amount of embryo clumps, were prepared. All the embryogenic tissue produced in each treatment, after the first month in culture, was subcultured into new vessels, maintaining the same amount of embryo clumps per vessel. The number of new vessels needed to culture all the tissue produced in each treatment divided by the initial number of vessels, was considered the "monthly multiplication rate". To determine the "differentiation ability" of each treatment, the number of single somatic embryos produced per vessel at the end of the 60 day period was counted. To test the "conversion ability" of the somatic embryos, the procedure that used PGR in the germination medium, described in Barra-Jiménez et al. (2014), was followed.

Dual culture bioassay

The dual culture bioassay was carried out by placing somatic embryos or embryo clumps from each one of the elicitation treatments, and a 10 sq mm PDA-agar medium with actively growing mycelium, in a 90mm Petri dish filled with SH basal medium. The embryos were placed on one side of the dish and the mycelium on the opposite side, both located a quarter of a dish-diameter from the respective edge of the dish. Dual cultures with somatic embryos of each genotype that did not undergo elicitation ("non-treated", NT) and cultures with just the mycelium of the oomycete on the same SH medium ("CONTROL") were also performed. Between seven to ten replicates per treatment were prepared. Data of differential growth of mycelium (DGM, i.e., growth towards the somatic embryos minus growth to the opposite side; L-l) and necrosis of somatic embryos using a predetermined scale (**Fig. 1**) were daily collected from day 2 to day 10.

Statistics

Data analysis was carried out by ANOVA, using the Statistica 5.1 software (StatSoft, Inc., Tulsa, OK, USA). Frequencies of germination were compared through the Fisher's Exact test using the GraphPad software (http://graphpad.com/quickcalcs/contingency1/).

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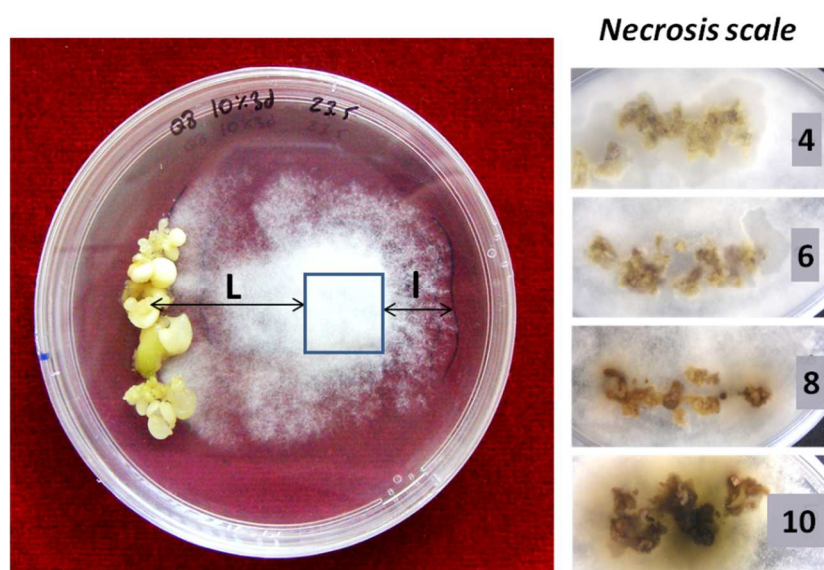
Figure 1. Dual culture bioassay of holm oak somatic embryos and embryo clumps with *Phytophthora cinnamomi*. Left, determination of differential growth of mycelium (DGM, L-l); right, Necrosis scale.

Results

Overall, treatments with the different tested elicitors did not significantly modify the multiplication rate of the embryogenic lines as regards to the untreated ones (**Tab. 1**).

Table 1. Effect of elicitors and way of application on the multiplication rate of three holm oak embryogenic lines (Q8, E2 and E00). NT, non-treated somatic embryos; MJA, 50 µM methyl jasmonate; BTH, 50µM benzothiadiazole; BABA, 50 µM β-aminobutyric acid; SA, 50 µM salicylic acid; FILT10 and FILT30, filtrate at 10% or 30% of an elicitin secretion medium in which the oomycete was cultured.

Elicitor	Short term elicitation				Long term elicitation			
	Q8	E2	E00	Mean	Q8	E2	E00	Mean
NT	1.8	1.6	2.3	1.9	2.4	3.0	1.8	2.4
MJA	1.5	1.0	1.5	1.3	3.0	2.4	2.0	2.5
BTH	2.5	0.2	2.0	1.6	1.8	2.2	1.0	1.7
BABA	1.7	1.6	2.0	1.8	2.8	2.8	2.4	2.7
SA	2.0	0.8	2.0	1.6	2.4	2.4	0.8	1.9
FILT10	1.7	1.4	1.5	1.5	2.4	1.2	0.4	1.2
FILT30	1.7	1.6	2.0	1.8	1.6	1.8	0.2	1.3
ANOVA, p			0.911				0.147	

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However, a decrease in the growth of lines was observed, mainly in those that were long-term treated with the FILT. Treatments also did not affect the differentiation ability of the embryogenic lines to form single embryos, except in the case of long term treatments with BTH that drastically hampered embryo differentiation (data not show). This effect of BTH was not observed in the short term treatments. During the course of the experiment with short term treatments the E2 line lost the ability to differentiate embryos. Interestingly, the treatment with methyl jasmonate avoided this loss (data not shown). Long term treatments with the different elicitors did not significantly influence conversion of somatic embryos from the Q8 line into plants. However, a reduction of conversion frequency was observed with most elicitors in the short term treatments (**Tab. 2**).

Table 2. Effect of elicitors and way of application on conversion of somatic embryos (se) of holm oak genotype Q8. Data are N, number of se; %, frequency of conversion (root and shoot formation); p, two-tailed P value of the Fisher's exact test comparison with NT. NT, non-treated somatic embryos; MJA, 50 µM methyl jasmonate; BTH, 50 µM benzothiadiazole; BABA, 50 µM β-aminobutyric acid; SA, 50 µM salicylic acid; FILT10 and FILT30, filtrate at 10% or 30% of an elicitin secretion medium in which the oomycete was cultured.

Elicitor	Short term elicitation			Long term elicitation		
	N	%	p	N	%	p
NT	45	24	na	50	38	na
MJA	17	6	0.1527	20	35	1.0000
BTH	36	22	1.0000	--	--	--
BABA	43	9	0.0881	29	31	0.6287
SA	21	10	0.1982	20	45	0.6010
FILT10	19	5	0.0901	46	35	0.2188
FILT30	48	8	0.0482	34	21	0.1000

In the dual culture with *Phytophthora cinnamomi* the presence of holm oak tissues enhanced the growth of mycelium (**Fig. 2**). The growth stimulation relative to the growth of the oomycete in control dishes without the embryogenic tissues was significantly dependent on genotype.

The DGM caused by this stimulation was affected by the elicitation treatments. In general, values of DGM were increasing up to day 4 after which it began to decrease. At this time point the DGM in dual cultures with somatic embryos from lines treated with some elicitors showed significant differences with the one of cultures with somatic embryos from untreated lines. In long term elicitation, DGM in cultures with the Q8 line did not show significant differences among elicited and non-elicited tissues. However, significant differences for DGM with regard to untreated tissues were recorded for BABA-treated tissues in the case of the E2 line, and OCF10, salicylic acid, BTH and BABA-treated tissues in the case of E00 line (**Fig. 2**). In short term elicitation, elicitors had no significant influence on DGM in cultures with somatic embryos of line E00, but BABA-treated tissues of line Q8 showed a significantly decreased DGM as regard the one of the non-treated tissues (data not shown).

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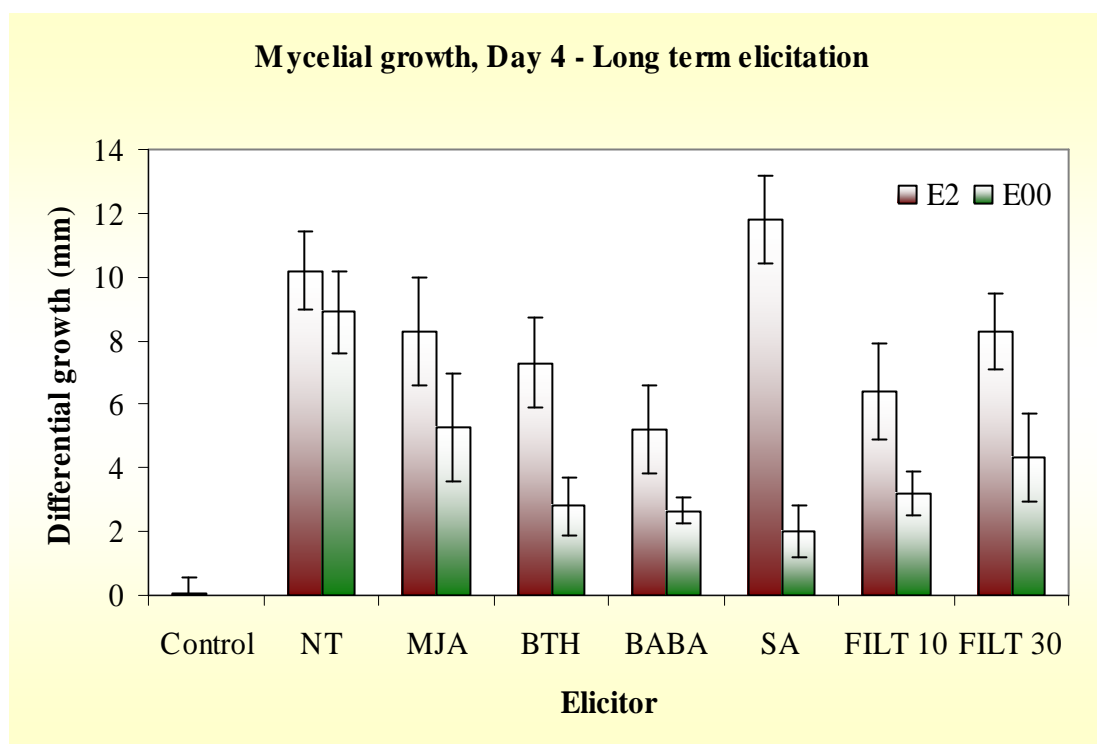
Figure 2. Effect of the presence of holm oak somatic embryos treated with different elicitors on the differential growth of mycelium of *Phytophthora cinnamomi* in dual culture. Embryos belonged to embryogenic lines E2 and E00. Control, culture of the oomycete without somatic embryos. NT, non-treated somatic embryos; MJA, 50 µM methyl jasmonate; BTH, 50 µM benzothiadiazole; BABA, 50 µM β-aminobutyric acid; SA, 50µM salicylic acid; FILT10 and FILT30, filtrate at 10% or 30% of an elicitin secretion medium in which the oomycete was cultured.

With regard to necrosis values, data collected at day 7 showed some significant differences among non-treated lines and the elicited ones (**Fig. 3**). In long term elicitation, no significant differences were observed between the non-treated embryos of line E2 and the elicited ones. However, necrosis was significantly reduced in genotype Q8 when the embryogenic line was treated with BTH and FILT30. Similarly, a significant decrease of necrosis values was recorded for genotype E00 when it was treated with BTH, FILT10 and FILT30. In short term elicitation, treatments to both tested embryogenic lines displayed significant reduction of necrosis values, namely if treated with MJA, BTH and FILT10 in the case of genotype Q8, and BABA and SA in the case of genotype E00 (**Fig. 3**).

Discussion

When applying several substances to seeds and plants, both chemical and biological, in order to induce resistance, the first step is to determine their possible toxicity in the plant material (Justyna and Ewa 2013; Ogórek 2016). In this study no major disturbances of the multiplication and conversion capacity of holm oak embryogenic lines were observed in response to the assayed substances. This could be due to the low concentrations applied, within or below the typical range used to elicit the production of plant metabolites

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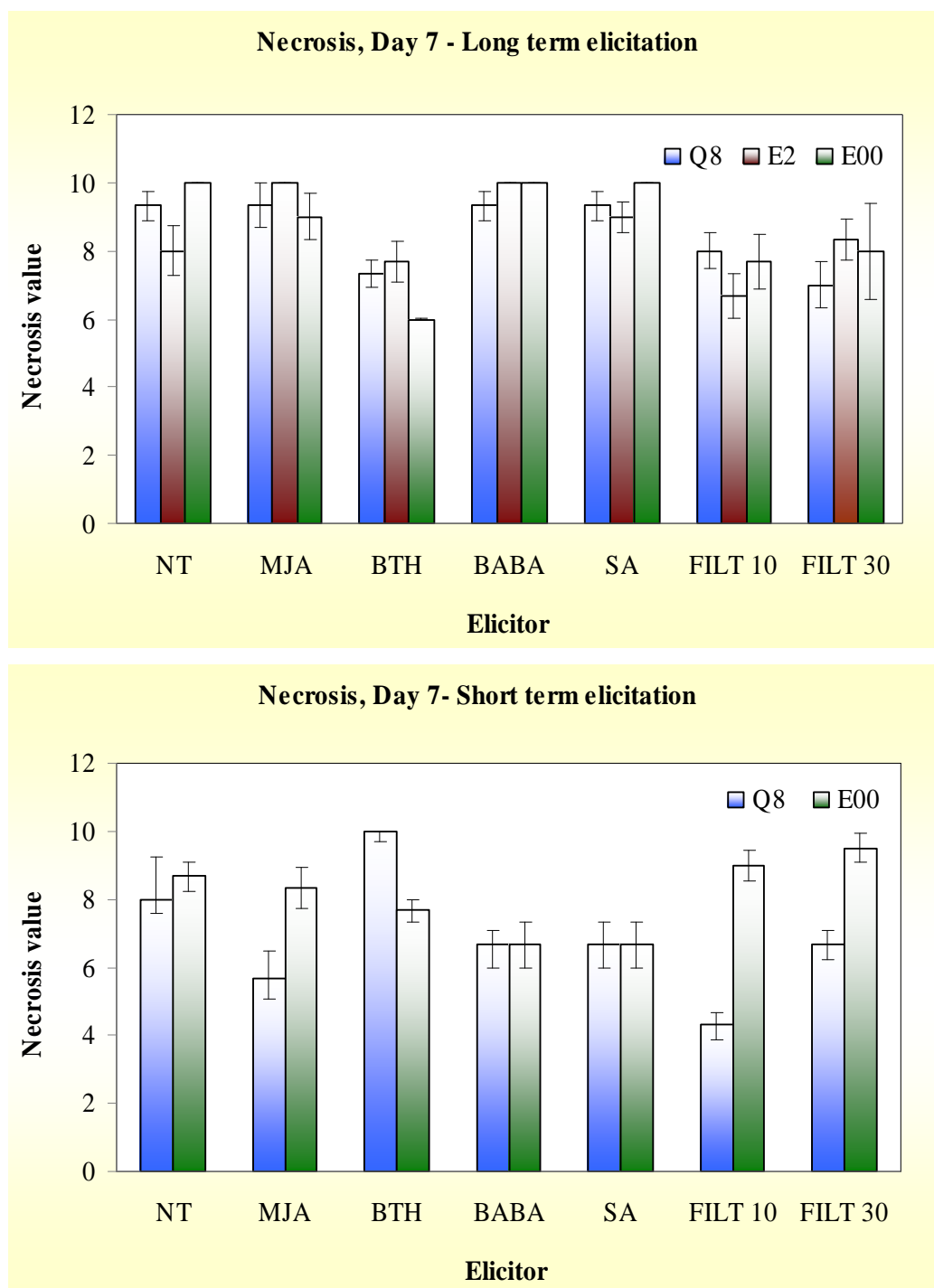
Figure 3. Effect of treating holm oak somatic embryos with different elicitors on the degree of necrosis in dual culture with *Phytophthora cinnamomi* measured at day 7. NT, non-treated somatic embryos; MJA, 50 µM methyl jasmonate; BTH, 50 µM benzothiadiazole; BABA, 50µM β-aminobutyric acid; SA, 50 µM salicylic acid; FILT10 and FILT30, filtrate at 10% or 30% of an elicitin secretion medium in which the oomycete was cultured.

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(Ramirez-Estrada et al. 2016). However, the almost complete loss of differentiation of single somatic embryos when using BTH in long term applications was noted, which precludes the use of this type of application in the case of this substance. Also a decline in multiplication rate was observed when embryogenic lines were cultured with oomycete culture filtrates during the whole 60 days period. Although the decreased rate observed was not statistically significant, the possibility of a true reduction of growth cannot be discarded. The culture of plant tissues with exudates of pathogenic agents has been used to select resistant variants *in vitro* (Ganesan and Jayabalan 2006; Flores et al. 2012). In the present case the cultures were at a selection pressure that could have caused the observed growth reduction. If this were the case, changes induced in the embryogenic lines might be of genetic rather than epigenetic nature.

The main purpose of this research was to determine whether somatic embryos belonging to individual genotypes that were under the influence of elicitors of defense response, developed a differential behavior when were challenged with the oomycete in dual cultures. There is increasing evidence that individual genotypes (clones) exposed to different environmental conditions acquire some kind of memory, mainly of epigenetic nature, that determines a differential behavior in response to other environmental stimuli (Raj et al. 2011). Although it is well established in plants that epigenetic marks are transferred to the offspring, which is the basis of transgenerational resistance (Holeski et al. 2012), the time of embryo formation is also a specific moment in the plant life in which reprograming and the establishment of new marks occur (Kawashima and Berger 2014). This phenomenon is behind the establishment of the epigenetic memory that takes place during seed formation described in conifers (Yakovlev et al. 2012). Therefore, when somatic embryos are forming by recurrent or cleavage embryogenesis under a controlled environment they may be at a suitable state for the induction of different epigenetic marks. This was demonstrated by Kvaalen and Johnsen (2008) when they induced the formation of different epitypes of the same genotypes of *Picea abies* by culturing embryogenic lines at different temperatures. In the present study holm oak embryogenic lines of the same genotype behaved differently in the dual culture bioassay according to the elicitation treatment they had previously received. This might open the door to the production of primed plants tolerant to *Phytophthora cinnamomi*.

The different behavior of elicited somatic embryos was detected in the dual culture with the oomycete. Therefore, this bioassay may be a tool to detect tolerant tissues. Good correlations between susceptibility of different forest species *in vivo* and *in vitro* to different pathogens have been demonstrated (Kvaalen and Solheim 2000; Nagy et al. 2005). Therefore in the present case it will be worth it to establish possible correlations of behavior in the dual culture with the response *in planta*. The dual culture could also be used as an experimental system to study the factors of chemotactic attraction of holm oak tissues to *Phytophthora cinnamomi*. This attraction has been observed during the infection process *in vivo* (Oßwald et al. 2014) and was one of the traits affected in the dual culture by the elicitation of holm oak somatic embryos.

Conclusions

Except long-term BTH treatment the elicitors did not greatly impair embryo differentiation and germination, although the decrease of germination ability of the short-term elicited embryos deserves further attention. Elicitation induced changes in the embryogenic lines that were detected after challenging holm oak tissues with *Phytophthora cinnamomi* in dual culture.

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Cloning drought tolerant *Eucalyptus globulus* in the Region of Bio-Bio, Chile

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Abstract

Three breeding populations were included as part of a strategy to improve drought tolerance of *E. globulus*: (A) open pollinated progenies with genetic ranking of cubic volume from a clonal seed orchard, (B) a population of 107 plus trees generated by mass selection of trees exposed to the drought conditions prevailing in the semiarid region of Chile, and (C) control pollinated progenies by intraspecific crosses between the B x A populations using a "one stop pollination" system. Micro propagation and rooting tests were carried out by somatic embryogenesis and organogenesis to make drought resistant clones available for operational multiplication in nurseries. *"In vitro"* protocols were developed to induce morphogenic responses and genetic transformation in adventitious buds and seeds of *E. globulus*. Traceability of superior genotypes was also made on the basis of molecular genetic markers and was physiologically evaluated in a greenhouse under water stress conditions to characterize their potential for drought resistance. It was possible to establish protocols through two pathways that allowed an appropriate transformation via *Agrobacterium tumefaciens* and that resulted in regeneration of two highly productive clones of *E. globulus*. Two morphogenic pathways were obtained indirectly from callus, one of these was from indirect somatic embryogenesis in seed explants, and the other pathway was by indirect organogenesis of adventitious buds in the explants. One of the objectives of the project was to evaluate the rhizogenic ability of clones of *E. globulus* and identify drought tolerant genotypes for propagation by conventional, operational rooting of cuttingsor mini cuttings and by using hydroponics. The results confirmed that *E. globulus* is a species with the low rate of adventitious rooting of stem cuttings reported for this species. The limited material available for evaluation (18 clones) did not allow us to identify individuals with a high average percentage of rooting. However, there are clones that in some particular assays exhibited a high rhizogenic response (up to 92%). The average rooting percentage obtained in all trials reached 14.2% but great variability was observed in the trials. However, some clones reached a high percentages of rooting. Among them are clones 87, 48, 62, 82 and 77 that obtained values ranging between 60 and 93%. Such percentages are compatible with operational cloning of these individuals.

Keywords: *Eucalyptus globulus*, breeding, somatic embryogenesis, genetic transformation, organogenesis, cuttings, mini cuttings, hydroponics

Introduction

Eucalyptus globulus Labill, Tasmanian blue gum, is a key plantation hardwood tree species in many temperate regions. It is widely planted for pulp and paper production due to its rapid growth, adaptability and exceptional Kraft pulp yield and fiber properties. In Chile there are now around 570,000 ha planted with this species (INFOR 2015)*,* representing 23% of the country's exotic forest plantation resource of around 2.4 M ha. *Eucalyptus globulus* was first introduced to Chile in the late 19th century near Colcura; a small town located south of Concepcion, with the early plantings being carried out to supply logs for coal mine supports. Today, most of the country's *E. globulus* plantations are concentrated in the Bío-Bío Region; this extends between latitudes of 36°00' to 38°30' S and longitudes from 71°00' W to the Pacific Ocean.

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Within this region, the preferred areas for *E. globulus* plantations are in the more coastal parts where rainfalls ranges from 1,000 to 1,240 mm yr^{-1}. In such areas, *E. globulus* achieves productivities of 25 m^3 ha^{-1} $year^{-1}$ and more without genetic improvement, due to relatively low temperature variation (8°C -10°C) between the mean temperature of the warmest and coldest months and absence of significant winter frosts (http://www.meteochile.gob.cl). Since 1990 many organizations in Chile started breeding programs to improve frost hardiness, growth, yield and fiber quality of *E. globulus* (Rojas Vergara and Griffin 1997). Drought hardiness as a trait was not included in such programs, but recent INFOR research has shown that climate change is likely to decrease the productivity of *E. globulus* in Chile's key plantation areas by 6-8% (Barros and Ipinza 2011) due to lower precipitation and more extreme temperatures.
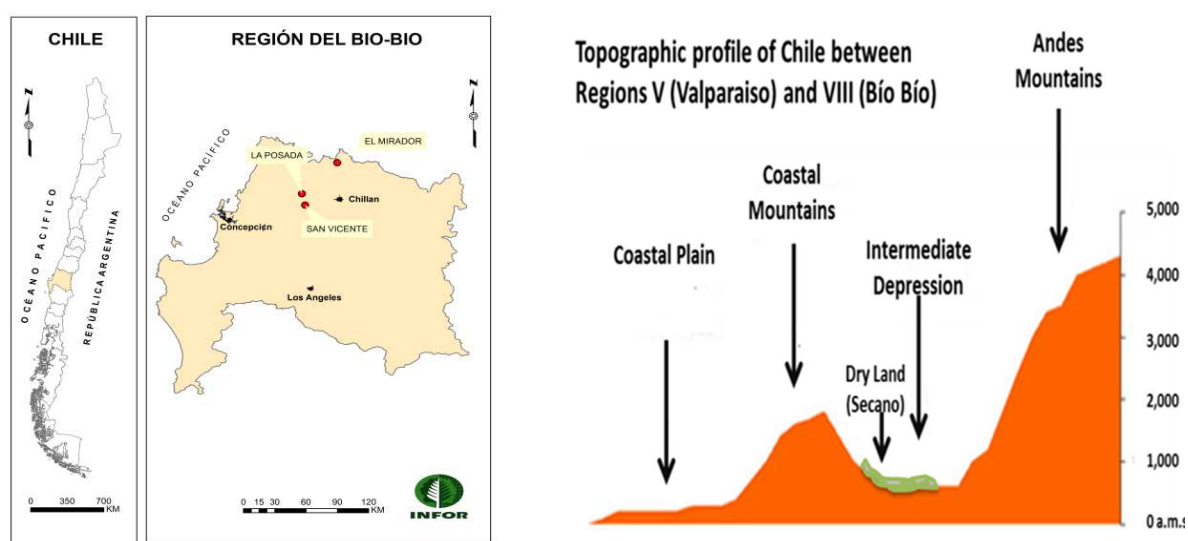
Figure 1. Topographic profile of Chile between Valparaiso and Bío-Bío Regions.

Chile's geography is characterized by four topographical forms: coastal plain, coastal mountains, intermediate (inland) depression and the Andes Mountains. The intermediate depression is a broad inland valley and is called the *"Secano"* (**Fig. 1**); its climate is characterized by a longer dry season (4-5 months with <40 mm rainfall $month^{-1}$) and higher annual thermal oscillation (ranging from 10 – 14°C) than in the coastal plain and mountains (http://www.meteochile.gob.cl). In winter, the minimum temperatures in the inner valley can drop below 0°C and in summer maximums can be close to 40°C. Water deficits, the difference between precipitation and potential evapotranspiration, can range from 12.5% to 36.8% or more (as a percentage of mean annual precipitation). Growers of *E. globulus* in the *Secano* have, through successive selection and propagation cycles, developed a Chilean landrace that seems somewhat better adapted to the somewhat drier climatic conditions in local areas where mean annual rainfalls ranges from 765 to 917 mm (INFOR, 1998).

Production advantages associated with vegetative propagation are closely associated with the genetic quality of individuals that are being multiplied. This technique is an essential component of forest genetic improvement programs (Zobel and Talbert, 1988). Due to the extensive life cycle of the tree a classical breeding program requires a long time to be developed. Therefore, vegetative propagation becomes a very useful tool for the breeder, allowing capture and quick transfer of genetic gain at any stage of breeding programs for commercial plantations (Kleinschmit et al. 1993; Mac Rae and Cotteril 1997). Furthermore, this ability to capture genetic gain (additive and non-additive), allows large-scale multiplication of plants

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exhibiting specific combinations of genes or hybrid plants, which is practically impossible to achieve by using seeds (Potts et al. 1992). In recent decades, vegetative propagation technologies have experienced intense development, realizing mass clonal production of commercially important species such as pine and eucalyptus, plus numerous hybrids (Bettinger et al. 2009; Assis 2011). However, there are still biological barriers that affect the process of cloning. The most serious barrier is related to the maturation of plant tissues in adult trees, which makes vegetative propagation very difficult for some species and virtually impossible for others (Rodriguez et al. 2005). This is a big problem for breeding programs because at the age at which trees are selected, based on the features that are intended to be improved or perpetuated, they have lost their ability to root (Gutiérrez 1995). Another limitation that restricts the operational application of clonal propagation is the higher cost of cloned plants in relation to those produced by seeds, due to the increased handling and infrastructure required for the vegetative propagation techniques developed to date. Depending on the complexity level, they are grouped into two categories, the first comprises conventional methods, also called macro propagation, and the second comprises the biotechnological technologies organogenesis and somatic embryogenesis, which are referred to generically as micro propagation.

A research and development program to increase drought hardiness of *E. globulus* plantations started in 2007, funded by INNOVA-CORFO http://www.english.corfo.cl/ and involved a technological network composed of the Chilean Agricultural Research Institute (INIA) and three private nurseries (Agromen, ViveroSur and Proplantas), which were linked to this project as partners, interested in the results and products. Its aim was to "*make available to the forestry companies and farmers in dryland areas of Chile, clones elites of Eucalyptus globulus, better adapted to drought and with better yields than those currently available, and transform some high productivity clones by incorporating specific genes, to be able to express a greater resistance to water stress*".

Objectives

1. Select families and genotypes of *E. globulus* that exhibit superior performance for drought tolerance in the Bio Bio region.
2. Develop macro and micro propagation systems to transfer genetic material to nurseries for operational multiplication.

Material and methods

Selection of drought tolerant E. globulus landrace plus-trees

Around 12,000 hectares of plantations were surveyed in drier environments between Ovalle (30°35' S, 71°12' W) and Ninhue (36°25' S, 72°26' W) in the *Secano* to identify plus-trees that exhibited superior volume and form in this environment where climatic conditions generally limit productivity in plantations of this species. From this work, 107 plus-trees with apparent drought tolerance were selected according to selection criteria described in **Tab. 1**. Three breeding populations were included: (A) open pollinated progenies of selected genotypes from a clonal seed orchard of INFOR's tree breeding program, mainly constituted by Australian *E. globulus*; (B) open-pollinated families of plus-trees selected from plantations of Chilean landrace origin grown under dry conditions, and; (C) control-pollinated families generated by intraspecific crosses between populations A and B using one stop control-pollination technology. These crosses were created to combine the genetic superiority of the clonal orchard genotypes and the site adaptation qualities of the Chilean landrace of *E. globulus*.

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Table 1. Criteria for plus-tree selection in Secano landrace plantations.

Selection trait	Criteria for acceptance
Stand Age	Present in even-aged stands of uniform density
Dominance	Being dominant (or exceptionally codominant) and not located near a tree plantation edge
D.B.H.	Above average DBH of the stand
Stem	Straight, cylindrical and no visible spiral growth; the tree should not be leaning
Crown	Balanced, small diameter crown
Branches	Short, small diameter and insertion angle on the stem close to 90° from the vertical.
Tree health	Absence of diseases and pests; largely free of attack from eucalypt longhorned borer (*Phoracantha semipunctata*)

Rhizogenic ability of drought tolerant genotypes of E. globulus clones

Rhizogenic ability of drought tolerant *E. globulus* clones in Population B was carried out by testing rooting of cuttings in the greenhouse. The cuttings consisted of stem segments extracted from mothers plants obtained through somatic organogenesis techniques and managed as such in the greenhouses of INFOR and companies associated with the project. Trials were established according to a methodology based on using a variable number of cuttings per clone based on mother's plant availability and their developmental stage. Those that have developed properly, possess pigment chlorophyll, show no evidence of damage and have a slightly flexible consistency while avoiding those that are too herbaceous, woody or highly axillary branched were used for the trials. From these stem segments with a pair of healthy leaves and a diameter not less than 2 mm were cut. The basal cut of each cutting was performed obliquely to increase the contact surface with the hormone that permits better distribution of the roots. The upper cut was straight. Additionally, leaves were cut crosswise in half to reduce perspiration and prevent overlap thus avoiding conditions of poor ventilation and excess moisture that allow fungal growth. The cuttings obtained were put in a fungicide solution (Benlate 0.6 g/l) and then, while keeping the clone's identification, hormone treatment was applied and the cuttings were then set in containers inside the greenhouse. Hormonal treatment consisted of applying exogenous auxin to the base of the cuttings, to improve their rhizogenic response. A mixture of indolebutyric acid (IBA) at 5,000 ppm in talcum powder was used for this purpose. The cuttings were inserted into a previously prepared substrate in plastic containers. Containers of 120-130 cc capacity were used and a mixture of peat and perlite (70:30 v/v) and enriched with slow release fertilizer at a rate of 5 kg/m^3 of substrate was added. Cuts on the leaves and apical ends of the cuttings were sealed with latex fungicide. The following clonal trials were established in greenhouses to assess rooting ability of clones.

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Initial trials (1,2,3,4,5)

For these tests the first set of shoots, obtained from the mother plants by pruning, were used. Test 3 includes a mixture of composted bark and sand as substrate, enriched with Basacote 6M (3Kg/m3) and treatment with fungicide at a rate of 50g per bag of substrate. In test 4 a hormonal treatment with IBA liquid preparation was used at the rate of 8.000 ppm. In test 5 a hormonal treatment with IBA liquid preparation was used at the rate of 2.000 ppm. Three different substrates were used; pure composted bark; pure perlite; and mixture of peat and perlite. Containers trays "teku" with six 200cc cavities were used.

Standard trials (6,7,8,9)

For these trials the second crop of shoots obtained from the mother plants were used. Clones were used that produced a large enough number of cuttings to establish an experiment with a proper statistical design and balanced structure. A randomized block design was used with three test blocks, each of which contained clones represented by 20 to 24 cuttings per clone per block. In these cases ANOVA and a multiple comparison test using Tuckey ($\alpha = 95\%$) were used. Trials No. 6, 7, 8 and 9 used IBA in talc at a rate of 8.000 ppm. During the preparation of the cuttings they were put in a bucket with water, not in fungicide solution. Fungicide was applied by spraying after the cuttings were set in containers with substrate.

Additional Trials (10,11)

Analogously to the standard trials surplus cuttings not used for the above standard tests were employed. Trials N°10 and 11 differ in the origin of the mother plants from which the cuttings were obtained. We used cuttings from mother plants in "tubetes" (plastic containers) that had already provided a crop of less developed, more succulent cuttings. These mother plants had not been previously harvested. Additionally, in test No. 11 IBA in talc was used at a rate of 5.000 ppm for all the cuttings. The substrate used from mother plants in tubetes was a mixture of perlite and peat at a volume ratio of 1: 1.

Mini Cutting Trials (12)

For that trial we used cuttings smaller (3 to 5 cm long) than usually used, taken from the smaller shoots of mother plants. These were cut with surgical scissors and stuck in thermoformed plastic trays of 130 cc capacity, with cavities 5 cm deep filled with a perlite and peat substrate (1: 3) that was previously sterilized in an autoclave. Three forms of hormone treatment were evaluated: IBA in talc (5,000 ppm); IBA in liquid (5000 and 8,000 ppm). The number of cutting per clone varied between 10 and 80 cuttings per clone and treatment, depending on availability.

Hydroponic culture (13)

It is a methodological variation of rooting method, where the traditional cuttings are replaced by small and succulent buds that are not suitable for the making of conventional cuttings or mini cuttings. These new propagules were placed in plastic trays without solid substrate, only with water. The effect on the rooting rate of various cutting types (apical internodes without leaves; and internodes with cut leaves), dose of hormone (0, 2,000, 4,000 and 8,000 ppm of IBA in liquid solution; 5,000 ppm of IBA in talc powder) and the effect of frequency of water renewal in the trays (no renewals; weekly renewals), were evaluated.

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Combined hydroponic culture (14)

Hydroponics were combined with techniques used in the conventional trials. Mother plants were used as a source of cuttings in pots and plants growing in tubes, to which previously were applied two fertilization treatments. The first was weekly application of the fertilizer Ultrasol 6/13/40 at a concentration of 2 g/l and the second was the use of a general formulation enriched with Ca, which was also applied weekly. Cuttings 6-10 cm in size were prepared, and kept in trays with hydroponics for 12 to about 14 days, IBA was applied at a concentration of 8,000 ppm in talc powder. They were then transplanted to 120 cc capacity tubes with a mixture of peat and perlite (1:1 v/v). During the rooting process the cuttings remained under normal glasshouse conditions, without applying sprinkler irrigation (**Fig. 2**).

Figure 2. View of rooting cuttings of *E. globulus* trials at greenhouses.

Combined Mini cuttings (15)

This trial took place under the same conditions described in the above test, but smaller (3-5 cm long) cuttings were transplanted into thermoformed plastic trays with 242 cc cavities and a 15 cc of substrate per cavity. IBA was applied in liquid form at a concentration of 1,000 ppm.

Variation Hydroponic culture (16)

This test was a variation of the hydroponics technique. Cuttings 6-10 cm in size were prepared and place in a mixture of 50% peat, 30% vermiculite and 20% perlite. The cuttings were placed in polystyrene tray seedbeds with 244 cc cavities with 25 ml substrate per well. During rooting of the cuttings they remained in a pool of hydroponics, filled with water up to 10 cm in height, to which was added the fertilizer Basacote 3M, in a dose of 20 g / l. No plant hormones were used. The cuttings were kept under greenhouse conditions

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for about 10 weeks, when the rooting rate was recorded. During this period they were kept at high relative humidity (above 70%) and an ambient temperature of 22 to 24 ° C. To avoid dehydration frequent short duration irrigation by sprinkler was used. They were sprayed with foliar fertilizer and preventive doses of fungicides. Dead leaves and dead cuttings were constantly removed.

Organogenesis

Thirty clones of adult trees of *E. globulus* were selected in dry areas called "secano" for *in vitro* culture during the spring season. Epicormic shoots were used as initial explants and these were obtained through flushing of buds on small pieces of branches collected in the field (**Fig. 3**).

Figure 3. Disinfected pieces of branches, kept in heated room (top left); epicormic shoots (top right); disinfection of shoots and culture (bottom left); starting *in vitro* cultures (bottom right).

Multiplication and elongation of buds

Nutrient medium described by Oller et al. (2004) was used for shoot multiplication. This consists of mineral salts of MS medium (Murashige and Skoog, 1962) complemented with 0.1 mg l^{-1} thiamine, 0.1mg l^{-1} pyridoxine 0.5 mg l^{-1}, nicotinic acid, 100 mg l^{-1}, m-inositol, 7 g/l agar, 2% (wt/vol) sucrose, 0.225 mg l^{-1} BAP and 0.018 mg l^{-1} NAA. The cultures were switched to fresh nutrient medium every 20-40 days and kept in a growth chamber at 22 ± 2°C with 16 hours photoperiod, using fluorescent lamps (57 mol m^{-2} s^{-1} PAR). At this stage, the explants were cultured in glass bottles of 500 ml capacity. The pH of the culture media was adjusted at 5.7 ± 0.05 before autoclaving at 121 °C and 0.1Mpa for 20 minutes.

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Shoot rooting

Elongated explants (2 to 3 cm long) with green leaves and without signs of vitrification were used; for poorly responding clones shorter and lower quality buds were also used. The basal leaves of explants were removed to prevent rooting of the leaf. The pH of the culture media was adjusted at 5.7 ± 0.05 before autoclaving at 121 ° C and 0.1Mpa for 20 minutes. Individual bottles were used in all phases for rooting. Explants were exposed to high doses of auxin (induction phase) and subsequently transferred to a hormone free nutrient medium (expression phase) where formation of adventitious roots occurs. For induction MS medium (Murashige and Skoog 1962) was used with mineral salts reduced to half and supplemented with White vitamins, supplemented with 0.25% (wt/vol) Gelrite, 1.5 % (wt/vol) sucrose and 3 mg l^{-1} IBA. During the expression phase MS nutrient medium free of hormones was used with mineral salts halved and ammonium nitrate was replaced by calcium nitrate (150 mg l^{-1}). The medium was supplemented with 0.1 mg l^{-1} thiamine, 0.1 mg l^{-1} pyridoxine 0.5 mg l^{-1} nicotinic acid, 100 mg l^{-1} m-inositol, 0.7 % (wt/vol) agar and 1.5 % (wt/vol) sucrose. The shoots remained between 1-4 days in the induction medium, depending on the clone, and then were transferred to expression medium. During rooting the explants were subjected to an initial period of 10 days of darkness, then were provided with a light photoperiod of 16/24 hours to complete the rooting phase. When a mild or moderate apical necrosis occurred during rooting, the explants were transferred to a culture medium called EI' to allow the formation of lateral buds and promote the growth of a new stem and root system. This medium was also used in the case of a low rooting rate. The EI' medium contained mineral salts of MS, 0.1 mg l^{-1} thiamine, 0.1 mg l^{-1} pyridoxine, 100 mg l^{-1} nicotinic acid, 100 mg l^{-1} myo-inositol, 0.1 mg l^{-1} calcium pantothenate, 2 mg l^{-1} glycine, 0.6 % (wt/vol) agar, 3.0 % (w/v) sucrose and 0.2 % (w/v) of activated charcoal.

Acclimation and growth of plantlets

Once rooted explants reached more than 2.5 cm in length and showed no apical necrosis. They were subsequently grown in hydroponics **(Fig. 4),** according to a methodology developed by Fair and Clapa (2009).

Figure 4. Climate Control of explants in hydroponics according to methodology proposed by Fira and Clapa (2009)

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Poorly elongated explants were transferred to sterile boxes containing a substrate composed of peat and perlite (1:3). MS liquid medium without sucrose and macronutrients was added. After further plant development they were transferred to hydroponics for acclimation. During the acclimatization process, the plants remained during the first four weeks at the hatchery, under the environmental conditions described for shoot multiplication. Then they were taken to a greenhouse where they were transplanted to 120 cc capacity containers with peat and perlite (1: 3) and the fertilizer Basacote ® Plus 3M (08/16/12 Relationship NPK plus trace elements) at a dose of 5 kg / m^3. Plantlets were placed for two weeks in a mini-greenhouse and provided with a relative humidity of 70%, through a system of nebulizers especially installed for this purpose. When they reached over 10 cm in length they were transplanted into 2,000 cm^3 bags (pots) with composted pine bark and the granular fertilizer Basacote 3M ® Plus (3 kg / m^3) and exposed to normal ambient conditions.

Traceability of drought-hardy genotypes

Samples of 54 *E. globulus* clones were analyzed. The samples were field material (n = 12), nursery stock (n = 17) and material cultured *in vitro* (n = 25). There were 6 samples of which it was not possible to obtain DNA or this was of insufficient quality for testing for SSR markers. DNA extraction was performed using the methodology described by Lodhi et al. (1994) with some modifications as described in previous reports. An amount of 0.1 g of plant tissue was ground in an automatic mortar for 2-3 min in the presence of 10 mg of PVP, using one or two stainless steel balls and 700 µl of extraction buffer (20 mM EDTA, 100 mM TRIS-HCl, 1.4M NaCl, 2% CTAB, 0.2% 2-mercaptoethanol, adjusted to pH 8.0). It was incubated at 60°C for 25 min, allowed to cool to room temperature and 0.6 ml of chloroform-isoamyl alcohol (24: 1) were added, mixed gently by inversion. This mixture was centrifuged at 8,000 rpm for 15 min in an Eppendorf microfuge; the supernatant was removed and transferred to another tube containing 0.5 volumes of 5 M NaCl and 2 volumes of pure ethanol. After mixing it was allowed to stand for 30 min at 4°C, centrifuged at 3,000 rpm for 3 min and then at 6,000 rpm for 3 min. The DNA pellet was washed with ethanol twice 76th and allowed to dry at room temperature. The pellet is then re-suspended in 100 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and Rnase (1 ul of a solution 10 mg / ml) was incubated at 37ºC for 30 minutes at 37ºC and stored in a refrigerator (for analysis in the following days) or freezer (for longer periods). This DNA was checked on agarose gels 0,8% to estimate the integrity and quantity, that is then measured by spectrophotometric absorption at 260 nm. For analysis of SSR 10 combinations of primers for amplification of the respective loci were used that had been obtained from Steane et al. (2001) (EMCRC series) and Brondani et al. (2002) (EMBRA series). The sequences of these primers were described in the earlier stages of this project. Similarly, PCR reactions and separation of the alleles of each marker was performed as was previously described, using polyacrylamide gel electrophoresis and silver staining.

Morphogenesis and genetic transformation studies

Induction of embryogenesis and organogenesis

Open-pollinated mature seeds and adventitious buds established *in vitro* from epicormic shoots of clones 82 and 33 of *E. globulus* from INFOR's breeding program were used as the source of explants for embryogenesis or organogenesis and transformation studies. Explants of seeds were surface-sterilized following the modified methods described by Pinto et al. (2002) and Nugent et al. (2001). Sterilized seeds and adventitious buds were cultured on three media: MS (Murashige and Skoog 1962), B5 (Gamborg et al. 1968), and modified DKW (Driver and Kuniyuki 1984) supplemented with different concentrations (0, 0.1, 3.0 and 10 mg l^{-1}) and combinations of growth regulator (NAA, IAA and Kinetin). A total of 150 explants were cultured for 3 weeks at 24±1°C.

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Strain and construct

Agrobacterium tumefaciens strain EHA 105 containing the binary plasmid pBIN m-gfp5-ER was used in the transformation experiments. The plasmid pBIN m-gfp5-ER carries the green fluorescent protein (GFP) gene and neomycin phosphotransferase (NPTII) gene. The *NPTII* gene conferring kanamycin resistance driven by the nopaline synthase promoter and the GFP reporter gene under the control of CaMV 35S promoter. *Agrobacterium* was prepared as described by Sambrook et al. (1989).

Transformation and regeneration of explants

The protocol was adapted from Ho et al. (1998) and Li et al. (2001). A total of 100 mature zygotic embryos were immersed in diluted bacterial suspension for 5-12 min, and co-cultivated at 25°C for 48 d in the dark on different solid media (MS, B5 and modified DKW). After incubation, the explants were transferred to callus induction and embryogenesis formation medium supplemented with 200 mg l^{-1} each carbenicillin and cefotaxime and 5 - 40 mg l^{-1} kanamycin sulfate for selecting transformed cells. The explants were transferred to fresh media every 3 weeks. Genomic DNA was isolated from putative transformants and wild type plants, using the method described by Steemkamp et al. (1994). PCR products were electrophoresed in agarose gel, stained with ethidium bromide and visualized under ultraviolet light. The expression of GFP was observed in callus tissue using a Zeiss Universal microscope equipped with epi-fluorescence illuminator and GFP filter system. Plantlets derived from somatic embryos were then transplanted to pots containing a mixture of vermiculite, peat, and perlite (2:2:1 in volume) and maintained in an isolated greenhouse. Plantlets were initially covered and gradually exposed to ambient humidity over a 3-week period.

Results

Open and control pollinated trials for drought hardiness selection

Growth parameters of *E. globulus* were obtained from the 24 control-pollinated families and 61 open-pollinated families included in these trials at age 5 to 6 years (**Tab. 3**).

Family performance was also compared to that of plants obtained from commercial *E. globulus* seed sources and seed lots of the drought tolerant species *E. camaldulensis* and *E. cladocalyx*. Whilst there were no significant differences between the average growth rates of the *E. globulus* plants obtained from seed sources at any of the three sites, there were large, significant differences among families within seed sources for average growth in the dry secano environment. In general, the top ranked groups of families were dominated by those having as parents plus-trees that had been selected for superior growth (and form) from the Secano plantations (Rojas Vergara et al. 2015).

Macropropagation

Rooting percentages as their variability among clones of *E. globulus* drought tolerant clones are similar to those obtained in other rooting experiments of this species, which is known for its low rhizogenic capacity, with large variability due to genotypic control (**Tab. 4**). The low rhizogenic capacity of *E. globulus* has been noted by several authors cited by Awad (1997). England and Borralho (1995) recognized the advantages associated with the use of rooted cuttings but noted that their use is limited precisely because of the difficulty to root *E. globulus* and they suggested, therefore, that intense selection based on rhizogenic ability is needed.

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Table 3. Growth parameters of *E. globulus* genetic trials.

Genetic source	Means by individual trial sites											
	La Posada (5 yr)				San Vicente (6 yr)				El Mirador (5 yr)			
	Survival (%)	H (m)	DBH (cm)	Index	Survival (%)	H (m)	DBH (cm)	Index	Survival (%)	H (m)	DBH (cm)	Index
Trial means	88.3	7.21	7.87		91.7	10.12	11.50		63.2	9.67	10.74	
Means – best 10 families[b]	88.0	8.13	9.08	0.246	90.0	11.67	13.17	0.408	65.5	10.64	11.79	0.002
Means – best 5 families[b]	88.0	8.30	9.46	0.318	88.3	12.09	13.22	0.503	68.3	10.98	12.23	0.013

Source	No. of families in top 5	No. of families in top 10	No. of families in top 5	No. of families in top 10	No. of families in top 5	No. of families in top 10
CSO families (OP)	1	2	2	5	1	2
Selected Chilean landrace families (OP)	4	8	3	5	–	–
E. globulus intraspecific hybrid families (CP)	–[a]	–	–	–	4	7
E. globulus seed orchard bulk	–	–	–	–	0	1[b]

[a] The "–" indicates no genetic material from the group included at this site.

[b] This is not actually a 'family'. This entry represents a bulked seedlot comprising seeds of many OP seed orchard families.

Organogenesis

The multiplication medium EC (**Fig. 5**) efficiently promoted shoot proliferation. While the multiplication rate is high and the shoots produced are vigorous and deep green in color, the rate of formation of elongated shoots is low. Commonly shoots with short stems and abnormal leaves are formed that are not suitable for rooting to increase the number of explants that could be rooted requires the use of nutrient media that promote shoot elongation. However, such media do not allow the multiplication of crops. Therefore, it is necessary to use the basal multiplication medium for reinvigorating the explants.

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Table 4. Results of rooting trials of drought tolerant *E. globulus* clones.

Type of trial	Trial number	Number of clones	Number of cuttings	Average (% rooting)
Initial	1	6	135	4.0
	2	7	57	7.0
	3	7	316	7.9
	4	7	212	44.8
	5	7	462	0.9
Standard	6	4	240	1.3
	7	3	650	3.7
	8	7	420	
	9	7	504	0.6
Aditional	10	17	758	5.7
	11	17	2083	12.0
Mini cuttings	12	13	492	13.3
	13	15	1058	9.7
Hydroponic culture	14	14	1272	23.4
	15	18	1157	16.7
Combined Mini cuttings	16	7	756	62.6
TOTAL	**16**	**18**	**10572**	**14.2**

Shoot elongation

To increase the number of rootable shoots requires that explants are grown in a nutrient medium which promotes the formation of elongated stems with normal leaves normal that are free of vitrification. Different media have caused marked differences in clonal elongation depending on the type of hormone used as was also found by Bennett et al. (1994) for *E. globulus in vitro*.

Rooting, acclimatization and plant production

Rooting results depended on previous activities, i.e., during earlier development and multiplication steps. To date, clones 48, 62 and 82 have shown higher percentages of rooting (up to 60%) than clones 57 and 79. These better clones provide the best options for operational propagation via organogenesis (micropropagation) because of their good aptitude for elongation and rooting.

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Figure 5. (a) Growing clone 103 in EC basal medium. (b) Clone 62, elongation medium EO9. (c) Explants from clone 85. (d, e, f) *In vitro* plants acclimated during the growth process.

Morphogenesis and transformation of seeds

Seeds of different eucalyptus clones showed different abilities with respect to induction rates of embryogenic calli after 45 days on Murashige and Skoog medium supplemented with 3 mg l^{-1} NAA. After eight weeks globular structures appeared in the embryogenic calli (**Fig. 6**). Also observed were clusters of embryos at different stages of development as well as some abnormal structures such as fused embryos and embryos with cup-shaped cotyledons (**Fig. 7**). Abnormal development of somatic embryos of *E. globulus* has been observed previously in embryogenic calli derived from mature seeds in the presence of 100 µM IBA (Nugent et al. 2001). In cotyledons of some somatic embryos red pigmentation (anthocyanins) occurred as has also been reported for the species by Pinto et al. (2002). In these embryos a small hypocotyl and an elongated root appeared after a few days and eventually they become seedlings.

Transformation assay of the callus phase showed GFP expression 21 days after infection with *Agrobacterium tumefaciens* (**Fig. 8**). Stable GFP expression in transformed callus of *E. globulus* was visualized under an epifluorecence microscope (40x) after six weeks of treatments. The cultures were exposed for 16 weeks to medium with 40 mg l^{-1} kanamicyn explants to eliminate non-transformants, 16 weeks presumably being long enough to rule out possible leaks as proposed by Ho et al. (1998). Plantlets regenerated from one hundred transformed cultures and that were subsequently multiplied without the selection agent were verified as transformed by PCR analysis. Six plantlets were checked for the presence of the transgene GFP but this was found in only three of them (**Fig. 9**). GFP positive plantlets were acclimatized and transplanted into pots containing sterile soil (organic soil: perlite, **Fig. 10**).

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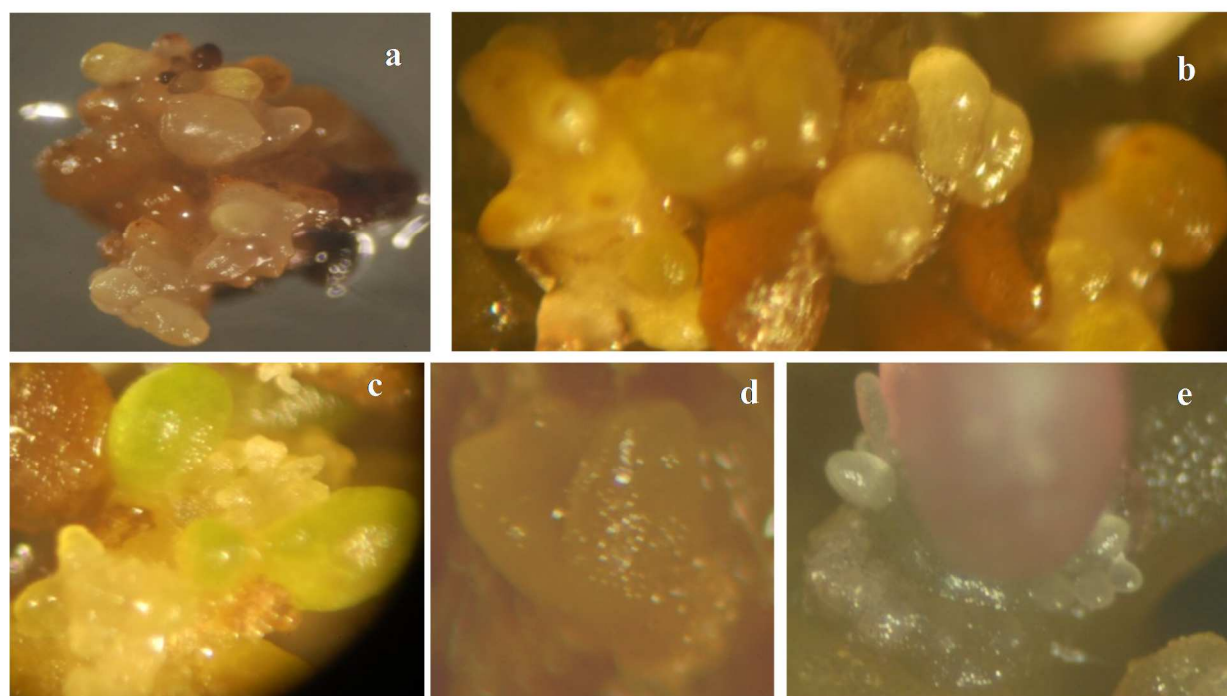
Figure 6. Induction of somatic embryogenesis in explants of *E. globulus* seed. Induction of embryogenic callus with phenolic compounds around the callus (a). Formation of yellowing globular structures (b). Development stages of embryogenic structures (c, d, e).
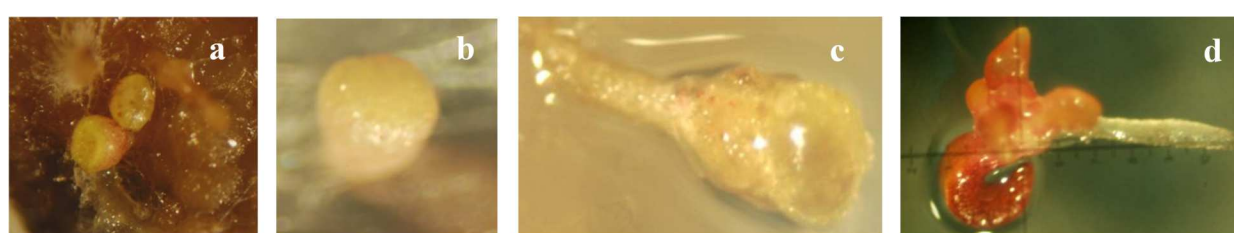
Figure 7. Different stages of somatic embryo development (a, b and c). Formation of embryos with fused cup-shaped cotyledons (d). Conversion of somatic embryo to plant with red pigmentation (anthocyanins) of cotyledons and root elongation was observed.

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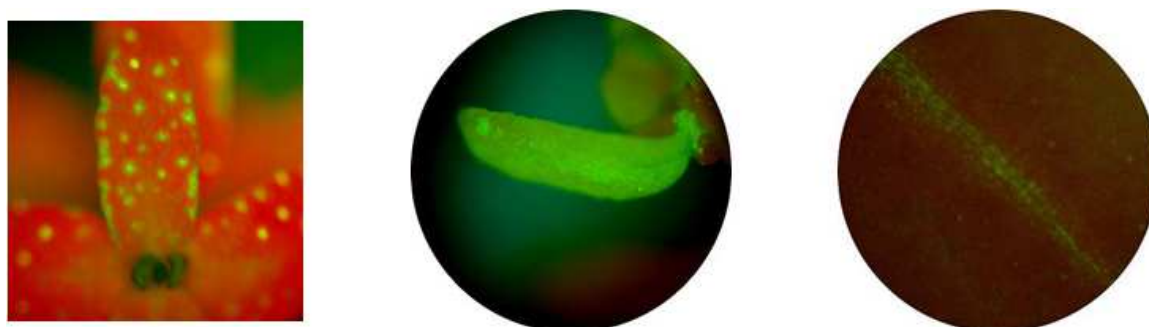
Figure 8. Expression of GFP in *Eucalyptus globulus*. Explants infected with *A. tumefaciens* carrying a construct with the gene encoding green fluorescent protein (GFP) are selected in kanamycin for 21 days and then evaluated for fluorescence emission by epifluorescence microscopy. Red indicates emission of chlorophyll; the green color corresponds to GFP expression.
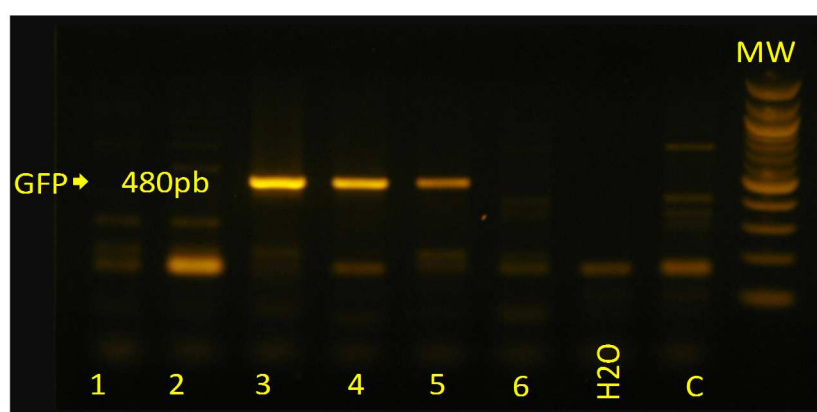
Figure 9. Electrophoresis of PCR products in 1% agarose gel. Plant genomic DNA isolated from young leaf tissues of regenerated somatic embryos in *E. globulus*. Lanes 1-6: plants transformed. The size of PCR-amplified *gfp* fragment was 480 bp. Lane C: negative control

Figure 10. Hardened transgenic PCR-positive plantlet grown in plastic pot

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Morphogenesis and transformation of adventitious buds

Similarly, transformation assays were carried out with adventitious buds, established *in vitro* from epicormic shoots of clones 82 and 33. **Fig. 11** shows the presence of incipient morphogenic structures in some of the explants of clone 82 and adventitious root formation on media containing the selection agent (kanamycin). Transformed callus of clones 82 and 33 was obtained after seven months. Morphogenic structures, possibly embryogenic, appeared in parts of transformed callus of clone 82 (**Fig. 12**). These should be analyzed histologically to determine their origin. Four months after infection with *Agrobacteri*um, bud primordia were evident, as well as adventitious roots in the media containing kanamycin. The developed shoots were transferred to elongation and multiplication medium. Samples were removed from elongated shoots to check by PCR the presence of the transgene. The results indicated that four explants were positive.
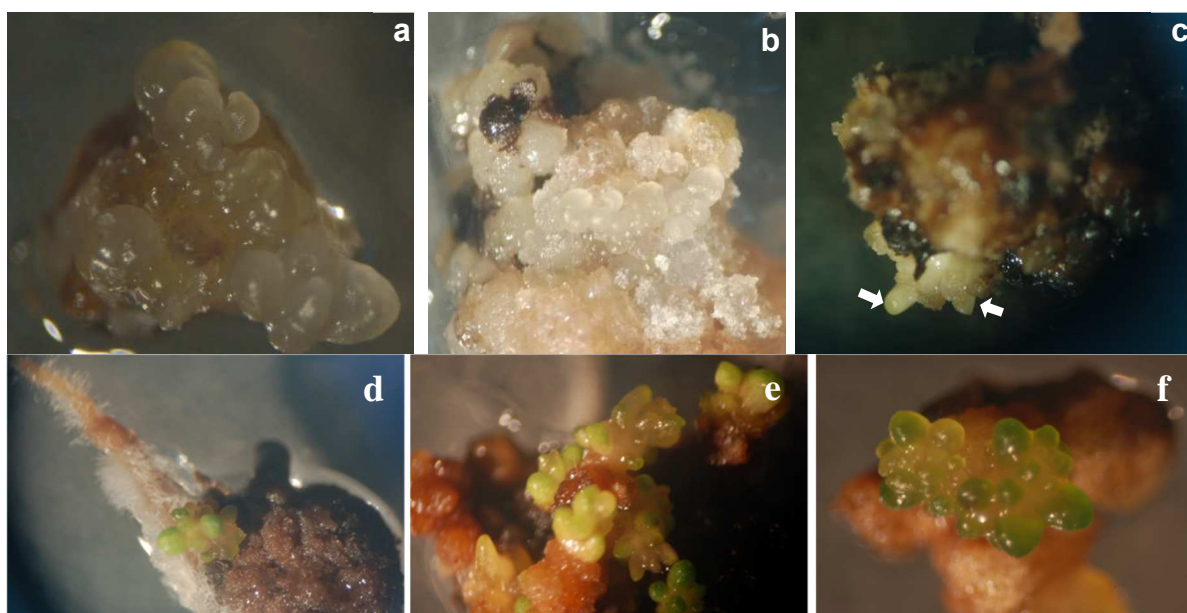
Figure 11. Genetic transformation assays in adventitious shoots established *in vitro* from epicormic buds of clone 82. Nodular morphological structures (a, b). Arrows indicate possible embryogenic structures formed indirectly from cultured explants (c). Formation of bud primordia in transformed explants of *E. globulus* clone 82 with the gene encoding GFP (d, e, f).

Molecular markers: traceabiity of drought-hardy E. globulus clones

All replicate samples from 54 *E. globulus* selected clones showed the same genetic pattern with any of the 10 SSR markers tested. An example of such electrophoretic analysis is shown in **Fig. 13** where the allelic patterns are shown for EMBRA-66 and EMBRA-45 markers. In these tests the replicas were analyzed in triplicate to the left of the gel (each outstanding triad with a green bar), followed by duplicate (each highlighted with a blue bar pair), and finally to the right of each gel samples are individuals in *in vitro* culture. It clearly shows that in each case the genetic pattern is identical in the clone and in its corresponding replicate while it is different from clone to clone.

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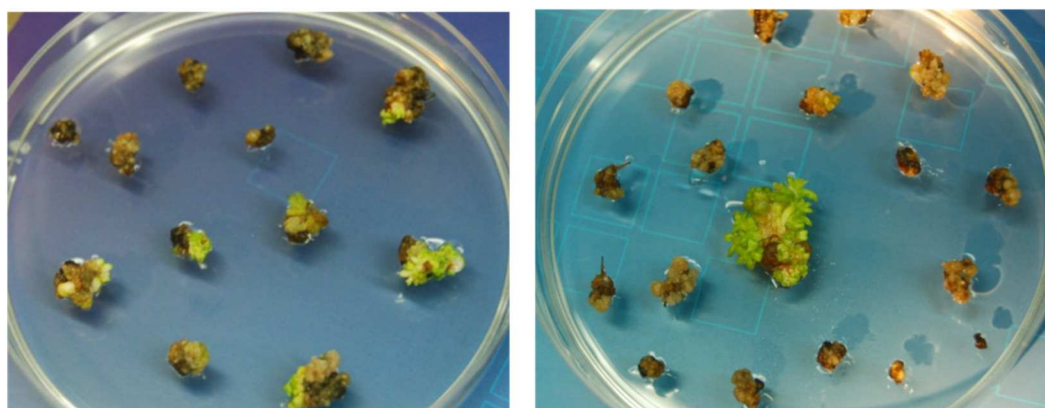
Figure 12. Indirect organogenic induction in transformed adventitious shoots of clone 82
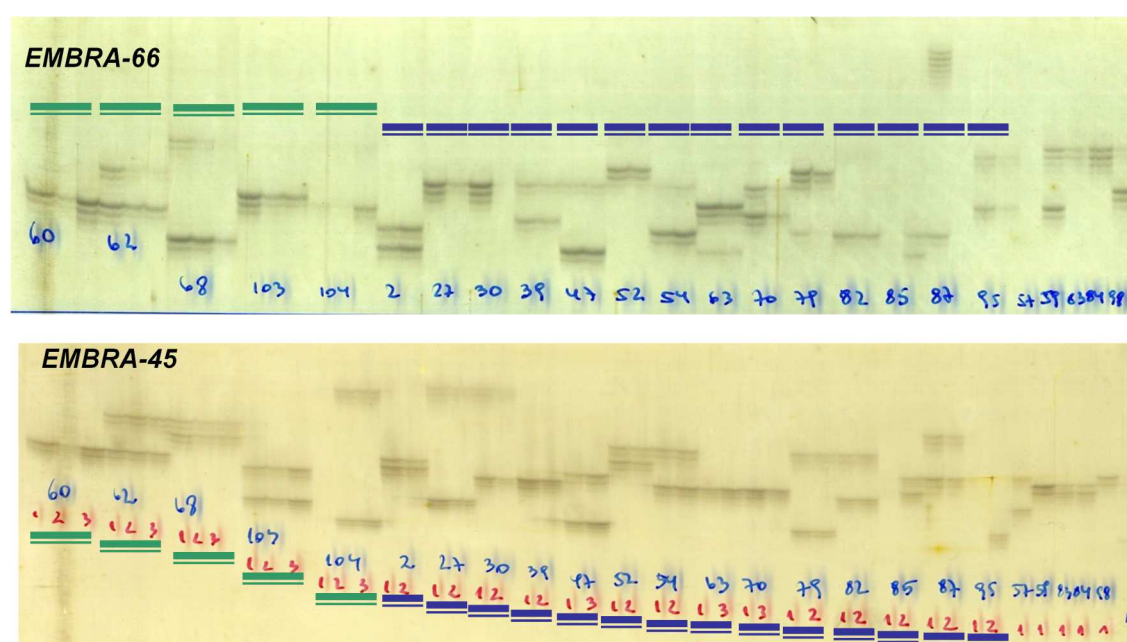
Figure 13. SSR patterns of selected *E. globulus clones using* EMBRA-66 and EMBRA-45 markers.

Discussion

Somatic embryogenesis produces bipolar structures that develop into embryos from cells that are not the product of a gamete fusion (Ammirato 1983). Organogenesis is another morphogenic event that is characterized by the development of structures that have an unipolar connection with the maternal tissue from which it originated (Hicks 1980). However, both morphogenic capabilities are variable and are, especially in woody species, conditioned by genotype, physiological age, environmental conditions, etc. Clonal propagation represents the most effective way of capturing both additive and non-additive genetic effects brought about by both traditional tree breeding and biotechnology, thus accelerating exploration of

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genetic gains in plantations (Mullin and Park 1992). Although industry demands an increase in *Eucalyptus* forest productivity, most species remain in an early stage of domestication. For example, most of the genetic parameters reported in *E. globulus* are based on open-pollinated progenies (López et al. 2002). However, due to inbreeding depression from self and/or related mating, observed genetic parameters derived from open pollinated eucalypt populations may be inaccurate or inflated. Therefore, most of the more recent breeding programs introduced control-pollinated approaches, which allowed more accurate estimations of the genetic parameters and the separation of additive from non-additive genetic effects (Silva et al. 2004).

In our research there were no significant differences between the average growth rates obtained with the *E. globulus* seed sources at any of the three sites. However, there were large, significant differences among families within seed sources for average growth in the dry Secano environment. In general, the top 5 and top 10 ranked groups of families were dominated by those having as parents plus-trees that had been selected for superior growth (and form) from the Secano plantations. The results we obtained with clonal propagation via cuttings are within the range of those published for *E. globulus* (Chaperon 1987) with an average rooting percentage of 14% and a rooting percentage that in some trials reached up to 92%. Note that although there are clones of *E. globulus* capable of 100% rooting, the species generally has a low rhizogenic capacity, the average rooting percentage being no greater than 15%. Indeed, other rooting studies of *E. globulus* (Gutiérrez 2006) obtained results that are similar to those documented by us, confirming the difficulty of the species for rooting. Gutiérrez (2006) evaluated the rooting ability of 1,300 clones of this species in a three year test, obtaining an average of 7% rooting and noted that most of the clones did not root or only at a low rate. However a few individuals (about 2% of the study population) showed a high rhizogenic response, which in some clones reached up to 86%. Similarly, studies by Uach (1997) reporting the rooting capacity of 112 clones of *E. globulus* indicate that 61.6% of the clones did not root; 31.2% of the clones had a low rooting rate ; 4.4% of the clones exhibited a medium rooting rate and only 2.7% showed a high rate. We evaluated drought tolerant cuttings of *E. globulus* that were obtained from micro propagated mother plants and found that micro propagation removed poorly rooting clones because these unable to multiply *in vitro* and thus did not give provide mother plants that could be used for making cuttings to be tested for rooting ability.

In vitro propagation of *E. globulus* was effective for the stages of establishment, multiplication, rooting and acclimatization of the plants. Clones 48, 62 and 82 and to a lesser extent clones 57 and 79, are capable of being propagated via organogenesis because of its good aptitude for elongation and rooting of the shoots. For those clones which showed difficulty in forming elongated shoots further research is needed before an increase in production of suitable shoots for rooting can be expected. This requires, among others, optimization of the duration of exposure to auxin in the root induction phase. In our study we established protocols that allow transformation via *Agrobacterium tumefaciens* and regeneration via indirect somatic and regeneration via organogenesis in two highly productive of *E. globulus* clones. The SSR markers selected in this study are suitable since they could differentiate any of the selections of *Eucalyptus* that were propagated. The protocols applied by the technical staff of INFOR for handling these samples was suitable to identify each selection, thus avoiding confusing the identity of genotypes in any of the cases analyzed.

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Sprout vigor of poplar cuttings from stoolbeds fertilized with nitrogen or phosphorus

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Abstract

Salicaceae stoolbeds produce propagating material for clonal plantations. With the annual shoot harvest, nutrients are exported from the system and in consequence, after successive production cycles, soil fertility can be reduced. Fertilization enables to compensate for extraction of nutrients. However, there is little information if mineral fertilization affects the early sprout of the cuttings. The aim of this study was to evaluate if fertilization with N or P in stoolbeds of two *Populus deltoides* clones -`Australiano 129/60´ and "150-82"- affects the early vigor of the sprout of cuttings. Commercial stoolbeds were divided into plots that received one of the following treatments: fertilization with N, fertilization with P and unfertilized. Cuttings harvested from each treatment were planted and root and sprout production during the first months after planting was measured. Fertilization with N increased the number of roots in `Australiano 129/60´ but increased the shoot: root ratio. Early vigor of "150-82" was not affected by fertilization. If cuttings from fertilized stoolbeds were subject to soil water deficit or flooding, their growth was affected similarly to those coming from unfertilized stoolbeds. Hence, fertilization of stoolbeds has no negative effect in the early vigor of the sprout of poplar cuttings, neither has it on their tolerance to water stress.

Keywords: *Populus deltoides*, mineral nutrition, fertilization

Introduction

Poplars (*Populus* spp) cover 80 million ha in the word, of which 7 million are plantations. Poplars have been extensively cultivated due to their rapid growth, easy vegetative propagation from unrooted cuttings, good re-growth capacity, adaptability to different sites, high plasticity in response to environmental changes and varied uses of wood (paper, timber, pulp, fiber and biomass for bioenergy) (Zsuffa et al. 1996, Balatinecz et al. 2001, Ceulemans 2011). In Argentina, the main area for poplar plantations is the Paraná River Delta where the main economic activity is Salicaceae (poplars and willows) forestry, with a total area for poplar plantations being approximately 15 000 ha (Borodowski 2006).

Poplar plantations are established from 60-100 cm in length un-rooted cuttings. These cuttings are obtained from stoolbeds, which are high density plantations that produce propagating material (cuttings) for clonal plantations. The stumps produce cuttings that are harvested yearly, over a 5-10 year period. Then, they can be replaced by new material (e.g., genetic improvement).

Phosphorus (P) and nitrogen (N) are the two essential macronutrient elements required for plant growth and development. P is a component of numerous metabolites in plants, such as DNA, RNA, phospholipids, adenosine triphosphate (ATP), adenosine diphosphate and nicotinamide adenine dinucleotide phosphate. Similarly, N is an essential component in proteins, nucleic acids, chlorophyll and many secondary metabolites in plants. However, although plants require these macronutrients, the supply of these essential

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nutrients is often limited in the soil (Gan et al. 2015). N and P are the nutrients that most frequently limit the productivity after reforestation and are extracted to a great extent by plants (Allen 1987).

Fertilization is important for sustaining the productivity of intensively managed forestations, since this requires maintaining soil fertility in the long term (O'Connell et al. 2004). The soil of the sites used to produce cuttings is subjected to heavy nutrient extraction, caused by the annual harvest of cuttings. The yield in poplar stoolbeds is about 4 kg of dry matter harvested per m^2 of land (Cortizo et al. 2006). This material contains approximately 40 g of N which are removed each year from the same place. In some stoolbeds the loss of soil fertility is evident, reflected in the lower productivity of the stumps. Fertilizer application can counteract nutrient extraction so that the soil regains fertility, while increasing the productivity of the stoolbed. In Argentina there is little knowledge about the response to fertilization of poplar plantations. The few background studies available have evaluated fertilization in plantations, but not in poplar stoolbeds (Achinelli et al. 2003a,b). High density plantations demand more nutrients so it is expected that stoolbeds also respond to fertilization. However, when stoolbeds of two clones were fertilized either with N or P, only one clone responded to N fertilization, while no response to P was observed (Faustino et al. 2016).

In addition, although accumulated reserves in the cutting do not affect rooting capacity, cuttings derived from stumps with better nutritional status may have greater vigor in root growth and greater energy in budding, since both processes depend directly on the amount of reserves (Cooke et al. 2005). Another aspect to consider when proposing fertilization is that plants with high growth rates generally have morphological and physiological characteristics that could result in lower productivity under stress conditions like drought (Fernández and Reynolds 2000). This commitment between growth potential and stress tolerance is explained by the fact that plants can develop several mechanisms to tolerate or avoid water stress, but these have a cost in terms of productivity (Pita and Prados 2001). Therefore, it is important to evaluate if the cuttings produced in fertilized stoolbeds, which are likely to grow at a high rate, are less tolerant to stressful situations such as flooding and drought occurring in the Paraná Delta region, where poplar plantations are widespread. This region can experience episodes of flooding in areas with no dykes (Luquez et al. 2012), while in the summer there is a low availability of water in the soil in areas with dykes

The objective of this work was to evaluate if fertilization with N or P of poplar stoolbeds, affects the physiological quality of the cuttings obtained in terms of sprouting vigor and survival.

Materials and Methods

In October 2013, a trial was started with commercial stoolbeds of *Populus deltoides* clones `Australiano 129/60´ and "150-82", installed in the year 2011 at the Experimental Station Delta del Paraná belonging to INTA. The site of the stoolbeds corresponds to a field lot that was used for the production of cuttings for more than 15 years, and it is presumed that this intensive use could have caused loss of soil fertility. Stoolbeds of *Populus deltoides* clones were either not fertilized or fertilized with N or P (see Faustino et al. 2016 for more details).

Evaluation of the nutrient concentration and basic wood density of the cuttings

In August 2014, once the growth stage was finished, the concentrations of N and P in stems were determined in 7 commercial cuttings from plots fertilized with N or P or non-fertilized plots. The determination of N concentration was made by the semi-micro Kjeldahl method and the concentration of P by acid digestion with subsequent reading by induced plasma emission spectrometry. Wood basic density was determined from the ratio of the anhydrous weight (stove drying at 100 ± 5 °C) and the water saturated volume in 2

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pieces extracted from the 20 to 25 cm basal end of each cutting. To obtain the 2 pieces, each cutting was split in two and the bark and pith were removed.

During the years 2014 and 2015, three experiments were carried out using cuttings from the fertilized with N or P, and non-fertilized plots of the stoolbeds. The aim of this set of experiments was to evaluate if there are differences in initial sprouting, growth and tolerance to water stress between cuttings from non-fertilized and fertilized modalities with the different mineral nutrients.

Evaluation of the physiological quality of cuttings harvested from fertilized stoolbeds

Early vigor

In August 2014, 1-year-old cuttings of 60 cm long were obtained from both stoolbeds and planted in 15-L pots filled with soil. One cutting per pot was planted and the pots were placed outdoors near the stoolbeds site, in a completely randomized design, with 30 replicates for each clone and treatment. Treatments were cuttings from nitrogen and phosphorus fertilized plants and unfertilized stumps for both clones.

One month after plantation, four samples spaced in time were taken to analyze the progress of sprouting and growth of cuttings. Six individuals per treatment were analyzed in each sample. In each sample the number and length of sprouts, dry weight and total leaf area, number and total dry weight of roots were determined.

Growth under field conditions

In July 2015, 1-year-old cuttings of 1 m long were obtained from the "150-82" stoolbed and planted in the field. Twelve plots of 36 plants each were planted with cuttings from the three treatments (N fertilization, P fertilization and unfertilized) with four replications. Plants were separated 2 m from each other.

In March 2016 different growth parameters were measured: number of shoots and diameter and height of the dominant shoot in every plant per plot; and dry weight of shoots, cuttings and leaves in six plants per plot.

Response of cuttings harvested from fertilized stoolbeds to water stress

At the end of August 2014, 1-year-old cuttings of 1 m long were obtained from the "150-82" stoolbed and planted in the 100-L pots filled with a mix of soil and sand (20:80). Five months later, eight cuttings of each treatment (N fertilization, P fertilization and unfertilized) where subjected to three levels of soil water content, well watered (WW), flooding (F) and drought (D), for five months. During these months, the development of leaves was followed (number).

Statistical analysis

Results were analyzed by factorial ANOVA, considering clone and fertilization as factors. In the experiment in which different water availability was evaluated, the factors were fertilization and water treatment. When ANOVA was significant ($p < 0.05$), means were compared by Duncan test ($\alpha = 0.05$).

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Results

`Australiano 129/60´ cuttings had a higher basic density than "150-82" cuttings in every treatment. Fertilization with N reduced wood density of the cuttings in both *Populus deltoides* clones compared to their respective controls, whereas the application of P did not modify this variable (**Fig. 1**).
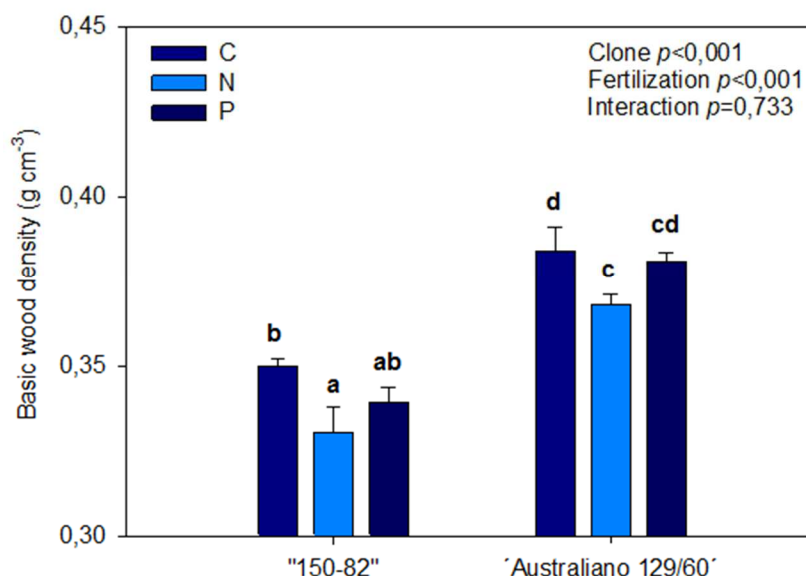
Figure 1. Basic wood density for "150-82" and `Australiano 129/60´ poplar clones, unfertilized (C) and fertilized with nitrogen (N) or phosphorus (P). Vertical lines on the bars correspond to the standard error of the mean. Means with the same letter do not differ significantly (p < 0.05).

N concentration of the cuttings was not affected by fertilization with N or P in neither of the two clones (**Fig. 2a**). However, P concentration was significantly reduced when stump were fertilized with N compared to non-fertilized and fertilized stumps with P, for both clones (**Fig. 2b**).

A few days after planting, the number of root insertions was significantly higher in cuttings fertilized with N in the stoolbed with clone `Australiano 129/60´, while "150-82" was not affected (**Fig. 3a**). This difference disappeared as the days went by.

By the end of the experiment the individual root dry mass in N or P fertilized cuttings of the `Australiano 129/60´ clone diminished in comparison to the levels observed in cuttings from non-fertilized stoolbeds. However, N fertilization increased individual root dry mass in clone "150-82" in comparison with that found after P fertilization or non-fertilization (**Fig. 3b**). As a consequence, total root dry mass was reduced by stoolbed fertilization with N or P in the clone `Australiano 129/60´, while it increased slightly in the other clone (**Fig. 3c**).

`Australiano 129/60´ had more shoot vigor than clone "150-82" in non-fertilized conditions. However in clone `Australiano 129/60´, the number of shoots and total shoot length decreased with fertilization (**Fig. 4**). Nevertheless, by the end of the experiment total leaf area (**Fig. 5**) and total dry mass (**Fig. 6**) were not affected by N or P fertilization in the stoolbed in any clone.

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Figure 2. N (a) and P (b) concentration for cutting of "150-82" and
`Australiano 129/60´ poplar clones, unfertilized (C) and fertilized with
nitrogen (N) or phosphorus (P). Vertical lines on the bars correspond to the
standard error of the mean. Means with the same letter do not differ
significantly (p <0.05).

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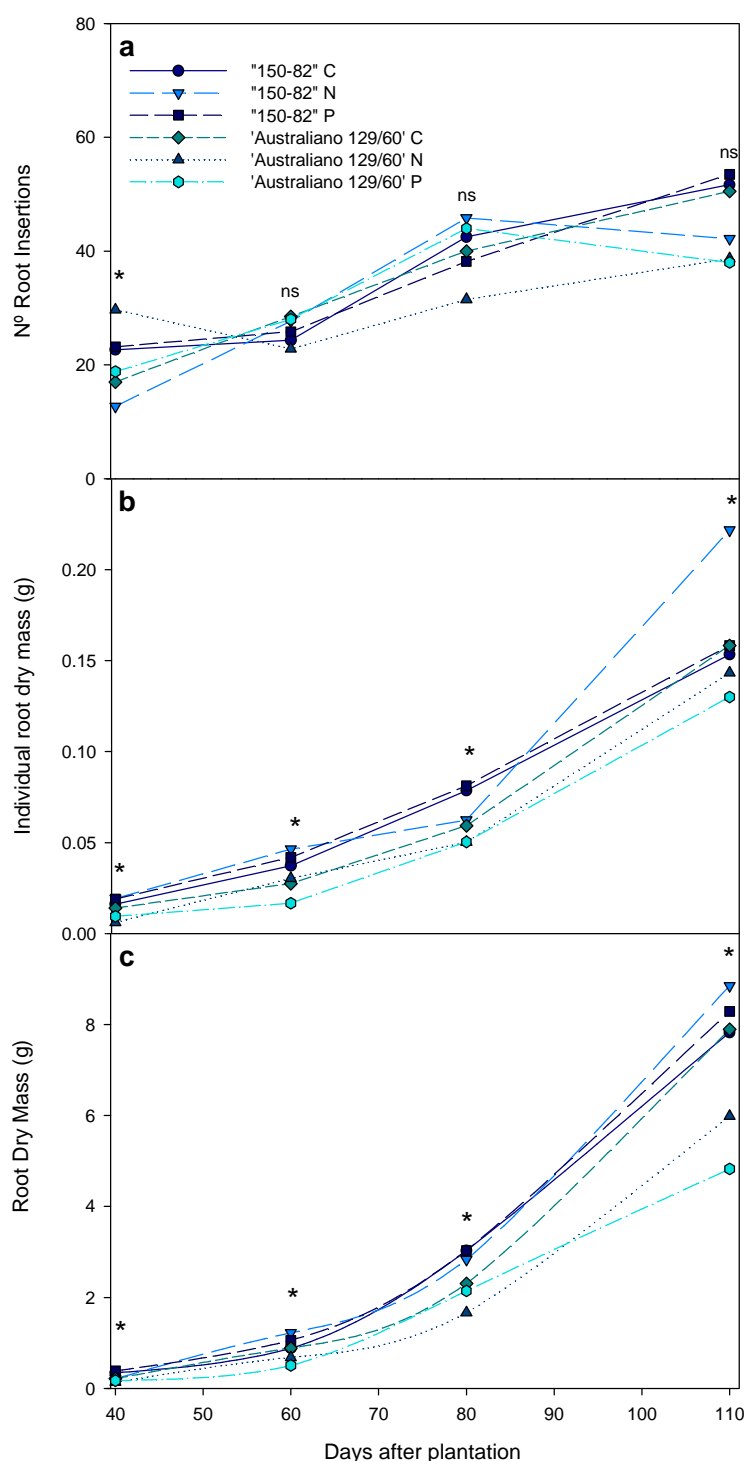
Figure 3. Root traits of cuttings from "150-82" and `Australiano 129/60´ poplar clones, unfertilized (C) and fertilized with nitrogen (N) or phosphorus (P). (a) Number of root insertions per cutting. (b) Individual root dry mass. (c) Total root dry mass. The asterisks indicate statistically significant differences between treatments for each date.

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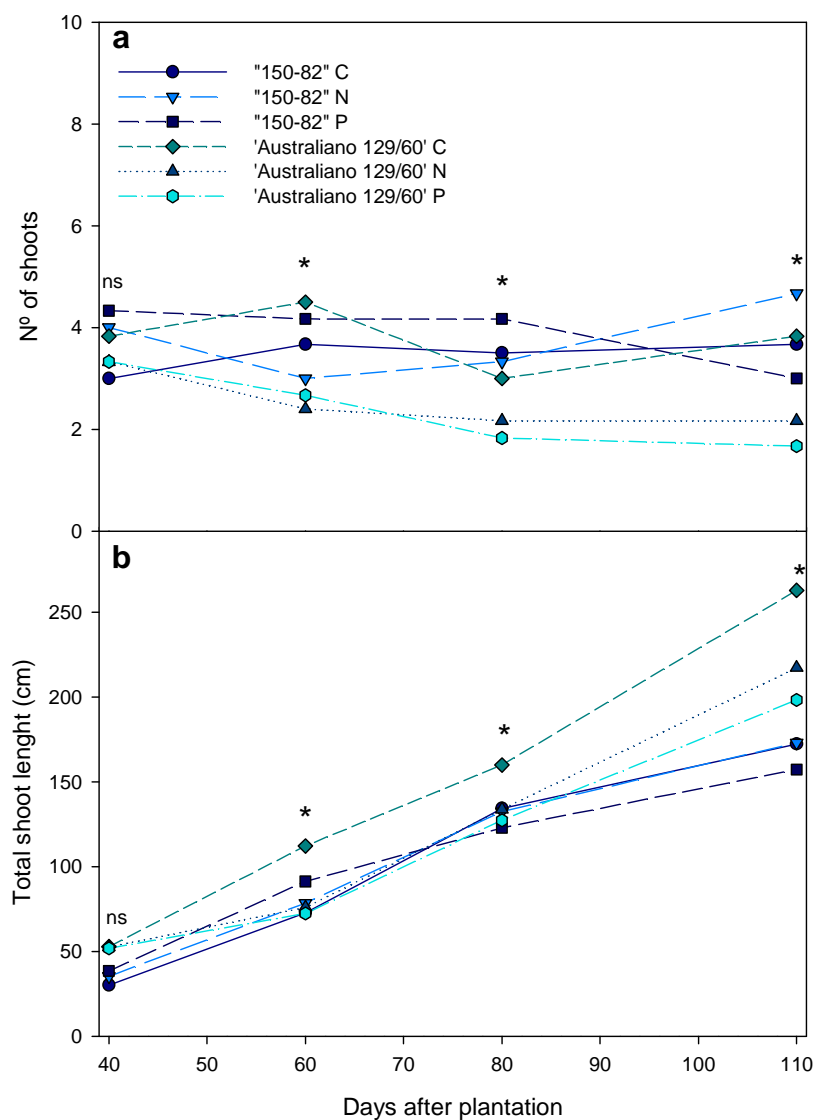
Figure 4. Shoot traits of cuttings from "150-82" and `Australiano 129/60´ poplar clones, unfertilized (C) and fertilized with nitrogen (N) or phosphorus (P). (a) Number of shoots of the cutting. (b) Total shoot lengtht. The asterisks indicate statistically significant differences between treatments for each date.

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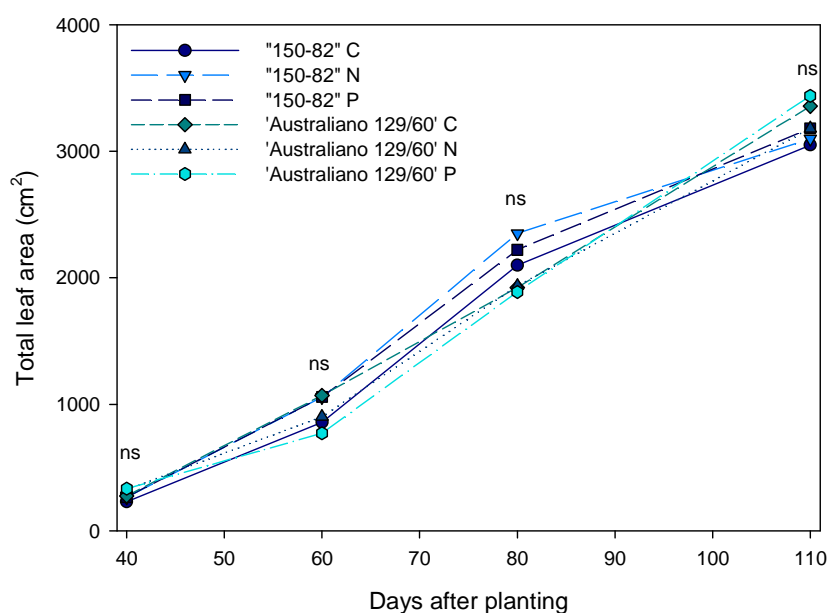
Figure 5. Total leaf area of cuttings from "150-82" and `Australiano 129/60´ poplar clones, unfertilized (C) and fertilized with nitrogen (N) and phosphorus (P).
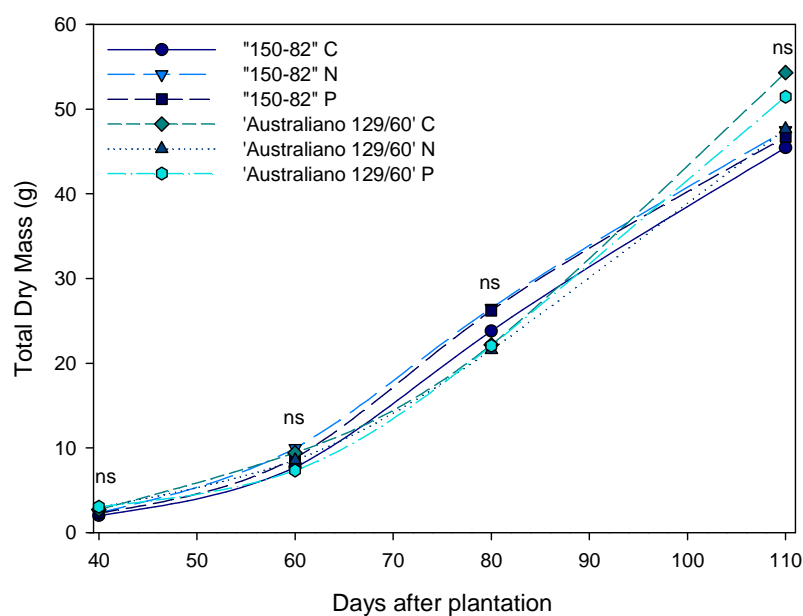
Figure 6. Total dry mass of cuttings from "150-82" and `Australiano 129/60´ poplar clones, unfertilized (C) and fertilized with nitrogen (N) and phosphorus (P).

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When cuttings obtained from non-fertilized and N and P fertilized stumps where planted in the field, no differences in growth where found. However, the number of shoots was reduced in cuttings of stumps fertilized with N, but this was not reflected in the dry weight of the aboveground biomass (**Tab. 1**).

Table 1. Growth traits of field-grown cuttings from "150-82" poplar clone unfertilized (C) and fertilized with nitrogen (N) and phosphorus (P) stumps. Means with the same letter do not differ significantly (p <0.05) for each trait.

Fertilization treatment	Height (cm)	Diameter (mm)	Number of shoots	Leaves dry weight (g)	Shoots dry weight (g)	Cuttings dry weight (g)
C	122,2 a	11,0 a	2,5 b	44,4 a	28,8 a	63,2 a
N	115,9 a	10,8 a	2,2 a	46,5 a	31,9 a	62,1 a
P	111,5 a	10,6 a	2,5 b	44,1 a	29,9 a	54,4 a

The next question was if drought or flooding can affect plants grown from cuttings harvested in fertilized stoolbeds. Cutting from stoolbeds fertilized with P had more leaves than non-fertilized plants, under well-watered conditions (**Fig. 7**). Drought reduced the number of leaves in cuttings grown in unfertilized stoolbeds. Flooding had little effect. Cuttings from stoolbeds fertilized with P were more defoliated in response to flooding. Those from N-fertilized stoolbeds tolerated flooding better than those from P-fertilized stoolbeds. Cuttings from stoolbeds fertilized with N produced a lower number of leaves since the beginning but also were more affected by drought.
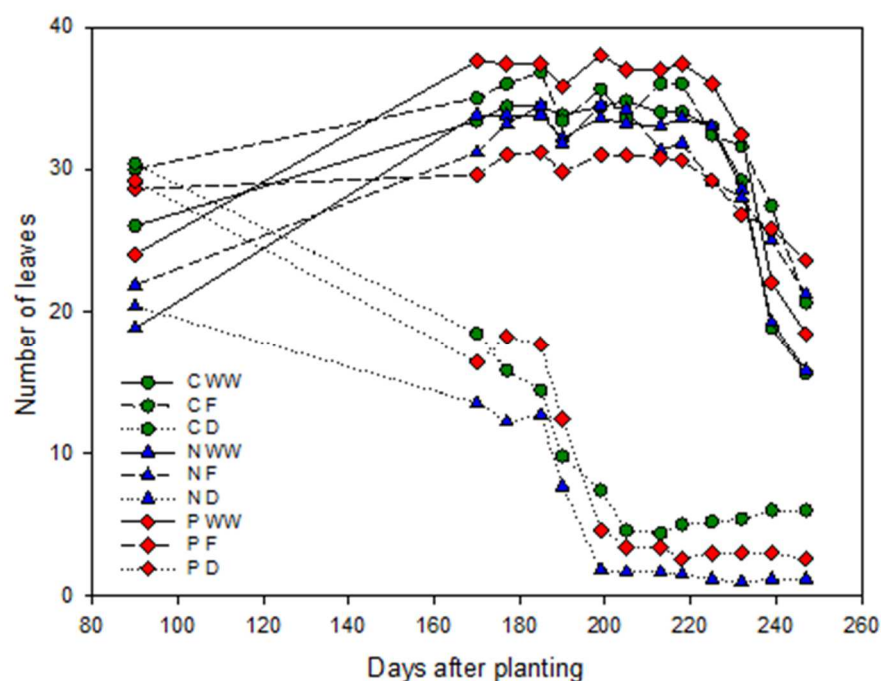
Figure 7. Number of leaves of cuttings from "150-82" poplar clone, unfertilized (C) and fertilized with nitrogen (N) and phosphorus (P), subjected to three level of water: Well watered (WW), Flooding (F) and Drought (D).

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Discussion

As fertilization affects wood density and has some impact in nutrient concentrations in the xylem, the question is: does mineral fertilization of the stoolbeds affect physiological quality of the cuttings?

The *physiological quality* of a cutting is the capacity to sprout and make a new plant. In the first experiment we evaluated the effect of fertilization with N or P in stoolbeds of two clones of *Populus deltoides*: "150-82" and `Australiano 129/60´. N fertilization reduced wood density (**Fig. 1**) and P concentration (**Fig. 2**) in wood of both clones. In general, lower wood density implies thinner cell walls in the xylem, lower reserves in the wood and/or wider vessel diameter. A general trend is that fast growing trees have lower wood density (Zanne et al. 2010). By contrast, any environmental factor that decreases the growth rate tends to increase wood density. Therefore, if fertilization increases growth rate, it was expected to decrease wood density (Pitre et al. 2007). N fertilization reduced wood density in Poplar clones (Hacke et al. 2010) and others latifoliated species (Bucci et al. 2006; Villagra et al. 2013). In general, wider vessels are related to lower water stress resistance, because the risk of embolism increases in wider conduits.

N fertilization increased growth in the stoolbed (Faustino et al. 2016). Under this condition, P was the limiting nutrient as is reflected in a diminished P concentration (**Fig. 2**). This modification, resulting from N fertilization can compromise the physiological quality of cuttings, because the reserve of nutrients to sustain bud-break is lower. Therefore, in a second step we showed the performance of cuttings from fertilized stoolbeds. The early vigor was measured in two experiments, in pots and in the field, conducted under well-watered conditions.

In the pot experiment N and P fertilization in the stoolbeds produced modifications in the early vigor of the cuttings. The responses were different between clones. `Australiano 129/60´ had less vigorous roots but more vigorous shoots than "150-82" (**Fig. 3** and **4**). However, in the first clone, fertilization resulted in decreased number of shoots and total shoot length. The same effect was also obtainbed for the roots. Despite these differences, total leaf area (**Fig. 5**) and total dry mass (**Fig. 6**) were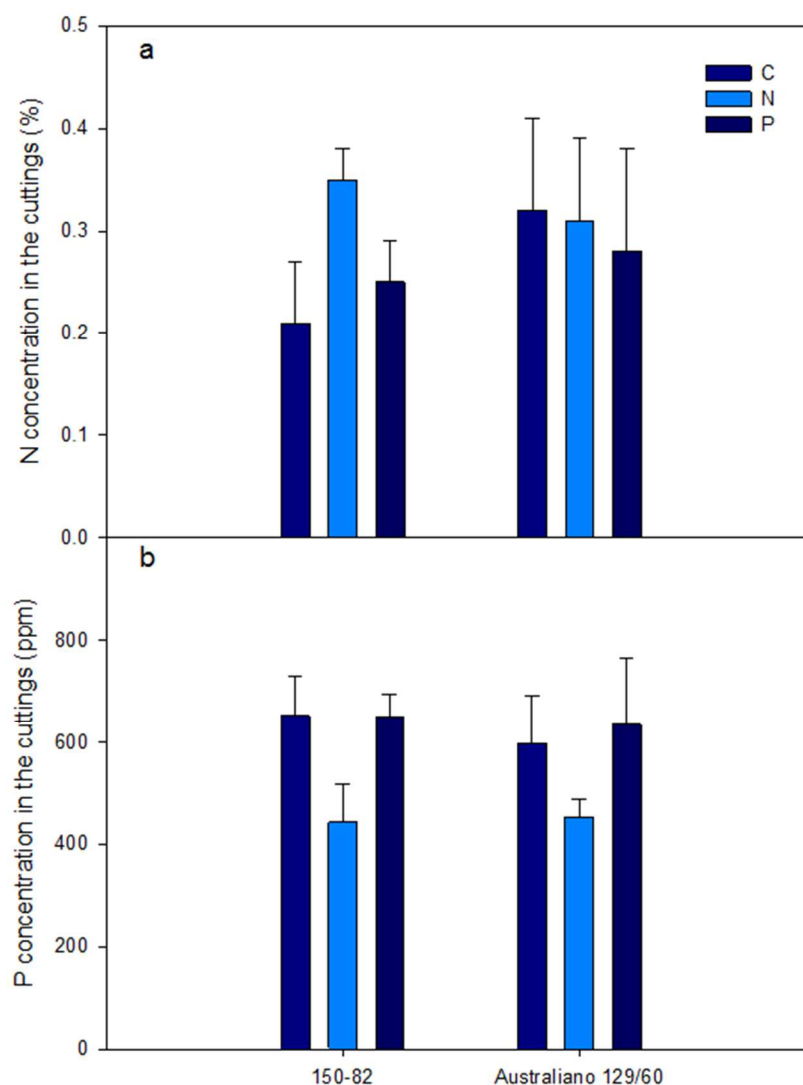 not affected by N or P fertilization in the stoolbed by the end of the experiment. Therefore, the early vigor of the cuttings was affected by fertilization, but the growth 3 months after planting was similar in fertilized and unfertilized cuttings, of both clones, if they grew under well-watered conditions.

After the field experiment, no differences were found in early vigor traits between cuttings obtained from non-fertilized and N or P fertilized stumps (**Tab. 1**). Therefore, there was no negative effect of applying N or P fertilizers in the stoolbed on the survival of clone "150-82" at field conditions. However, these measurements were made a few months after planting, without exposure to flooding or drought.

The third step was to show if drought or flooding can affect plants derived from cuttings harvested from fertilized stoolbeds of clone "150-82". We measured leaf development through counting leaf number in plants grown in pots under water stress conditions. The number of leaves is a good indicator of stress (Kramer and Kozlowski 1979; Luquez et al. 2012). If this trait is considered, N fertilization in stoolbeds increased susceptibility to drought and improved flooding tolerance. P fertilization increased the defoliation produced by flooding. These results should be considered when the possibility of fertilizing a stoolbed is evaluated, at least for clone "150-82", since the occurrence of water stress is frequent during the establishment of the new plantation in the Paraná River Delta. However, although there is some effect of stoolbed fertilization in the defoliation under drought or flooding, N or P fertilization had no effect on plant survival.

Conclusions

When stoolbeds were fertilized with N or P the early vigor of the cuttings of clone "150-82" was little affected. Some traits of the early vigor of clone `Australiano 129/60´were negatively affected by

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fertilization (less roots and shoots). However, growth was not reduced if plants grew under well-watered conditions during 3 months after planting. Fertilization with N reduced wood density of the cuttings, reduced leaf production in the middle term and reduced the tolerance to drought of the new plants. Fertilization with P had no effect on wood density, number of leaves and drought tolerance, but slightly reduced tolerance to flooding. Neither N nor P fertilization reduced the survival of the plants under stress.

The effect of fertilization on the tolerance to drought and flooding should be taken into account if fertilization is adopted as a practice to increase the yield of the stoolbeds or to compensate nutrient extraction. Genotypic variability should be considered.

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Mass production of self-rooted *Hevea brasiliensis* industrial clones by tissue culture and nursery methods

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Abstract

Industrial clonal plantations of *Hevea brasiliensis*, more commonly known as rubber tree, have been established for several decades with grafted/budded plants as an alternative to the mass clonal propagation of the mature selected industrial clones on their own roots. Substantial investments have been devoted for many years to the development of tissue culture techniques focussing on somatic embryogenesis and micropropagation by axillary budding to reach this goal but with limited success. The recent mass production of self-rooted *H.brasiliensis* industrial clones by nursery methods provides the opportunity to assess the respective pros and cons of *in vitro* versus nursery vegetative propagation methods for mass producing industrial clones of the rubber tree.

Key words: bud grafting, clonal plantations; *in vitro* culture, micropropagation, rooted cuttings, rubber tree

Introduction

Hevea brasiliensis, of the Euphorbiaceae family, is a diploid (2n=36) monoecious allogamic tree species that can reach more than 40m in height in its natural environment, the Amazon basin forest (Webster and Paardekooper 1989). Its rhythmic growth, endogenously controlled, follows Rauh's architectural model (Hallé and Martin 1968; Combe and du Plessix 1974). Its root system consists of one vigorous vertical taproot and several lateral roots (Compagnon 1986).

H. brasiliensis is the main source of natural rubber, which has warranted the domestication of the species since the XIXth century. The first rubber tree plantations were established in Ceylon in 1876 just after the second industrial revolution with seedlings from the Wickham collection in the Brazilian amazon basin forest (Compagnon 1986; Baulkwill 1989). Suitable growing conditions are characterized by annual rainfalls of 1,800 to 2,500 mm with an optional drier season which should not be too pronounced. Soils, slightly acidic, must be well drained as the species is sensitive to waterlogging, root diseases and diebacks (Baulkwill 1989; Watson 1989).

At the beginning of the XXth century, a program of genetic selection was initiated by Dutch researchers in Java and Sumatra from this Wickham seedling population (Baulkwill 1989; Simmonds 1989). Clonal propagation of these materials by rooted cuttings and by grafting techniques was attempted. Only the grafting of hardwood or softwood buds onto seedling rootstock in active growth, developed in 1918 by Van Helten, was efficient enough to be used operationally for selecting and then mass planting the rubber tree clones (Dijkman, 1951). In the 1930's, the superiority of industrial rubber tree clonal plantations over seedling-derived ones was definitely acknowledged (Compagnon 1986; Dijkman, 1951).

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Rubber tree clonal plantations are usually planted in monoclonal blocks of budded trees that grow upon a genetically heterogeneous population of seedlings rootstocks (Clément Demange et al. 2007). Despite a much higher and uniform productivity than seedling-established plantations, grafted clones are exposed to:

i. Graft-induced drawbacks encompassing imbalanced aerial development as well as various kinds of scion X rootstock incompatibilities which can be one cause of within-clone variability and of the bark necrosis syndrome (Masson and Monteuuis 2017).

ii. Maturation-induced negative effects of the material produced by conventional budwood gardens (Masson and Monteuuis 2017).

Rationale and historical background of producing *H. brasiliensis* self-rooted clones

As early as in the 1910's, researchers observed that the grafted clones failed to perform as well as the original mother trees they derived from (Compagnon 1986). Further experiments (Dijkman 1951) demonstrated that cutting-derived clones were more vigorous, less prone to early production of lateral and axillary shoots, and yielded more latex than the same clones produced by grafting.

Graft-induced negative effects were assumed to be the cause of such differences. The production of the same clones on their own roots appeared logically as the most rationale way of investigating further this issue.

Rooting *H. brasiliensis* cuttings was attempted at the end of the XIXth century using softwood terminal shoots averaging 30cm in length but these shoots, especially when collected from mature materials, died before they could be rooted (Warburg 1902). This situation remained unchanged for nearly 50 years till Stahel (1947) improved significantly the rooting rates by using permanent fine water sprays. Soon after that, Dijkman (1951) succeeded in rooting shoots taken from the crown of 3 to 4 year-old seedlings that were placed in open air rooting beds under a similar water misting system. The adventitious roots formed looked like tap roots, which was considered as a promising indicator of suitability for field planting. Levandowsky (1959) became the first to root mature material cuttings under an open air intermittent mist-system in nursery conditions. This technique was soon after applied successfully with up to 90% of rooting rates to fifty mature rubber trees including representatives of industrial clones PB86 and GT1, the most widely planted genotypes in Malaysia at that time (Tinley and Garner 1960). Surprisingly these activities were not pursued leading Compagnon (1986) in the mid 80's to state that none of the numerous attempts to mass propagate industrial rubber clones cost-effectively by rooted cuttings had really succeeded so far. From the 70's, attempts to produce self-rooted rubber trees by cuttings were gradually abandoned to give preference to tissue culture that was actively booming at that time.

Background, prospects and current limitations of the tissue culture option

In vitro micropropagation by axillary budding of *H. brasiliensis* has been initiated using shoot apices or shoot nodes as primary explants preferably because shoot apical meristems are too tiny in this species to be successfully inoculated (Venkatachalam et al. 2007; Monteuuis, unpublished results). Protocols developed since long ago for *in vitro* multiplying and rooting *H. brasiliensis* microshoots have been reported to be not efficient enough for mass producing industrial rubber tree clones (Lardet et al. 1987). Despite substantial investments during the past 40 years this situation has not radically improved lately (Venkatachalam et al. 2007). Moreover, the microshoots rooted *in vitro* cannot be easily acclimatized (**Fig. 1**) to natural environment to be planted ultimately, especially when the tissue culture facilities are distant from the nurseries and the planting sites (Dibi et al. 2010; Masson 2017).

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Figure 1: Transfer in SoGB nursery facilities of *H. brasiliensis* microcuttings produced *in vitro* in Europe and sent under proper conditioning 4 days before.

Somatic embryogenesis was first attempted on rubber trees at the Rubber Research Institute of Sri Lanka in 1972 using anther-derived calli from which embryoids then shoots were obtained a few years later (Satchutanantavale and Irugalbandara 1972; Paranjothy and Gandimathi 1975). Subsequent works have established that *H. brasiliensis* is one of the few woody species for which somatic embryogenesis can be obtained from sporophytic tissues - mainly from immature anthers, seed integuments and roots - of mature trees (Carron et al. 1989; 1995). However, the success rates are still very low and strongly genotype-dependent, irrespective of field performance. Thus it can be assumed that only about 20 of the 50 industrial rubber tree clones planted worldwide have successfully responded to somatic embryogenesis initiation so far with a much lower proportion being able to fully develop into somatic embryos. These are prone to somaclonal variation risks liable to vary according to the genotypes and the protocols used, somatic embryogenesis procedures maintained long-term being more exposed (Compagnon 1986; Montoro et al. 2012). Moreover only small quantities of somatic embryos can be produced using the procedures developed so far, but their numbers can be amplified by micropropagation by axillary budding, notwithstanding the foregoing limitations of this technique. Because of this very low efficiency, it is estimated that a ready for planting *in vitro*-issued rubber tree costs nowadays 30 times more than the same material traditionally produced by grafting.

As regards field performance, a few observations indicated that self-rooted tissue culture plants had a better growth and a higher latex production than grafts of the same age and of the same genotype (Dibi et al. 2010; Montoro et al. 2012). However, these statements were based on insufficient numbers of plants and of clones assessed during a too short period for drawing definitive conclusions regarding the superiority of self-rooted vs grafted materials. Besides, between clone-differences may exist and the clones that are best in West Africa conditions like PB217 and IRCA331 can still not be produced by tissue culture (Carron et al. 2009; Masson unpublished results).

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Prospects and current limitations of the rooted cutting option

These *in vitro* limitations spurred the Société des caoutchoucs de Grand Béréby (SoGB) in the Ivory Coast to revive the experiments on the production of industrial rubber tree clones by rooted cuttings in local nursery conditions. Soon after, SoGB was able to root several hundred cuttings of two mature clones with a 75% success rate, benefiting from good nursery facilities equipped with a reliable mist system (Masson et al. 2013, **Fig. 2**). Since 2013, the technique has kept improving and all the 36 industrial clones tested so far could be rooted by the SoGB, especially the best ones, PB217 (**Fig. 3** and **4**) and IRCA331, which could be considered as a meaningful follow up of the work initiated by Levandowsky in the 50's. Although the best rooting scores were obtained from *in vitro*-derived plants, suitably managed as responsive stock plants, it has to be mentioned that average rooting rates of 60% were recorded for shoots collected from field growing mature industrial clones. The first observations tend to indicate that adventitious rooting capacity varies from one mature clone to another. Efforts are being pursued to check if these clonal differences cannot be lessened by applying rejuvenating practises to the stock plants. Such physiological conditioning is also expected to increase the number of shoots with a high rooting capacity in order to produce more rooted cuttings in shorter delays for greater overall efficiency.

The SoGB experience shows that industrial *H. brasiliensis* clones can be self-rooted by rooted cuttings in local nursery conditions at a much cheaper cost and for a greater number of clones than by tissue culture. Nursery-produced rooted cuttings are also more robust, adapted to natural conditions and can thus be more successfully field planted.

Figure 2: Rooting cuttings of *H.brasiliensis* industrial clones in SoGB nursery facilities.

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Figure 3: Three-month-old container-grown rooted cutting of clone PB217 at SoGB.

Figure 4: Root system of a 3 month-old rooted cutting of clone PB217 at SoGB.

Rationale of combining tissue culture and nursery techniques for mass producing self-rooted *H. brasiliensis* industrial clones

Although first attempted with disappointing results, the possibility of mass producing *H. brasiliensis* clones by rooted cuttings has soon be abandoned in favor of grafting which has rapidly been adopted as an alternative and more efficient way for developing, on a large scale, industrial clonal plantations of rubber trees (Dijkman 1951; Webster and Baulkwill 1989). Grafted *H. brasiliensis* clones however combine advantages but also drawbacks (Masson and Monteuuis 2017), and the prospects of field testing clones on their own roots have remained a prevailing objective at the research and development levels (Dijkman 1951; Compagnon 1986; Webster 1989). During the past few decades, substantial efforts have been devoted to tissue culture for achieving this goal while nursery techniques have been neglected untill SoGB decided recently to reconsider their usefulness and demonstrate their efficiency (Masson et al. 2013). Although the

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tissue culture and nursery protocols currently available are very likely amendable, it is warranted to consider whether nursery and *in vitro* vegetative propagation methods can complement each other synergistically for greater overall efficiency, as has already been demonstrated for other tree species (Thompson 2014). Tissue culture could be useful for vegetatively multiplying selected genotypes with shorter delays and in bigger amounts than in nursery. The microshoots produced *in vitro* can be rooted preferably in *ex-vitro* conditions, as is routinely practiced, with great success, with teak for instance (Goh and Monteuuis 2016).

Adapted *in vitro* protocols can help also for physiologically rejuvenating the mature selected clones in a more efficient way than in nursery conditions by miniaturizing the sporophytic tissues used. In this respect, the possibility to get somatic embryos from mature selected genotypes on *H. brasiliensis* is real advantage. However, the rejuvenation achieved by somatic embryogenesis, although undoubtedly recognized as complete from an ontogenetical standpoint, may be more questionable physiologically as possible negative ageing influence of non-optimal medium components on tissue cultured soft and permeable isolated cells or group of cells cannot be ruled out (von Aderkas and Bonga 2000).

Rubber tree tissue culture has been for a long period of time disconnected from the trees in their natural environment, far away from most of *in vitro* facilities. Closer connections and location proximities between field, nursery and tissue culture activities should be encouraged for introducing plant material that can be physiologically preconditioned for greater culture initiation success (Monteuuis et al. 2011). Deeper knowledge of *H. brasiliensis* physiology in natural environment may also help in simplifying tissue culture protocols to make them more operational and cost effective. In this respect, benefitting downstream from nearby nursery facilities must be considered as a real asset for the *ex-vitro* rooting and acclimatization process, as demonstrated for teak for instance (Monteuuis 2000; 2016).

Conclusion

The recent progress, especially in nursery techniques, made by SoGB have promoted the mass production of industrial rubber tree clones on their own roots, with particular mention for the outstanding clones PB217 and IRCA331 which could not be produced by tissue culture so far. This has given the possibility to set up well–designed with big enough sample size trials to compare reliably the field performances of self-rooted vs grafted *H. brasiliensis* clones (**Fig. 5**). Efforts are pursuing along this line in SoGB on an increasing number of industrial clones in order to take into account possible clone X clonal propagation technique interactions. These activities are expected to soon provide a definite answer to the old question of knowing whether latex yield can be significantly increased using clones on their own roots instead of grafts. In the affirmative, the future of self-rooted rubber-tree clonal plantations will then be ultimately dependent on economic aspects. In this regard , the possibility to improve the efficiency of tissue culture and nursery techniques separately or combined for mass producing self-rooted *H. brasiliensis* industrial clones will have a major impact.

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Figure 5: Five-month-old rooted cuttings of clone IRCA331 field tested at SoGB.

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Scaling-up of Cherry rootstocks in a temporary immersion bioreactor system

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Abstract

In this research, we have developed a Temporary Immersion Bioreactor System (TIBS) to scaling up the propagation rate of Cherry rootstocks Maxma 14, Colt and YQW1. These rootstocks are key to obtain grafted plants with commercial yields for export. The best conditions for the TIBS were acquired using an experimental design with factorial treatment of 2^3 with 6 replicates and the variables being the number of immersions, BAP concentration and air supply in order to determine what conditions produce the highest number of commercial quality plantlets. The rooting of the plantlets, using the best conditions for each variety, was carried out in *in vitro* media supplemented with IBA and, finally, the acclimatization was achieved in humid tunnels on soil at 25°C. The results for the rootstocks Maxma 14, Colt and YQW1 with an initial inoculum of 40 plantlets reached on average 74 ± 8, 88 ± 6 and 54 ± 6 plantlets within 21 days (duplication rate 2), respectively, in comparison with *in vitro* cultures with a duplication rate 2 of 30 to 45 days to obtain a similar number of plants. The methodology of rooting and acclimatization reached 90% of success for rootstocks of Maxma 14, Colt and YQM1. During the entire process, we used 56 bioreactors, which provided us the opportunity to evaluate the needs and capabilities of the TIBS production yields and creating a platform for massive propagation of Cherry rootstocks.

Keywords: temporary immersion bioreactor system, Cherry rootstock, cytokinin, aeration, immersion.

Introduction

Chile is one of the main countries which exports Cherries off-station to the northern hemisphere, with a high potential of business growth because of an increasing demand for this fruit by North American, Asian and European markets. To cover this need, thousands of hectares should be cultivated using rootstocks that improve the agronomic features of the fruit and increase the yield of the crops for export. Although, the conventional propagation, or *in vitro* propagation, of diverse varieties of cherries is possible with these procedures, propagation of the rootstocks is recalcitrant when using both the conventional and *in vitro* methodologies. To overcome the above problems, temporary immersion systems in micropropagation are beneficial for the proliferation of shoots and cuttings, microtuberization and somatic embryogenesis. The immersion time, i.e., the duration and frequency, is the most important and critical parameter for the efficiency of the system. By optimizing the volume of the culture medium and the vessel a substantial improvement in efficiency is also observed, especially for shoot proliferation. Generally, the temporary immersion also improves the quality of the plant tissue, resulting in greater vigor and a normal morphology of the seedlings. Hyperhydricity, which affects crops in liquid medium is eliminated with these cropping systems by controlling or adjusting the immersion duration. Furthermore, the propagated plant material in these systems has a better performance during the acclimation phase than the material obtained on a semi-solid medium or in liquid (Berthouly and Etienne, 2005). Another comparative advantage of TIBS is that it also helps to significantly increase yield by eliminating the problems mentioned above. The observed

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improvement is due to early autotrophic development of the aeration-stimulated plant, which may involve both the continual renewal of the gaseous environment or the presence of elevated CO_2, and the dosage of nutrients (Aragón et al. 2010). The objective in this work was to evaluate three cherry rootstocks, Maxma 14, Colt and YQW1 in different culture conditions in a TIBS that included as variables, aeration, cytokinin concentration and immersion for scaling-up production.

Material and methods

The methodology is based on three different aspects of tissue culture that will be described below.

In vitro *culture*

All the *in vitro* plantlets of the cherry rootstock Maxma 14, Colt and YQW1 are obtained from the germplasm collection of the Instituto de Investigaciones Agropecuarias (INIA). To begin the scaling-up of the *in vitro* culture process by the Temporary Immersion System (TIBS), 200 plantlets of each rootstock were micro-propagated *in vitro* with a propagation rate of 2, which was achieved after 40 days, when the plantlets reached 4 cm and were ready to divided in two new plantlets. The rootstocks were maintained in the semi-solid basal media DKW (Driver and Kuniyuki, 1984) modified with the addition of 0.01mg/L of Indolbutyric acid (IBA) (Phytotechnology Laboratories®), 0.6 mg/L of 6-Benzylaminopurine (BAP) (Phytotechnology Laboratories®), 250 mg/L of ascorbic acid (Calbiochem®), 25 g/L of sucrose (Sigma-Aldrich®) and 7 g/L of Agar-Agar (Sigma-Aldrich®). The pH was adjusted to 6.0 and the photoperiod was 16h light/8h darkness at 25°C ±1°C.

Scaling-up by TIBS

The inoculum of the 0.5 L TIBS, that was developed at INIA, was 40 plantlets, 2 cm long, without roots and with one large leaf, produced *in vitro* for each bioreactor and rootstock. The number of explants that had achieved a propagation rate of 2, was determined after 21 days post inoculation (dpi) in the bioreactor. To find the best growing condition for each rootstock, a statistical factorial design 2^3 was carried out, including two cytokinin concentrations (MA: 0.6 mg/L of BAP and MB: 0.1 mg/L of BAP) in the same *in vitro* medium that was described in the previous section but in liquid form with immersion occurring 2 or 4 times per day for a period of 1 minute each, with or without supplementary aeration. The photoperiod was 16h light/8h darkness at 25°C ±1°C.

Rooting and plant acclimatization

For rooting, the plantlets produced by the TIBS were collected after 21 dpi, and the root and large leaf were removed. In this step, the plantlets were not divided and 4 cm long shoots were used for re-introduction to *in vitro* semi-solid basal rooting medium DKW (Driver and Kuniyuki, 1984) modified with the addition of 0.1mg/L of IBA (Phytotechnology Laboratories®), 250 mg/L of ascorbic acid (Calbiochem®), 25 g/L of sucrose (Sigma-Aldrich®) and 7 g/L of Agar-Agar (Sigma-Aldrich®) and the pH was adjusted to 6.0. After this step, the plants were transferred, in their flasks, to a warm bed with a surface temperature of 30°C. The plantlets had rooted after 21 days. Finally, the rooted plants were removed from the flasks, cleaned of culture medium and transplanted into containers with a 3: 2: 1 mixture of peat, leaf soil and perlite, covered

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with a plastic bag that was cut at its ends every 5 days on the warm bed with constant humidity. After that the plants were ready for delivery to nurseries or for storage.

Results and discussion

Plant number production in different culture conditions

By assessing the results for each culture condition and by genotype, the best condition for Maxma 14 was Condition 2 (C2): 2 immersions, 1 min in MB and aeration. For YQW1 it was C6: 4 immersions, 1 min in MB and aeration, and for Colt it was C8: of 2 immersions, 1 min in MA and aeration. It appears that each genotype needs a unique culture condition. However, in the case of YQW1 the process increased the number of plant but also caused hyperhydration. All plantlets produced were rooted under *in vitro* conditions, reaching 95% efficiency. The plantlet yields produced by the experimental matrix for each genotype are presented in **Fig. 1**. Similarly, in semi-solid *in vitro* culture of *Actinidia deliciosa*, the evaluation of three concentrations of the cytokinin 6-benzyladenine (BA) showed that the minimum concentration was adequate and gave a better rate of survival than high concentrations of BA (Moncaleán et al. 2009). Moreover, in *Tectona grandis* L., cultivated in TIBS, Quiala et al. (2012) showed that the diminution of BA in the liquid culture medium in the bioreactor improved propagation and reduced hyperhydricity. Similar results were obtained by Garcia-Ramirez et al. (2014) in *Bambusa vulgaris*, where again, the low concentration was the better. In our case, for Maxma 14 and YQW1 the low concentration of Cytokinin (0.1 mg/L of BAP) was the best condition and for Colt, 0.6 mg/L of BAP was adequate. In the case of Colt, the C2 (MB) and C8 (MA) results showed no significant statistical differences. However, we chose the C8 condition because it resulted in less variability, as shown by the lower standard deviations for this condition than for the C2 one. It is possible that with more replications or adjustment C2 could be the adequate condition.

The results indicate that inclusion of aeration improves the tissue culture in the bioreactor. Use of aeration avoids hyperhydration in high rotation cultures in bioreactors. Especially with YQW1 rootstock the lack of additional aeration resulted in hyperhydration and necrosis of the biomass (**Fig. 1**). The aeration of the bioreactor favors the renewal of the atmosphere inside the TIBS (Aragón et al. 2010) and is a new study to develop.

Duplication rate 2

Using the best conditions, as determined in the previous experimentation, the number of times a propagation rate of 2 was achieved at 21 dpi were made was determined. The results are presented in **Fig. 2**, where Maxma 14, C2, reaches 74.38 seedlings, YQW1, C6, reaches 546 and Colt, C8, reaches 88.36. These results showed that the culture conditions selected for Maxma 14 and Colt, on average along with their standard deviations, reach rate 2 in the 21d period. For YQW1, the amount of biomass required was not reached due to hyperhydration, and, therefore, research on the optimization of media and growing conditions should be continued.

In summary, **Fig. 3** presents the different steps performed in this work to establish a mass propagation platform on a pilot scale.

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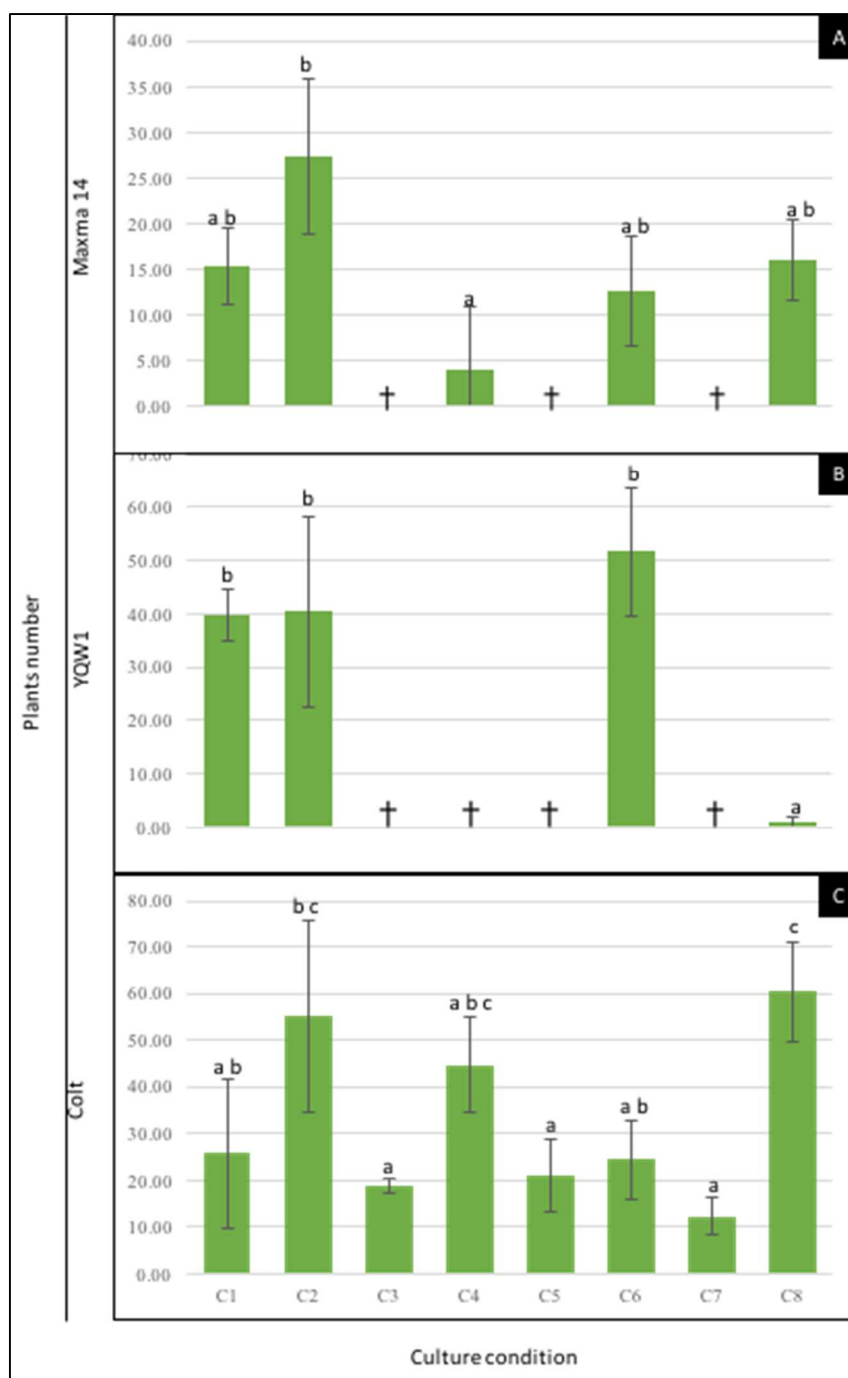
Figure 1. Plant number production in different culture condition. For the selection of the best culture condition (C) of each germplasm at 21 days, we tested 8 conditions that included a different number of immersions, concentration of hormone in the medium, and air supplied. The results included plants produced and acclimatized. A, In Maxma 14 the best condition was C2. B, In YQW1 the best condition was C6 and finally, C, In Colt the best condition was C8. The bars represented the average of 6 replicates and the whiskers are the SD. Different letters indicate significant differences in Tukey´s test (α=0.05).

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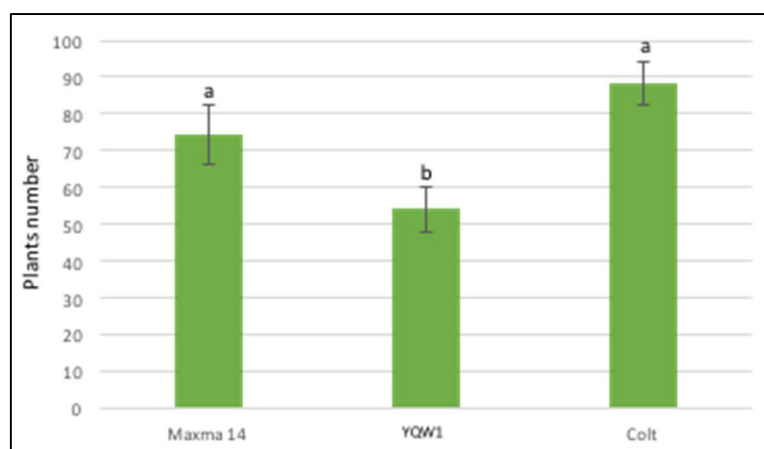
Figure 2. Duplication rate 2. For this experiment, we selected the culture condition that had produced the highest number of plants and had reached a duplication rate of 2 at 21 dpi. For Maxma 14 we obtained 74 plantlets ± 8, for YQW1 54 plantlets ± 6 and for Colt 88 plantlets ± 6. The bars represented the average of 6 replicates and the whiskers are the SD. Different letters indicate significant statistical differences in Tukey´s test (α=0.05).
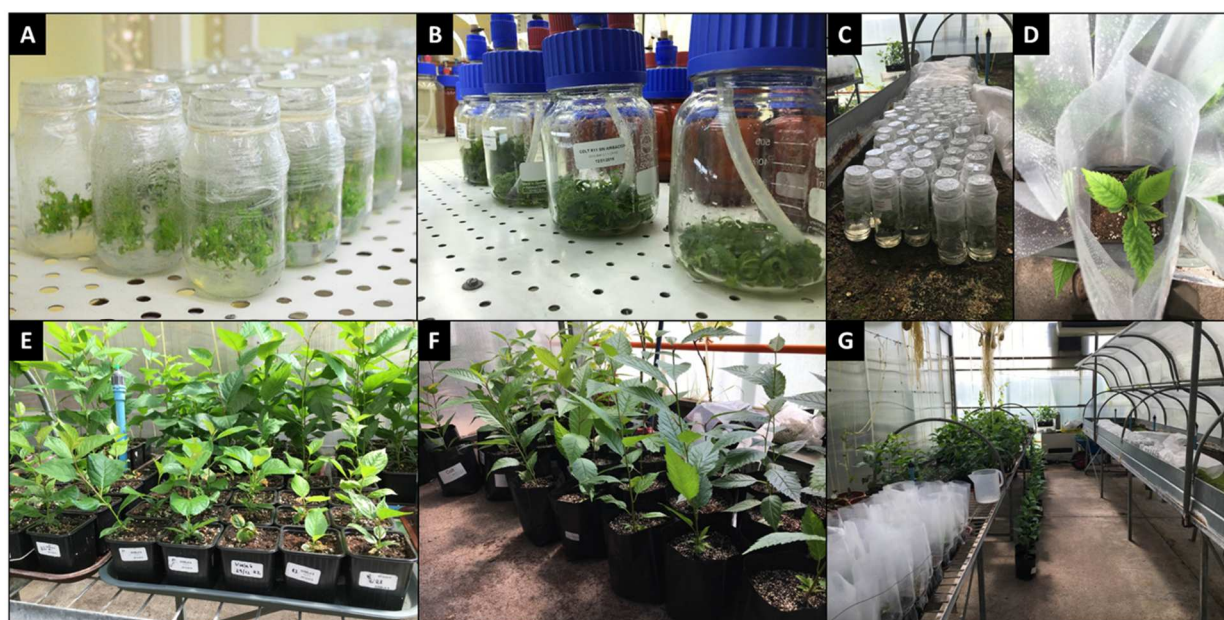
Figure 3. Scaling-up of Cherry Rootstock Production from *in vitro* culture to the greenhouse. A, *in vitro* culture. B, Propagation in Temporary Immersion Bioreactors. C, Rooting in a heated bed. D, acclimatization in a controlled environment tunnel. E, acclimatization in the greenhouse. F, Plants ready for nursery delivery. G, Work place in the greenhouse.

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Conclusion

Resulting from this work, we can conclude that the production of plantlets of cherry rootstocks in a TIBS is possible, provided a correct experimental design is followed that allows to find the suitable conditions of culture. First, the use of a bioreactor generated a reduction in the duration required to achieve a propagation rate of 2 on semi-solid *in vitro* culture medium (Duplication rate 2: 30 days for Maxma 14 and Colt and 45 days for YQW1) to 21 days for Maxma 14 and Colt, and for YQW1. We need to improve the media and culture conditions to obtain a duplication rate of 2 at 21 days. Second, the lowering of cytokinin concentration and the inclusion of aeration was significant in the success of the bioreactor cultures. Third, it is necessary to include a system for *ex vitro* rooting to decrease the time needed for the whole process. Finally, in continuation of this work, we are doing a physiological characterization of the process with the aim of establishing a model production control system for industrial scaling-up.

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Industrial implementation of somatic embryogenesis for the production of *Coffea canephora* P. plantlets

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Abstract

Somatic embryogenesis has been investigated on *Coffea* species since the late 70s with the aim to become an alternative method to conventional vegetative propagation of selected varieties produced by methods such as rooted cutting or grafting. During years 2000s, protocols were optimized and defined for *Coffea canephora* P. and a pilot production unit (10 million somatic embryos per year) was set up in Nestlé R&D Tours facilities to validate the technical feasibility of large scale production. The somatic embryos were then shipped to coffee producing countries to be sown, grown and acclimated before their distribution to farmers.

The following and logical step was to transfer the technology and the somatic embryo production to the coffee producing countries. NSIP (formerly AGROMOD SA de CV), a renowned company specialized in vitroplant production and commercialization in Mexico demonstrated interest in acquiring the process developed by the Nestlé R&D center. The technology was transferred and the embryo production in the laboratory was rapidly mastered. Callogenesis was observed on semi-solid medium for all of the selected clones chosen for the project. In liquid medium, embryogenic cell lines could be established and used to produce torpedo embryos. Further development of torpedo embryos was obtained in bioreactors fed by automated temporal immersions into liquid medium. On the other hand, the plantlet acclimation *ex vitro* was the most difficult part of the process to achieve and important losses were reported at the beginning of the project. The technical issues were finally solved allowing to reach an embryo-to-plantlet conversion rate of 50% and beyond, as initially expected. By end of 2016, NSIP produced more than 9 million coffee plantlets.

Keywords: plantlet production, mass propagation, somatic embryo, conversion, technology transfer

Introduction

Since 1990, the Nestlé R&D Center of Tours developed an accelerated propagation method for the production of coffee plantlets at a pilot scale (Ducos et al. 2007a). This cloning method is based on indirect somatic embryogenesis *in vitro*, which preserves the agronomic performance among regenerated plantlets. A great advantage of the technique stands in the fact that the plantlets pass through a juvenile phase (rejuvenation) comparable to seedlings, and display a good root-stem connection compared to rooted cuttings. Moreover, compared to *in vitro* micro cutting, this technology is highly effective and does not require a lot of hand labor due to partial automation. It is assumed that using this technology in a suitable laboratory setting, one FTE would produce up to 1 million pre-germinated somatic embryos per year (Ducos et al. 2010). Finally, this method presents a high versatility that allows quick changes in the varieties to be propagated, compared to extensive clonal gardens used for cutting production. In *Coffea canephora* P., this

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process allows reliable propagation as proved by the high phenotypic conformity of the propagules as well as the good field performance compared to the donor tree (Ducos et al. 2003).

This process can be divided into 7 main steps:

i.   Callogenesis on semi solid medium: The process starts with a callus production phase, during which the dedifferentiation and multiplication of totipotent cells occurs from pieces of leave collected from the mother plants to be propagated.

ii.   Callus multiplication in liquid medium: In order to accelerate the biomass production of callus, small amounts are inoculated in liquid culture medium. Each inoculum represents an individual cell line, with proper growth and morphogenetic capacity. After a cycle of 4 subcultures in increasing volumes of medium, cell lines are evaluated for their ability to regenerate pre-germinated embryos.

iii. Embryo "Torpedo" production: For each cell line, callus produced in liquid medium is poured and subcultured at low density, into a liquid medium promoting cell differentiation and the embryogenic pathway. Then globular-shaped somatic embryos start to develop, followed by formation of torpedo-shaped embryos.

iv.   Somatic embryo pre-germination: Torpedo suspensions are placed under a temporal immersion regime inside disposable bioreactors called "box in bag" developed by R&D Tours (Ducos et al. 2010). Light and a cytokinin are required to stimulate photosynthesis. Then, torpedo embryos start to elongate, they become green and develop cotyledons. Roots can emerge at the end of this step. Finally embryos are ready to be transferred to the greenhouse to convert into plantlets.

v.   Embryo storage in the greenhouse: pre-germinated embryos are placed on the substrate for a period of 6 to 8 weeks. The objective is to remove the vitrified appearance and to harden to a degree the embryos. During this step, some of them start to germinate (i.e., root emission and true leaves formation). After this period, embryos are planted one by one into the substrate.

vi.   Germination and growth: Embryos, germinated or not placed inside a clean substrate. Culture conditions (climate, fertilization and sanitization) must be carefully monitored to promote germination (roots and first leaves emission) and initial growth of the plantlets.

vii. Hardening: Once plantlets are close to commercial size, climatic conditions are progressively moved towards real nursery conditions, which means higher light intensity and lower relative humidity. This step should be successfully realized without growth arrest or stress. During this hardening phase, the cuticle is formed on the leaf surface and a strong root system is produced. Plants are then ready to be delivered to the nurseries.

The coffee culture in Mexico is suffering from low levels of technical ability (outdated practices and varieties), resulting in insufficient bean production to support domestic requirements. At the same time, consumer demand is still increasing in this country, especially with regard to the soluble and Robusta-based coffee products. However, *Coffea arabica* is traditionally the main cultivated coffee species in Mexico, while *C. canephora* (Robusta) beans are widely imported from Brazil and Vietnam. As a result, the competition for green coffee from manufacturers is intensifying. To face this issue, the Nescafé Plan was launched in 2010 in Mexico in order to optimize the coffee supply chain. The farmers and coffee communities are taking benefits from this initiative which is based on the principle of "creating shared value", i.e., price stability and better farmer's income, social care for workers and technical supports in the field. Among others, one of Nestlé's contributions is to deliver to the farmers high-yielding and disease-resistant coffee varieties. One concern for Nestlé is to make these plantlets rapidly available to renew old and unproductive plantations.

An opportunity appeared to transfer to a local company and implement there the process for the production of Robusta plantlets. A contract was signed in 2011 between Nestlé Mexico, the Mexican institute of Agronomic Research INIFAP and Agromod (now NSIP), a private company with well-known expertise in plant production by *in vitro* micropropagation. Such a transfer was reported previously for the propagation

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of Arabica F1 hybrids in Nicaragua (Etienne et al. 2012). Authors reported the feasibility and also the hurdles they faced when transferring a whole *in vitro* process close to the coffee plantation areas.

For all the selected varieties, the objectives of the transfer of technology were i) to ensure a reliable production of embryogenic callus on solid and in liquid medium, ii) to ensure an efficient differentiation of the embryogenic callus into somatic embryos and iii) to implement an effective acclimation protocol that would allow a 50% embryo-to-plantlet conversion rate.

Material and method

Preliminary work regarding the technology transfer

A building was fully renovated for *in vitro* production in 2011 by NSIP inside their facilities located near Frontera Hidalgo (state of Chiapas, **Fig. 1**). The constraints were numerous and required specific equipment. Maintaining a high sanity level was achieved through the use of positive pressure inside the laboratory and by filtering (HEPA quality) the air entering into key areas such as the inoculation room (laminar flow hoods) and the culture rooms while a chilling unit produced air conditioning that maintained a constant temperature of 24°C inside the laboratory as well as a significant reduction of the relative humidity (40% against 60% and more outside). Water quality, for the preparation of the culture media and for steam production for the autoclaves (2 x 1m^3 Systec, Germany), was assured by a reverse osmosis demineralization station. Electricity was permanently delivered to the laboratory even in case of global breakdowns (auxiliary power supply). A first culture room was designed for the embryogenic callus culture on semi solid medium and liquid medium. Petri dishes were stored inside cabinets, whereas flasks containing embryogenic callus suspensions were cultured on orbital shakers (110 rpm, Sartorius, Germany). The second culture room was equipped for pre-germination of the somatic embryos inside the bioreactors under a temporal immersion regime. The compressed air flow that allows the temporal immersion system to function was driven automatically by controlled openings and closings of the solenoid valves (Festo). High pressure sodium bulbs were used as light sources. The work started by mid-2011 and the laboratory was operational in March 2012.

Embryogenic callus formation and multiplication

For the Mexican market was defined a set of 4 clones of robusta coffee (*Coffea canephora* var. Pierre), proceeding from Nestlé's breeding program, and selected through multi-site and farmers trials. Once deployed in the field, respecting a balanced mix of these clones ensures that the farmers will achieve suitable pollination, a prerequisite for consistent harvests in this allogamous species. These clones, FRT06, FRT07, FRT09 and FRT23 were previously established in a clonal garden managed by INIFAP for the production of rooted cuttings.

In order to initiate, locally, callus production on semi-solid medium, 10 mother plants of each of the 4 clones were planted in 2011 at the INIFAP center located in Tuxtla Chico (Chiapas-Mexico). They were obtained by rooted cuttings and then installed in the NSIP greenhouse. To validate the genetic conformity of the donor plants, a PCR-based test was performed on leave samples before any were used in production. First collection of leaves and disinfection was planned in April 2012.

The protocols for disinfection, explant and embryogenic callus culture on semi solid medium, callus production on liquid medium, expression and pre-germination of the somatic embryos were as previously described by Ducos et al. (2007a and b, 2010).

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For each clone, donor trees were sampled 4 times over a year to generate different pools of embryogenic callus. To initiate somatic embryo production (expression), a previous cycle of embryogenic callus multiplication should be performed in liquid medium, the resulting biomass of callus being used as the inoculum for the production. This cycle is started 8 weeks before embryo production and consists in a successive culture of the callus in increasing volumes of culture medium. A stable cell line must support the subcultures without displaying precautious differentiation (i.e., somatic embryo formation in multiplication medium). Otherwise, the cell line is discarded from the production process. For each cell line, the growth rate was calculated as the ratio of the final biomass (fresh weight, FW) after 8 weeks of multiplication, versus the inoculum biomass. We followed the growth rate data of 759 cell lines used for embryo production over a 22 months period to assess the embryogenic callus performance in terms of biomass production per genotype.

Embryo production and productivity parameters

A new embryo production cycle started every 4 weeks (13 batches per year), using the amount of callus produced by the multiplication cycles. In the expression medium, the embryo differentiation was promoted by the removal of the plant growth regulators as well as by a drastic reduction (x 0.05) of the cell density compared to that density during multiplication. Fully developed, torpedo-shaped embryo were obtained after a 5 to 11 week period in expression medium. Then, the torpedo embryos were further developed in the disposable bioreactors under a temporal immersion regime. The bioreactors were inoculated with 30 to 60 g of torpedo-shaped embryos and placed under light for a 8 to 12 week period. During this period, the embryo axis and the cotyledons started to elongate, chlorophyll was synthesized and a white root started to develop. At the end of this development phase, the embryos were harvested, weighed (final biomass, B) and moved to the greenhouse for the acclimation step. Samples of 1 g of FW (b) of somatic embryos were collected to determine the total number and the selectable number of somatic embryos (n). Based on these data, an estimation of the total number of selectable embryos (N) was calculated per bioreactor according to the formula:

$$N = \frac{(B * n)}{b}$$

This first key indicator of the laboratory performance was used to measure the monthly somatic embryo production of the laboratory and was calculated by summing all the data of bioreactor harvests on a monthly basis. Also, this estimation of the selectable somatic embryos allowed the measurement of the embryogenic callus productivity, which is the ratio between N and the inoculum biomass (g of embryogenic callus) used to start the production. For the 4 genotypes we followed this callus productivity in terms of embryo differentiation over 25 batches (almost 2 years) to compare their relative efficiency.

Acclimation and embryo-to-plantlet conversion

Several protocols were implemented during the first 2 years of production to try to acclimate and convert the pre-germinated somatic embryos into commercial plants. Different substrate mixes, container sizes, confinement protocols and fertilization types were evaluated for their efficiency. At the beginning of 2014, an optimized protocol was set up based on those preliminary tests. Harvested embryos were washed to remove culture medium and placed inside plastic trays on a layer of coco fiber mixed with slow release fertilizer (1.5 g.l^{-1} of mini osmocote, Everris). Trays were wrapped with transparent plastic bags and stored inside microtunnels under dim light for a 6 to 8 week period. This storage phase aimed at stimulating the root and shoot development of the embryos prior the planting step. Germinated embryos responding to the selection quality criteria were then sorted by size and planted individually into trays containing a mix of black and blond peat moss (45% and 35% respectively) as well as perlite (20%). Fertilization was achieved

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by mini osmocote. The trays were closed by an acrylic tape which allows a tight confinement of the embryos. They were then placed inside microtunnels with a tight control of the light, temperature and relative humidity. Foliar fertilizers and fungicides were applied to stimulate plantlet growth and to protect them against pathogens. After several weeks (from 8 to 32) of growth, the plantlets that reached the standards for commercialization were transplanted and hardened inside specific microtunnels where they were exposed to higher light intensity as well as to lower relative humidity. This step promoted the formation of the foliar cuticle. Finally, well rooted, green and healthy plantlets with 4 to 5 pairs of leaves were harvested, washed and packaged bare root to be sent to the nurseries where they would be transplanted for another period of growth. The number of delivered plantlets was also recorded on a monthly basis as the key indicator for the acclimation performance and compared with the expected plan. Delivery curves were also established for each genotype to understand how they performed in the acclimation phase.

We were highly interested in calculating the global embryo-to-plant conversion rate to assess the adequacy of the embryo quality and the acclimation protocol efficiency. Considering the embryo and plantlet production as a single flow, we based our calculation on sliding means over 3 months of delivered plantlets (P) that were divided by sliding means over 3 months of embryo productions (E) that were harvested 6 months earlier (i.e., average time to acclimate plantlets). Thus, the calculation of the conversion rate for the current month (C_0) was given by the formula:

$$C_0 = 100 * \frac{\overline{(P_0 + P_{-1} + P_{-2})}}{\overline{(E_{-6} + E_{-7} + E_{-8})}}$$

We calculated the conversion rate starting from March 2013 to December 2016 to study its evolution and the impact of the acclimation protocol changes. Finally, the acclimation performance per genotype was assessed by the analysis of the conversion kinetics, i.e., the percentage of the planted embryos that converted into sold plants as a function of the time (in months) spent under acclimation in the greenhouse.

Statistical analysis

Data collected from embryogenic callus and somatic embryos cycles of production were analyzed (ANOVA, general linear model) to detect any effect of the genotype, followed by means pairwise comparison tests (Tukey, $\alpha = 0.05$) using the software Minitab® 16.1.1.

Results

First months of work in the laboratory

After all equipment was successfully tested, the laboratory was delivered to the production team on March 2012 (**Fig. 1**). Despite a significant seismic activity in the area (more than 3 events > 6.0 on Richter scale in 2 years, Servicio Sismologico Nacional http://www.ssn.unam.mx/), and challenging climatic conditions (heat and heavy rainfalls), the building structure was fully validated for routine embryo production. The auxiliary power supply demonstrated its effectiveness in case of power breakdowns, which happened quite often in this area, and prevented any disruption in the production. The deionized water production unit was working well, as was confirmed by daily measurements of water electrical conductivity as well as by pH measurements, allowing the production of the culture media and proper autoclave functioning. The building sanitation was well managed, based on daily cleaning (floors), weekly fumigations and corrective applications of biocides. Air conditioning was controlled for each part of the laboratory and especially in culture rooms to always reach the set points of temperature. Thanks to efficient maintenance, any technical issue was solved on time and never impaired the production plan. Thus, the contamination level, a strong

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preoccupation when transferring *in vitro* technology to the tropics, was quite low (< 5%) at any step of the process, indicating a good management of the sanitation and good operators skills during media preparation, inoculation and subculture.
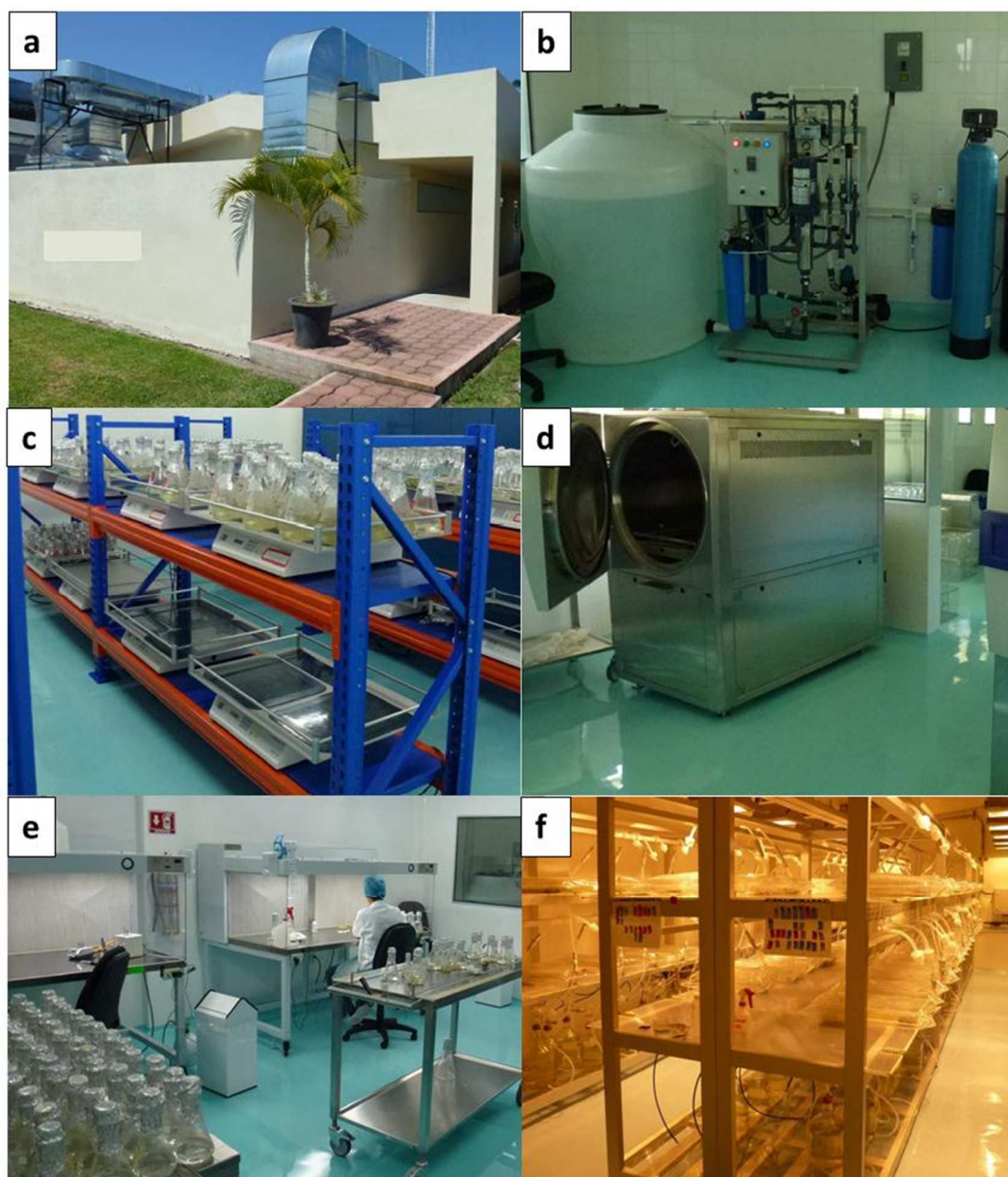
Figure 1. NSIP laboratory for pre-germinated coffee embryo production. a) Exterior view with part of the air conditioning equipment on the roof. b) Deionized water production and storage station. c) Orbital shakers with 1 l-flask for embryogenic callus and torpedo-shaped embryo production. d) Sterilization unit (autoclave, Systec). e) Laminar flow hoods in the inoculation room. f) Bioreactors room for the embryo pre-germination step.

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Embryogenic callus production

The donor plants were maintained under culture conditions that promote young and soft leaves formation throughout the year, thus making it possible to induce callogenesis more frequently (i.e., specific fertilization and flower buds removal) than was possible at the Nestlé R&D center in France. Frequent pruning also promoted the growth of young orthotropic shoots bearing new collectable leaves. The induction of embryogenic callus was successfully and routinely achieved from the explants collected on the donor trees for the 4 genotypes (**Fig. 2**). For all of them, a grey to yellow, friable embryogenic callus started to grow on semi solid medium. This allowed the constitution of embryogenic callus stocks in Petri dishes, usable for constant initiation of production batches in liquid medium. No negative aging effect was observed (i.e., regarding growth issues or regeneration abilities) from the embryogenic callus maintained up to 24 months on the semi solid multiplication medium.
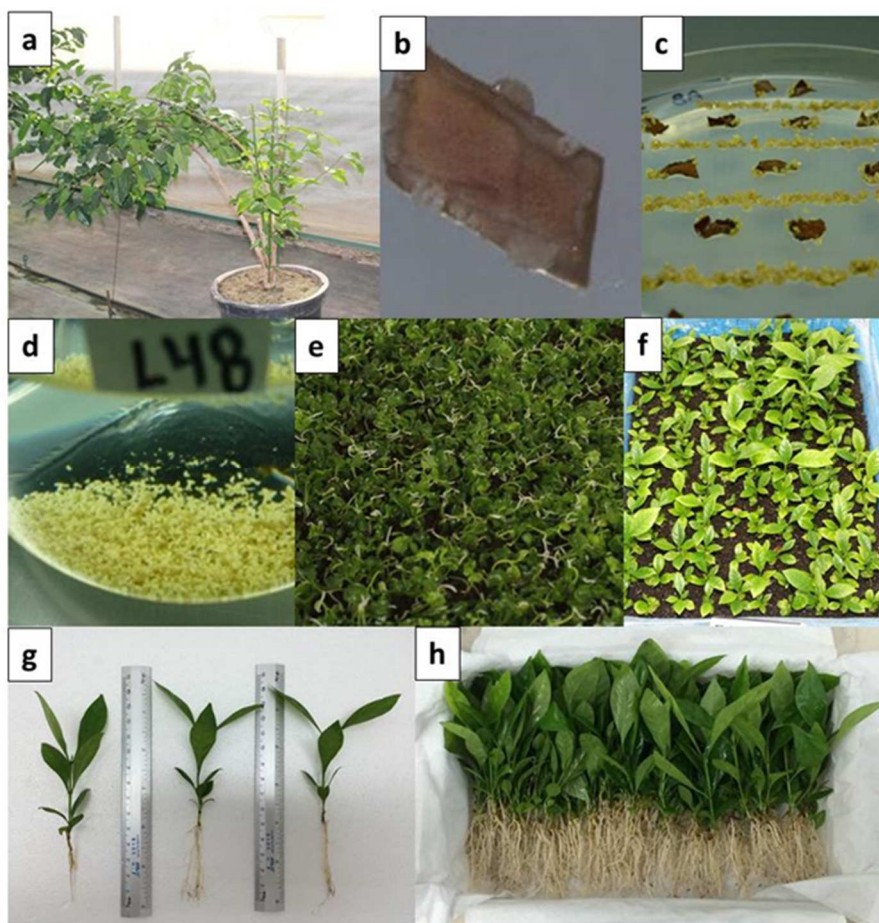
Figure 2. Plant propagation process by somatic embryogenesis. a) Mother tree to be propagated. The tree is bent to promote the development of new shoots. b) Leave explant from mother tree placed *in vitro* to initiate callus production. c) Explants and callus cultured on semi solid medium. e) White somatic embryos at the torpedo stage cultured in liquid medium. f) Green, pre-germinated somatic embryos with developed cotyledons and emerging roots. f) Germinated embryos growing on substrate in the greenhouse. g) Fully hardened plantlets ready to be delivered. h) Packaging of plantlets prior delivering to the nurseries.

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Performance of the embryogenic callus growth in the liquid multiplication medium was studied over the 24 first batches of production. Comparing the growth rates calculated at the end of the 8-weeks callus production cycle, revealed a genotype effect (F=10, p<0.001) on the ability of the callus to grow in liquid medium. Three genotypes displayed a comparable and mean growth rate of 12 to 13.5, whereas FRT23 displayed a significantly lower growth rate, close to 9 (**Fig. 3**). This lower growth ability was compensated for by a higher number of inoculated flasks at the beginning of the production cycle in liquid medium. It could also be useful to adapt the inoculum density at the beginning of the callus production cycle as some genotypes were more prone to differentiation. For instance, genotype FRT23 should be inoculated at rather high density (15 to 20 g of FW.l^{-1}) compared to the standard density (10 g of FW.l^{-1}) used for a more stable genotype such as FRT06.
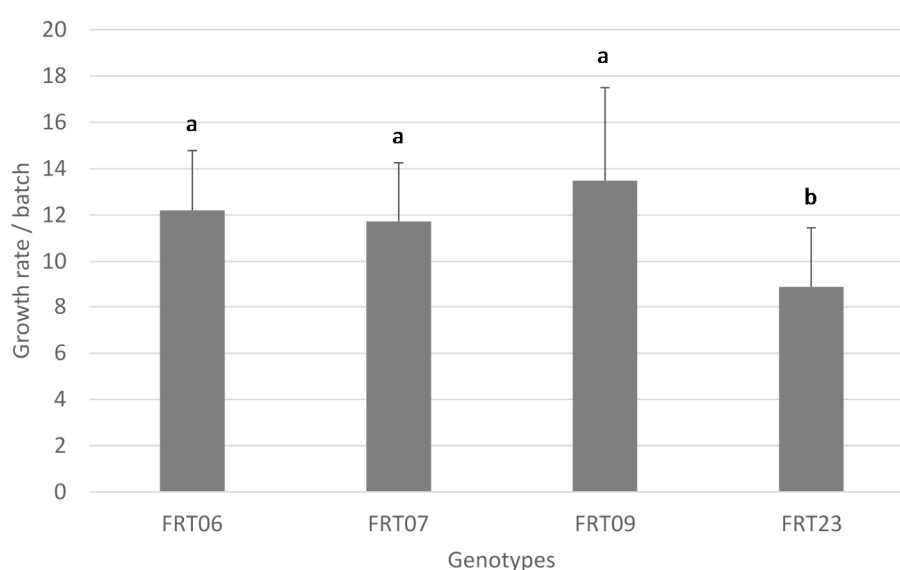
Figure 3. Embryogenic callus growth rate per genotype. Means are calculated based on the results (fresh biomass) collected during the 24 first production batches. For each genotype, one batch consists in 5 to 10 cell lines grown by consecutive 2-weeks subcultures over 8 weeks into increasing volumes (10, 20, 50 and 100 ml) of liquid medium. A total of 759 cell lines was used. Genotypes with different letters are significantly different ($\alpha = 0.05$) according to the Tukey's pairwise comparison test.

Pre-germinated somatic embryo production

Based on the selection of the best embryogenic callus, somatic embryos were efficiently produced and delivered as soon as May 2012 to the greenhouse for further acclimation (**Fig. 4**). Considering the produced numbers of embryos, the expected targets were surpassed for 2012 (3M vs 1M) and 2013 (8.4M vs 3M). During this period, the callus productivity in terms of regenerated and selectable embryos reached values between 14,000 and 26,600 embryos per gram of inoculated callus. The 4 genotypes displayed significantly different (F=7.73, p < 0.001) productivities (**Fig. 5**). FRT23 embryogenic callus was the most productive whereas FRT07 and FRT09 were low producing genotypes. FRT06 was an intermediate genotype, as callus productivity was not significantly different from any other genotype.

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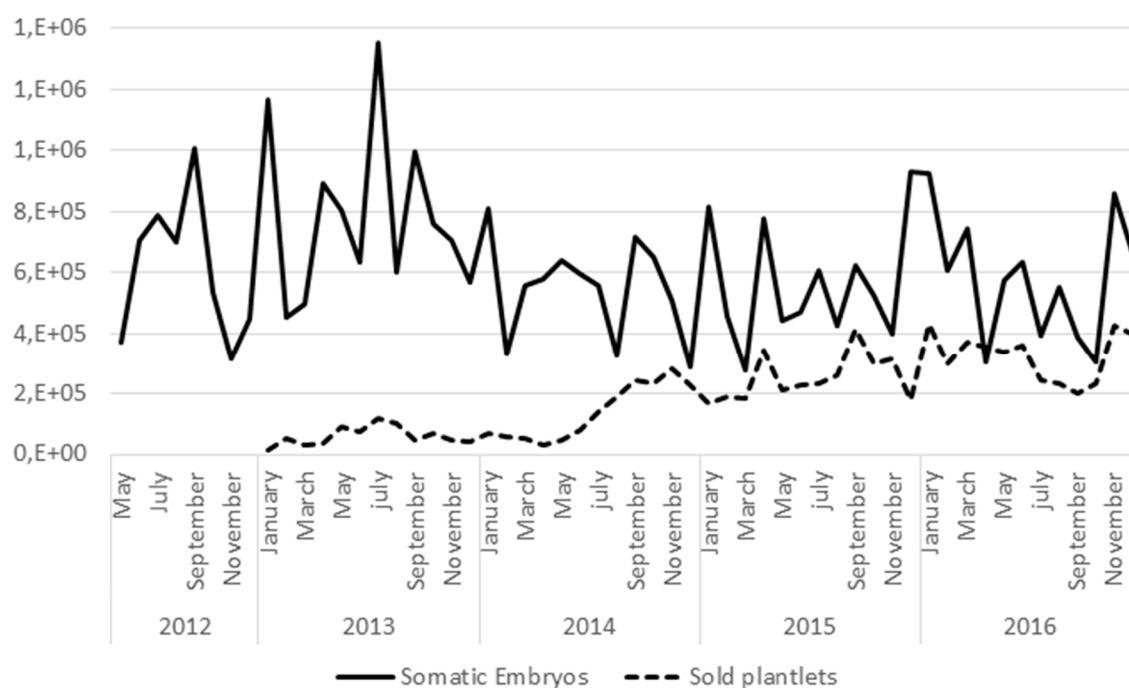
Figure 4. Evolution of the monthly somatic embryo production by the laboratory and the monthly plantlet delivery over 5 years. Monthly somatic embryo production data are obtained by adding the estimated numbers of selectable embryos harvested for the 4 genotypes month by month. Sold plantlets data represent the sums of the fully acclimated plantlets from the 4 genotypes that could be delivered month by month.
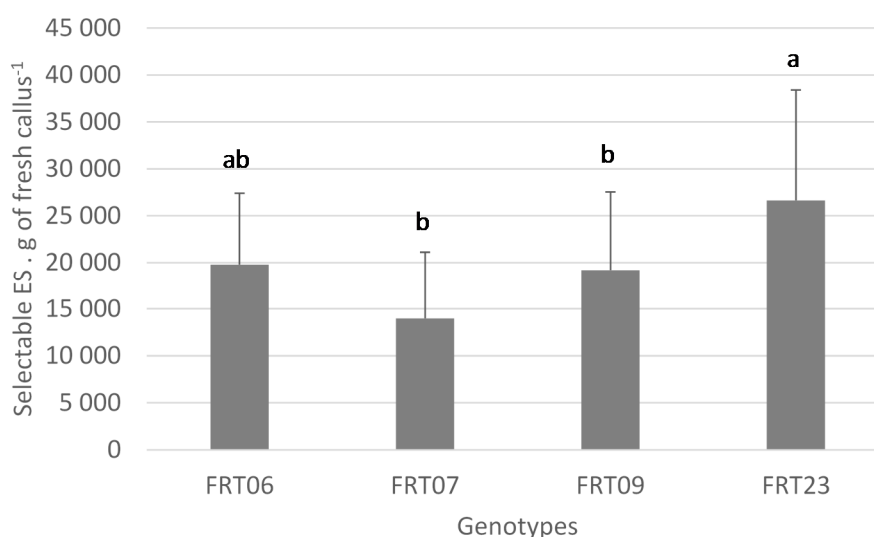
Figure 5. Embryogenic callus productivity expressed as the number of selectable somatic embryos (ES) regenerated per gram of callus inoculated into the expression medium. For FRT06, FRT07, FRT09 and FRT23, data represent the mean productivity of the callus calculated from 177, 188, 113 and 205 harvested bioreactors, respectively, corresponding to 25 batches of production. Genotypes with different letters are significantly different ($\alpha = 0.05$) according to the Tukey's pairwise comparison test.

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Despite these good *in vitro* results, embryo quality was challenged and particularly the embryo size was identified as a possible cause for low conversion in the greenhouse. Thus, bioreactors were inoculated at lower density to allow the embryos to develop bigger cotyledons and hypocotyl. This adjustment was reflected in the global embryo production since 2014 with a total of 6.5M (vs 8.4M in 2013, **Fig. 4**). The embryo production level was maintained in 2015 (6.7M) and slightly increased in 2016 (6.9M).

Acclimation and embryo-to-plantlet conversion

The somatic embryos produced in 2012 were acclimated and converted into plantlets that were delivered at the beginning of 2013 (**Fig. 4**). Several protocols were tried to germinate embryos and grow the plantlets up to commercial size, however, with low success during the first months. Indeed, the conversion rate at this time was as low as 10% on average (**Fig. 6**) and far from the expected 50%. By the end of 2013, some protocol modifications were implemented to address this issue, improving the embryo quality being one of them. In the greenhouse, the acclimation process was fully redefined and implemented early 2014 to fix the high mortality and the slow growth observed. Positive effects were observed as soon as July 2014 by increasing the number of delivered plantlets (**Fig. 4**) and conversion rate. In September 2014, a 40% conversion rate was routinely reached, 50% and even more since May 2015 (**Fig. 6**).

For the 4 genotypes, a period of 8 months was necessary to convert a maximum of planted embryos into sold plantlets (**Fig. 7**). After this period of acclimation in the greenhouse, the conversion rate dramatically decreased. Due to the selection prior to planting a high proportion (more than 83%) of the planted embryos converted into sold plantlets during this period, except for the FRT07 genotype (only 58%). Also, the genotypes behaved differently during the acclimation process. FRT23 converted quickly compared to any other genotype so that at the end of the third month of acclimation, 46% of the planted embryos were delivered versus 15%, 14% and 18% only for FRT06, FRT07 and FRT09 respectively. For these three genotypes, the highest conversions occurred between the 4th and the 6th months of acclimation, even if in a lower proportion for FRT07.
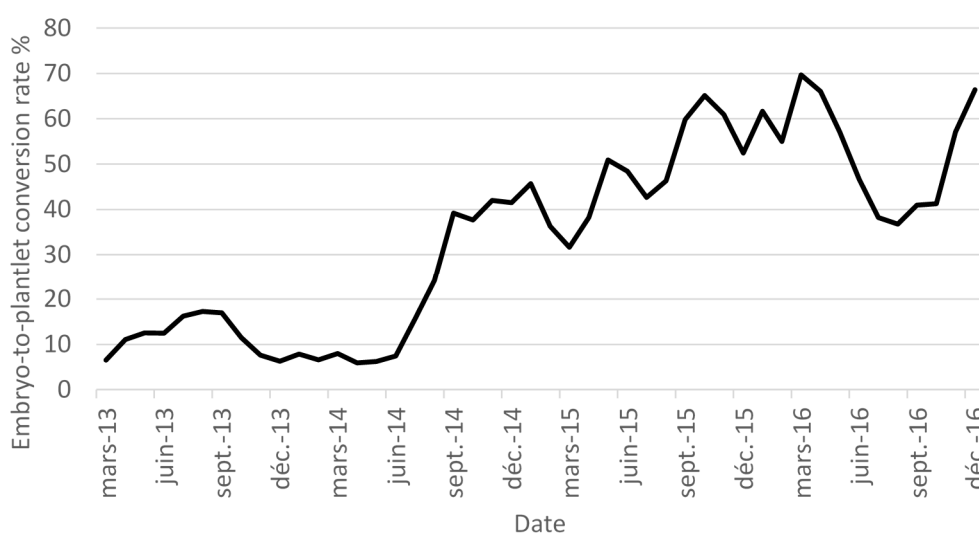
Figure 6. Evolution of the monthly, embryo-to-plantlet conversion rate from March 2013 to December 2016. Data are calculated based on the monthly plantlet sales versus the somatic embryo productions from the laboratory.

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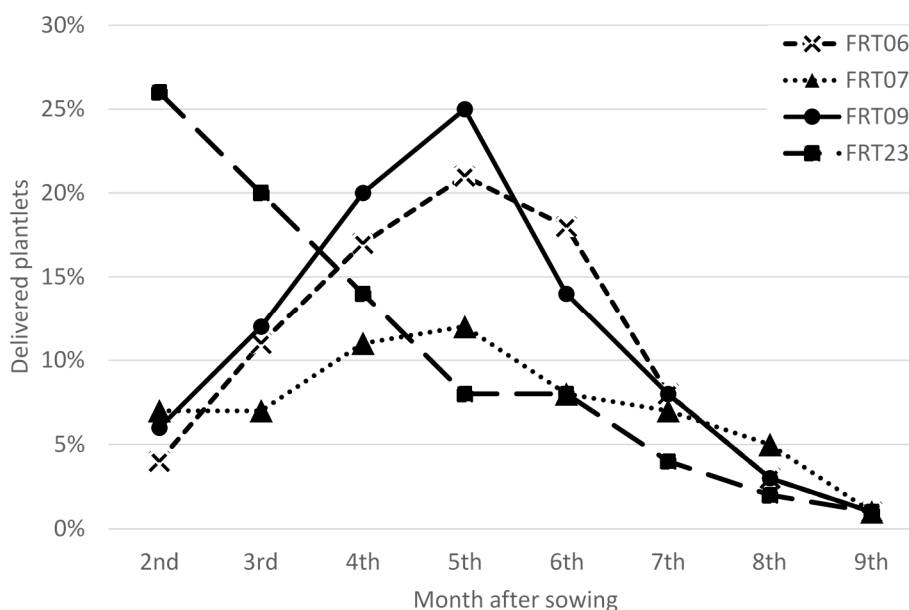
Figure 7. Planted embryos conversion into sold plantlets as a function of the acclimation duration. Percentages calculated based on cumulative acclimation results recorded since 2014.

Discussion

Looking at the performance of the embryo production in the laboratory as well as the embryo-to-plantlet conversion results, the technology transfer could be fully validated. 3.8M plantlets were sold by end of 2016 and more than 5M (100% production capacity) are expected to be 2017. Considering the quality of the plantlets, a high clonal conformity was observed and only rare phenotypic variants were observed from time to time in the nursery, such as variegated plants for instance. For *C. canephora*, regenerating embryogenic callus without the supply of exogenous auxins is of particular interest. This species belongs to the minority of species for which somatic embryogenesis induction does not require a balance of auxin and cytokinin. The lack of auxins during the callus induction and proliferation as well as the limited period of culture (24 months) are thought to reduce the risks of generating such somaclonal variations. This issue is a major concern for *C. arabica*, which requires the addition of synthetic auxin like 2,4-Dichlorophenoxyacetic acid to produce embryogenic callus (Bobadilla Landey et al. 2013, 2015).

The genotypic effect was not observed for the induction of callogenesis meaning that no recalcitrant genotypes were used in this production. However, looking at the embryogenic callus proliferation and regeneration abilities, clear differences could be observed between genotypes. We confirmed that FRT23 is a rather poor producer of callus and a fast regenerating (embryo) and converting (plantlets) genotype. Interestingly, embryos of this genotype were bigger in terms of cotyledons than the other genotypes. Conversely, FRT06 genotype was a highly stable genotype regarding callus production and regenerated thin embryos bearing short cotyledons. The most difficult genotype to regenerate was the FRT07, especially because of its low conversion performance. One problem with the FRT07 embryos was their hyperhydric aspect after the pre-germination phase. This could explain the poor germination ability of this genotype.

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Promoting embryo size (hypocotyl length and cotyledon development) by reducing bioreactor density or enriching the culture medium was suspected to have improved the conversion ability of the embryos into plantlets. Bigger embryos geminated faster and gave more vigorous plants, probably due to a higher availability in storage compounds. This is of particular relevance as the somatic embryos do not possess the seed maternal tissue to sustain early plant development. Previous publications on *C. arabica* and on the hybrid *C. racemosa x C. arabica* also mentioned the correlation between the conversion ability and the embryo size, but these publications, however, focused on cotyledon length (Barry-Etienne et al. 2002; de Rezende Maciel et al. 2016).

As previously mentioned for *C. arabica* technology transfer, the more sensitive step of the production process was the acclimation of the pre-germinated somatic embryos after direct planting (Etienne et al. 2012). A high confinement was required at the beginning of the *ex vitro* culture, to maintain a saturated relative humidity but also to keep a favorable gaseous environment. Elevated concentration of carbon dioxide was demonstrated to promote germination and early growth of the somatic embryos (Ducos et al. 2009). At the same time, such conditions promoted the development of opportunist pathogens such as *Myrothecium roridum* which impacted dramatically the survival rate of the young coffee plantlets during the first months of the production. This coffee disease, previously described for *C. arabica* has recently been reported for *C. canephora* in Brazil (Silva and Pinho 2014). The fungus was controlled through a tight management of sanitation of the greenhouse, combined with a fertilization application plan that strengthened the defenses of the plantlets. Also of particular importance was the progressive hardening step, which ensured a good survival rate of the plantlets in nurseries where the climatic conditions were less favorable than in the controlled greenhouse.

The production cost of the plantlets using somatic embryogenesis is still a matter of concern for companies interested in massive propagation. Compared to traditional rooted cuttings, somatic plantlets remain significantly more expensive despite the clear advantages in using the somatic embryogenesis propagation method (i.e. juvenility and fast production). Improvements in the process efficiency are sought by decreasing callus and embryo suspensions heterogeneity for instance. Also, a higher level of automation in the acclimation process could lead to significant decreases in production costs. Finally, combining propagation techniques is another way to reduce the production costs of the somatic plantlets. This is now the case for *C. arabica* since juvenile somatic plantlets were efficiently used as mother plants to produce softwood cuttings (i.e. mini cuttings, Georget et al. 2017).

Conclusion

The process was fully transferred and adapted for efficient plantlet production. Some efforts remain to be done to make this propagation method fully competitive with rooted cuttings. Since the beginning of the plantlet production, more than 9 million of highly productive plantlets were delivered for coffee plantation renovation, which represents an area of 4500 Ha. It is expected that Robusta bean importations will be impacted as soon as 2017.

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Extended abstracts

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The development and application of conifer tissue culture and somatic embryogenesis protocols in New Zealand: The *Pinus radiata* D. Don story.

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Keywords: *Pinus radiata*, cuttings, organogenesis, somatic embryogenesis, commercial forestry

Introduction

Pinus radiata D. Don. is the dominant forestry species in New Zealand, where it occupies almost 90% of the current area of planted forests. Breeding programs are advanced and seed production is well organized. A 2009 New Zealand nursery survey found that of the 37.7 million *P. radiata* tree stocks produced, 70% originated from control-pollinated seed (cuttings 25%, seedlings 45%). The remainder (30%) were produced from open-pollinated seed (Forest Owner's Association, & Ministry of Agriculture and Forestry, 2009). New Zealand also supplies the Australian radiata pine seed market. Propagation protocols to increase the availability of elite-cross material have been developed and tissue culture strategies have been tested and used commercially since the mid 1980's. The tissue culture work initially focused on adventitious shoot production from cotyledons of non-germinated control-pollinated seed. The advent of somatic embryogenesis (SE) in combination with cryogenic storage quickly superseded the earlier organogenic approaches. New and emerging technologies in forest-tree breeding include genomic selection where the potential to use this technology in combination with SE is attractive. Somatic embryogenesis is the underpinning technology for genetically modified *P. radiata* and potentially the pathway to producing rejuvenated planted stock directly from mature trees. More recently, research and application efforts have been focused on integrating organogenic tissue culture protocols with SE and on current nursery practices. This presentation (extended abstract) will focus briefly on which companies currently employ somatic embryogenesis as part of their research and production strategies. An outline of some of the early propagation research at Scion will be given and I will discuss how these technologies were developed and are employed today not only in research initiatives but commercially in New Zealand.

Who uses somatic embryogenesis in New Zealand now?

The three main forest companies are Timberlands (www.tll.co.nz), Arborgen (www.arborgen.co.nz) and Scion (www.scionresearch.com) which is principally a forest research institute funded via the government and various commercial collaborations and partnerships (project dependent). These companies are the result of a trail of mergers, sell-offs, and rebranding over the previous 3-4 decades. Timberlands manages the largest forest estate in New Zealand at 198,000 ha. It was previously known as the Kaingaroa Timberlands Partnership. Their somatic embryogenesis initiative was a separate entity within this and a partnership between Forest Genetics and CellFor (www.forest_genetics.com). Arborgen is now the owner of the original Tasman Forestry Ltd tissue culture facility (1980's to early 1990's). This then became part of/or was part of Fletcher Challenge Forests, then Rubicon, Horizon 2, followed by Arborgen who also took up Carter Holt Harvey's somatic embryogenesis capability around 2006. I am not sure of the exact dates of mergers and takeover status of the entities mentioned but certainly these have been some of the forestry
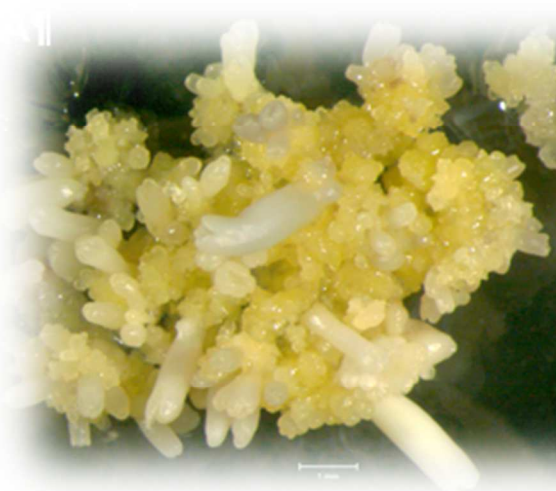
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business associated names attributed to what is now called the Arborgen facility. The basis of the Scion facility was developed back in the era of the Forest Service, pre-1988, when the New Zealand forest estate was largely government owned and administered. It was known as the Forest Research Institute (FRI) and was later renamed Forest Research as the shift from government funding to more commercial engagement increased. FRI was rebranded as Scion and for a while the propagation and tree breeding units formed a joint venture with CSIRO in Australia called ENSIS, subsequently dissolved. Interestingly, for the three companies Timberlands, Arborgen and Scion, the key propagation staff remained the same despite the considerable changes in ownership, management and restructuring. A further company should be mentioned, the Radiata Pine Breeding Company (RPBC, www.rpbc.co.nz), whose primary focus is the provision of superior radiata pine germplasm to shareholders and customers in Australasia. (Timberlands, Arborgen and Scion are, or in Scion's case, have been, shareholders). Scion is currently a significant research provider for the RPBC, which in recent years has significantly funded propagation projects. These include zygotic embryo-rescue from stored seed of native *P. radiata* populations, organogenesis from mature selections to facilitate seed orchard production and development of protocols for improved initiation of somatic embryogenic cell lines from elite breeding programs.

Cuttings

The development of cutting methodologies for *P. radiata* deserve special mention in their own right but are also of significance in the current implementation of somatic embryogenesis in New Zealand. The first in-vitro experiments were instigated to study adventitious root formation in cuttings in the 1970's. Subsequent tissue culture work was instigated using both field grown seedlings and zygotic embryos. The cuttings work led to an understanding of the impact of physiological age on rooting, growth and form (Menzies et al. 1988, 2000). Current practice is to grow stock plants in the nursery and to harvest cuttings from these over 1- 4 years. Cuttings can be directly set into nursery beds or containers. There is still some harvest of cutting stock from field-grown production material, again up to a maximum of 4 years after planting. Cuttings have become the preferred stock, especially for harsher or very fertile sites and in the winter of this year (2016) demand in the South Island for cuttings outstripped supply. Cuttings, in comparison to seedlings, often are sturdier stock with larger root collar diameters. Costs for cuttings are approximately $370/1000 for control pollinated (CP) seedlings, $450/1000 for CP cuttings, $900/1000 for SE derived, clonal stock.

Adventitious shoots: key developments and outcomes

The major impetus to the intensive development and commercialization of adventitious protocols for *P. radiata* was the limited supply of CP seed. Zygotic embryos were used either as germinated or non-germinated explants. The adventitious shoots arose primarily from cotyledonary tissue with the adventitious shoot meristems produced in sub-epidermal cells. The cotyledonary tissue on cytokinin-containing media produced distinctive meristematic nodules. In some genotypes, these nodules could give sustained proliferation of meristematic tissue. Aitken et al. (1988) postulated it would be possible to produce 260,000 shoots from meristematic nodules of a single clone in a year. Further developments included liquid culture of the meristematic nodules and cool storage of shoots (10-12 Celsius). Simultaneously the challenges of shoot vitrification, or hyperhydricity as it was later termed, became apparent with continuous organogenic culture (Debergh et al. 1992). *Pinus radiata* shoots would appear wet or translucent and most often arose in adventitiously-derived cultures. Images of these shoots taken with a scanning electron microscope showed the cuticular wax formation on the surface of the needles was very different between the normal and hyperhydric shoot types (Aitken-Christie et al. 1985). Non-hyperhydric shoots (waxy shoots) have needles covered in large amounts of tubular epicuticular wax. The hyperhydric (wet shoots) showed no tubular wax formation and very small amounts of globular epicuticular wax. Though the wet shoots multiplied well in-vitro, they were challenging to establish ex-vitro, often with poor survival and lower

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rooting percentages than the waxy shoots. These observations led to research on hydric control which resulted in better management of gelling agents in culture media and was attributed to a thirty-fold increase in propagation rates (Nairn et al. 1995). Scion (then FRI) facilitated transfer of the adventitious technology to Tasman, who improved protocols, commercialized production and established 1000's of ha of clonal forestry derived from adventitious shoots (Gleed 1993, Nairn 1993). At peak production the Tasman team was able to produce 2.5 million plants from 32 clones over a 2.5 year period, and up to 2,500 shoots/operator/day, with production incentives. Cool storage was used to hold the shoot cultures while field testing took place, though there were reservations about how long-term this could be and the space requirements were significant. This lead to work on cryopreservation with whole zygotic embryos, cotyledons and meristems (**Fig. 1**) (Hargreaves et al. 1999, 2004 Hargreaves and Menzies 2007). Via the interest in the adventitious methodology for *P. radiata*, a robust organogenic skill set was developed for this species.
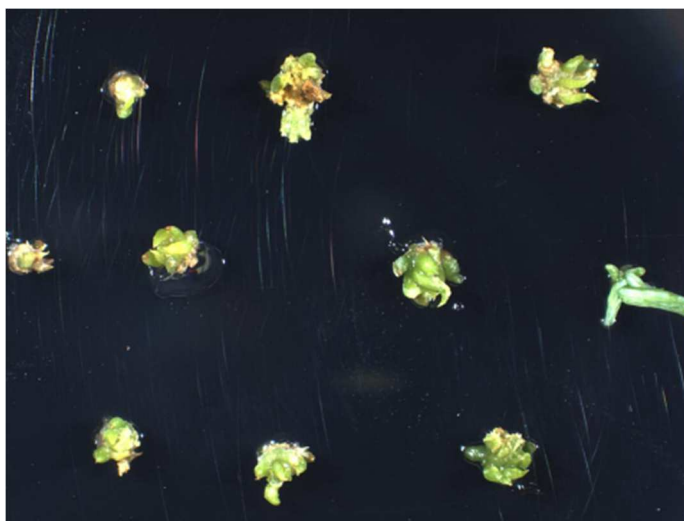
Figure 1. Regrowth from cryopreserved meristems of *P. radiata.*

Notably at this stage of the evolution of in-vitro protocols, in the early to mid-1980's, SE protocols for conifers were beginning to appear. There were significant challenges, especially with *Pinus* spp. Initiation rates were low, not all crosses were amenable, cryogenic protocols needed optimizing, maturation of embryo initials and conversion to plantlets was problematic and the growth rate of the resultant plants in the field looked compromised. This meant that despite the potential of this new technology, an organogenic approach still had many advantages, so both Scion (then FRI) and Arborgen (then Tasman Forestry Ltd) continued research in both organogenesis and SE.

Finally, a comprehensive study of adventitious versus axillary shoots was set up in a way that controlled for many variables (Hargreaves et al. 2005). A sample of cotyledons was abscised from zygotic embryos for adventitious shoot production and the remaining epicotyl meristems were used to establish a shoot culture. Except for the initial 3 week exposure to cytokinins, the adventitious and epicotyl origin shoots had the same culture conditions and transfers. Wet shoots mentioned earlier were simply selected against and not transferred. The result of this was clear. Adventitious-origin shoots exhibited slower growth in-vitro,

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were slower to root, showed increased physiological age in the nursery bed and earlier male/female cone formation after 3-5 years in field (Hargreaves et al. 2012).

Despite these challenges, this was not the end for adventitious tissue culture and the methodology has been used to rescue stored seed of native population *P. radiata* (Hargreaves et al. 2007). This seed had been in storage for 30 years and was showing only 3% germination rate. The megagametophyte tissue had broken down but the zygotic embryos still retained some viability and were able to form adventitious tissue which resulted in a 70% genotype capture (**Fig. 2**). Use of these adventitious tissue culture methods facilitated the trial establishment in both NZ and Australia of the same genotypes. Adventitious methods have also recently been used for early screening for disease resistance and as a tool for understanding some of the mechanisms of infection (Hargreaves et al. 2013).

Figure 2. Established plants derived from adventitious shoots formed on rescued zygotic embryos (the 1978, stored *P. radiata* seed of the native population).

Organogenesis from mature tissues, why?

There were a number of drivers for our work with mature shoot tissue of *P. radiata*. These included early amplification of field selections for seed orchard establishment (**Fig. 3**) and the possibility that the in-vitro treatments would result in rejuvenation (Horgan and Holland 1989 and Horgan et al. 1997). Dale Smith and his Scion (FRI) team, using the knowledge gained from the SE work, experimented with initiating rejuvenation in meristem slices placed on nurse tissues (Hargreaves et al. 2002). Mature tissue culture has been successfully used to transfer seed orchard material to Australia but the challenges of diseases, fire, and hungry cockatoos made New Zealand a better place to produce seed for Australian forest growers.

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Arborgen supported some of this rejuvenation research with *P. radiata* for several years but so far, with no publicized breakthroughs.
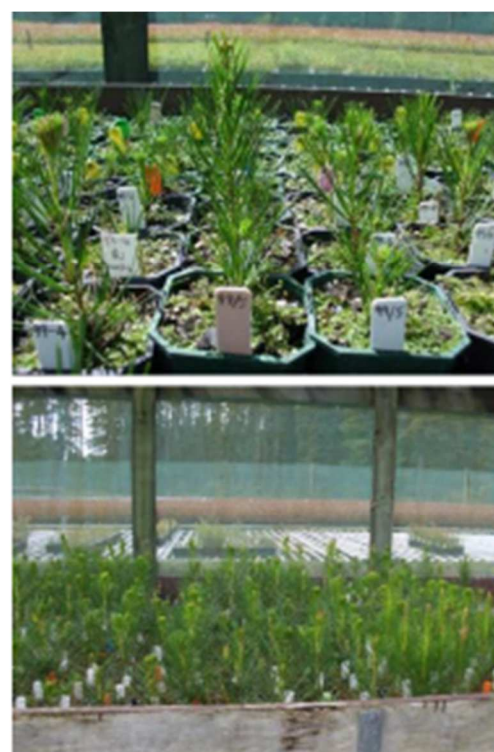
Figure 3. Shoots derived from 10-year-old, field-grown selections to be used for seed orchard stock plants.

Somatic embryogenesis

There are many reasons why the SE technology rapidly became the favorite propagation tool for the development of multi-varietal forestry in New Zealand. Cryogenic storage while field testing took place was more effective and economic than cool storage. The amplification of embryogenic tissue seemed to be unlimited and the potential to use molecular markers for early screening very attractive. It should also be noted that SE is the primary enabling technology for genetic engineering (GE). Dale Smith was instrumental in developing protocols and media formulations for SE of *P. radiata* and facilitated the early transfer of this technology to the companies that preceded Arborgen and Timberlands in New Zealand (Smith 1996, Smith et al. 1994 and Smith 1997). Another positive attribute for SE with *P. radiata* in New Zealand was that it could be successfully integrated with organogenesis and stool bed production (**Fig. 4**).

However, industrial support for SE technologies was at first reluctant. The difficulties of initiation of cell lines from all CP crosses was discouraging and often there was poor representation of cell lines. These problems caused a resistance to SE and explain why SE and the other technologies of adventitious x organogenesis and meristem cryopreservation discussed earlier, were all pursued for so much longer in New Zealand. In the end, the RPBC supported significant research into improving initiation with their breeding populations and this resulted in high cross and cell-line capture (Hargreaves et al. 2009, 2011).

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The SE methodologies are still not without challenges. Problems with weakness in the root collar region of somatic embryos, especially in some clones, means that germinants cannot be used directly as stock plants for risk that they will blow over in the nursery bed. Controlling water in the in-vitro ex-vitro environments is a precise art. Aging via cell division/subculture frequency, exposure to ABA, number of organogenic transfers pre-stoolbed establishment are all aspects that need to be monitored to avoid loss of somatic embryo maturation ability and overt physiological age in stock plants. Plastic and organic waste also needs to be managed. The potential for automation of culture via liquid culture and robotics is proving difficult with *P. radiata*. However, do not be discouraged, it may be that some combination of liquid culture for cell line proliferation, and in the short term, an in-vitro rooting protocol for organogenic shoots may further improve the economics of the process. It should be noted, Arborgen has a successful in-vitro rooting protocol for *P. radiata* shoots, unfortunately they are not sharing the technique.

Figure 4. Organogenic shoots set in the glasshouse, derived from SE via organogenesis.

Conclusions

The future of SE, being an integral part of production forestry in New Zealand, is assured. We now have extensive SE field trials, in excess of 10 years old and in several countries (**Fig. 5**). These trials have had a positive impact on forest grower's perceptions of SE, as they can now see the trees and order cutting stock of them for their replanting programs. The Timberlands estate (198,000 ha) already has upwards of 25% clonal material in their current planting program and intends that 25-33% of the estate will be dedicated to

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clonal forestry. Arborgen has purchased Edendale nursery (in the south of the South Island) and hybrid pines derived from SE are likely to be added to varieties for sale in NZ (Hargreaves et al. 2016). This is part of their future strategy with demand for cutting stock already high and more land likely to be available for afforestation resulting from poor economic returns from its agricultural use. Scion has a continuing strong research commitment to both genetic engineering and the implementation of molecular marker aided breeding, SE.

Figure 5. Timberlands/Forest Genetics clonal trials (5-7 years), plants produced via cuttings taken from SE stock (Cathy Hargreaves, Fabricio Biernaski and Mike Carson in the figures).

The enduring friendships created over the frustrations and triumphs of tissue culture research, provide joy in my life today. If I have to close with any message for you all, I wish I had published more!

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Integration of selection, breeding, somatic embryogenesis and cryostorage to conserve and restore threatened North American forest trees

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The consequences of global climate change, as well as globalization of trade, include rapid increases in the number and scale of menaces to North American forest trees from insect pests and pathogens, loss of habitat and other stresses. These pressures make critical the application of biotechnological approaches to supplement conventional programs aimed at conserving germplasm of threatened forest species and restoring forests. *In vitro* propagation, in particular somatic embryogenesis (SE), could make major contributions to forest tree conservation and restoration programs. First, embryogenic cultures of forest trees can be readily cryostored, with high recovery rates. Thus, the genetic diversity of threatened species populations could be conserved for decades by cryostoring embryogenic cultures initiated from these trees, using minimal space and low levels of maintenance. Second, because embryogenic cultures make excellent target material for gene transfer, candidate genes that may confer resistance or tolerance to pests or pathogens can be tested in transgenic trees, potentially leading to deployment of trees engineered with the most effective protective genes or combinations of them. However, even if such trees are produced, other factors, such as the current regulatory environment and public opinion in the U.S. and elsewhere, may delay release for deployment by decades. The uncertainty associated with deployment of transgenic trees raises the potential of a third route by which somatic embryogenesis can make an immediate, rapid contribution to species restoration by combining its powerful mass clonal propagation capacity with traditional selection and breeding. Summarized below are three cases of North American forest trees under threat from exotic pests or pathogens and descriptions of how we are collaborating with tree selectors and breeders to attempt to generate resistant or tolerant varieties of these trees that can be rapidly propagated for species restoration.

American chestnut (*Castanea dentata*), once a dominant component of eastern U.S. forests, was devastated by the accidental introduction of the chestnut blight fungus (*Cryphonectria parasitica*) from Asia in the early 20th century. In collaboration with the American Chestnut Cooperators Foundation (ACCF), we have employed the SE protocol we developed for American chestnut (Andrade and Merkle 2005) to conserve large surviving American (LSA) chestnut trees and to propagate germplasm derived from crosses between the LSAs, so that clones can be tested for chestnut blight resistance. We have already produced somatic seedlings from clones derived from crosses between the LSAs "Ragged Mountain" and "Thompson", and these trees have been planted out on two National Forests in Virginia by ACCF collaborators (**Fig. 1A**). Similarly, we have collaborated with The American Chestnut Foundation (TACF) to use SE to implement clonal testing of conventionally bred material from their hybrid backcross breeding program for resistance to chestnut blight. Because TACF's Backcross 3-F3 (BC3F3) material has, on average, 15/16 American chestnut genes and 1/16 Chinese chestnut genes, we were unsure if our protocol, developed for pure American chestnut, would be effective with hybrid material. In fact, previous attempts by us to initiate

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embryogenic cultures of pure Chinese chestnut, using our American chestnut protocol, had all failed. However, culture initiations from thousands of open-pollinated and control-pollinated BC3F3 seeds from BC3F2 seed orchard parents showed no significant difference in SE induction percentage from that of pure American chestnut explants (Holtz et al. 2017). The first BC3F3 somatic seedlings (**Fig. 1B**) have already been deployed in clonal field tests. The American chestnut SE protocol also induced somatic embryogenesis from Backcross 2 material (7/8 American chestnut genes and 1/8 Chinese chestnut genes), but failed to induce repetitively embryogenic cultures from Backcross 1 or F1 hybrids, which have higher proportions of Chinese chestnut genes (Holtz et al. 2017).

Figure 1. A. Somatic seedling derived from culture initiated from cross between two large surviving American chestnut parents, three years following out-planting on national forest land. B. BC3F3 hybrid backcross chestnut somatic seedlings growing in the shade house.

Eastern hemlock (*Tsuga canadensis*), a member of the Pinaceae, is a keystone species that shades mountain streams in the Appalachian Mountains of the Eastern U.S. Carolina hemlock (*Tsuga caroliniana*) is a relatively rare tree found on rocky outcrops in the southern Appalachians. Both species are under threat of extinction by the exotic insect pest, hemlock woolly adelgid (HWA; *Adelges tsugae*). We are working with breeders at North Carolina State University and the Forest Restoration Alliance to conserve and restore eastern and Carolina hemlocks, using SE to clonally propagate putatively HWA-resistant eastern hemlocks and hybrids between Carolina hemlock and HWA-resistant Asian hemlock species. The hemlock SE protocol we developed for eastern and Carolina hemlocks is similar to ones reported for other Pinaceae species (Merkle et al. 2014). Using this protocol, embryogenic cultures were initiated from seeds derived from crosses between Carolina hemlock and Chinese hemlock (*Tsuga chinensis*) and between Carolina hemlock and southern Japanese hemlock (*Tsuga sieboldii*). Putative hybrid somatic seedlings were produced from the hybrid hemlock cultures and are currently being grown in a nursery by NC State University collaborators in preparation for screening for resistance or tolerance to HWA infestation (**Fig. 2**).

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Figure 2. Hybrid hemlock (*Tsuga caroliniana* x *Tsuga chinensis*) somatic seedlings growing in the greenhouse in preparation for screening for resistance to hemlock woolly adelgid.

Embryogenic cultures were also started from open-pollinated seeds collected from eastern hemlocks growing in the "bulletproof stand", a small population of eastern hemlocks in New Jersey that appears to be largely unaffected by HWA infestation, although all other eastern hemlock trees around them have been killed by HWA.

White ash (*Fraxinus americana*) and green ash (*Fraxinus pennsylvanica*) are valuable forest and landscape trees native to the eastern U.S. White ash wood, in particular, is highly valued for furniture and tool handles, and is the wood of choice for baseball bats. Ash populations in the U.S. and Canada have been devastated by emerald ash borer (EAB; *Agrilus planipennis*), an insect pest introduced from Asia, which has killed millions of trees in the past decade. Some native ash trees appear to possess natural resistance to EAB. In Michigan, white ash trees that have survived EAB infestation that has killed surrounding trees are known as "lingering ash." As part of the effort to conserve and restore ash populations that are being devastated by EAB, we have produced embryogenic cultures of both green ash and white ash. We first developed the SE protocol for ash using immature seeds collected from local Athens, Georgia green ash trees, since EAB had not yet reached Georgia. The same induction-maintenance medium (IMM; Andrade and Merkle 2005) that we use for American chestnut worked very well with green ash, generating cultures with massive somatic embryo production capacity (Li et a. 2014). Then, we used the green ash protocol to initiate embryogenic cultures from seeds collected from multiple lingering white ash parents in Michigan by Ohio

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State University collaborators. There were no controlled pollinations performed between lingering ash parents, but we reasoned that whatever the pollen source was, it must be another lingering ash parent (ash are dioecious), so we essentially allowed natural selection accomplish the breeding for resistance. Multiple lingering white ash embryogenic cultures (**Fig. 3A**) were initiated and somatic seedlings have been regenerated (**Fig. 3B**), some of which will be installed in a test planting at UGA in 2017.
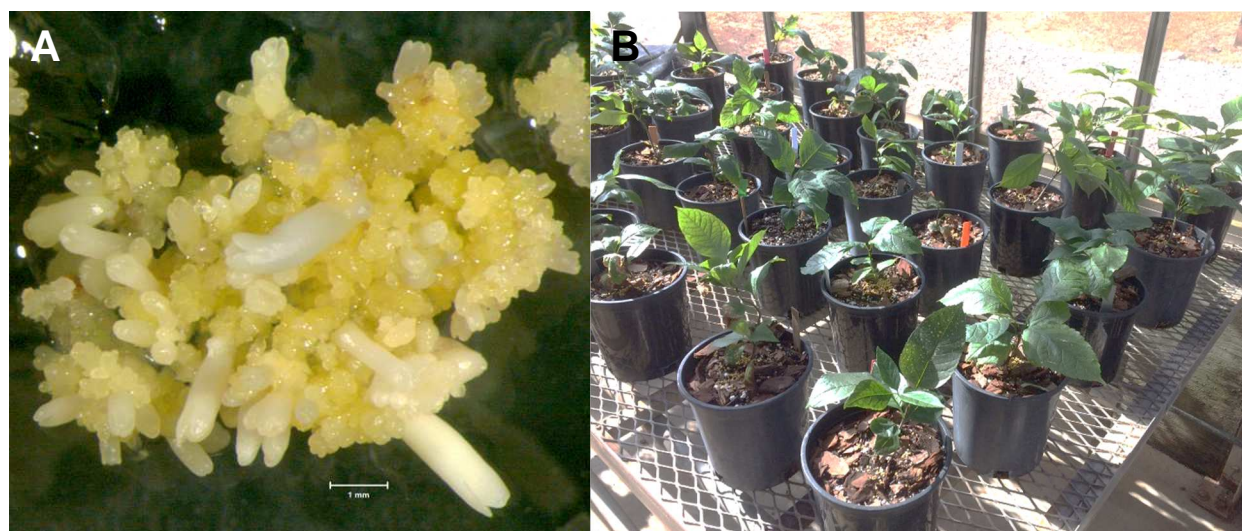
Figure 3. A. White ash somatic embryos derived from seed explant from a "lingering" ash parent. **B.** Lingering ash somatic seedlings in the greenhoues.

Chestnut, hemlock and ash embryogenic cultures have been successfully cryostored and recovered using a standard protocol (Merkle et al. 2014). Thus, individual clones that show promise in field tests for pest or pathogen resistance, as well as other desirable traits, can be recovered from cryostorage and production of somatic seedlings from those genotypes can be scaled-up for restoration plantings. Integration of selection and breeding programs with *in vitro* culture tools like SE and cryostorage creates a powerful combination to aid in conserving germplasm and restoring threatened trees to the forest.

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In vitro biotechnology of *Melia volkensii*, a high potential forestry tree from eastern Africa

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Keywords: *Melia*, reforestation, Mahogany, *in vitro* culture

Introduction

Melia volkensii belongs to the Meliaceae family. It is endemic to the semi-arid and arid areas of Kenya, Somalia and Tanzania. Its natural range spreads from dry bush land to wooded grassland, situated between 400 and 1600 metres above sea level. Remarkably, it combines drought resistance with fast growth. The main purpose of developing new plantations is for production of termite-resistant mahogany-like timber to be harvested after 15-20 years. Furthermore, its leaves and seeds contain medicinal and insecticidal compounds which can supply an additional income to the farmers. The species has been overexploited and both habitat fragmentation and loss of wild elite trees have been reported (Runo et al. 2004). Even though its local importance, *M. volkensii* is a challenging tree to propagate. The fragile seeds shelter in a woody endocarp which is difficult to open without damaging the fragile seeds. The seeds germinate poorly (Stewart and Blomley 1994) and despite all efforts, seedling production is not able to satisfy demand. Therefore, during the last decade, plant tissue culture tools have been explored for propagation, somatic embryogenesis and adventitious shoot and root induction.

In vitro culture initiation

Seedlings

After cracking the endocarp, fresh seeds with an intact seed coat are rinsed in 70% ethanol, sterilized for 15 min in a 10% NaOCl solution with 0.005% detergent (teepol), and subsequently rinsed three times in sterile distilled water. Usually it's enough to remove the seed coat at the micropylar end but often longitudinal incisions are made through the seed coat, as a scarification treatment. The seeds start to germinate within one week on MS medium (Murashige and Skoog 1962) supplemented with 20 g l^{-1} sucrose at pH 5.4 (**Fig. 1**). Each cotyledon has an axillary meristem that produces extra shoots when the main shoot axis is damaged. Without cotyledons, the development of the seedling is very poor. About two weeks after germination, the seedling can be subdivided into nodes to start micropropagation.

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Figure 1. *M. volkensii* seedlings, 14 days after sowing *in vitro*.

Plus trees

Shoots from plus trees are grafted on seedlings and grown under hygienic conditions or forced from branches (**Fig. 2**). When good growth is achieved, the shoots are prepared for *in vitro* culture initiation by removing the leaves while remaining a stub of the petiole. Then the shoots are rinsed in 70% ethanol, sterilized for 5 min in a 0.5% $HgCl_2$ solution and then 15 min in a 10% NaOCl solution (both with 0.005% Tween 20) and subsequently rinsed three times in sterile distilled water.

Figure 2. New shoots are forced from branches of elite trees.

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Micropropagation

Although *Melia volkensii* can be propagated on MS medium supplemented with BA, more shoots of higher quality are produced with the topolin derivatives MemTR and mTR (Olchemim.cz) (unpublished results). Excessive curling of the leaves can be avoided by using containers with filter lids. A better general quality of the shoots is obtained in this way. Well-closed polypropylene Microbox ® (Saco2, Belgium) with or without integrated filter of different pore size (L, XXL or XXL+) where compared in this regard. The medium was MS supplemented with 20 g l^{-1} sucrose and 10 µM mTR. The largest leaf surface area was obtained when a filter was present with no effect of the filter pore size (**Fig. 3**). Without aeration of the headspace, the plant growth is slow and plants developed curly leaves as well as white calluses on the stem.
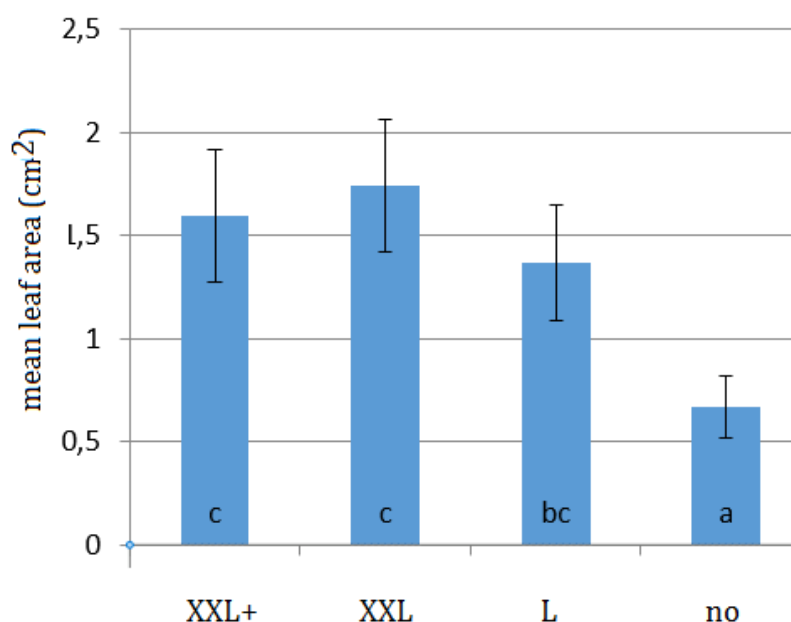
Figure 3. Effect of integrated filter on mean leaf area (cm^2). Filter pore size is decreasing from 'XXL+' to 'XXL' and 'L'. Bars: standard deviation. Significant differences between means (Tuckey's tests, 95%) are indicated by different letters.

Callus induction

Melia is known to easily develop calluses *in vitro*. Mulanda (2016) succeeded in inducing callus on carpels, leaves, petioles/rachis, mature zygotic embryos, root segments and seedling epicotyls on several media including i) MS medium supplemented with 0 – 8 mg l^{-1} BAP combined with 0.05 - 0.4 mg l^{-1} of either 2,4-D or NAA, or ii) MS medium supplemented with 0 – 1 mg/l TDZ with or without 0.2 mg l^{-1} 2,4-D, and B5 medium supplemented with 2 mg l^{-1} kinetin.

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Somatic embryogenesis

Indieka et al. (2007) regenerated somatic embryos on cotyledons derived from fresh, immature seeds. After 4 weeks *in vitro* culture on MS medium with 0.5 mg l^{-1} BA and 0.2 mg l^{-1} 2,4-D, up to 60% of the cotyledonary explants initiated embryogenic cultures. In this work, we confirmed these results. Direct somatic embryogenesis after 5 weeks induction is illustrated in **Fig. 4**. The somatic embryos germinate easily on hormone free MS medium (**Fig. 5**).
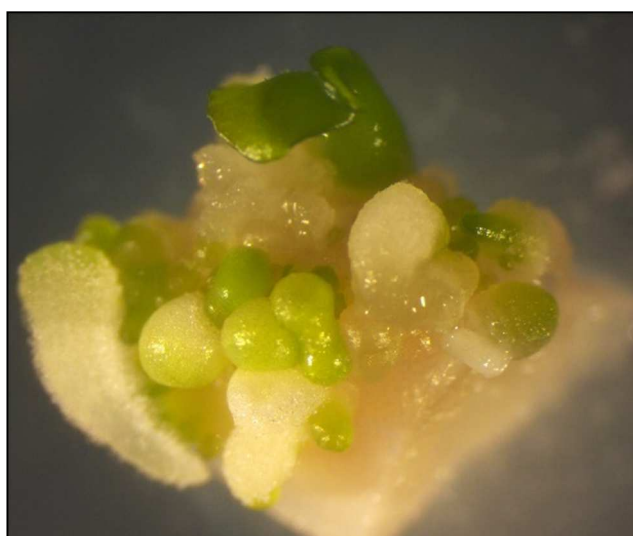
Figure 4. Somatic embryos developing on an isolated cotyledon from *M. volkensii* seedling.

Figure 5. Germinated somatic embryo of *M. volkensii* developing on MS medium without plant growth regulator.

Somatic embryogenesis was also induced by Mulanda et al. (2012) on zygotic embryos excised from mature seeds using the B5 medium (Gamborg et al. 1968) supplemented with MS vitamins, 20 g l^{-1} sucrose and 0.05 mg l^{-1} – 4 mg l^{-1} TDZ. After 21 days, callus masses were subcultured to hormone-free B5 medium. Multiple somatic embryos are initiated within 14 days and further developed into shoots. Shoot elongation was achieved when transferred to ½ MS medium supplemented with 0.1 mg l^{-1} BA and 10% coconut water.

Since the performance of seedlings is unknown, efforts should be made to obtain somatic embryos derived from explants of selected, elite trees.

Adventitious shoot regeneration from leaf explants of elite trees

Werbrouck (2017) suceesfully induced adventitious shoots on leaf explants with 10 µM INCYDE-Cl, INCYDE–F, TDZ, 2iP or CCPU. TDZ and 2iP were however not very effective compared to INCYDE-Cl, INCYDE–F and CPPU. The well-known synthetic phenyl urea cytokinins CPPU and TDZ are inhibitors of cytokinin oxidases/dehydrogenases (CKX) (Chatfield and Armstrong 1986; Laloue and Fox 1989). But INCYDE-Cl or –F and CKX are also involved in the catabolism of endogenous cytokinins such as Z and 2iP by oxidative removal of their side chain (Zatloukal et al. 2008). Remarkably, the INCYDEs could awaken dormant meristems in the secondary axils of the *M. volkensii* composite leaves (**Fig. 6**).

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Figure 6. Adventitious shoots developing on leaf explants from elite trees of *M. volkensii*.

In vitro rooting

Rooting of *in vitro* shoots is not self-evident. When 0.1-1 mg l^{-1} IBA was added to ½ MS medium, the stem bases reacted by producing abundant callus but no roots (**Fig. 7**). On a hormone free ½ MS medium, only 10% of the shoots produced adventitious roots. But at least they grow out of the stem base, with minimal callus formation (**Fig. 8**).
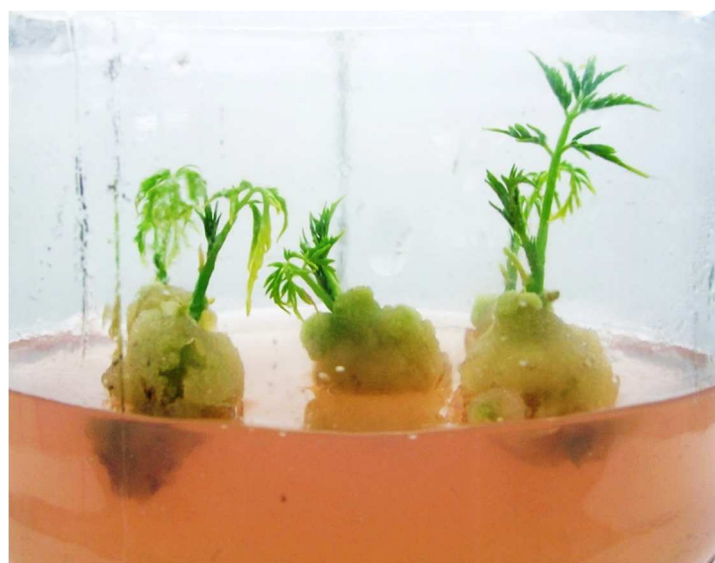
Figure 7. Excessive callus formation on MS medium with 2 mg/l IBA.

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Moreover, the leaves turned yellowish and easily dropped. Pulse treatments with high IBA concentrations were also tested. When the *in vitro* shoots were placed in a semi solid ½ MS medium with 200 mg l^{-1} IBA for 4 h, and afterwards were transferred to the same hormone free medium, 56% of the shoots rooted (unpublished results). Often the roots grow very fast, and apparently at the expense of the shoot. Shoots are very brittle and separated from the shoot vascular system by a large callus mass (**Fig. 9**). Such a root system is not functional during acclimatization.

Figure 8. Normal roots on hormone free medium.

Figure 9. Abnormal root and shoot after pulse treatment with IBA.

Acclimatization

Since *in vitro* roots show a shallower adventitious root system than seedlings (Mulatya et al. 2002), it can be expected that roots of *in vitro*-derived plantlets should be pruned in such a way that orthotropic root growth is stimulated. Three different main roots are shown in **Fig. 10**: a hook-shaped root, split root and tap root. Only the straight tap root promises a good tree anchorage. All types show the typical thickened morphology, illustrating the storage function of the root.

After root development, acclimatization was undertaken at KEFRI in a growth chamber set at 30°C. The rooted shoots were transferred into sterile sand covered with a polythene tube and were then kept in growth chambers for 30 days (**Fig. 11**). The plantlets were then transferred to coconut peat in a greenhouse and allowed to grow for about 30 more days to obtain fully acclimatized plants (**Fig. 12**). Overall, about 25% of the plantlets converted into plants of sufficient quality for field plantation.

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Figure 11. Plantlets acclimatizing in sterile sand

Figure 10. Different morphology of the main root of *in vitro plants*: hook-shaped root (left), split root (center) and tap root (right).

Fig. 12 Fully acclimatized plants at KEFRI

Conclusion

Because of its precious termite-resistant timber and fast growth under harsh semi-arid conditions, there is a high demand for *Melia volkensii* plants. Current research is focusing on translating the gathered knowledge to commercial micropropagation of mature elite trees and to develop a robust rooting and acclimatization protocol. This will open up opportunities for planting massive multiclonal forests that could somewhat change the view of the savannahs.

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Propagation of ornamental forms of Norway spruce (*Picea abies* L. Karst) using rooted cuttings and chip-budding

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Keywords: vegetative propagation, grafting, landscaping

Among the normal trees in our forests there are, as rare whims of nature, a variety of peculiar tree forms: spruces with globular crown, golden and red needle trees, and narrow-crowned, weeping spruces (**Fig. 1**). The phenomenon is caused by a mutation, i.e., a change in genetic material. The change in genetic code can take place in gametes prior to fertilization, or in vegetative buds.

Some of the genetically changed forms of trees are very decorative and can be utilized as ornamental trees. The utilization of special ornamental tree forms usually requires vegetative propagation. In this way, the traits of the parent tree are passed on as such to the cloned offspring. Methods used, include grafting, rooted cuttings and tissue culture.

In this work both bud grafting and rooting of cuttings methods are applied for various ornamental forms of Norway spruce (*Picea abies* L. Karst). Preliminary results showing differences in propagation success between the methods, and among forms and clones are presented.

Figure 1. Three different ornamental forms of Norway spruce: golden spruce (normal growth, yellow needles), globular spruce (reduced growth, dense branching) and globular red needle spruce (reduced growth, dense branching, red needles).

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Bud grafting (chip-budding)

Grafting is the most utilized method when the special tree forms, found in the nature, have been propagated to clonal archives or arboretums. However, in grafting there are different kinds of methods, and there is potential for improvement. Scion grafting (either as side or slice grafts) is in most cases an applicable method, but is work and material consuming. For bud grafting (i.e., grafting of adventitious buds with a chip of wood left attached = chip budding) less material is needed. In some cases bud grafting could be a suitable method for ornamental spruce forms as well (**Fig. 2**). In bud grafting a chip of wood is excised from the rootstock stem and replaced by a piece of donor plant stem with a bud. If grafting has succeeded the bud starts to grow.

Figure 2. In the middle picture a bud grafted on the 20th of August 2015, and at the right the one grafted on the 13th of May 2015. Both of them were photographed on the 10th of May 2016.
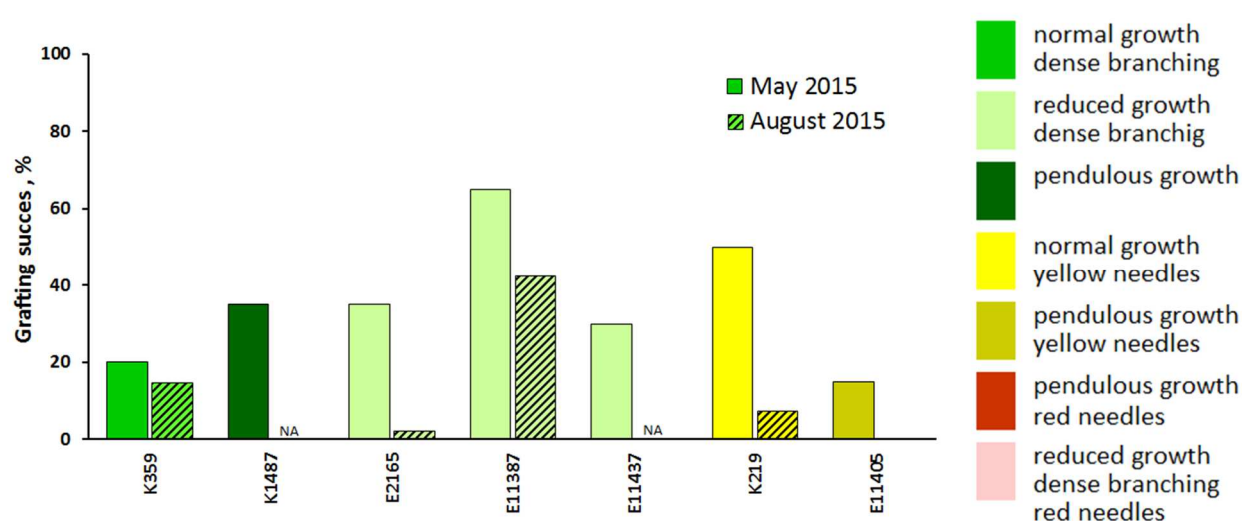

Figure 3. Grafting success in different clones of Norway spruce when bud grafting was made in the spring and late summer. Different ornamental forms are marked in different colors shown on the right side of the figure.

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The applicability of bud grafting for propagation of ornamental forms of spruce was tested using 7 different clones, in two different season (**Fig. 3**). Grafting success was better in the spring. There were clear differences between the clones. Compared to scion grafting, the results were worse: grafting success for scions, made by a skilled expert in the spring is normally over 80 %, whereas overall success in bud grafting in our experiment was 36 %.

Cutting propagation

Cutting propagation can be applied in Norway spruce. Rooted cuttings have, however, not been used in practice for propagation of ornamental forms, at least not in our Northern conditions. At present, there is no cutting propagation method efficient enough for affordable production of ornamental forms. The rooting success is influenced by many factors: the taxa, donor age, timing of propagation, rooting substrates (Nikkanen et al. 2013).

In cutting propagation applied in spruce in Finland, shoots have been collected in winter and put into rooting media in early spring. In our experiments other methods, especially timing of propagation were also studied. Rooting experiments were started with shoots still growing in mid June, and with shoots already preparing for winter in late August (**Fig. 4**). For the present, the results of the rooting success are presented only for the generally applied method, i.e., dormant shoots collected in winter and put to substrate in March (**Fig. 5**).

Differences in rooting success of cuttings between different age and type of donor plants were clear. In addition, rooting success varied among the clones and forms tested. The rooting media did, however, not affect rooting in this experiment. The rooting success was above 90 % when young SE plants were used as donor plants (Tikkinen et al. 2017), while it was in most cases less than 20 % when using grafts in old collections and in a propagation garden as donor plants (**Fig. 5**). The lowest rooting success was found in the forms with reduced growth and dense branching.

Figure 4. Different methods of cutting propagation tested in Norway spruce. At the left there are shoots collected in late June and put into peat substrate immediately (photo taken two weeks later). In the middle shoots were collected in mid August and put into sand within two days (photo taken one week later). At the right shoots were collected in winter and rooting in peat substrate started in March (photo taken 14 weeks later).

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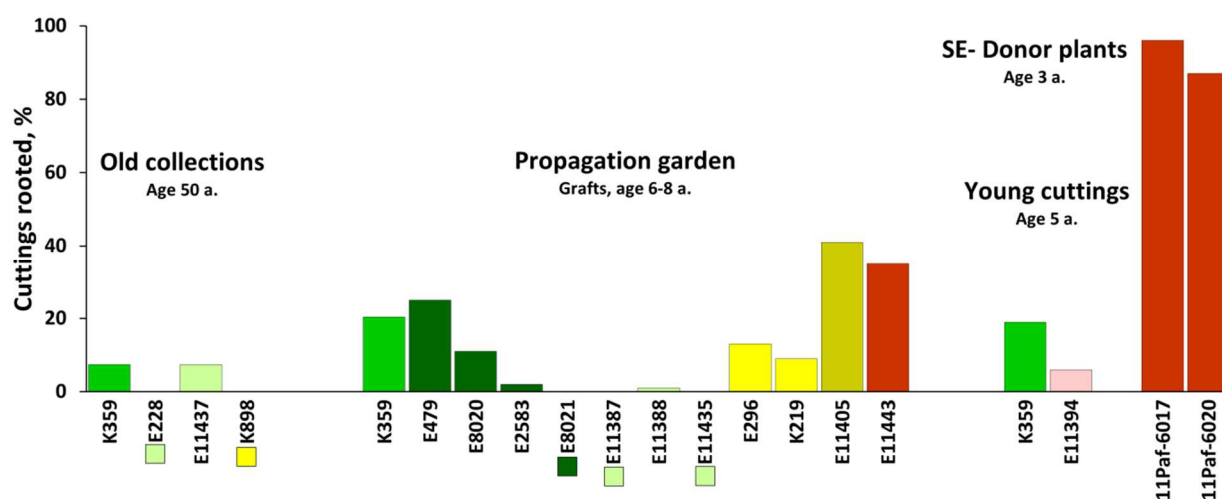
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Figure 5. Rooting success in different clones, collected from different propagation sources. Different ornamental forms are marked in different colors (see **Fig. 3**).

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Shoot proliferation and organogenesis of the strawberry tree (*Arbutus unedo* L.): physiological analysis under water stress

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Keywords: drought, organogenesis, shoot proliferation, physiology, trees

Strawberry tree (*Arbutus unedo* L.) is a small evergreen tree that grows spontaneously in several countries of the Mediterranean basin and Atlantic coast, including Portugal (Piotto et al. 2001). Due to its ability to grow on poor soils and regenerate after forest fires, it is a very important species in Mediterranean ecosystems by avoiding erosion and helping the recovery of marginal lands (Piotto et al. 2001; Quevedo et al. 2013). Strawberry tree is a very attractive ornamental and can be used for honey production (Tuberoso et al. 2010). The fruit is a spherical edible berry (**Fig. 1a**) that is commonly used in the manufacture of traditional products, such as jam and jelly. However, its main application is the production of an alcoholic distillate. Once considered a "Neglected or Underutilized Crop" (www.cropsforthefuture.org/), the demand for strawberry tree by producers and stakeholders has increased, and in order to make this species more attractive, an intensive propagation and breeding program is being carried out. Our group has been working in the improvement and breeding of strawberry tree using biotechnological tools (Martins and Canhoto 2014) and several micropropagation protocols have also been developed (Mereti et al. 2002; Gomes and Canhoto 2009; Gomes et al. 2010; Martins et al. 2015). These kinds of protocols have several advantages when compared to conventional methods. Nonetheless, the evaluation of quality and performance of the micropropagated plants is a key step.

Adequate plant quality, among other factors, is influenced by the propagation method and growing conditions up to the moment of planting. The evaluation of physiological performance is an essential procedure not just to validate the quality of the plants obtained *in vitro* but also to predict their performance under the sub-optimal growing conditions that can occur in the field. This is of prime importance in order to select the most stress- tolerant plants and to meet the demands of farmers, issues that we intent to restrict ourselves to with this work.

Within a changing climate context and because strawberry tree orchards are usually established on poor and dry soils, drought tolerance of the plants is particularly important. Considering the Mediterranean climate and the predicted climate change (Guiot and Cramer 2016), it is imperative to monitor, select and breed stress resistant plant stock material. We aim to test the influence of the propagation method and of the environmental growing conditions on strawberry tree performance, particularly under drought. For that, acclimatized *in vitro* plants produced by different micropropagation methods (shoot proliferation on solid and liquid medium and organogenesis) were submitted to different watering regimes (well-watered and water limited) and some morphological and physiological parameters were evaluated. Statistical significance of the results was assessed by two-way analysis of variance (ANOVA).

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A selected adult clone was established *in vitro* through the use of epicormic shoots that were micropropagated on a modified De Fossard medium (De Fossard et al. 1974) supplemented with 2 mg/L benzylaminopurine, by axillary shoot proliferation on solid medium (**Fig. 1b**) and by axillary shoot proliferation and organogenesis (adventitious shoot formation from calli) on liquid medium. No significant differences were found in the number of shoots obtained by shoot proliferation between solid (2.2±0.4 shoots/explant) and liquid medium (5.9±0.4 shoots/explant). However, the number of shoots obtained through organogenesis was substantially higher when compared to shoot proliferation (15.6±2.2 shoots/explant). Shoots were rooted with 2 mg/L indole-3-butyric acid, with rates higher than 80%, and were successfully acclimatized (**Fig. 1c**). Even though higher multiplication rates were achieved, hyperhydricity was observed on some of the shoots obtained on liquid medium, either by shoot proliferation or organogenesis, causing phenotypic differences as well as lower levels of chlorophyll (data not shown). Three months after acclimatization the size of the plants obtained through organogenesis was smaller, not only in height but also in leaf area, when compared to those obtained by shoot proliferation. Nevertheless, at this point plants have recovered normal levels of chlorophyll and no signs of hyperhydricity were observed.

Figure 1. (a) Adult strawberry tree with mature fruits. (b) Micropropagation of a clone of an adult strawberry tree by shoot proliferation on solid medium. (c) Three months old acclimatized strawberry tree plants.

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Six month-old plants obtained by the three methods of *in vitro* cloning were then submitted to two different water regimes: WW – well watered (watered to 70% field capacity every day) or WS – water stress (water withholding). After 10 days under these conditions plant growth was determined by measuring plant height and biomass. Other assessed physiological parameters included relative water content (RWC), concentration of chlorophyll a and b, effective (Φ_{PSII}) and maximum (F_v/F_m) quantum yield of photosystem II, CO_2 assimilation rates (A) and stomatal conductance (g_s). These were analyzed following the procedures described in Correia et al. (2014).

Results are available in **Tab. 1**. No significant differences were found in terms of plant biomass, plant height or chlorophyll a and b levels between plants submitted to different watering regimes. Moreover, most of the physiological parameters evaluated showed very similar responses under optimal conditions, under stress, and among the different cloning methods. The stress did not significantly affect photosystem II (F_v/F_m and Φ_{PSII}), neither were marked differences found on relative water content (RWC). Stomatal conductance (g_s), on the other hand, showed a reduction in water limited plants.

Table 1: Morphological and physiological parameters evaluated on acclimatized, micropropagated strawberry tree plants under two different water regimes. Values (mean ± SD) with different letters have significant statistical differences (*p<0.05*).

	Well watered			Water stress		
	SM	LM	LMO	SM	LM	LMO
Plant height (cm)	20.2±1.9a	19.3±3.1a	17.1±3.8a	20.5±0.9a	22.6±3.0a	19.2±1.2 a
Plant biomass (g)	1.53±0.36a,b	1.48±0.16a,b	0.88±0.17b	1.33±0.27a,b	1.73±0.31a	1.19±0.45a,b
RWC (%)	87.25±4.89a	83.94±3.90a,b	85.61±4.66	81.32±3.44a,b	76.88±8.50b	80.59±5.87a,b
Chlorophyll a (μmol mg^{-1})	0.0135±0.0017a	0.0110±0.0033a	0.0105±0.0028a	0.0122±0.0017a	0.0116±0.0018a	0.0134±0.0026a
Chlorophyll b (μmol mg^{-1})	0.0064±0.0009a	0.0054±0,0015a	0.0049±0.0013a	0.0057±0.0007a	0.0054±0.0008a	0.0062±0.0013a
φ PSII	0.644±0.042b	0.706±0.024a	0.660±0.034b	0.706±0.025a	0.713±0.016a	0.697±0.021a,b
Fv/Fm	0.813±0.011a	0.810±0.008a	0.793±0.024a	0.802±0.005a	0.801±0.009a	0.783±0.012a
gs (mol H$_2$O m^{-2} s^{-1})	0.083±0.010a	0.084±0.040a	0.053±0.040a,b	0.030±0.020b	0.028±0.010b	0.027±0.020b

SM: axilary shot proliferation on solid medium, LM: axilary shot proliferation on liquid medium, LMO: liquid medium organogenesis, RWC: relative water content, Φ_{PSII}: effective quantum yield of photosystem II; F_v/F_m: maximum quantum yield of photosystem II, gs: stomatal conductance.

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The reduction in growth reported for other assays (Ogaya et al. 2003; Ogaya and Peñuelas 2004; Vasques et al. 2013) was not observed in this work probably due to the time required for the plants to activate the complex mechanisms involved in the response to drought stress (Chaves 2002; Chambel et al. 2005). The physiological parameters evaluated were in line with those described in previous reports (Castell and Tarradas 1994; Vasques et al. 2013), and the lower stomatal conductance has also been reported elsewhere as part of the strategy used by the strawberry tree (Castell and Terradas 1994; Gratani and Varone 2004; Navarro-García et al. 2011), a typical sclerophyllous species, in order to cope with drought stress (Chaves 2002).

Overall, the micropropagation of strawberry tree on liquid medium was the most efficient and cost effective, and the response of the acclimatized micropropagated plants under drought stress was very similar among plants obtained by the different propagation methods used. This indicates that the different micropropagation methods employed do not induce any particular abnormality that may alter the way plant cope with water deficit conditions, and foresees their potential performance and productivity under sub-optimal field conditions.

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In vitro propagation of mangroves for greenbelt development to mitigate climate change

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Keywords: mangroves, *in vitro* propagation, greenbelt model, carbon sinks, climate change

Introduction

Global warming is mainly caused by the accumulation of greenhouse gases in the atmosphere. Anthropogenic activities such as fossil-fuel combustion, cement production and land-use/land-cover results in the emission of greenhouse gases such as Carbon dioxide (76%), Methane (13%), Nitrous oxide (6%) and Fluorocarbons (5%). The increase in greenhouse gases (GHGs) in the atmosphere is recognized to contribute to global climate change (IPCC, 2001). The inexorable rise of Earth's temperature is expected to increase biodiversity loss, sea level rise, land use and land cover changes, notable shifts in ecosystem and alter global biogeochemical cycles all of which can significantly impact human and natural systems (McCarthy et al*.,* 2001; Parry et al*.,* 2007; Greene & Pershing, 2007). Mangrove trees act as sinks, which concentrate pollutants such as sewage, toxic minerals, carbon dioxide, pesticides, herbicides, etc. They sequester much more carbon per unit area than even tropical rain forests. Mangroves are salt tolerant trees found mainly in the tropical and subtropical intertidal regions of the world. They are viviparous, hermaphroditic and pollinated by animals. Mangrove forests currently occupy 14,650,000 ha^{-1} of coastline globally. Mangroves play an important role in ecosystem functioning and are economically important (Kathiresan and Qasim, 2005). They protect coastal areas by maintaining the marine food chain, they preserve water quality, provide habitat for fish and birds, prevent erosion and maintain the health of coral reefs. Currently they are under pressure due to various reasons such as increasing temperature, tidal mixing, coastal current changes, human activities like conversion to aquaculture or agriculture, and release of effluents and sewage. Therefore, conservation of mangroves is urgent inorder to protect the world's most productive mangrove forest ecosystems. Greenbelt development around the coastal area is helpful to mitigate climate change, and will protect the coastal ecosystem and conserve mangrove species. The aim of the present study is to induce development of callus from root explants using different growth regulators. Afterwards, the *in vitro* grown plants will be used in the development of a greenbelt model around the coastlines and riverbanks.

Materials and Methods

Sample collection

The mangrove species selected for the study were *Acanthus ilicifolius*, *Callophyllum inophyllum* and *Excoecaria agallocha.* These three species have bioactive compounds with various medicinal properties.

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Fresh mangrove roots were collected from the Pichavaram mangrove forest, Tamil Nadu, India and the species was scientifically identified.

Sterilization of glassware

All the glassware were washed thoroughly with detergent (2% Teepol) and rinsed with double distilled water twice and dried in an oven at 30ºC. The distilled water and other accessories were autoclaved and they were kept in an oven until use.

Preparation of MS medium

For the preparation of the basal MS medium, separate stock solutions of macro nutrients, micro nutrients, iron supplements, vitamins and boric acid were prepared and stored in the refrigerator. Individual growth regulators such as BA, kinetin, NAA, IAA, IBA and 2,4-D were also prepared and kept at 4 ± 1ºC. From the stock solution working standard media was prepared.

Sterilization and transfer of explants

The root explants were surface sterilized with a detergent solution (2% Teepol) for 5 min, 0.1% mercuric chloride for 1.5 min followed by 70% ethanol for 45 seconds and then with autoclaved double distilled water. After sterilization, all the explants were aseptically transferred to the MS medium supplemented with various concentrations and combinations of cytokinins and auxins, 3.0% sucrose and 0.8% agar.

Results

Under *in vitro* conditions the root callus was raised in MS medium supplemented with different concentrations and combinations of auxin and cytokinins such as BA, kinetin, NAA, IAA, IBA and 2,4-D.

The presence of phytohormones at different concentrations and combinations in MS medium improved the development of callus (**Fig. 1**). Various concentrations of phytohormones were used to find out the optimal concentration which supports the callus development. Using NAA and IAA alone in the medium was found to be less effective for induction of *in vitro* root callus than the use of 2,4-D (**Tab. 1**). The MS medium with 2,4-D shows maximum callus growth has been achieved with 0.3 mg/L and 0.5 mg/L concentration in *Acanthus ilicifolius and Callophylum inophylum.* Whereas the combination of medium with these auxins and 2 different cytokinins was most effective for the development of *in-vitro* root callus.

Roots and shoots were regenerated from the callus in the presence of auxin and cytokinin. Afterwards the plantlets were transferred to the greenhouse for acclimatization and survival and then subjected to the field. The *in vitro* grown plants were used for the development of a greenbelt model around the coastlines and riverbanks for protection from typhoons, erosion, tidal surges, cyclones and geomorphic erosion.

The combination of 0.5 mg/L NAA and 0.5 mg/L kinetin showed maximum response in *Acanthus ilicifolius.* The combination of 0.5 mg/L NAA and 0.5 mg/L BAP achieved maximum response in *Acanthus ilicifolius*. Whereas the combination of 0.5 mg/L IAA and 0.3 mg/L BAP showed maximum response in *Callophylum inophylum.* The present study revealed that the combination of 0.3 mg/L 2,4-D+0.5 mg/L KIN and 0.3 mg/L 2,4-D+0.5 mg/L BAP was more effective than all the other different concentrations and combinations of phytohormones for the induction of *in vitro* root callus for the three different species *Acanthus ilicifolius*, *Callophylum inophylum* , *Excoecaria agallocha* (**Tab. 1**).

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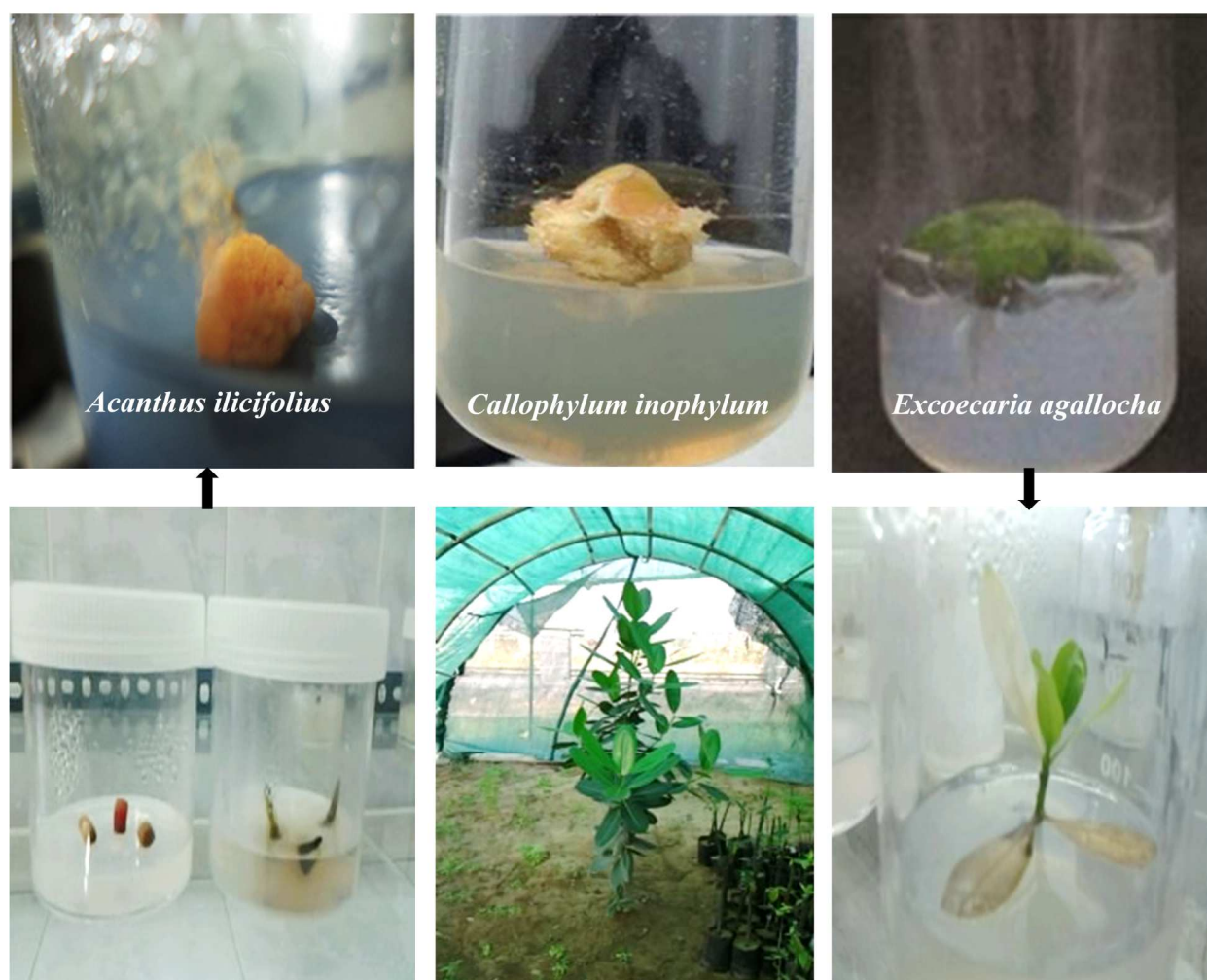
Figure 1: Development of root callus and plantlet of *Acanthus ilicifolius*, *Callophyllus inophyllum* and *Exocaria agallocha.*

Conclusion

Mangrove greenbelt development provides protection to the ecosystem by stabilizing sediments and nutrient rich run-off, by filtering freshwater discharge from land for coral reef growth, by enhancing the biomass of coral reef fish species, by providing nursery habitats between seagrass beds and by stimulating patch reefs that increase young fish survival. Also, mangroves have the ability to store more carbon faster, permanently and deep in the soil by their entangled root system. It will be helpful to mitigate climate change and environment.

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Table 1: Effect of different concentrations of auxins and cytokinins on root callus growth in *Acanthus ilicifolius*, *Callophyllus inophyllum* and *Exocaria agallocha*

Growth hormones (mg/l)	% of callus response in *A. Ilicifolius*	% of callus response in *C. inophyllum*	% of callus response in *E. agallocha*
NAA			
0.1	11±0.1	22±0.2	12±0.0
0.3	20±0.3	28±0.1	15±0.3
0.5	27±0.2	21±0.3	16±0.1
IAA			
0.1	10±0.1	14±0.1	21±0.2
0.3	14±0.5	11±0.2	22±0.0
0.5	17±0.2	30±0.5	22±0.5
2,4-D			
0.1	25±0.2	29±0.4	31±0.2
0.3	48±0.1	31±0.0	33±0.4
0.5	69±0.7	47±0.6	35±0.3
NAA+KIN			
0.5+0.1	11±0.2	12±0.3	35±0.1
0.5+0.3	29±0.6	26±0.2	32±0.3
0.5+0.5	51±0.2	28±0.4	25±0.3
NAA+BAP			
0.5+0.1	21±0.8	19±0.1	29±0.5
0.5+0.3	37±0.7	30±0.2	23±0.0
0.5+0.5	52±0.5	31±0.5	25±0.7
IAA+KIN			
0.5+0.1	0.7±0.3	15±0.1	10±0.1
0.5+0.3	12±0.2	19±0.1	10±0.6
0.5+0.5	21±0.2	17±0.4	11±0.1
2,4-D+KIN			
0.3+0.1	20±0.2	17±0.6	34±0.4
0.3+0.3	49±0.0	43±0.9	33±0.2
0.3+0.5	70±0.8	51±0.1	36±0.3
2,4-D+BAP			
0.3+0.1	39±0.3	31±0.3	44±0.3
0.3+0.3	57±0.1	59±0.2	47±0.0
0.3+0.5	89±0.6	81±0.0	58±0.6

Note: data (mean frequency ± standard deviation) are expressed as fresh weight of callus; 50 explants were taken for each experiment. Each experiment was repeated five times.

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Seedlings production of *Toona ciliata* by serial minicutting

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Keywords: Red cedar; vegetative propagation; reinvigoration

The Red cedar (*Toona ciliata*) presents no difficulty in rooting when propagated by minicuttings, but the root system has fewer first order roots and shorter length of roots than seminal seedlings. Thus, this study aimed to evaluate the quality of rooted minicuttings and seedlings produced by serial minicutting and by field rescue cultivation, using adult strains of three *Toona ciliata* clones (TC3, TC9 and TC15) in the field.

The minigarden was established in a suspended seedbed with clonal seedlings produced from rescued sprouts obtained from strains of three matrices that suffered a shallow cut (field rescue); with seedlings from clonal minigarden minicuttings previously established and marked as subculture 1 (SUB1); and with seedlings multiplied by serial minicutting of SUB1 rooted minicuttings, marked as subculture 2 (SUB2). Successive sprouts were collected from the clonal minigarden at 30-day intervals. Thirty days after staking, the minicuttings were evaluated for rooting percentage, number of first and second order roots, and total root length. Basal fragments were collected from minicuttings of TC9 and TC15 clones for anatomical characterization. After 105 days of staking, the seedlings were evaluated for survival rate, height, diameter at lap height, leaf area, shoot dry mass, number and length of first roots, total root length, roots dry mass and Dickson quality index. The root system obtained for clonal seedlings after 30 and 105 days is illustrated in **Fig. 1**.

Based on cross sections of the cutting's base of TC9 and TC15 after the different forms of cultivation (SUB1, SUB2 and field rescue), it was found that both clones had transitioned from primary to secondary growth. The results verified that SUB1 and SUB2 resulted in a sheath around the phloem composed of undifferentiated cells, but because these tissues did not mature, it's not possible to say whether this sheath prevents root development or not. Subculture type did not increase the rooting of clones (**Tab. 1**). There was not difference in the number of first and second order roots and in root length between subculture type and between clones. The TC9 clone had a greater number of first order roots and total root length in SUB1 than in SUB2. No differences were observed for the other clones according to cultivation type (**Tab. 2**). In SUB 1 the TC15 clone produced clonal seedlings of better quality than TC3. The subculture process resulted in TC9 and TC15 clonal seedlings of better quality than those produced by direct field rescue (**Tab. 3**). Therefore, the response obtained with the two serial minicutting programs was the same with regard to the rooting of *Toona ciliata* clonal seedlings and their quality, however, there were differences between clones. There were no anatomical barriers to rooting of clones exposed to different subculture types.

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Figure 1. Root system of clonal seedlings produced by serial minicutting at 30 and 105 days after the staking.

Table 1. Rooting minicutting of *Toona ciliata* clones (TC3, TC9, TC15), 30 days after staking according to cultivation type (SUB1, SUB2 and rescued sprouts from field (RC)).

Cultivation type	Rooting (%)			
	TC3	TC9	TC15	Mean
SUB1	57.4 b B	58.3 b B	83.3 a A	66.3
SUB2	68.5 ab A	69.4 ab A	63.9 a A	67.3
RC	83.3 a A	83.3 a A	72.2 a A	79.6
Mean	69.7	70.4	73.1	
CV (%)	17.5			

Means followed by the same capital letter in rows and lower case letter in column do not differ by Tukey (5%)

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Table 2. Root number of first order (RNFO), total length of roots (TLR) and dry mass of the root system (DMRS) of *Toona ciliata* clonal seedlings 105 days after staking, according to cultivation type (SUB1, SUB2 and rescued sprouts from field (RC)).

Cultivation type		RNFO			
		TC3	TC9	TC15	Mean
SUB1		3.4 a B	7.3 a A	8.1 a A	6.4
SUB2		3.8 a AB	2.7 b B	5.2 a A	3.9
RC		5.5 a A	7.7 a A	5.8 a A	6.4
	Mean	4.3	5.9	6.4	
	CV (%)		14.93		
		TLR (cm)			
SUB1		816.7 a A	985.3 a A	1373.1 a A	1058.4
SUB2		809.5 a A	693.1 b B	1012.3 a A	838.3
RC		836.7 a A	559.8 ab A	770.5 a A	722.3
	Mean	820.9	746.1	1052.0	
	CV (%)		7.91		
		DMRS (g)			
SUB1		0.38 a B	0.51 a B	0.79 a A	0.56
SUB2		0.48 a A	0.41 a A	0.56 ab A	0.48
RC		0.49 a A	0.31 a A	0.43 b A	0.41
	Mean	0.45	0.41	0.59	
	CV (%)		22.35		

Means followed by the same capital letter in rows and lower case letter in column do not differ by Tukey (5%)

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Table 3. Dickson quality index (DQI) of *Toona ciliata* clonal seedlings 105 days after staking, according to cultivation type (SUB1, SUB2 and rescued sprouts from field (RC)).

Cultivation type	DQI			
	TC3	TC9	TC15	Mean
SUB1	0.34 a B	0.46 a AB	0.61 a A	0.47
SUB2	0.46 a A	0.42 ab A	0.51 ab A	0.46
RC	0.46 a A	0.29 b A	0.39 b A	0.38
Mean	0.41	0.39	0.51	
CV (%)		21.34		

Means followed by the same capital letter in rows and lower case in column do not differ by Tukey (5%)

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The effect of serial minicutting on productivity and morphological characteristics of *Toona ciliata* ministumps

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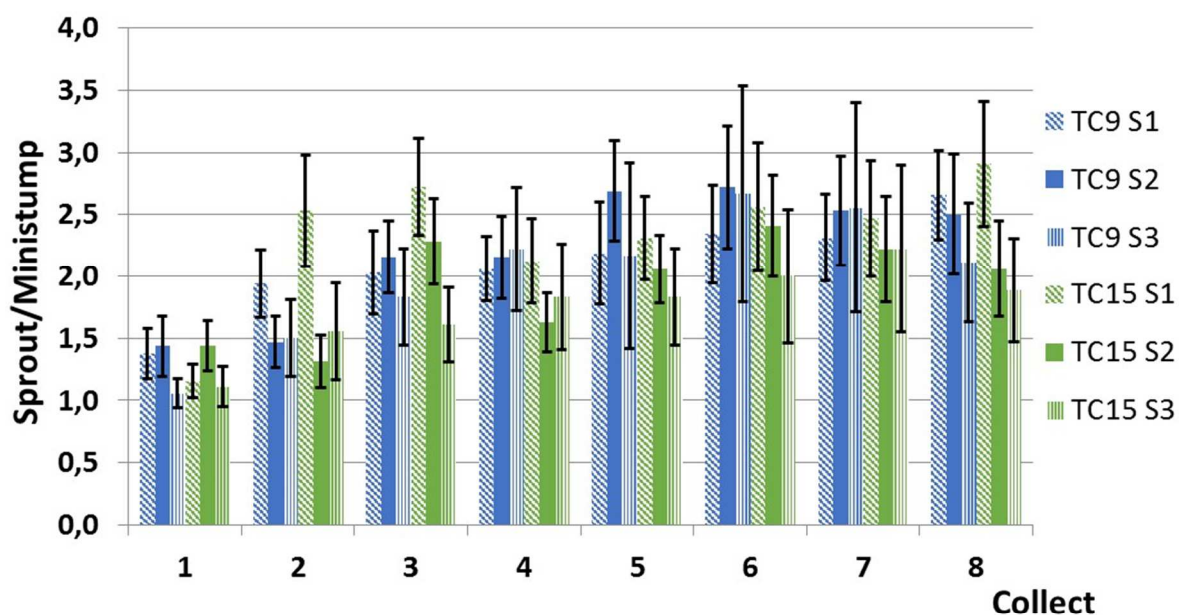**Keywords:** Red cedar, vegetative propagation, clonal minigarden

The Red cedar (*Toona ciliata*) stands out in the Brazilian forest sector due to its relatively short production cycle, good timber productivity and high marketing value. Studies show that the vegetative propagation of this species is feasible for commercial production as well as for rescue and multiplication of adult genotypes with interesting features.

However, the physiological age of the plant matrix may impact growth of the vegetative propagules, as these may have unfavorable morphological characteristics for the handling in minigardens. Studies suggest that serial minicuttings can be used to promote the strengthening of the propagules and for the induction of juvenile characteristics. This study aims to evaluate the productivity of ministumps and of the morphology of shoots of Red cedar clones submitted to serial minicutting.

In a clonal minigarden implanted with stakes of adult matrices, rescued after harvest, sprouts of two clones were collected (TC9 and TC15). From each sprout, minicuttings (5 cm in length) containing a leaf and a couple of leaflets reduced by 50%, were made. The minicuttings were placed in tubes (280 cm^3 in size), with a commercial substrate based on pine bark and slow release fertilizer. Rooting was carried out under intermittent mist for 30 days. At the end of this period, they were transferred to the greenhouse and on the day 195 after staking the cuttings were transferred to suspend seedbed, where they had the apex pruned to 8 cm from the root collar, for the formation of minitumps of the first subculture (S1). Sprouts of S1 were collected to form the second subculture (S2) and these provided sprouts for subculture 3 (S3). Monthly evaluations on the productivity were carried out regarding the number of sprouts and minicuttings produced by each ministump (**Fig. 1**).

To evaluate the morphology, 4 ministumps of each clone were selected and evaluated at 6, 7 and 8 months after the implantation of each subculture (**Fig. 2**). The number of leaves and leaflets leaf area and dry mass were determined in the harvested sprouts. No changes in the ministumps productivity or in the biometric characteristics of the sprouts, due to three subcultures in a serial minicutting, were observed. This work will be continued to assess whether more subcultures may result in the reinvigoration of these clones and in the reestablishment of juvenile characteristics.

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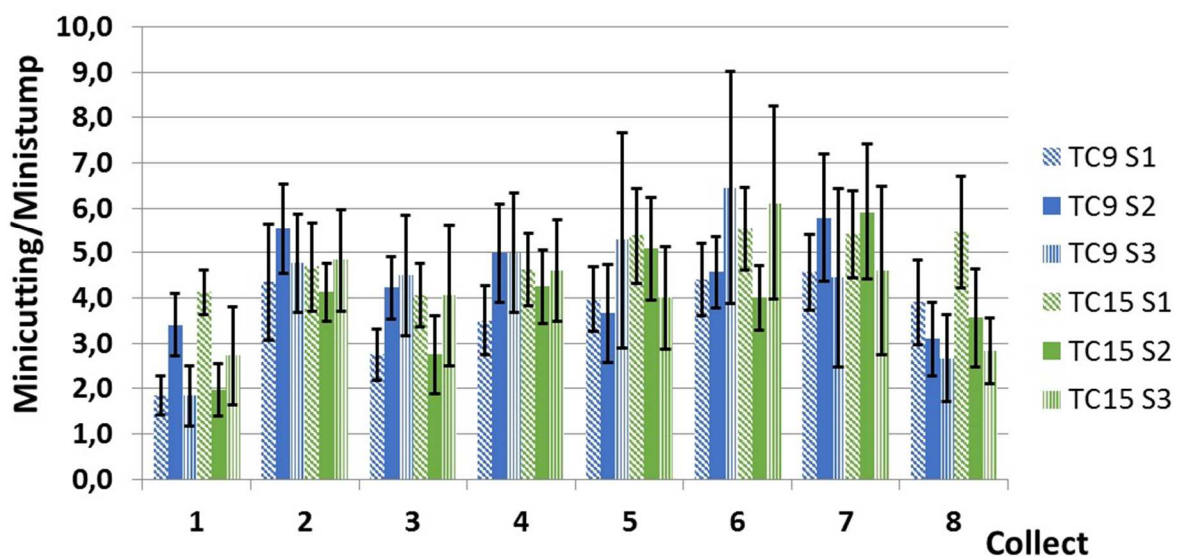Campos Mamede Weiss de Carvalho G et al.

A

B

Figure 1. Average number of sprouts (A) and minicuttings (B) produced by ministumps of *Toona ciliata* clones (TC9 and TC15) over 8 collections in three subcultures (S1 and S2 with N = 32; S3 with N = 18). Vertical bars represent confidence limits (Cl: 95%).

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Figure 2: Clonal minigarden established in a suspended seedbed and sprouts harvested of ministumps selected for morphological evaluation in which, the number of leaves and leaflets leaf area and dry mass were quantified.

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González P & Barth S

Vegetative propagation of *Cordia trichotoma, Cabralea canjerana* and *Picrasma crenata* species with potential for productive diversification

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Keywords: macropropagation, native forest, conservation.

Misiones has around 1.700.000 hectares of native forest that belongs to restricted areas of national, provincial or private reserves. Due to the large exploitation of forests, this area has witnessed a steady decline in the supply of raw material and timber products. There are native species of great economic and social value in the Misiones rainforest such as *Cordia trichotoma* (black parrot), *Cabralea canjerana* (cancharana) and *Picrasma crenata* (Palo bitter). These species have suffered deforestation and selective use of the best individuals, resulting in a reduction in the size of populations under natural conditions. This has adversely affected the genetic bases of the first two species.

Previous work carried out by INTA (*National Agricultural Technology Institute*) identified individuals with desirable characteristics from the forestry point of view, such as fruiting period, pre-germination seed treatment and nursery behavior of these species.

It is necessary to seek alternatives for propagation of these species to increase the availability of plants. A potentially effective tool to carry out multiplication asexually is by macropropagation. To help in minimizing the exploitation of native forests and to generate a sustainable balance in economic, ecological and social terms, we made it our objective to study different techniques of macropropagation.

For vegetative propagation we selected young trees obtained by seed germination followed by decapitation of the seedling to allow lateral bud proliferation. New cuttings were disinfected with the fungicide (Captan 2 g/L) and used in a rooting experiment thereafter. Treatments consisted of assessing the response to exposure to different concentrations of the hormone IBA. The cuttings were set in containers (trays and tubes), with a mixture of substrates (50% composted pine bark + 50% lateritic soil) and slow-release fertilizer (NPK plus micronutrients). The experiment took place under greenhouse conditions with controlled irrigation (**Fig. 1**). These experiments were conducted in the laboratory and field Annex of Laharrague EEA Montecarlo (INTA). A randomized design with 3 replications (plots) was used for each species. The results were interpreted statistically by Analysis of Variance using Mixed Models.

With cancharana the highest percentages of rooting obtained in apical cuttings were 95.8% with IBA 0 ppm and 91.6% with IBA 1000 ppm. With subapical cuttings, the rooting percentages obtained were 33.3% with IBA 0 ppm and 29% with IBA 500 ppm. In black parrot, the best rooting percentages were observed with doses of IBA ranging from 0 to 1000 ppm, yielding 96% rooting with apical cuttings. There were no significant differences in rooting rates between those of apical and subapical cuttings since for both about 80% rooting rates were obtained for the same concentrations of IBA. In the case of Palo bitter, obtained the best results (45%) when cuttings were treated with IBA 2500 ppm in inert powder or 40% when treated with 3000 ppm IBA in an inert gel (**Fig. 2**).

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González P & Barth S

Bitter stick

Cancharana

Black Parrot

Figure 1. Stakes of bitter stick, cancharana and black parrot.

Figure 2. Rooted cuttings of bitter stick, cancharana and black parrot.

In conclusion, these species can be clonally propagated but to obtain the maximum number of rooted plants, the use of growth regulators such as IBA and their optimally effective concentrations have to be determined.

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What is the best way to maintain embryogenic capacity of embryogenic lines initiated from Douglas-fir immature embryos?

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Keywords: cytology, embryonal masses, morphology, multiplication, *Pseudotsuga menziesii,* non-embryogenic cells, somatic embryogenesis.

Introduction

Douglas-fir (*Pseudotsuga menziesii* (Mirb) Franco) is a native conifer from the Pacific North-West of the US and Canada, and is one of the most important timber species used in the world. Douglas-fir is highly productive in a range of climatic conditions and has valuable wood properties (quality) as well as a strong tolerance to diseases and insects. In Europe, Douglas-fir is a major species for reforestation with increasing demand for its wood. Adaptation of new varieties to climate change and associated stresses is one challenging question for ongoing breeding programs. Efficient selection and vegetative propagation of improved varieties are key issues to maintain productivity in plantation forestry (Lelu-Walter et al. 2013). However, as in many other conifers, early maturation is preventing clonal forestry through conventional multiplication methods in Douglas-fir (Bastien et al. 2013). Somatic embryogenesis from immature seeds, coupled with cryopreservation, is a promising retroactive clonal propagation system of selected trees. This technology has been developed for an increasing number of conifer species (Klimaszewska et al. 2016). Excluding patents, there are only a few published studies on Douglas-fir somatic embryogenesis (Durzan and Gupta 1987; Pullman et al. 2005, 2009). One recurrent problem is the sustainable multiplication of initiated embryogenic material, i.e., embryonal masses (EMs). Yellowish, non-embryogenic cells (NEC), which are interspersed with EM, is frequently observed during this process. In this work, we describe EM morphology and cytology (light and confocal microscopy) of different embryogenic lines. A suggested method to reduce the formation of NEC and sustain EM proliferation is presented.

Materials and methods

Plant material

Somatic embryogenesis was initiated from immature zygotic embryos of Douglas-fir obtained from 3 controlled crosses (74 x 44, 56 x 47, 55 x 46). Experiments were conducted with three embryogenic lines

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(D1, D2, D3), each originating from one of these genetically unrelated full-sib families. Proliferation was performed according to Reeves et al. (submitted) with the following modifications: the basal multiplication medium used was Glitz medium (Litvay basal medium: Litvay et al. 1985 modified by Hargreaves et al. 2009) supplemented with 1 mg L^{-1} 2,4-D, 0.5 mg L^{-1} BA, 30 g L^{-1} maltose, and 4 g L^{-1} gellan gum (phytagel). EMs were routinely subcultured every two weeks in clumps on proliferation medium in darkness at 23°C. However, to ensure EMs proliferation, we also applied the proliferation method previously developed for pine species, i.e., EMs dispersed onto filter paper (Lelu-Walter et al. 2016). Filter paper with attached EMs was placed on the surface of fresh multiplication medium.

Histology

In this work we used high-resolution optical and confocal microscopes for cytological observations of the three proliferating embryogenic lines. Fresh EMs were collected (only the whitish parts), stained with trypan blue and observed using transmission light microscopy. The viability was assessed using confocal microscopy after double staining with fluorescein diacetate (FDA) and propidium iodide (PI) (Vondráková et al. 2010). Green fluorescein fluorescence reveals viable cells, whereas red fluorescence of PI-DNA complexes occurs in dead cells.

Results and discussion

Morphological and histological aspects of embryogenic lines

According to their macro-morphology EMs were classified under two types (**Fig. 1A**): i) "pure lines" (D1, D2), i.e., white-translucent EMs with immature somatic embryos (SE) as typically described in conifer species, or ii) a mixed line (D3), i.e., EMs with immature SE and NEC (brown parts). Clusters of NEC cells did not produce any cotyledonary SE after maturation treatment (Reeves et al. submitted). These NEC erratically appeared among EMs during the culture process and were found to reduce EMs proliferation.

Transmission light microscopy (**Fig. 1B**) confirmed that line D1 is only made up of embryogenic cells whereas line D3 is a mixture of immature SE and round cells (NEC). Interestingly, line D2 initially classified as a "pure line" also appeared to be a mixture of SE and NEC. NEC is tightly interspersed with SE, and it is difficult to dissociate them. We concluded that macro-morphology is far from being sufficient to describe EMs.

Using confocal microscopy with FDA/PI staining (**Fig. 1C**), dead cells were observed in embryo suspensors from all lines suggesting actively occurring programmed cell death (PCD) that is required for normal embryo development. For mixed lines such as D2 and D3, persistent non-embryogenic cells of unknown origin presented only viable cells.

Proliferation method

For one mixed line (D3), the transfer of EMs onto a filter paper greatly reduced the formation of NEC. Different methods are possible to reduce the production of non-embryogenic cells: frequent subculture of EMs (no aging), vigorous dissociation in liquid medium (Reeves et al. submitted), and transfer on filter paper (homogenization).
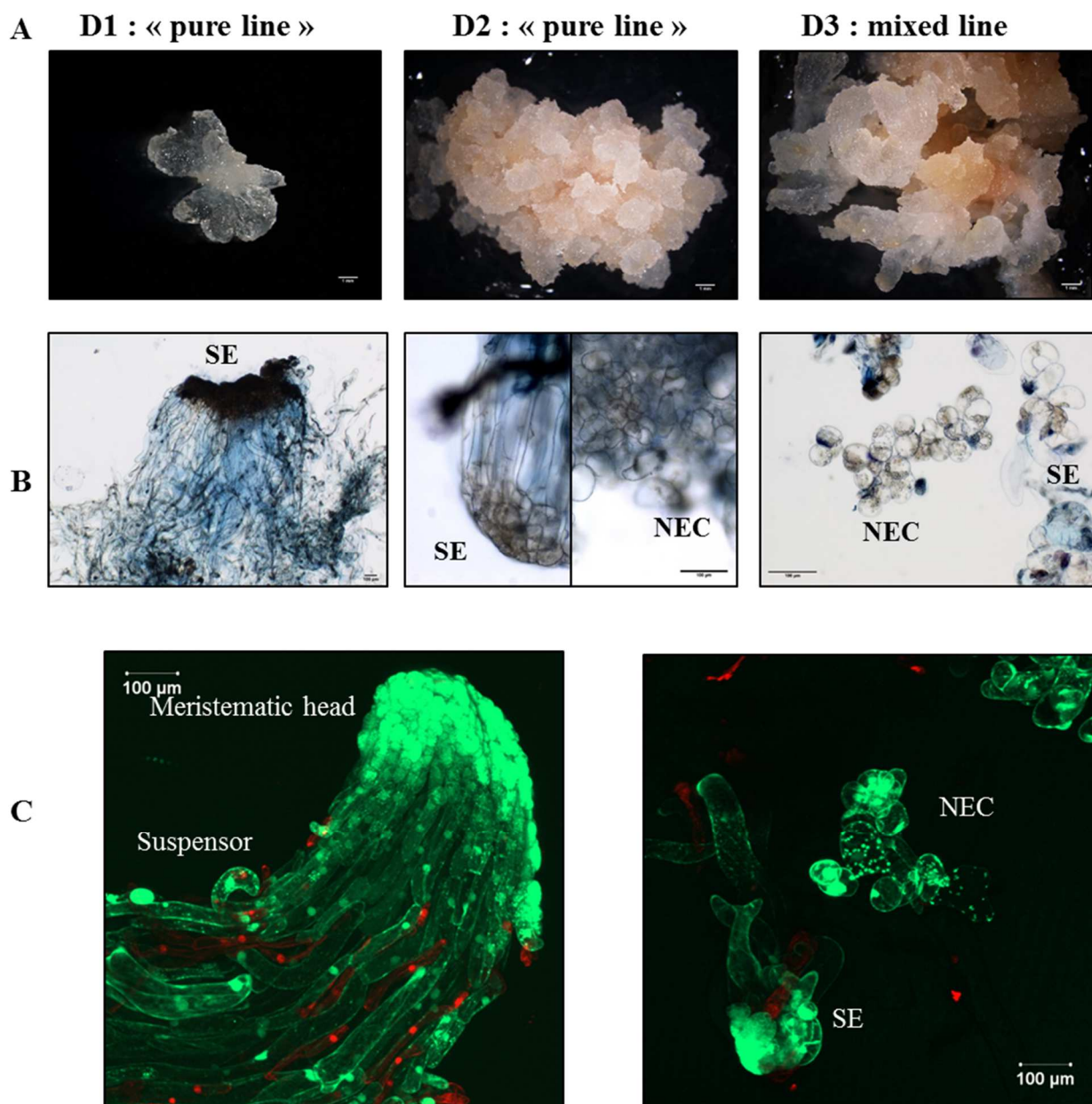
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Figure 1. Observation of three Douglas-fir embryogenic lines (D1, D2, D3).

Macro-morphology (A). Transmission light microscopy: EMs stained with trypan blue (B). Confocal microscopy: viability was determined by double staining with fluorescein diacetate (FDA) and propidium iodide (PI). Green fluorescein fluorescence reveals viable cells, whereas red fluorescence of PI-DNA complexes accumulates in dead cells (C). SE: somatic embryo; NEC Non-Embryogenic Cells.

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Conclusions

In some lines, the whitish parts of proliferating EMs were shown through microscopy to contain both immature SE and NEC. Viable non-embryogenic cells are of unknown origin.

To tentatively reduce the occurrence of non-embryogenic cells, it is recommended to frequently subculture EMs (each week), to vigorously dissociate them in liquid medium (Reeves et al. submitted) before transferring the resulting homogenate onto filter paper as a thin cell layer.

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Possibilities of somatic embryogenesis for production of hybrid pine and loblolly pine

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Keywords: somatic embryogenesis, *Pinus taeda*, hybrid, megagametophyte.

Introduction

Clonal propagation allows to increase productivity of forest plantations by capturing non-additive genetic effects. In conifers, somatic embryogenesis is an alternative technique for cloning genetically improved trees.

In this study, immature seeds from controlled crosses of loblolly pine and *Pinus elliottii* var. *elliottii* x *Pinus caribaea* var. *hondurensis* F1 hybrid were used to determine the most appropriate culture mediun for induction, multiplication and maturation of embryogenic masses.

Materials and methods

Immature cones were collected from ten clones from the first week of November 2015 to first week of January 2015 in the clonal seed orchard located at San Antonio, Misiones, Argentina. The megagametophytes with zygotic embryos were cultivated in 1253 induction medium (Pullman et al. 2005).

Results and discussion

Loblolly cultivated zygotic embryos, at developmental stages 2 to 4 (Pullman et al. 2003), had better responses than hybrid pine embryos cultured at those stages.

Two media compositions were tested for optimal multiplication of embryogenic suspensor mass (EM). One with standard concentration of plant growth regulators (Lelu et al. 2006) and medium 1133 (Pullman and Bucalo 2011). In both media they were subcultured as masses, thereby oxidation mass was avoided. A greater number of EMs were obtained in *Pinus taeda*, whereas in the hybrid only one EM was obtained.

Both PEG and gelling concentration in maturation medium gave better results with higher concentrations. The gellan gum generated lower water availability in the medium, thus promoting maturation of immature embryos. The best results in the maturation media were achieved when the medium contained 90 µM ABA and 10 mg l^{-1} of gellan gum (**Fig. 1**).

The same medium tested for loblolly pine were used for the hybrid pine. For the latter species, the numbers of achieved EM was very low.

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Figure 1. Loblolly pine EMs in maturation conditions on a nylon cloth showing developing cotyledonary somatic embryos. Bar: 5 mm.

Germination was considered successful when the elongation of both the root and shoot meristems had occurred. **Fig. 2** shows the plantlets before they were transplanted to composite substrate.
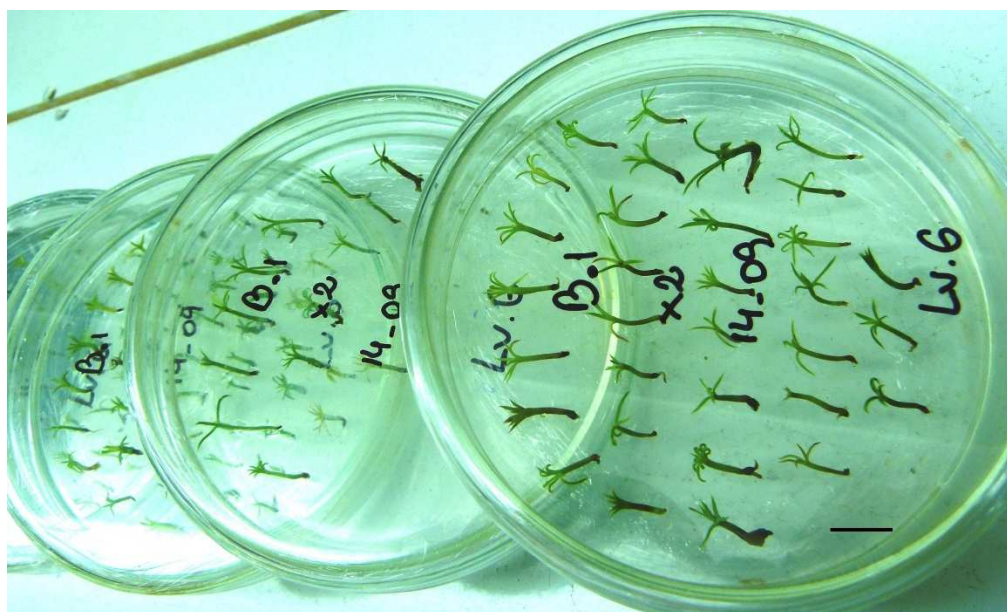

Figure 2. Germinating somatic embryos of *Pinus taeda*. Bar: 1 cm.

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Conclusions

The optimum window for the harvesting of immature zygotic embryos for successful somatic embryogenesis intiation occurs for the F1 hybrid two to three weeks earlier than for *P. taeda*. However, the culture medium that gave the best induction, multiplication and maturation results were the same for both species.

When the optimal stage of development of the zygotic embryo for somatic embryogenesis differs between tested families, the optimal stage should be determined for each family.

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Strategies for the early detection of embryogenic competent cells in tamarillo somatic embryogenesis

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Keywords: embryogenic cells, FACS, IAA, immunolocalization, *Solanum betaceum*

Introduction

Somatic embryogenesis (SE) is a developmental pathway in which complex regulatory networks end in the acquisition of embryogenic competence and further somatic embryo development (Yang and Zhang, 2010). Embryogenic cultures are usually formed by a heterogeneous cell population whose cells differ in size, shape, morphogenetic fate, and also express different molecular or cellular markers as they interact in a complex network of signals and transduction pathways. Which of those cells are able to enter into an embryogenic pathway and what happens before their transition into an embryogenic state remains poorly understood. Thus, the early identification of subpopulations of embryogenic cells among a mass of cultured cells and the study of their features may provide new clues for better predicting or inducing the embryogenic pattern of development. For this purpose, it is crucial to develop tools that might be used to detect the embryogenic cells in the earliest stages of culture, and to distinguish them from those that are unable to regenerate (Ochatt et al. 2010).

Lately we have been using the SE induction system of tamarillo (*Solanum betaceum* Cav. syn. *Cyphomandra betacea*) to characterize the very early stages of somatic embryo formation (Lopes et al. 2000, Canhoto et al. 2005, Correia et al. 2011, Correia et al. 2012a, 2012b). The rationale behind the use of tamarillo as a model is that it has some advantages over other embryogenic systems, particularly for molecular analyses and experimental embryology approaches, since: 1) SE can be induced in several juvenile organs and in explants derived from adult trees, 2) long-term embryogenic *calli* can be maintained in culture without loss of embryogenic potential, 3) somatic embryo conversion into plantlets is easy and hundreds of plantlets can be obtained from a single embryogenic callus, 4) embryogenic (EC) and non-embryogenic (NEC) cell lines can be obtained from the same explant exposed to the same culture conditions. This last feature is particularly relevant since the two types of cell lines can be grown independently and molecular comparisons can be made between two lines with the same genetic background, yet expressing different morphogenic abilities, due to differences in gene regulation. In addition to the protocols of *in vitro* plant regeneration, our group developed also an efficient transgenesis system for tamarillo, with plant regeneration through SE, as well as through a protoplast isolation protocol (**Fig. 1**) which are important tools for functional genomics studies in tamarillo, particularly for cell-type-specific transcript and protein profiling. Using this system we have already identified differentially expressed proteins between auxin induced ECs and NECs (Correia et al. 2012b) and a protein with a putative inhibitory role in the acquisition of embryogenic competence, NEP-TC (GenBank JQ766254), was identified and characterized. NEP-TC is a 26.5 kDa protein identified as being consistently present in

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tamarillo's non-embryogenic cells, this protein having been associated with the SpoU SAM-dependent RNA methyltransferase family of proteins.

These first approaches to clarify the particularities of ECs and NECs in tamarillo through proteomic analysis confirmed that they are of interest as an alternative model system, particularly for molecular analyses. With the availability of next-generation sequencing (NGS) techniques and the continuous improvement and growth of data sets, more complete surveys of the transcriptome dynamics during the SE process will be possible, and can be extended to other model organisms than tamarillo.

Strategies for the early identification of embryogenic competent cells

Previous analyses showed that embryogenic cells of tamarillo are isodiametric, with a dense cytoplasm usually organized into globular clumps, whereas NEC cells are elongated, largely vacuolated, and growing isolated or forming small linear groups (**Fig. 1A**). While NEC callus are usually formed by a homogenous population of non-embryogenic cells, embryogenic tissues are more heterogeneous since among the isodiametric embryogenic cells, non-embryogenic cells are also present.

Based on this system, we have attempted to target and select the specific cells that undergo the pathway of embryogenic competence acquisition and to detect gene expression modifications occurring in them (**Fig. 1B**), namely by: 1) close monitoring of embryogenic cell behavior as a function of the endogenous auxin indole-3-acetic acid (IAA) levels and distribution patterns; 2) functional characterization and localization of a putative SE's inhibitory protein (NEP-TC); and 3) fluorescence-activated cell sorting (FACS) of embryogenic and non-embryogenic protoplasts for transcriptome profiling.

For monitoring cell behavior during SE induction, based on the expression and/or localization of specific molecules, optimized protocols for cryofixation and immunohistochemistry were developed (**Fig. 1B**). Particular features of the samples were studied, namely their cell heterogeneity and high water content. The obtained sections were successfully incubated with different primary antibodies (anti-IAA and anti-NEP-TC) and labelled with secondary antibodies for immunolocalization.

The immunohistochemical analysis showed that the accumulation of IAA occurs in very localized niches of embryogenic cells, which is in accordance with the significantly higher endogenous IAA contents in embryogenic samples. The IAA content tended to increase as the dedifferentiation of the original explant evolved. These results are an important additional guidance for the identification of embryogenically competent cells by their correlation with the location of IAA accumulation niches.

Because the NEP-TC distribution pattern is specific for non-embryogenic cells, we can separate NEP-TC labelled cells from the embryogenically competent ones for further studies of regulatory pathways in the process of embryogenic competence acquisition.

The FACS analysis of protoplasts derived from cultured tamarillo embryogenic cell cultures allowed the identification of cellular subpopulations of embryogenic and non-embryogenic cells, according to the distribution of light-scattering intensities at different angles. For the efficient application of fluorescence cell sorting techniques to tamarillo somatic embryogenesis explants, starting from the early stages of the induction process until the separation of embryogenic and non-embryogenic cells (**Fig. 1B**), an optimized protocol for protoplast isolation and purification had to be developed. After sorting, these subpopulations were further characterized in terms of their cytological features before proceeding with the transcriptome profiling by RNA sequencing.
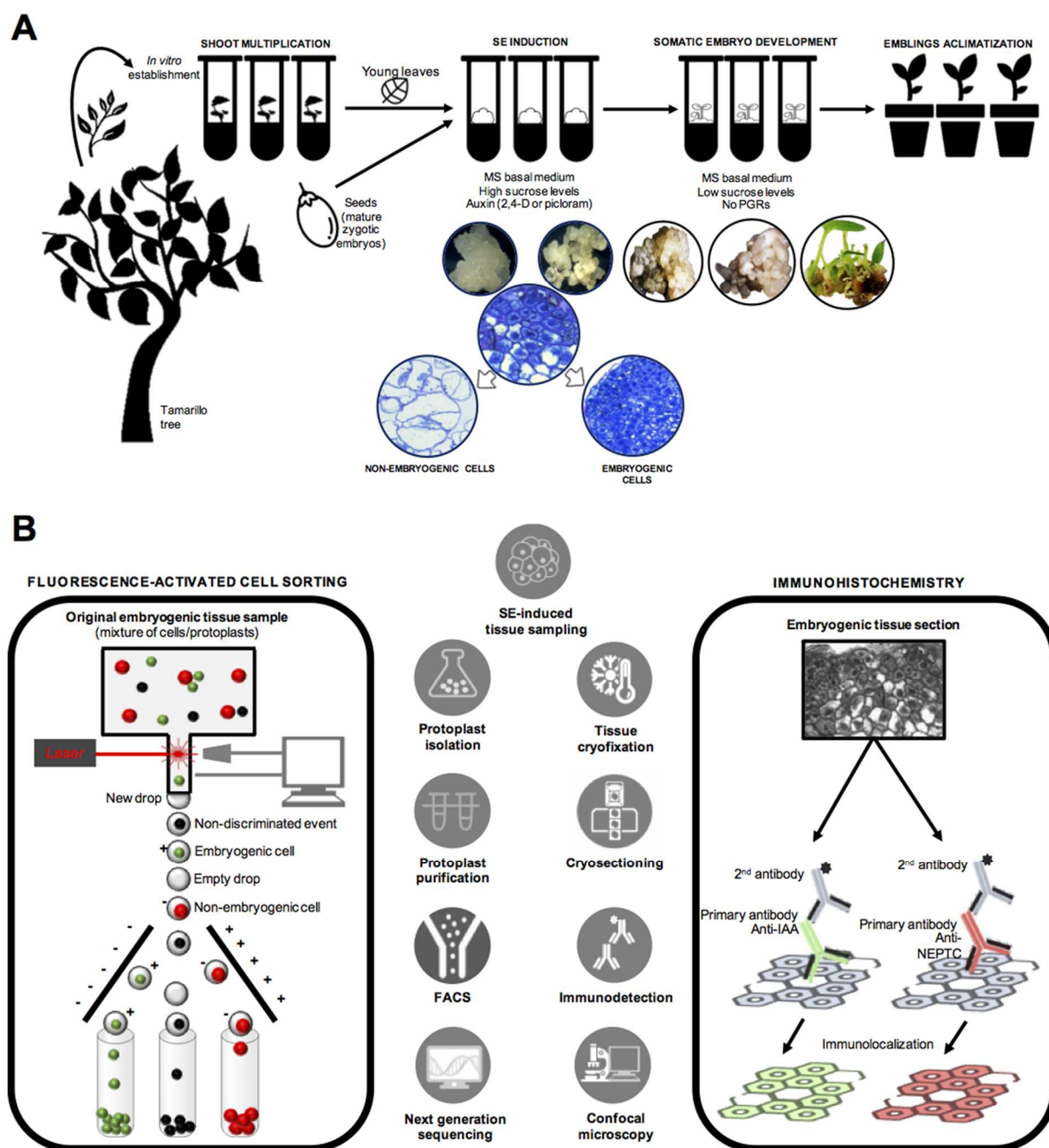
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Figure 1 – Somatic embryogenesis (SE) in tamarillo by a two-step process (A) with SE induction from young leaf explants or zygotic embryos (step one) and embryo development from induced embryogenic cells (step two). Embryogenic and non-embryogenic cells are induced side-by-side from the same explant, under the same culture conditions. (B) Fluorescence-activated cell sorting (FACS) and immunohistochemistry techniques developed for the efficient targeting and isolation of tamarillo embryogenically competent cells.

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Conclusions and future prospects

The results obtained led to the first-time establishment of optimized protocols for the: cryofixation of embryogenic and non-embryogenic samples for immunohistochemistry applications; immunolocalization of IAA during somatic embryogenesis induction in tamarillo; isolation and purification of protoplasts from tamarillo embryogenic and non-embryogenic explants; fluorescent activated cell sorting (FACS) of embryogenic cells based on light-scattering intensities. The establishment of these completely novel protocols was necessary to set the basis for the early and correct identification of embryogenically competent cells. These results will contribute to the identification of differentially expressed genes in very specific and localized cells during the early stages of embryogenic competence acquisition, which would allow the construction of models to describe the changes associated with cell differentiation during SE. A better understanding of the mechanisms of plant embryogenic competence acquisition could not only be a scientific breakthrough, but also give important insights into the way to increase plant production through SE.

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Identification of regulatory miRNA-target nodes across embryo development in *Pinus pinaster*

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Keywords: conifer, embryogenesis, microRNA, microRNA target, non-coding transcriptome, degradome

Introduction

Small non-coding RNAs (sRNA) play major roles in gene expression regulation associated to plant growth and development, and in the response to abiotic and biotic stresses, either through transcriptional or post-transcriptional gene silencing. Most plant sRNAs are produced as 21 to 24 nucleotide long molecules and, based on their mode of biogenesis and function, have been classified in different categories (Axtell 2013).

The microRNAs (miRNAs), produced from single stranded hairpin precursors, and the short interfering RNAs (siRNAs), deriving from double-stranded precursors, are two major sRNA classes. The siRNAs can be divided in further classes which include for instance the trans-acting siRNAs (ta-siRNAs).

The sRNA landscape of a species in specific tissues or developmental stages can be very informative of the gene expression control mechanisms in place. Although many sRNA studies have been performed in the last few years in angiosperm model and non-model plants, still little is known about the sRNA transcriptome of gymnosperms and its putative role in the distinct characteristics exhibited by these species when compared to the angiosperms. During the plant life cycle, the embryogenesis stage, during which the embryo development and patterning take place, followed by embryo growth, accumulation of storage reserves, desiccation tolerance acquisition and entry into dormancy, is crucial for the establishment of a fully functional individual. Knowledge of the molecular players and the signalling pathways controlling this process can provide relevant information in a biotechnology framework for establishing and/or optimizing large scale propagation systems based for instance on somatic embryogenesis.

Microarray analysis of *P. pinaster* zygotic embryogenesis highlighted several epigenetic regulation mechanisms and showed that functions related to sRNA pathways appeared differentially regulated across development with a prevalence of miRNA functions in mid to late embryogenesis (de Vega-Bartol et al. 2013). As a way of expanding our current understanding of the molecular regulation of embryo development, we have generated a first overview of the sRNA transcriptome of the developing embryo of *P. pinaster*.

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Material and methods

A set of sRNA libraries spanning the maritime pine zygotic embryo development as well as other reproductive tissues were prepared. Zygotic embryos at five developmental stages, from the early globular embryo to the mature embryo, as well as the corresponding megagametophytes, were obtained by the dissection of seeds of an open-pollinated mother tree (Escaroupim, Portugal). Additionally, female and male cones were also collected. After RNA extraction using "Plant/Fungi Total RNA Purification Kit" (Norgen Biotek Corp.) and quality evaluation, libraries were prepared and sequenced using Illumina technology for generation of sRNA datasets representative of the described tissues/developmental stages. By using an in-house sRNA pipeline called miRPursuit (Chaves et al. 2017), centered on publicly available tools such as the UEA small RNA workbench (Stocks et al. 2012), sRNA raw data were processed and sRNAs from different classes were identified. Processing of the raw data included trimming of adapter sequences, filtering based on read length (18-26nt) and abundance (at least 5 reads), and low complexity and ribosomal (rRNA) and transfer RNA (tRNA). Retained reads were mapped against an available conifer genome (*P. taeda*), and then searched against the miRBase (Kozomara and Griffiths-Jones 2014) for identification of conserved miRNAs. Non-conserved reads were then run through different tools for the prediction of novel miRNAs and ta-siRNAs, using selected parameters. Additionally, the putative targets of the identified sRNA were predicted using psRNATarget software (Dai and Zhao 2011) and the reference transcriptome of *P. pinaster* (Canales et al. 2014). A few conserved miRNAs have been selected for validation of expression profile by RT-qPCR using TaqMan probes. Moreover, degradome sequencing of pooled samples was performed in order to validate sRNA target genes regulated by cleavage.

Results and discussion

From the over 800 million reads generated, less than 50% were kept after filtering steps and approximately 30% have been mapped against the *P. taeda* genome. Less than 5% of the total initial reads were classified as conserved miRNAs, and a similar percentage as novel miRNAs, while approximately 3% were predicted to be ta-siRNAs. In terms of non-redundant reads, the most numerous elements were the novel and the ta-siRNAs. Although the conserved miRNAs were the less numerous concerning the non-redundant reads, they were the most highly expressed. Over 40 families of conserved miRNA were identified, with the MIR166 and MIR159 being the families with a higher number of isoforms. Most identified MIRNA families are conserved across major plant groups, but gymnosperm-specific, and particularly conifer-specific MIRNAs, have also been identified, such as MIR3701 and MIR949. The expression profiles obtained by RT-qPCR were in general agreement with the sequencing data.

miRNA target genes have been predicted against the reference transcriptome of *P. pinaster* (Canales et al. 2014) and further validated by degradome sequencing analysis. This strategy allowed us to identify close to 100 conserved miRNA-target pairs in embryo sequencing libraries and many other novel miRNA-target pairs. Among the target genes identified, a number of transcription factors with known roles in plant embryo development have been identified for further characterization.

Based on the analysis of the sRNA datasets here generated, several miRNAs have emerged as potential regulators of pine embryogenesis.

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Shoot proliferation of chestnut (*Castanea sativa* Mill.) and *in vitro* protective effect of endophytes against *Phytophthora cinnamomi*

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Keywords: coculture, endophytes, PCR, *Phytophthora cinnamomi*, shoot proliferation, symbiont

Chestnut (*Castanea sativa*) is a widely cultivated forest deciduous tree that produces an edible nut that is especially appreciated in southern Europe. Plant growth and fruit production have been greatly affected by several plagues and diseases, such as ink disease caused by the fungus *Phytophthora cinnamomi* that grows in the soil leading to crown and root rot (Vannini and Vettraino 2001). Because chestnut is severely affected by this pathogen, which usually leads to the dead of host plants, it is crucial to develop control strategies at different levels, in order to mitigate the disease and minimize economic losses. Although several strategies have been implemented, according to Rodrigues and Martins (2005), chemical control and a search for resistant host plants has been ineffective. Moreover, there is an increasing social demand for a more sustainable approach, and different strategies must be taken into consideration in order to ensure crop productivity (Cazorla and Mercado-Blanco 2016). Biological control might be challenging in the control of woody plants diseases, but several authors have pointed out that bacteria and both Ascomycota and Basidiomycota fungi can be successfully used to mitigate the problem (Malajczuk 1983; Gouveia 1993; Zabalgogeazcoa 2008), using different strategies, such as the production of secondary metabolites, antibiotics and enzymes (Dutta et al, 2014). Therefore, the main objective of this work is to access the tolerance of selected clones of chestnut to this pathogen, and evaluate the effect of symbionts isolated from natural growing trees in order to develop a biological control strategy.

For this purpose, roots of selected chestnut clones have been sterilized with calcium hypochlorite (2% for 5 min), washed in sterile distilled water and plated on PDA medium. Colonies were isolated based on morphology and time of growth and fungal DNA was extracted with a NucleoSpin® Plant II kit (Macherey-Nagel, Bethlehem, PA, USA). The amplification of rDNA was carried out with JumpStart™ Taq DNA Polymerase with $MgCl_2$ (Sigma-Aldrich, St. Louis, MO, USA), and primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTTATTGATATGC-3') synthesized by Eurofins (Ebersberg, Germany), on a Arktik™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) under the following conditions: 94 ºC for 5 min, followed by 35 cycles at 94 ºC for 1 min, annealing at 57 ºC for 30 s and extension at 72 ºC for 45 s, before a final extension at 72 ºC for 10 min. Detection of PCR-amplified products was performed by electrophoresis on a 1.5% (w/v) agarose gel and PCR products were sequenced by Eurofins. The identification of the isolates was performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Most of the isolates were identified as Ascomycota and to predict its behavior when exposed to the pathogen, amylase, cellulase, lipase, pectinase and protease activities have been assessed according to Maria et al. (2005), with cellulase activity being the most evident among the tested isolates.

The antagonistic effect of the endophytes was tested when co-cultured *in vitro* with two different strains of *P. cinnamomi* isolated from chestnut trees showing symptoms of the disease. Five isolates have been

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identified based on the ability to reduce the growth of the pathogen, either by causing a dead-lock or by substitution, and its protective effect against *P. cinnamomi* was tested. In order to test the protective effect of the endophytes, selected clones of chestnut have been established *in vitro* and multiplied through shoot proliferation, according to the protocol of Baltazar (2015), on WPM medium (Lloyd and McCown 1981) supplemented with 0.2 mg/L zeatin (Duchefa Biochemie B.V, Haarlem, The Netherlands) and 3% sucrose (w/v, Duchefa). Shoots were successfully rooted (98%) on Knop medium (Gautheret 1959) with 2.0 mg/L indole-3-butyric acid (IBA, Sigma-Aldrich). Some plants were also dipped in IBA and placed in sterile jiffy pots *in vitro* and acclimatized for further analysis.

Plants from the different clones produced by this method have been cultured *in vitro* with the selected endophytes and a highly virulent strain of *P. cinnamomi*. The mycelium growth of both pathogen and endophyte and the appearance of visible symptoms on the plant have been registered during a month, and compared to a non-infected control group. Without endophytes, the pathogen severely affected the plants, causing death in less than two weeks. Although some of the endophytes tested showed to minimize the symptoms, they were unable to avoid plant death. Nevertheless, two of the symbionts (*Trichoderma harzianum* and *Diaporthe* sp.) successfully protected the plants and no symptoms of the disease were observed. *T. harzianum* has already been identified to confer protection against pathogens, including *P. cinnamomi* (Gouveia 1993), and according to Hakizimana et al. (2011), both *T. harzianum* and *Diaporthe* sp. were effective against *P. cinnamomi* by deadlock with mycelia contact, and were able to minimize disease symptoms on avocado. The results obtained with this work may open the way to develop a strategy that minimizes the effects of ink disease in chestnut, based on the protective effect of symbiont microorganisms.

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Evaluation of induced tolerance to *Phytophthora cinnamomi* in holm oak somatic embryos

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Keywords: biotic stress, dual cultures, elicitor, holm oak, induced tolerance, *Phytophthora* spp.

Introduction

Holm oak trees (*Quercus ilex* L.) are challenged by some biotic stresses like fungal infections. The main disease is caused by the oomycete *Phytophthora* spp., which is responsible for forest decline and dieback in evergreen oak forest areas of the southwestern Iberian Peninsula (**Fig. 1**).

Our study is based on the possibility of applying elicitors or oomycete extracts to holm oak somatic embryos (SE) in order to induce epigenetic memory, priming, that may increase tolerance to the pathogen in future infections. To this end, we will first determine the effect of priming treatments on SE development and oxidative stress production. At the end of the experiment regenerated plants will be tested for tolerance by inoculating roots with *Phytophthora cinnamomi* mycelium.

Figure 1. Holm oak decline caused by *Phytophthora cinnamomi.*

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Material and methods

Elicitation assays

The holm oak embryogenic line Ha13 (**Fig. 2**) obtained as described in Blasco et al. (2013) was cultured for 3 days in 40 ml ESM medium (Elicitin Secretion Medium, Horta et al. 2008) with either *Phytophthora cinnamomi* 1630 strain (**Fig. 3**) extracts (30 or 50%) or the elicitors methyl jasmonate (MeJA), benzothiadiazole (BTH) and para-aminobenzoic acid (PABA) at 0, 5, 10, 25 and 50 µM. Extracts were obtained by filtration and subsequent filter-sterilization of *P. cinnamomi* cultures previously established in liquid medium. Then, embryos were transferred to proliferation medium (MS + STS + CA, Martínez et al, 2015). Data on SE growth and development were recorded after 60 days.

Figure 2. Holm oak embryogenic line Ha13.

Figure 3. *P. cinnamomi* 1630 strain.

Defense responses to Phytophtora

As a measure of primary defense response, formation of hydrogen peroxide (H_2O_2) was determined. Elicited material was soaked for 3 hours with 40 ml ESM liquid medium containing 20% of OCF exudate and then transferred to proliferation medium. After 3 days, H_2O_2 concentration was measured according to Chong et al. (2005).

Dual cultures as preliminary assays for Phytophtora tolerance

Elicited SEs and controls were cultured in opposite positions in the same petri dish containing PDA medium with a piece of *P. cinnamomi* mycelia ($0.5cm^2$) placed in the middle (**Fig. 4**). The ratio of mycelium growth towards the control and the elicited material was evaluated daily.
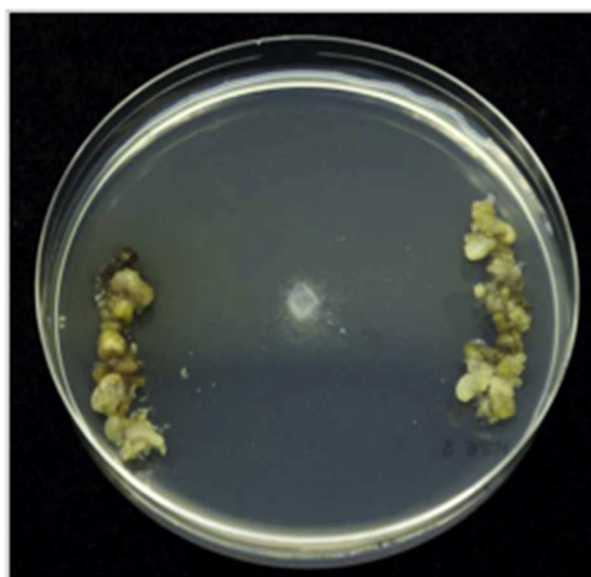
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Figure 4. Double dual culture of elicited SEs and controls with *P. cinnamomi* mycelia

Results

Effect of oomycete extract (OCF) and elicitors on holm oak SE growth and development

The application of sterile oomycete extracts did not affect growth and further development of the embryogenic line (**Fig. 5A** and **5B**).
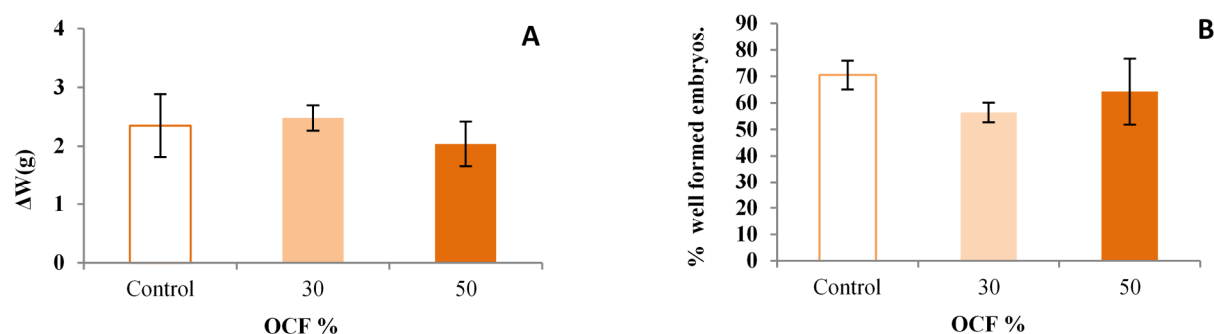

Figure 5. Effect of oomycete extracts on (A) weight increase (ΔW g); and (B) frequency of well-formed SE. Embryogenic line Ha13 was elicited with OCF (30 and 50%) and then transferred to proliferation medium. Data are means ± SE of 3 replications after 60 days of elicitation.

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Among the elicitors tested, MeJA was the least harmful for further SE growth (**Fig. 6A**). In fact, the growth of the embryogenic line increased with MeJA concentrations up to 25 µM. Nevertheless, the highest concentration of this elicitor negatively affected this parameter. Elicitation treatments with 25 and 50 µM of BTH and PABA, produced a decrease in fresh weight of the Ha13 embryogenic line (**Fig. 6B** and **6C**).
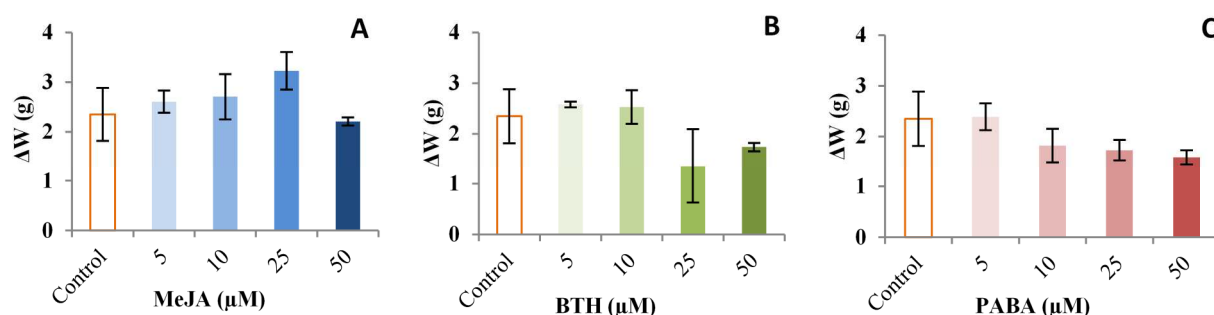

Figure 6. Effect of elicitor (MeJA, BTH and PABA) concentrations on weight increase (ΔW g) of the embryogenic line Ha13. Data are means ± SE of 3 replications after 60 days of elicitation assay.

As is shown in **Fig. 7**, all elicitors even when applied at low concentrations negatively affected further development of SEs. This effect was especially evident when the embryogenic line was treated with 50 µM BTH and PABA (**Fig. 7B** and **7C**).
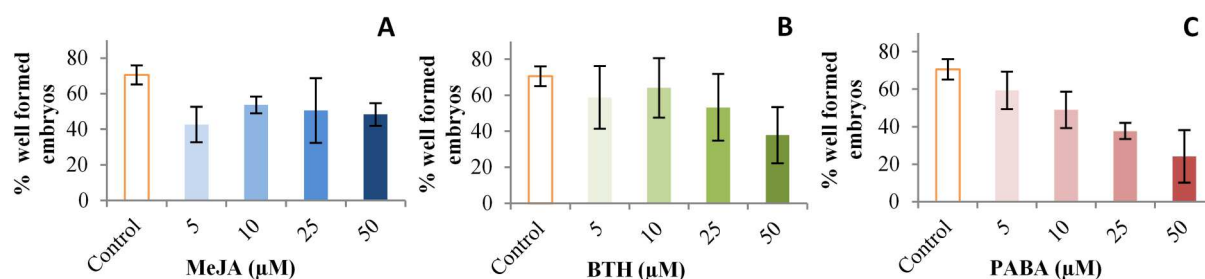

Figure 7. Percentage of well formed somatic embryos after elicitation with MeJA (A), BTH (B) and PABA (C). Data are means ± SE of 3 replications after 60 days of elicitation assay.

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Biotic stress responses

Hydrogen peroxide production of the control and elicited embryogenic lines were determined after challenging the tissue with liquid oomycete mycelia extract (20%). In general, H_2O_2 production was significantly higher in lines previously elicited with 5, 10 and 50 μM of MeJA or BTH (**Fig. 8B** and **8C**) and 25 μM of PABA than in the control (**Fig. 8D**).
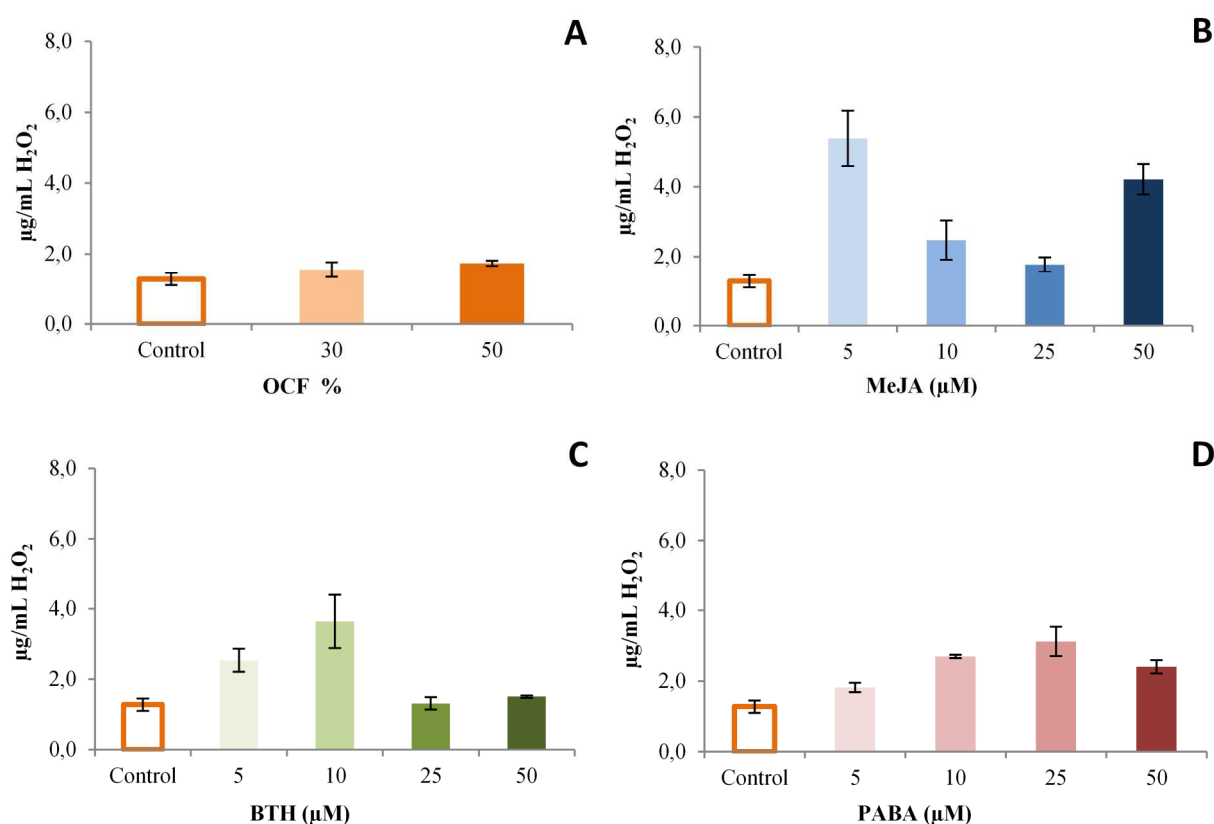

Figure 8. Oxidative stress measurement by means of peroxide concentration (μg/mL). Samples in liquid medium with 20% oomycete extract assay. Material previously treated with (OCF) (A) and chemical elicitors MeJA (B), BTH (C) and PABA (D). Data are means ± SE of 3 replications after 60 days of elicitation assay.

Preliminary assays for Phytophtora tolerance

In the dual cultures the mycelia growth ratios higher than 1 showed that the elicited material inhibited mycelium growth and thereby that these treatments confer some degree of tolerance. Best results were obtained after elicitation with 5 or 50 µM of MeJA (**Fig. 9B** and **Fig. 10**).
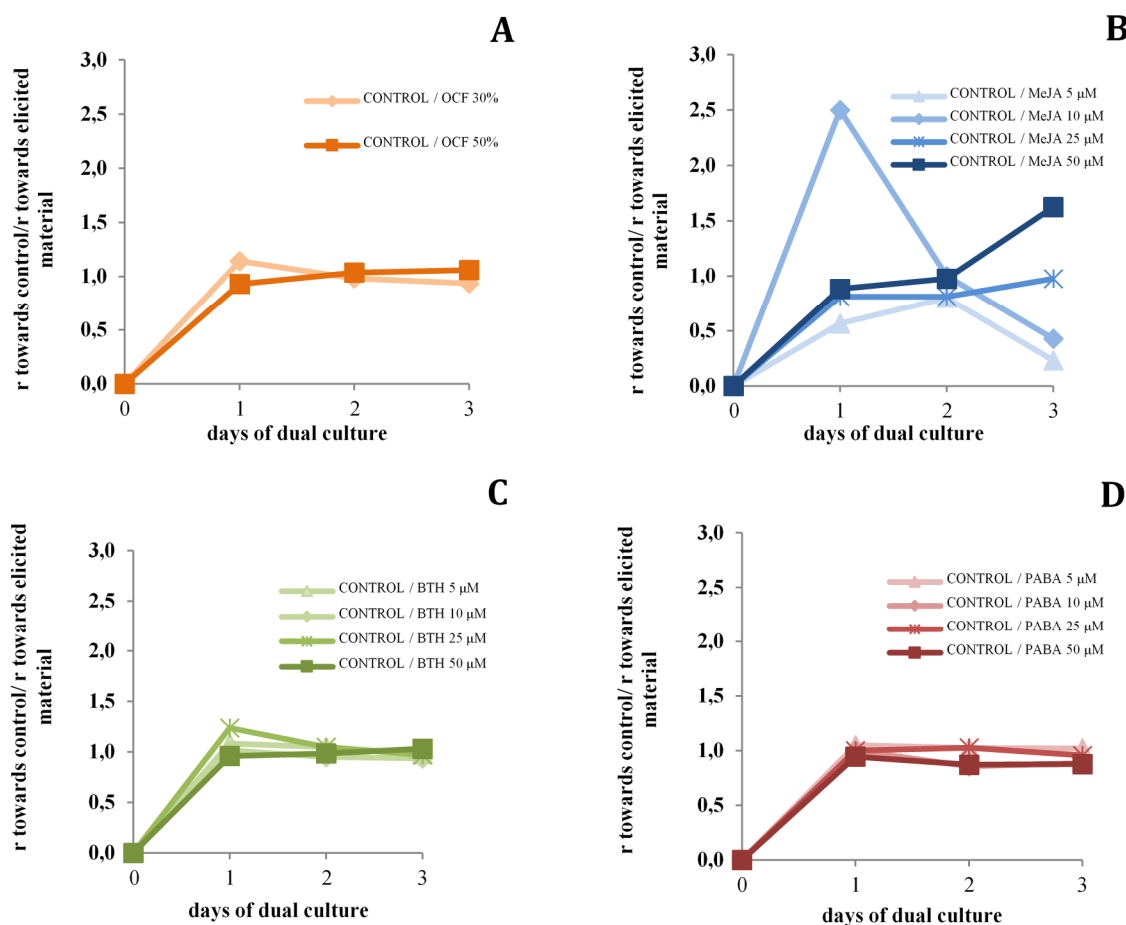
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Figure 9. Mycelia growth ratio (growth towards no elicited SEs, control/ growth to elicited SEs) in dual cultures. Embryogenic lines were previously elicited with oomycete extract (OCF) (A) or the chemical elicitors MeJA (B), BTH (C) and PABA (D). Data are means of 3 replications measured for 3 days. Ratios > 1 show some degree of tolerance to *P. cinnamomi*.
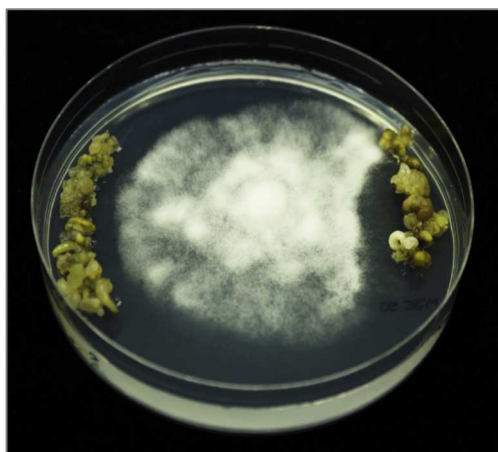

Figure 10. Dual culture. Control (left) and SE elicited wih MeJA (right).

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Conclusion

In conclusion among the elicitation treatments tested, 50% OCF and 5, 10 or 50 μM de MeJA seem to be the best to induce tolerance to *P. cinnamomi* in *Q. Ilex* embryogenic lines. These treatments do not affect SE growth and induce some tolerance in dual cultures. Three months after elicitation, we were able to regenerate shoots from SE treated mainly with MeJA, and to a lesser extend with PABA or OCF, whereas those treated with BTH produced necrotic tissue and did not develop into plants. The shoots are being rooted and further tolerance assays will be performed.

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Productivity in various pedo-climatic conditions of cold-hardy *Eucalyptus* clones developed by FCBA for plantation forestry in southern France

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Keywords: micropropagation, frost-tolerance, *Eucalyptus gundal*, climatic zones, growth, volume

Breeding cold-hardy *Eucalyptus* clones in France

FCBA (and formerly AFOCEL) is developing a long-term research and breeding program of *Eucalyptus* species since the early seventies with the objective of clonal plantation forestry in southern France (Franclet and Boulay 1989, Cauvin and Melun 1994, Nguyen The et al. 2004, 2010a, De Morogues et al. 2011). Developments include both traditional breeding and vegetative propagation of selected clones (Durand-Cresswell et al. 1982) and operating biotechnological inputs such as micropropagation through axillary budding (Boulay 1983, Franclet and Boulay 1989, Trontin et al. 2004, Da Silva Perez et al. 2011), cryopreservation of shoot apices (Pâques et al. 2002, Harvengt et al. 2015) and DNA fingerprinting (Da Silva Perez et al. 2011). Significant developments in *Eucalyptus* genetics and genomics are also ongoing in the frame of national partnerships (e.g., EUCANET project, http://www.agence-nationale-recherche.fr/?Projet=ANR-06-ERAP-0010) and transnational collaborations (e.g., TREE FOR JOULES project, http://www.agence-nationale-recherche.fr/?Project=ANR-10-KBBE-0007) towards increased knowledge about genes involved in cold-hardiness, growth, development and wood properties (Nguyen et al. 2017). *Eucalyptus* short-rotation coppicing is of economic interest in France (De Morogues et al. 2011) for both the pulp industry (Da Silva Perez et al. 2011) and biomass/bioenergy production (Melun and Nguyen The 2012, Gabrielle et al. 2012).

The main objective is to select and deploy fast-growing varieties that are well-adapted to the various pedo-climatic conditions found in southern France, from oceanic to more Mediterranean and arid conditions (Moreaux et al. 2012). One critical issue for breeders is to improve cold-hardiness of new varieties developed for foresters to cope with erratic severe winter or early autumn frost (Potts et al. 1987, Marien 1988, Cauvin and Potts 1991).

Selected *Eucalyptus* species for this program, namely *E. gunnii* Hook. F. and *E. dalrympleana* Maiden, originated from the mountainous regions of their natural distribution area in Australia, especially Tasmania. *E. gunnii* was identified as one of the most appropriate species for cold hardiness. *E. dalrympleana* exhibited much less tolerance to frost but significantly better growth behaviour compared to *E. gunnii*. Breeding efforts were, therefore, mainly focused on *E. gunnii* as well as *E. gunnii x E. dalrympleana* hybrid species (*E. gundal*) using well-selected provenances (**Fig. 1**).

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FCBA-121

Adult, age 15 · Smooth/reddish-pink bark · Narrow/elongated juvenile leaves

FCBA-208

Adult, age 14 · Smooth/whitish bark · Rounded juvenile leaves

FCBA-645

Adult, age 12 · Smooth/whitish bark · Pointed juvenile leaves, red twigs

Flowering & Seed production

Flower buds and flowers · Maturing capsules · Mature capsules and seeds
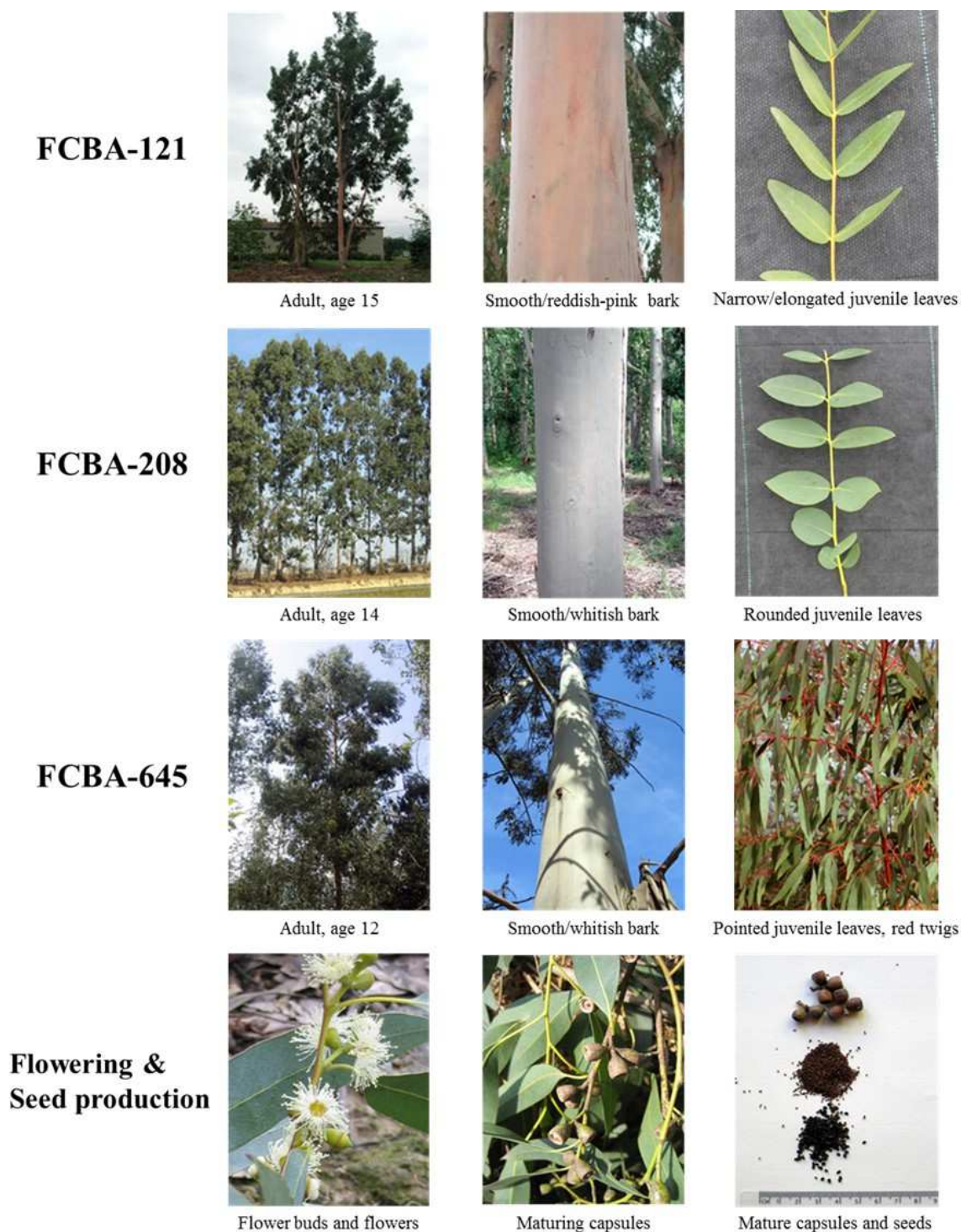
Figure 1. Some features of *E. gundal* hybrid clones (*E. gunnii* x *E. dalrympleana*) selected by FCBA (121, 208, 645) for plantation forestry in southern France. *E. gundal* clones exhibited both cold-hardiness (up to -12°C at a 50% cut-off threshold for damages) and high productivity (15-20 t/ha/year at age 9-13, up to 30-35 t/ha/year in favourable conditions, see **Fig. 6**).

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Deployment strategy of selected cold-hardy *Eucalyptus* clones

The best selected *E. gunnii* and hybrid *E. gundal* clones for both good growth and significant cold-hardiness (up to -18°C or -12°C at a 50% cut-off threshold for damages, respectively) were successfully introduced *in vitro* for conservation purposes (including cryopreserved collections, (Pâques et al. 2002, Harvengt et al. 2015), reactivation or maintenance of organogenic capacities (especially rooting ability) and rapid initial micropropagation through axillary budding (Franclet and Boulay 1982, 1989, Boulay 1983, Trontin et al. 2004, Da Silva Perez et al. 2011).

After *in vitro* establishment, suitable *E. gunnii* or *E. gundal* clones are further evaluated for micropropagation performance (from multiplication rate to delivery rate to customers) in order to select suitable varieties for cost-effective pre-development as rootstocks (**Fig. 2**). Micropropagated plants are then used by commercial forest nurseries (e.g., FORELITE, France) to implement large stool beds for the production of cuttings from selected varieties (**Fig. 3**). Following this strategy, around 2000 ha of pilot clonal plantations have been established in southern France (100-150 ha/year), mainly for the purpose of short-rotation coppices (3 rotations of ca. 10 years, **Fig. 4**).
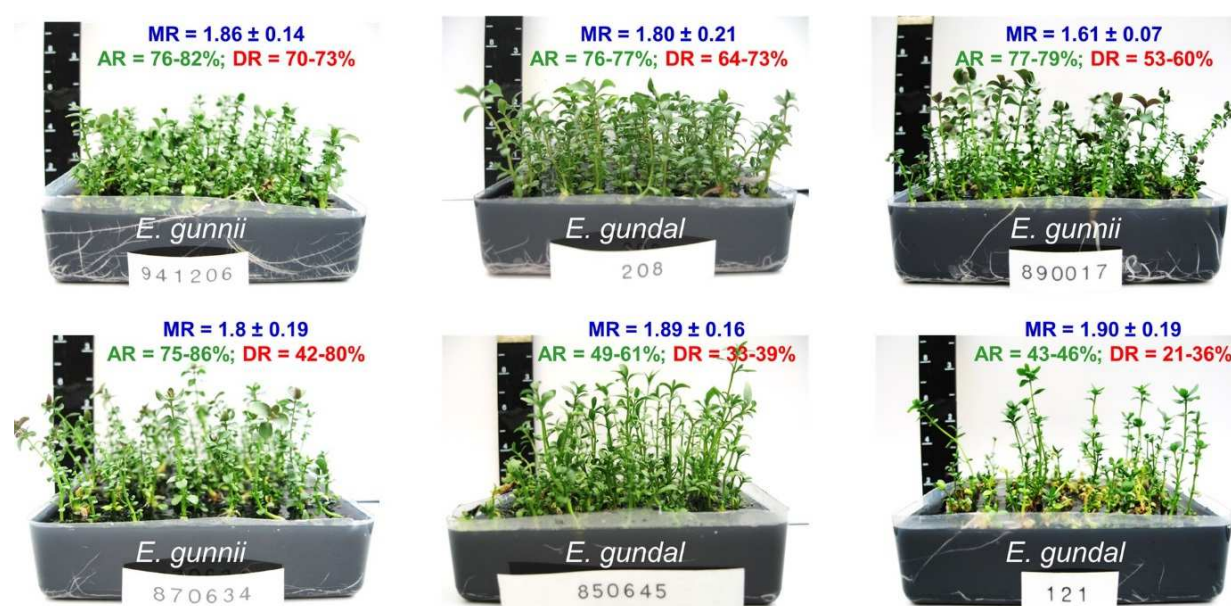

Figure 2. Micropropagation performance of 6 selected *E. gunnii* (941206, 890017, 870634) and *E. gundal* clones (208, 850645, 121) in standard conditions developed at FCBA. Some *E. gundal* clones (208, 850645) are commercially available in France for rootstock production and deployment in plantation forestry (FCBA-208, FCBA-645, see **Fig. 1**).

MR = Multiplication Rate (mean ± 95% confidence limits of 6 consecutive biweekly subcultures)
AR = Acclimatization Rate (range, %) – Rooted plantlets vs. shoots in rooting induction
DR = Delivery Rate (range, %) – Delivered 2-month-old plants to customers vs. shoots in rooting induction

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Figure 3. Acclimatized *in vitro* rootstocks (2 months old) of cold-hardy *E. gundal* FCBA clone (left, XYLOBIOTECH greenhouse) and outdoors stoolbeds (right) established at commercial forest nurseries for cuttings production (FORELITE, France).

Figure 4. Short rotation coppice (SRC) of *E. gundal* (clone FCBA-121) grown as a wood biomass crop for ca. 10 years in southern France (Lautignac, climatic zone 2, see **Fig. 5**).

A. SRC at age 1 year; B. SRC at age 3 years; C. SRC at the time of harvesting (10 years).

The significant testing network for monitoring productivity of cold-hardy *E. gundal* clones in various pedo-climatic conditions from southern France

Pedo-climatic constraints in France to grow cold-hardy *Eucalyptus* varieties have been precisely mapped (Nguyen The et al. 2010b, **Fig. 5**) considering:

i)   The estimated risk for pH-related chlorosis using integrated information from the European Soil Data Base (Panagos 2006, http://esdac.jrc.ec.europa.eu/), the BDAT (mapping tool Geosol, GisSol, INRA,

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http://estrada.orleans.inra.fr/geosol/), and forest soil mapping from IFN/Lerfob (http://inventaire-forestier.ign.fr/spip/). Selected *Eucalyptus* spp. typically grow at pH in the range 4.5-7.5 with some tolerance to pH higher than 8 in well-watered conditions.

ii) The estimated risk for severe winter frost using data from Météo France (frost occurrence below -12°C between Dec. 1st and March 15, mean 1975-2005) and FCBA field data to select iso-value lines of temperature. The -12°C temperature is the minimal temperature tolerated by mature *E. gundal* stands with optimal winter hardening.

iii) Topographic information about altitude (up to 400 m) and slope areas of more than 20% (BD ALTI ®, IGN) which is the upper limit for easy (mechanized) management of forest plantations.
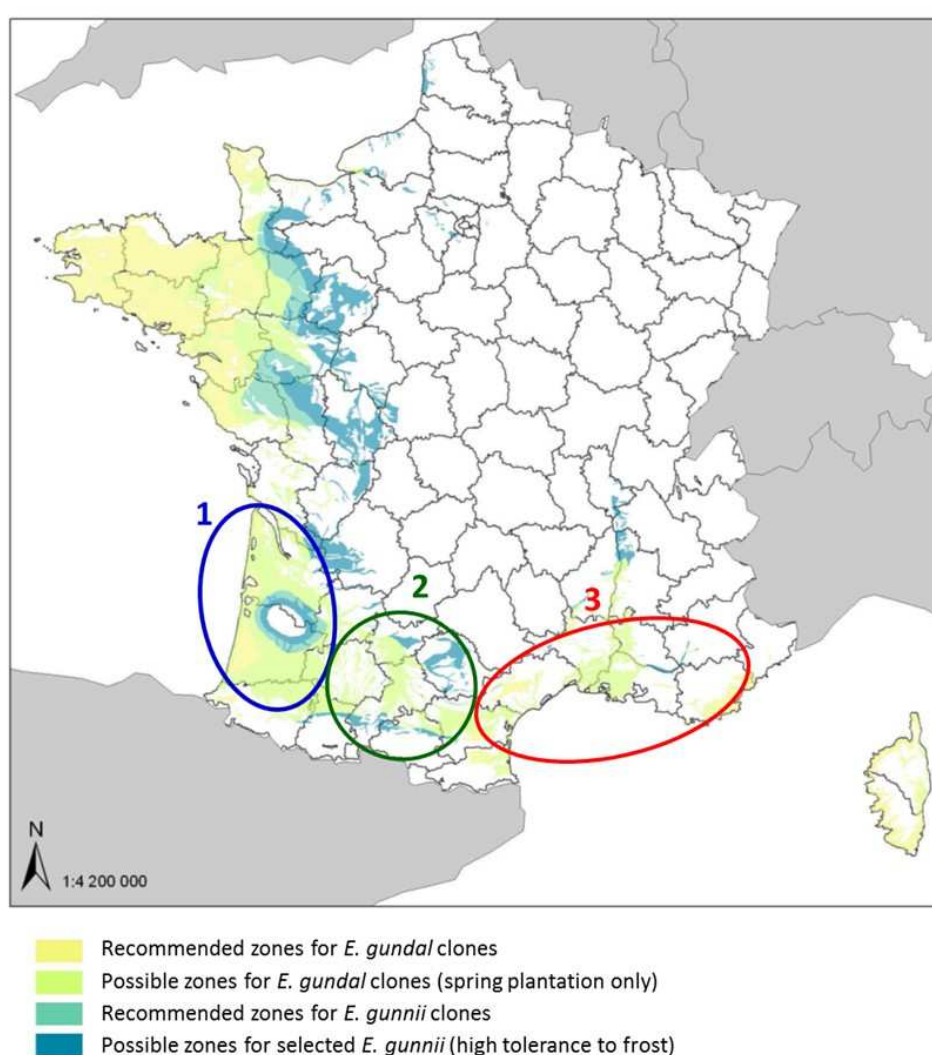

Recommended zones for *E. gundal* clones
Possible zones for *E. gundal* clones (spring plantation only)
Recommended zones for *E. gunnii* clones
Possible zones for selected *E. gunnii* (high tolerance to frost)

Figure 5. Map of pedo-climatic constraints in France to grow cold-hardy *Eucalyptus* varieties (adapted from Nguyen The et al. 2010b). Three main climatic zones were defined for plantation in southern France, i.e., oceanic (climatic zone 1, blue), transitional oceanic (climatic zone 2, green) and Mediterranean (climatic zone 3, red). Background map source: European Soil Database (Panagos 2006).

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Based on these data as well as climate consideration and soil characteristics, 3 large climatic zones for *E. gundal* plantation forestry in southern France were defined (oceanic, transitional oceanic and Mediterranean). The climatic zone 1 (oceanic, South-West regions, mostly poor, sandy and dry soils) is characterized by high atmospheric moisture, significant annual rainfalls (1000–1500 mm), hot summers and mild winters with low risks for severe winter frost. In climatic zone 2 (transitional oceanic, South-Central regions, mostly heavy soils), atmospheric moisture is lower as well as rainfalls (700-1000 mm/year) with quite hot and dry summers and possible cold winter with high risk for severe winter frost. Heavy soils in this zone also increased the risk for temporary flooding. In climatic zone 3 (Mediterranean, South-East regions, mostly calcareous soils), there is usually low atmospheric moisture with rainfalls often less than 700 mm a year, very hot and dry summers, stormy autumns and mild winters. As a result, the risk for severe winter frost is quite low in this zone.

In order to estimate field performance (especially productivity) of selected cold-hardy *E. gundal* clones in various pedo-climatic condition from southern France, a total of 75 experimental plots have been established in these 3 climatic zones (11 000 monitored trees). All stands are referenced into BAOGREFF, the FCBA database for genetic origin and forest field experiments information (http://base-sylviculture-genetique.fcba.fr/?lang=en).

The East-West productivity pattern of cold-hardy *E. gundal* hybrid clones in southern France

One reference (FCBA-121) and 2 commercially available varieties (FCBA-208, FCBA-645) were evaluated at various field plots (47 stands) established in climatic zones 1 (8 stands), 2 (26 stands) or 3 (13 stands). Productivity, expressed as fresh tons of salable wood per hectare at harvesting age, is shown in **Fig. 6**. Data were recorded during 1st (30 stands), 2nd (13 stands) and 3rd (5 stands) rotations at ages 4 to 13 years (74 data sets overall).

Selected clones grew well over 3 short rotation coppices in the variable pedo-climatic conditions found in southern France showing that these varieties can tolerate periods of winter frost. Overall, mean productivity at the end of rotation (9-13 years) significantly increased (by over 50%) from rotation 1 (15.1 t/ha/year, range 3.0-21.3) to rotations 2 (23.2 t/ha/year, range 16.5-36.1) and 3 (23.4 t/ha/year, range 19.2-27.6). Considering climatic zones, an East-West productivity pattern was observed (**Fig. 6**), from a mean of 8.5 t/ha/year in Mediterranean zone 3 (3.0–17.8), to 19.1 t/ha/year in transitional oceanic zone 2 (15.2-28.9) and 30.2 t/ha/year in oceanic zone 3 (23.0-36.1). A similar productivity pattern is observed at early harvesting ages (4-8 years).

Conclusion

We concluded that cumulated annual rainfall and soil water availability (in interaction with soil quality) are the main factors affecting the productivity of cold-hardy *Eucalyptus* varieties in southern France. From these data it appeared that *E. gundal* hybrids clones are capable of regulating water use both positively (in favourable climatic zones with good water availability, 1000-1500 mm/year) and negatively (in more Mediterranean zones with dry periods).
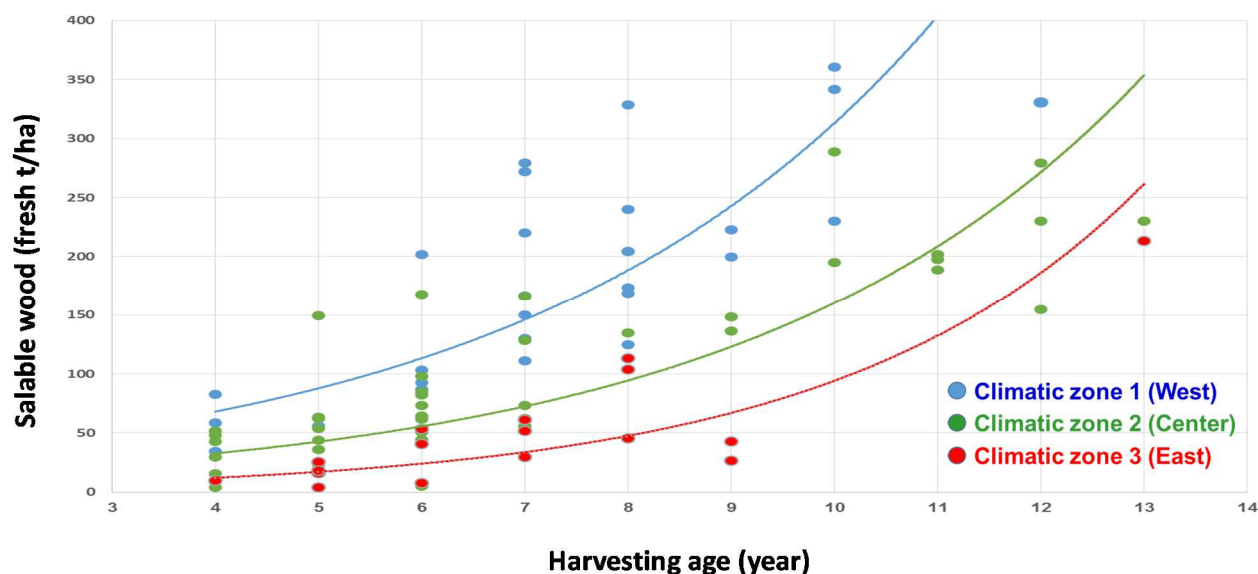
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Figure 6. Productivity of cold-hardy *E. gundal* FCBA clones (FCBA-121, FCBA-208, FCBA-645) in 3 pedo-climatic conditions found in southern France (see **Fig. 5**) from oceanic (climatic zone 1, West) to transitional oceanic (climatic zone 2, Center) and Mediterranean (climatic zone 3, East).

Productivity is expressed in fresh tons of salable wood per hectare at harvesting age (years) for 47 stands in their first, second or third rotation coppice (74 data).

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From Petri dishes to bioreactors – First experiences on optimization of Norway spruce SE-process for bioreactors

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Keywords: somatic embryogenesis, *Picea abies*, temporary immersion system, cost efficiency

Introduction

The project *Vegetative propagation of spruce –towards future plant production,* carried out at Natural Resources Institute Finland (Luke), is focused on paving the way for practical application of Norway spruce (*Picea abies* (L.) Karst.) somatic embryogenesis (SE). One important aspect related to this goal is improving cost efficiency of the process. Currently the SE-process used at Luke is based on manual labor, which is time consuming and expensive. Using bioreactors for multiplication of embryogenic tissue (ET) and for maturation of somatic embryos could increase efficiency of propagation by reducing the amount of manual labor and, as a result, reduce the cost of a single plant significantly.

Use of a temporary immersion system (TIS) is one of the most commonly used bioreactor types in *in vitro* culturing. There are several commercially available TIS models in which liquid medium is applied at intervals to plant material that is located in a compartment that is separate from the medium. The advantages of the TIS systems in embryo germination and plant production have been documented, and benefits have been shown to include the reduction in workload and thus in cost, and better plant performance by allowing improved contact between the plant material and the medium because of repeated, temporary total immersion and by renewing the culture atmosphere during each immersion (Etienne & Berthouly 2002, Hanhineva & Kärenlampi 2007). However, most of the studies have been done with shoots or germinating embryos and only limited data are available on the effect of the different culture parameters, such as the immersion frequencies, on embryogenic tissue culture during the proliferation stage (Etienne & Berthouly 2002).

Besides productivity of the cultures, there are several other factors to be taken into account when choosing the type of TIS. Because tissue cultures need aseptic conditions, an autoclaving or other sterilizing treatment for TIS is needed, and often the space for sterilization is limited. Because of repeated autoclaving TIS components should be made of heat-stable durable materials. Also the easiness of aseptic handling during the culturing process is important. For example, if there are many parts of the TIS to be handled, or parts do not fit each other well enough after repeated autoclaving cycles, the risk for contamination increases.

The aim of this work was to evaluate different commercial TIS models for somatic embryogenesis of Norway spruce. The optimization of the tissue culture process was started by testing ET proliferation on various support materials and by searching the optimal frequency of medium immersion.

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Material and methods

Embryonic cell lines of Norway spruce used as test material (see **Tab. 1**) were initiated from immature seed embryos following Klimaszewska et al. (2001) method. Initiations were made in July 2014 and established ETs were subcultured every two weeks. ET clumps were maintained on semi-solid Litvay's medium (mLM) (Litvay et al. 1985) as modified by Klimaszewska et al. (2001). Medium for bioreactors was the same mLM without gelling agent.

Three commercially available TIS models were evaluated for somatic embryogenesis: Rita® (Vitropic, France), SETIS™ (Vervit, bvba, Belgium) and Plant Form (Plant Form, Sweden). Rita® and Plant Form consist of one vessel where media and ET are located in different compartments, while SETIS™ consists of two connected vessels where media is in one vessel and ET in the other. In all these TIS models, the medium is forced upwards by air pressure provided by air pumps controlled by programmable timers to immerse the plant tissue. For mass production of spruce somatic embryos the Rita® vessels were evaluated to be too small for practical use, thus only the two other models were tested further. SETIS™ and Plant Form bioreactors were autoclaved and loaded aseptically according to manufacturer's instructions.

To the SETIS™ system 500ml media was added into the medium vessel and it was closed while waiting for the culture vessel to be filled. Six embryogenic lines were used in two separate experiments. In the first experiment, an average of 800 mg of ET was suspended in 10 ml of liquid mLM and sprayed, using 25 ml syringe, onto sterile filter paper (Whatman #2) placed on the internal support pad in the culture vessel. In the second experiment, 800 mg of ET clumps were spread straight onto the internal support pad using the syringe.

To the Plant Form system 200 ml of medium was added to the bottom of the vessel. Eight lines were used in separate experiments as shown in **Tab. 1**. On average, 1200 mg of ET was suspended in 10 ml of mLM media and spread either 1) onto filter paper (Whatman #2) placed on sterile paper towel to drain the extra liquid, and then the filter paper with ET was placed in a basket inside the vessel; 2) ET was placed in the basket without use of filter paper 3) ET was placed onto a dense metal net (Ø 0.15 mm); or 4) ET was placed onto filter paper deposited earlier in the basket inside the vessel. In the experiments 1-2, ET was immersed for 2 minutes at 6 hour intervals, in the experiment 3 the immersion interval was 4 hours. In experiment 4, three different immersion intervals were tested: 4, 6, or 8 hours. Experiment 1 started on 11th March 2015, experiment 2 without filters on 31st March, experiment 3 with metal net on 22nd April, and experiment 4 with different immersion intervals on 17th, 21st and 22nd April.

As a control, the same embryogenic lines were proliferated also on semi-solid medium. On average, 200 mg of ET was suspended in 3 ml of liquid mLM and poured onto a filter paper (Whatman #2) placed on a Büchner funnel. The liquid was drained off by suction and the filter was placed on mLM medium in a 9 cm petri dish. All bioreactors and petri dishes were kept in a dark room at +21°C for two weeks. ET mass was weighted before and after every experiment, and growth rate was calculated as: GR= weight after experiment/ weight before. The differences in growth rate between bioreactors and control plates were statistically tested using the Independent Samples Mann-Whitney U test, and between immersion intervals using the Independent-Samples Kruskal-Wallis test (IBM SPSS statistics 22).

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Table 1. Growth rates of the Norway spruce embryogenic lines in the proliferation experiments in Plant Form (PF) bioreactors and on control plates with semi-solid media, tissue proliferation measured for 2-week period.

Experiment / Embrygenic line	1-Paper filter and drying		2-Without filter		3-Metal net		4-Paper filter and varying immersion intervals					
	PF 6 h interval	Semi-solid media	PF 4 h interval	Semi-solid media	PF 4 h interval	Semi-solid media	PF 4 h interval	Semi-solid media	PF 6 h interval	Semi-solid media	PF 8 h interval	Semi-solid media
14Pa 1606	7.1	5.0	2.0	1.3	6.6	6.8	7.5	6.3	4.2	4.0	7.1	2.7
14Pa 1813	16.8	12.6	0.8	2.4								
14Pa 4639	11.0	9.9	2.7	2.8								
14Pa 4027	2.9	3.5	1.0	0.2								
14Pa 5109	8.5	4.9	1.4	1.6								
14Pa 645	12.8	9.0	0.7	2.0								
14Pa 4623					10.8	9.4	11.2	8.9	8.4	6.4	4.8	3.8
14Pa 1087					9.1	6.4	8.4	5.4	7.3	5.4		
Mean	9.8	7.5	1.4	1.7	8.8	7.5	9.0	7.7	6.7	5.3	5.9	3.3

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Results

Autoclaving of TIS proved to be more laborious and time-consuming than expected. This is because of the autoclave used at Luke, (Systec VX-75), can accommodate only 2 SETIS™ or 4 Plant Form vessels at a time, i.e., autoclaving of numerous vessels for a bigger experiment would take several working days.

With SETIS™ we had handling problems: aseptic spreading of ETs onto the internal support pad in the deep culture vessel having a narrow neck proved to be difficult, and this lead to serious contaminations. Tissue growth was visually detected, but because of contaminations it was not weighed. By using Plant Form only one bioreactor became contaminated in experiment 4.

There were no significant differences in growth rates of Norway spruce ETs between their growth in Plant Forms TIS and in the controls on semi-solid media in any of the experiments, even though in most of the cases ETs proliferated more in TIS than on the control plates (**Tab. 1**). The growth in Plant Forms without any filter in experiment 2 was, however, remarkably low compared to that in other experiments, and also lower than on the control plates. When comparing immersion intervals, growth rate of ETs was higher at 4 hour than at 6 or 8 hour intervals, however the difference was not statistically significant.

Discussion

Although Norway spruce ET has been shown to grow in suspension culture (Hakman et al. 1985, Boulay et al. 1988), it is usually grown in semi-solid media (Högberg et al. 1998, Klimaszewska 2001, Högberg & Varis 2016). In many of the published studies on suspension cultures of Norway spruce ET the number of tested genotypes or the frequencies of lines proliferating successfully is not given. In our previous experiments, only two out of eleven lines proliferated successfully in liquid media rotating 100 rpm in Erlenmayers, which is not acceptable for commercial plant production where the protocol has to be suitable for wide range of genotypes, reliably, and cost-effective.

Instead of suspension cultures, Norway spruce ET proliferates well when it is only temporary immersed into liquid media, as shown in this study. In all but one experiment ET proliferated better in a TIS bioreactor than on semi-solid media, even though the differences were not statistically significant. Similar growth rates in TIS and on controls indicate that the low growth rate in the experiment 2 without a filter is probably due to quality of the ETs in this particular experiment, rather than the lack of a filter. It was, however, observed that in the basket without a filter some medium remained between the immersion times and that may have reduced the growth of ET, because in this experiment ET growth was higher on the control plates. In all the experiments ET clumps tended to float in the medium if there was too much media and it was not properly drained from the inner basket of the Plant Form. The handling of paper filters was difficult, whereas a metal net was easier to handle but it did not remain evenly flat when autoclaved. In order to ensure maximal proliferation of ETs, the supporting structure (filter or net) should be as flat as possible, since medium tends to remain between clumps, and growth of ETs is visibly inferior on these wet areas.

In TIS bioreactors, frequency and duration of medium application will affect the availability of nutrient and growth regulators, and thus the development of cultures. Optimal frequency and duration of immersion may vary between species (review see, Etienne and Berthouly 2002). For example Tisserat and Vandercook (1985) had 12 h cycles and 5-10 min immersion durations for both date palm and carrot embryogenic callus proliferation. In our study, more frequent (4h interval) immersion was better for the proliferation of Norway spruce ETs than longer (6 to 8 h) intervals. Optimization should, however, be continued by testing varying amounts of ET inoculum and lengths of proliferation time in order to determine conditions for the most efficient multiplication of the cultures.

From the efficiency of propagation point of view, the use of time and resources should be reduced by the transfer from plate cultures to bioreactors. Based on the present experience with the tested commercial TIS

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models, sterilizing and loading of the bioreactors requires more work than expected, making the overall work load comparable or even bigger than manufacturing and using semi-solid media in single-use petri dishes. This could be solved by designing bioreactors specifically for SE cultures, taking into account space requirement of the ETs as well as space available in sterilizing apparatus. Another issue is the efficient use of media: bioreactors should preferably use less media per gFW of produced ET or per number of somatic embryos than propagation on semi-solid media in petri dishes. This was not yet achieved in the present study, but could probably be reached by focusing on the factors having the major effect, i.e., amount of inoculum and medium, length of proliferation period and medium content.

It can be concluded that optimizing a tissue culture method, such as somatic embryogenesis of Norway spruce in bioreactors may be challenging and time consuming, with all the culture process steps having to be optimized separately and for a wide variety of genetic materials. In Norway spruce SE, the proliferation of ETs in TIS bioreactors has been achieved but could still be further optimized to increase propagation efficiency. The next step in somatic plant production, i.e., maturation of somatic embryos, requires separate optimization that may include not only the factors tested already for proliferation (TIS model, support structure, immersion frequency) but also others, such as gas exchange or medium applied.

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Short abstracts

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Nisa M et al.

Cloning cork oak trees selected as tolerant to *Phytophthora cinnamomi*

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Abstract

Vegetative propagation is a main tool in forest breeding that captures all the genetic potential of selected trees and produces uniform offspring. This way of propagation allows the simultaneous capture of different traits and therefore its transfer to the improved populations, resulting not only in better growth performance and product quality, but also in tolerance to pests and diseases. The cork oak (*Quercus suber* L.) is a Mediterranean tree of ecological and economic interest. This oak sustainably produces cork, a renewable product used as stoppers of high quality wine bottles, but also in other industrial applications. Acorns to feed cattle and gastronomically appreciated mushrooms are other products of interest. This species is threatened by a decline syndrome called "la seca" that is causing high mortality among cork and holm oak trees in the "dehesa" and "montado", agroforestry systems of Spain and Portugal. Among the causes of this syndrome, one of the most important is the infection by the oomycete *Phytophthora cinnamomi* Rands. The production of varieties tolerant to this pathogen could be one way to address this problem. Clonal heritability of tolerance to *Phytophthora* sp. has been demonstrated in several species. In the framework of a project in which one of the objectives was to determine whether the tolerance trait can be transferred to cork oak clonal progenies, adult trees are being cloned by somatic embryogenesis (SE).

Ten trees were selected as tolerant on the basis that they were standing in a low-lying zone in which the oomycete was detected, and that the mortality of surrounding trees was high. Samples from five trees growing in the same area but at higher altitude, where mortality is rare, were collected as controls. Following procedures previously established by our team, SE induction was performed in leaves from these trees. Embryogenic lines were obtained from 8 putative tolerant trees and from 4 control trees. Frequencies of induction varied with genotype, ranging from 2 to 74%. Proliferation was carried out by secondary embryogenesis. A decline of single embryo production with time in culture was observed, although a significant interaction with genotype was also recorded. Germination of somatic embryos was also influenced by genotype with mean frequency of 47% ranging from 16 to 65%. In order to increase the production of plants from the selected trees, multiplication by organogenesis from shoots of germinated somatic embryos as rejuvenated explants was accomplished. Rates of multiplication depended on genotype. The mean number of buds per nodal explant after 6 week of culture ranged from 2.5 to 9.5 and the mean number of shoots per explant longer than 15 mm, suitable for rooting, from 0.3 to 2.6. Microshoots rooted after a 24 h treatment with IBA at frequencies between 10 and 47% depending on genotype. An effect of the length of the IBA treatment on rooting frequencies was recorded, doubling when it was increased to 48 h. Somatic embryogenesis has been a suitable tool to clone selected cork oak trees. Work is in progress to test the tolerance of the regenerated plants.

Keywords: breeding, forest biotechnology, disease tolerance, *Quercus suber*, somatic embryogenesis

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Integrating low cost vegetative propagation techniques with a domestication and conservation strategy for multipurpose native species of Misiones, Argentina

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Abstract

The Atlantic Forest or *Selva Paranaense*, represents one of the most biodiverse regions of Argentina, currently covering an area of 1,183,791 hectares. However, selective logging of native timber species, the advance of the agricultural frontier and the overexploitation of natural resources have led to the degradation of the remaining forest, with the consequent loss of genetic variability. A direct consequence is the continued underutilization of the genetic potential of plant species and seeds are increasingly more scarce and difficult to harvest. Representative of this situation are the legume tree species *Peltophorum dubium* (caña fistola), *Enterolobium contortosiliquum* (Timbó), and native fruit trees like *Eugenia involucrata* (Cerella or native cerezo), *Acca sellowiana* (native Guayabo) and *Rheedia brasiliensis* (Pacurí), who have all been selected for our studies. The value of using native tree species in ecosystem restoration is receiving growing recognition, not only globally, but also locally. However, insufficient attention has been given to genetic variation within and among native tree species (Thomas et al. 2014).

The aim of our research was to facilitate and increase the availability of planting material and making accessible their genetic diversity for local restoration programs. To achieve this goal, we have carried out a project to establish a network of seed trees and areas in the province of Misiones, and initiated the study of the genetic structure of two native species (*Peltophorum dubium* (caña fistola) and *Enterolobium contortosiliquum* (Timbó)), as well as the development of clonal propagation techniques. So far, we have obtained four microsatellite markers for *Peltophorum dubium*, and eight for *Enterolobium contortosiliquum*, and started a short-term provenance and progenies test for both species, to assess the genetic diversity and to contribute to their conservation and sustainable management. We also propose to develop a methodology for *in vitro* and *ex vitro* propagation. For the *in vitro* studies, different sources of explant, nutrient media, hormones, culture conditions and acclimation were studied. For *ex vitro* propagation, a mini-stumps/mini-cuttings methodology was addressed. Different container sizes, fertilization and the use of semi-hydroponics systems, and growing environments, were studied for ministumps management. For rooting of minicuttings, different inductive treatments (pre-treatments of ministumps, application method and auxin concentrations) were considered. Through this study, we generated 1) an *in vitro* germination and establishment protocol for the proposed species, 2) an axillary multiplication methodology for *Peltophorum dubium, Enterolobium contortosiliquum, Eugenia involucrata* and *Acca sellowiana*; and 3) a method for induction of organogenic and embryogenic tissue from explants of *Peltophorum dubium* and *Enterolobium contortosiliquum*.We also developed an operational management system for ministumps and protocols for rooting of minicuttings for *Peltophorum dubium, Eugenia involucrata* and *Acca sellowiana*.

Keywords: multipurpose species, micropropagation, macropropagation

Reference: Thomas E, Jalonen R, Loo J, Boshier D, Gallo L, Cavers S, Bordács S, Smith P, Bozzano M (2014) Genetic considerations in ecosystem restoration using native tree species. Forest Ecology and Management 333: 66-75, https://doi.org/10.1016/j.foreco.2014.07.015

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"DendroMax" – a cornerstone to integrate biotechnology into traditional German forestry

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Abstract

International efforts have resulted in protocols for somatic embryogenesis (sE) in woody plants to be applied not only as a tool for clonal propagation but also to accelerate current breeding strategies. These are fundamental prerequisites for the project "DendroMax", which aims at an operative integration of sE of coniferous timber species into established breeding programs.

Specifically for hybrid larch *Larix x eurolepis* we are aspiring to implement sE within the public enterprise for forestry in the state of Saxony (Staatsbetrieb Sachsenforst). This entails a chain of operations: Controlled crossings between selected parental trees for sE-initiation, maintenance, storage and characterization of sE-material, plantlet production, acclimatization and transfer to nurseries and evaluation in field trials (for ecological adaptation, superior yield and wood characteristics). The challenges we face not only involve the incorporation of the entire process of sE into routine breeding procedures under economically competitive conditions, but also in the fulfillment of national guidelines and prevailing laws, e.g., regarding the marketability of newly developed varieties, particularly with respect to breeding for a modern and flexible forestry. The collaboration is supported by the Federal Ministry of Food and Agriculture (BMEL) in Germany and will allow public access to know-how and *in vitro* breeding material while addressing foresters of both, the private and the public operating sector. Currently, field trials with clonal hybrid larch are being assessed in Saxony and these represent the first systematic plantations of sE-derived plants of *Larix x eurolepis* in Germany.

Keywords: hybrid larch, *Larix x eurolepis*, somatic embryogenesis, clonal propagation, forestry breeding, field trials

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Duarte ER et al.

In vitro shoot induction and multiplication from nodal segments of *Austrochthamalia teyucuarensis* H. A. Keller

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Abstract

Austrochthamalia teyucuarensis H.A. Keller is a species endemic in the province of Misiones. It belongs to the Apocinaceae family and blooms from September to February. Its fruit is a follicle and seeds typically show high germination rate (90%). *A. teyucuarensis* has great potential as an ornamental species but, unfortunately, the only known population is restricted to about 100 specimens that are located in an area of high anthropic incidence. The species is therefore critically endangered and the development of suitable propagation techniques to enable restoration and conservation *ex situ* is required.

The aim of this work is to propose an alternative seed germination method in controlled conditions followed by vegetative propagation from nodal segments using *in vitro* culture techniques. The mature seeds were harvested in Teyú Cuaré Spot, San Ignacio, Misiones (27° 16' 43.9" S - 55° 33' 44.9" W) in June. Disinfection of seeds was achieved in two steps under the laminar flow chamber: 1) seeds were immersed in a solution of hydrogen peroxide (H_2O_2) 10 volumes for 30 min and then in 70% alcohol for one min; seeds were subsequently transferred to a solution (1.5%) of sodium hypochlorite (NaOCl) supplemented with Tween 20® (two drops) for 20 min; 2) seeds were soaked in H_2O_2 10 volumes for 16 hours, then in 70% alcohol for one minute and finally in NaOCl 1.5% with two drops of Tween 20® for 20 min. The culture media used for germination were original MS (Murashige and Skoog 1962) and SH (Schenk and Hildebrandt 1972) formulations with 6 g.L^{-1} agar used as gellan gum. The media were free of sucrose and growth regulators.

Two seeds were grown in 50 ml tubes, with 10 ml culture medium, incubated for 30 days and exposed to laboratory light conditions (PAR: 116 μmol.m^{-2}.s^{-1}; photoperiod: 14 hours) and controlled temperature (27 ± 2°C). Nodal segments, obtained from the *in vitro* germinated seedlings, were cultured on MS medium supplemented with BA (0, 2.2, 4.4 and 8.8 μM), and incubated under the same conditions as stated above for 45 days.

The results showed that the germination percentage was 100% in both media and the morphogenic process began two days after the start of the *in vitro* cultivation. Furthermore, the disinfectants and the disinfection process used were effective in the elimination of pathogens (fungi and bacteria). The multiplication treatments with 2.2 and 8.8 μM BA showed the best results with 3.00 ± 0.10 and 3.17 ± 0.95 shoots per explant, respectively.

In conclusion, we can state that the disinfection procedures and culture media that were evaluated were appropriate to achieve the establishment and *in vitro* germination of mature embryos from *A. teyucuarensis* HA Keller, and that BA induces growth of multiple shoots, thus generating an alternative reproduction method for *ex situ* conservation of this endemic species.

Keywords: endemic, Apocinaceae, seed, shoots

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Duarte ER et al.

Adventitious bud regeneration and plantlets production of *Balfourodendron riedelianum* (Engl.) Engl.

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Abstract

Balfourodendron riedelianum (Rutaceae) is distributed between the latitudes of 10° S and 30° S comprising the south of Brazil and northern Argentina. Unfortunately, the high quality of its wood, used in luxury furniture, construction and carpentry in general, has triggered the overexploitation of this natural resource. Actually, *B. riedelianum* is included in the category of "endangered species" in the Red Book of the International Union for Conservation of Nature.

In order to develop a protocol for adventitious bud formation and large-scale plant production of *Balfourodendron riedelianum*, hypocotyls and cotyledons, obtained by the *in vitro* germination of seeds, were cultured on Murashige and Skoog (MS) semisolid medium (Phytagel®, 3.5 g·L^{-1}), plus sucrose at 30 g·L^{-1} and containing different combinations of α-naphtalenacetic acid (NAA, 0.05 µM), 6-benzyladenine (BA, 0.44 µM), and thidiazuron (TDZ, 0.004-0.4 µM). The cultures were incubated at 27 ± 2°C and exposed to a photoperiod of 12 h (116 µmol·m^{-2}·s^{-1} PPFD) for 30 days. Thereafter, the regenerative explants were transferred to bioreactors containing 100 mL MS plus gibberellic acid (GA$_3$, 0.5; 1 µM), BA (0.8, 1.5 µM) for elongation for 30 days. Subsequently, the elongated shoots were rooted in MS semisolid (agar 0.65%) medium with either indole-3- butyric acid (IBA, 2.5, 5, 7 µM) or NAA (0.5, 1.4, 2.7 µM) under the environmental conditions described above.

The regeneration frequency (40 ± 10%) and the number of adventitious buds per responsive explant (8.00 ± 6.15) was greatest when the cotyledons explants were cultured in MS plus NAA 0.05µM and TDZ 0.04 µM. During the elongation phase, 48.79 ± 4.01% of shoots grown in MS plus BA 0.8 µM provided shoots with more than 5 mm in length. All plantlets raised *in vitro* were phenotypically normal and successfully hardened to *ex vitro* conditions.

Keywords: *Balfourodendron*, cotyledon, hypocotyl, temporary immersion

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Zanella L et al.

Effect of folic acid and culture medium on somatic embryogenesis initiation in *Pinus caribaea* var. *hondurensis*

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Abstract

Somatic embryogenesis is a tissue culture technique which consists in embryo formation from somatic cells in a process similar to that of zygotic embryogenesis. Some advantages of the technique are mass propagation of elite clones, cryopreservation of embryogenic tissues and *ex situ* conservation of germplasm of endangered species. This study aimed at evaluating the effect of folic acid on the embryogenic potential of mature megagametophytes of *Pinus caribaea* var. *hondurensis* cultured on different media.

Excised megagametophytes were placed on induction medium containing QL (Quoirin & Lepoivre 1977), DCR (Gupta & Durzan 1985) or WV5 (Coke 1996) salts and vitamins, sucrose, myo-inositol, glutamine, casein hydrolysate, 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP) and agar, with or without the addition of 100 mg L^{-1} folic acid (FA) in Petri dishes. The explants were incubated in the dark at $23 \pm 2°C$ with subculture to new, fresh induction medium every 21 days. After 90 days the explants were evaluated regarding the percent of embryogenic tissue initiated through staining with Evans blue in acetic acid. After 120 days, we evaluated the embryogenic tissues for the presence of somatic embryos. The treatments were compared by Tukey's test ($p < 0.05$). The coloring test indicated the presence of embryogenic tissue in all treatments, and the formation of pro-embryos was observed under the microscope. Besides, qPCR analyses comparing embryogenic and non-embryogenic tissues revealed the expression of *AaSERK1* gene only in embryogenic tissue.

The percentage of initiated embryogenic tissue was higher in all treatments containing FA (up to 48% on QL + FA). However, initiation rates obtained for the DCR and WV5 media with FA were not statistically different from the QL without FA (26%). After 120 days, somatic embryo-like structures were observed on media with FA and on QL medium without FA. The largest number of embryogenic tissue with embryo-like structures (10%) was observed on QL medium + FA. The largest average of embryo-like structures per embryogenic tissue was observed on both QL media with or without FA (5). Histological analyses showed that the observed structures have no vascularization within the embryogenic tissue and no meristem. Therefore, these data pointed to the formation of abnormal embryos.

Keywords: QL, WV5, DCR, megagametophyte, qPCR, *AaSERK1*

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Rocha SP & Niella F

Induction of embryogenic tissue from apical meristems of *loblolly pine*

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Abstract

Family forestry is currently implemented on a commercial scale in the province of Misiones; however, the genetic gains are lower than those obtained from clonal forestry. Therefore, the potential of the somatic embryogenesis technique to generate rejuvenated tissue from meristems of adult trees could be an appropriate technology to advance *Pinus sp.* clonal forestry.

Our research focused on the development of embryogenic lines from apical meristems of juvenile and adult tissues of *Pinus taeda.* Different studies were carried out, such as: 1) *In vitro* establishment, which involved donor plant sprouting as pre-treatment and growing apical meristems from potted plants, 2 and 4 years old, and from grafts 18 years of age *in vitro*. 2) Embryogenic induction from cross sections of 5 mm in thickness, obtained from sterile shoot apices of 2- and 4-year-old seedlings and from grafts established *in vitro*. Basic medium WV5 (W) described by Coke et al. (1996) and the common DCR (D) medium described by Gupta and Durzan (1985) were used. The concentrations and combinations of growth regulators used for both the pre-treatment and the stages of initiation and maintenance, were the ones described by Malabadi and van Staden (2005). The inductive pre-treatments studied were: a) the sterilized explants were cultured in pre-inductive medium, at 3°C, in darkness, for three days previous to being sub-cultured in the inductive medium and b) without inductive pre-treatment, explants were sterilized and grown directly on inductive medium, at room temperature in darkness. A completely randomized design was used with a factorial arrangement of treatments with five replications with 10 explants each in every test conducted at each stage of study. The variables evaluated were number of surviving explants (vigorous and sterile)/treatment; number of induced explants/treatment; induction type and place, as well as cytological and histological observations.

It was shown that stock plant isolation for at least 15 days, while spraying the plants weekly with a fungicide and bactericide solution, followed by surface sterilization with sodium hypochlorite, resulted in $80 \pm 12\%$ explant survival, ready to be used for the somatic embryogenesis studies. Moreover, the pre-cultivation of explants, at 3°C, prior to culture on induction medium in the dark at 27°C, was a necessary step for the induction of embryogenic tissue. The developmental stage of the apical shoots used as explants, affected the cytology and histology of the generated embryogenic tissue.

Keywords: *Pinus taeda*, *in vitro* culture, histology

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Vondráková Z et al.

The effect of UV-B radiation on the development of Norway spruce somatic embryos

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Abstract

Norway spruce (*Picea abies* (L.) Karst.) is a native European conifer that is widely planted both in cool and temperate regions and that is one of the economically most important coniferous species within Europe. Due to its adaptability, it has been introduced around the world. Various environmental conditions bring about a wide range of abiotic stresses that affect the growth and the development of the plants and affect their morphology. We focused our investigation on the effect of various doses of UV-B irradiation on spruce somatic embryos (SE). The effects were evaluated biochemically and morphologically.

We have used a model system of Norway spruce somatic embryogenesis to follow the stress effect on SE in desiccation, when embryos mature biochemically. The stress induced by cultivation without any medium during desiccation is the prerequisite for a subsequent successful germination of SE. The most general plant response to UV-B radiation is the activation of polyamine and flavonoid biosynthetic pathways as polyphenolic compounds and polyamines possess free radical scavenging properties that can improve the plant's stress tolerance. We examined the kinetics of the polyamines and the enzymes involved in the polyamine biosynthesis. Content of phenolic acids was assessed; polyphenolics were localized histochemically. The level of malondialdehyde (MDA) indicated the extent of lipid peroxidation. We found changes in polyamine content and in the ratio between putrescine, spermidine and spermine induced by irradiation with high UV-B doses. The detected increase of MDA content points to an increase in oxidative stress.

HPLC-MS analyses revealed the presence of eight phenolic acids in extracts of SE: two cinnamic acid derivatives - *p*-coumaric and ferulic acids and six benzoic acid derivatives - *p*-hydroxybenzoic, protocatechuic, vanillic, gallic, salicylic and anisic acid. Total content of phenolic acids in irradiated embryos increased by about 25% relative to the control. The effect of irradiation was most clearly manifested in the accumulation of glycosides of benzoic acid derivatives. Polyphenolics accumulated in the epidermis and idioblasts of cotyledons and hypocotyls and in cells of the root cap of irradiated SE. This is contrary to what happened in the control SE, where polyphenolics occurred only in cells of the root cap. These findings, together with increased autofluorescence of flavonoids in the epidermal layer of irradiated SE, detected under a confocal microscope, represent avoidance mechanisms important for protecting embryo tissue against UV-B. These results will be published this year. A prior study by us showed the severe effect of different UV-B doses on the embryogenic suspensor mass of Norway spruce (Cvikrová et al. 2016).

Keywords: conifer, somatic embryogenesis, oxidative stress, malondialdehyde, polyamines, polyphenolic compounds.

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González-Cabrero N et al.

Effect of culture conditions on DNA methylation in stone pine embryogenic lines

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Abstract

Somatic embryogenesis is a way to regenerate clonal plants through the formation of somatic seeds. As a cloning technique it is a main tool in forest breeding that captures all the genetic potential of selected trees and produces uniform offspring. Seed formation in Norway spruce has been identified as a key point to establish environmentally induced epigenetic marks. These marks form an epigenetic memory in plants that affects long lasting adaptive traits. Therefore, somatic embryogenesis could also be used to produce primed plants that are conditioned to cope with different kinds of stress.

DNA cytosine methylation is one of the main processes underlying these epigenetic marks. Stone pine (*Pinus pinea* L.) is a genetically uniform but highly phenotypically plastic species of ecological and economic interest. Variability in cytosine methylation among genotypes and populations of the species has been demonstrated.

The aim of this study was to determine the effect of culture environment, temperature and water availability, on the cytosine methylation profile of the whole genome of stone pine embryogenic lines. Two embryogenic lines, at the proliferation stage, were analysed with the Methylation-Sensitive Amplification Polymorphism (MSAP) technique.

Embryo-suspensor masses were subcultured at 18, 23 or 28 °C on media with 4 or 10 g/l of Gelrite for six weeks. After that, samples were harvested for MSAP analysis. A total of 202 markers from which 83 were classified as Methylation Insensitive (MI) and 119 as Methylation Sensitive (MS) were scored. The degree of methylation, 58.91% of the cytosines at CCGG motifs, is in line with what has been reported for the species. Significant differences for frequencies of methylation were recorded between genotypes and among treatments within one of the genotypes. Out of the 83 MI markers, 77 were found to be Monomorphic Methylation Insensitive. The remaining 6 MSAPs (2.97% of the total number of MSAPs) were identified as Polymorphic Methylation Insensitive (PMI). Within the MS markers, 86 were identified as Monomorphic Methylation Sensitive and the remaining 33 were identified as Polymorphic Methylation Sensitive (PMS, 16.34% of the total number of MSAPs). Of these, 5 PMS showed a different pattern in relation to culture conditions during proliferation. Preliminary studies with embryogenic masses under maturation conditions and cotyledonary embryos showed an increase in intensity of fragments, suggesting a higher degree of demethylation associated with the differentiation processes.

Keywords: epigenetic memory, forest biotechnology, MSAP, *Pinus pinea*, somatic embryogenesis.

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Navarro BV et al.

Cell-to-cell trafficking patterns of Brazilian Pine (*Araucaria angustifolia* Bertol. Kuntze) cell lines with contrasting embryogenic potential

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Abstract

Besides biotechnological application, somatic embryogenesis also constitutes an important tool to study early stages of embryogenesis that are not possible to be performed *in vivo*. At the cellular level, the acquisition of embryogenic competence involves aspects related to cell communication and signaling, such as receiving the correct environmental signal and its subsequent cellular internalization, and the regulation of cytoplasmic trafficking. Transcriptomic and proteomic studies of Brazilian Pine (*Araucaria angustifolia*) embryogenesis, a native conifer currently classified as a critically endangered species, have demonstrated that there is a differential expression of genes and proteins related to cell-to-cell communication, especially in the vesicular transport and regulation of plasmodesmata (Pds) regions. In this context, we carried out a transmission electron microscopy (TEM) analysis of embryogenic cell cultures used for Brazilian Pine transcriptomic/proteomic studies (responsive [SE1] and blocked [SE6] embryo formation cell lines) in order to find new evidence about the importance of cell-to-cell trafficking in embryo formation.

Eight cell-to-cell communication and transport-related proteins were identified with a significantly different abundance in SE1 and SE6. *Beta-adaptin-like protein c, Vesicle-associated membrane protein 722* and *Ap-1 complex subunit gamma-1,* proteins related to the clathrin-mediated endocytosis, were more abundant in SE1, while *Gtp binding protein* was observed exclusively in this cell line. Images obtained by TEM showed a large presence of Golgi bodies, vesicles, lipid bodies and mitochondria in SE1 embryogenic cells, suggesting an intense metabolic activity. Otherwise, Pds regulation-related proteins were identified in SE6 cell line, such as *Glucan endo-beta-glucosidase-like* (overexpressed protein) and *Beta-glucan-binding protein* (exclusively observed in SE6), that degrade callose in the Pds edges, used to maintain the open and close control in this area. The presence of callose was confirmed by confocal microscopy using aniline blue to contrast callose, and propidium iodide to contrast the nucleus. Beside this, numerous amyloplasts with starch grains and an increase in the Pds regions were also observed in this cell line. In addition, *Dynamin-related protein 3b* and *Endomembrane family protein 70* were more abundant in SE6, indicating that for this cell line the vesicular transport was restricted to intracellular organelles. These results imply that the increase in SE1 cell-to-cell trafficking centered on transport is important in embryogenic differentiation, while SE6 displayed cells with poor intercommunication selectivity and the accumulation of substances (e.g. starch), which might indicate that energy storage is more predominant in this cell line. Therefore, it is crucial to understand the mechanisms that regulate cell-to-cell information trafficking, in order to apply this knowledge in improving the somatic embryogenesis tool.

Keywords: vesicular transport, plasmodesmata, cellular communication, conifer

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Raschke J et al.

Same, same but different – closely related conifer species and even clones vary in their optimal culture conditions

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Abstract

Somatic embryogenesis (sE) in conifers is considered a promising tool that offers great advantages in modern breeding strategies in forestry. Nevertheless, there is a constant need of adjusting the system, not least because the mechanisms behind its operation remain largely unknown. For individual species (*Larix x eurolepis, L. decidua, Pseudotsuga menziesii*, *Abies nordmanniana)* our group adapted protocols to generate sE clones under standardised conditions. Subsequently, a characterisation and evaluation of the single clones enabled us to create a multi-trait repository of genotypes. At this stage we are selecting clones based on their handling characteristics (maintenance and proliferation rate; quality and quantity of mature embryos as well as of plantlets after conversion). In addition, we are continuously analysing parameters to improve upon the results of induction, maturation and conversion: e.g., concentration and ratio of plant growth regulators (PGR); nutritional factors; duration of maturation; subculture intervals; variation in temperature regimes; the influence of crossing partner combinations. Comparisons of the *in vitro* processes for each species reveal their strengths and bottlenecks. For *P. menziesii* and *L. x eurolepis* we confirmed that induction is most efficient using globular and early cotyledonary stages of the zygotic embryo as starting material, whereas in *A. nordmanniana* sE is reliably induced from mature zygotic embryos, which ensures a seasonally independent induction period. Taken together, a shortened, calculable induction schedule with yet a reasonable yield has been developed for each species. Following selection of mature embryos with defined morphological qualities, we currently obtain an average conversion rate of above 60% of vigorous *L. x eurolepis* and *A. nordmanniana* plantlets. The conversion efficiency is considerably lower in *P. menziesii* and still requires further adjusting. However, this is partially compensated for by the production of a fairly large number of embryos per gram fresh weight of embryogenic culture during maturation.

Despite advances in the field of sE, the yield of somatic embryos varies strongly across clones, as many regulatory influences remain poorly understood. The formation of proper somatic embryos during maturation remains to be of great interest as this requires a timely and exact sequence of developmental steps. Drawing on the diverse characteristics of established genotypes, we intend to identify marker genes for proper development of numerous embryos. We also intend to screen for known embryogenesis related factors (e.g. *LdLEC1, LdWOX2, LdBBM, LdSERK, LdABI3, LdKN-sSTM*), using *Larix spp.* as a model species for (somatic) embryo development. Thereby, we aim to further our basic understanding of key developmental processes.

Along with improvement of the workflow, our results may offer the possibility to control the efficacy of the sE process, which would be fundamental to reliably generate plantlets ready to be acclimatised to support forestry.

Keywords: somatic embryogenesis, induction, maturation, conversion to plantlets, gene expression

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Rico S et al.

Use of *in vitro* chestnut clones to characterize candidate genes for resistance to *Phytophthora cinnamomi*

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Abstract

European chestnut (*Castanea sativa* Mill.) is a broadleaved tree species native to Europe and of great economic importance for the Mediterranean region. However, among other threats, chestnut populations are affected by "ink disease" caused by *Phytophthora cinnamomi*, a fungus-like eukaryotic microorganism belonging to the class oomycota. This disease has contributed to the drastic decline of chestnut distribution in Europe, with the greatest reduction in the warm southwestern and southern regions of central Europe. It can also affect seedlings, thus causing severe economic losses in nurseries. Hybrids between European and Asiatic chestnut species have long been used because of the high degree of natural resistance to the pathogen shown by the Chinese and Japanese species. The disease-related resistance genes play an important role in plant defence mechanisms, including the plant response to recognition of the pathogen and regulation of plant immune responses to infection by the pathogen.

In this study, we analyzed the expression patterns of nine chestnut genes after *in vitro* inoculation with *P. cinnamomi* into microshoots from 4 clones of chestnut showing different levels of resistance to this pathogen: Cs12 (a pure *C. sativa* clone highly sensitive to infection) and three *Castanea* hybrids, P043, P042 and P011, with different percentages of alleles of Asiatic origin, in which P011 is the most resistant clone. The analyzed genes encode transcription factors (*CsERF1*, *CsSCL1*), members of the GH3 family (*CsGH3.1*; *CsGH3.2* and *CsGH3.5*), a glycine-rich protein (*CsCPE*), an actin-depolymerizing factor (*CsADF*), a LRR-RLK receptor kinase (*CsRLK*) and a transcriptionally controlled tumour protein (*CsTCTP*). Rooted plantlets were inoculated with a virulent strain of *P. cinnamomi,* and leaf samples were collected 24, 48 and 72 hours after inoculation for qPCR analysis. Two hormone-signalling genes responsive to auxin, *CsGH3.1* and *CsGH3.2*, were strongly upregulated in the most resistant clone 24 h after infection. The expression levels increased throughout the course of infection, indicating that these genes are involved in defence mechanisms. Expression levels of *CsGH3.5, CsTCTP* and genes encoding the transcription factors *CsERF1* and *CsSCL1* also increased after infection in the most resistant clone, although they exhibited different expression profiles. On the other hand, induction of these genes was lowest in the most sensitive clone (except for *CsSCL1)*. Strong upregulation of the *CsCPE* gene observed after 72 h of infection in the most resistant clone indicates a role for this gene in the plant response to infection and may prevent the progress of pathogenesis through modifications in cell wall structure. The slightly early upregulation of *CsLRK* gene in the most resistant clones (P011 and P042) 24 h after infection, together with the downregulation of the gene in the most sensitive clones (Cs12 and P043), also suggests involvement of the gene in pathogen recognition. Our results suggest that inoculation triggers a general shift in the pattern of gene expression in the plantlets. In general, greater transcriptional activation is directly related to a higher degree of resistance in the clones.

Keywords: chestnut, ink disease, leaves, resistance genes

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Varas E et al.

Use of *in vitro* chestnut leaves as a model system for studying auxin regulation and gene expression during the regeneration of adventitious roots

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Abstract

The clonal propagation of ornamental plants and woody species is highly dependent on their ability to form roots, which is frequently lost during plant development. In chestnut, cuttings and shoot cultures derived from mature tissues are difficult to root using auxin treatments. Characterization of auxin-regulated genes involved in the control of adventitious rooting is required in order to improve the rooting of recalcitrant species. However, as these genes may also be involved in other developmental processes that occur simultaneously in response to auxin, the expression levels may not necessarily be correlated with root organogenesis. To address this issue, we developed an experimental system consisting of detached leaves from microshoots, to minimize the tissue complexity and also to exclude other developmental processes that may be triggered by auxin in microshoots.

The aim of the study was to analyze the physiological and molecular responses during the induction of adventitious roots in the leaves excised from juvenile and mature microshoots. We compared the rooting ability of juvenile and mature leaves in response to auxin in order to test the suitability of the system to study the loss of rooting capacity associated with the maturation process. The effect of the auxin transport inhibitor NPA (N-1-naphthyl-phthalamic acid) on the rooting response was also investigated in juvenile leaves. To determine the period required for reprogramming the fate of certain cells that can establish the root differentiation pathway, NPA was applied at different times after the initiation of auxin treatment. We also identified three genes: *CsSHR2* (Short-root 2), which encodes a GRAS transcription factor; *CsEND093*, which encodes an early-noduline; and *CsUGT*, which encodes an UDP-glycosyltransferase that catalyzes the transfer of sugar to specific acceptors. Expression of these genes was analyzed by qPCR in leaf samples harvested at different times (6, 12, and 24 h) after treatments.

The results indicate that the leaf system is a suitable method for studying the adventitious rooting process, as the rooting response was similar to that of microshoots, which is also ontogenetic-stage dependent. The strongest inhibition of rooting rates by NPA was directly correlated with the earliest application of this auxin polar transport inhibitor. Reprogramming of rooting competent cells to root initials occurs during the first 48 h of auxin induction and requires polar auxin transport, as root initiation is strongly inhibited by NPA during this period. The early auxin-related induction of *CsSHR2* and *CsENOD93* observed only in rooting-competent leaves, together with the inhibitory effect of NPA on gene expression and root induction, suggests an important role for these genes in the initial steps of adventitious rooting.

Keywords: adventitious rooting, auxin, chestnut, gene expression, NPA

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Trontin J-F et al.

Somatic embryogenesis as an enabling technology for reverse genetics: achievements and prospects for breeding maritime pine (*Pinus pinaster* Ait.)

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Abstract

As a major pine species of great ecological and socio-economic interest in Southern Europe, various approaches are concurrently developed for *Pinus pinaster* towards enhanced selection efficiency and deployment of improved, better-adapted varieties. Strong synergies are expected between traditional breeding, DNA-based selection (especially genomic selection) and somatic embryogenesis (SE) as a scalable vegetative propagation method of tested varieties for implementing multivarietal forestry. SE has been shown for more than 15 years to be an effective support for stable *Agrobacterium*-mediated genetic modification of selected genotypes (FCBA, INRA and iBET developments). Validating marker associations with specific properties before transfer into breeding selection models is still challenging. We developed reverse genetic studies (French and multinational/European initiatives) aimed at establishing direct associations between gene expression (including transcription factor genes) and adaptively significant phenotypes through overexpression or loss-of-function strategies such as by RNA interference silencing (RNAi). Data are accumulated for various genes involved in wood formation, carbon and nitrogen metabolisms, stress resistance, embryogenesis and plant development. Based on this collective effort, we will highlight some major achievements, discuss weaknesses of current technology and new opportunities.

Keywords: genetic transformation, *Agrobacterium*, transgene, transcriptomics, proteomics, off-target effect

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Graça D et al.

Cryopreservation of embryogenic cell lines of *Solanum betaceum* Cav.

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Abstract

Germplasm preservation has a very important role in current breeding and conservation programs. Due to either the genetic alterations that plant breeding may impose and to the extinction rate that numerous plants are facing, it is crucial to store genetic resources in order to avoid the loss of biodiversity. *In vitro* conservation methods depend on the storage duration that is intended. For long-term storage, cryopreservation is the only method currently available (Ozudogru and Lambardi 2016). A vitrification procedure that allows the cryopreservation of tamarillo (*Solanum betaceum*) embryogenic lines has been developed for the first time.

Tamarillo is a solanaceous tree from the Andean region and economically important due to the nutritional qualities of its fruits. An important biotechnological tool that can be applied to this species is somatic embryogenesis, using a protocol that was established at the Plant Biotechnology Laboratory of the University of Coimbra (Guimarães et al. 1988). In this procedure leaf segments are first exposed to MS media with an auxin and high concentrations of sucrose, resulting in the formation of embryogenic and non-embryogenic masses. Embryogenic masses are then transferred to auxin-free medium to allow somatic embryo development (Canhoto et al. 2005). However, maintenance of the embryogenic masses requires frequent subcultures. Furthermore, in long-term cultures (more than 2 years), karyotype aberrations and other events causing somaclonal variation occur resulting in atypical embryos (Currais et al. 2013).

Four different lines of embryogenic masses (EM) were submitted to a 5-day cold hardening stage and a 3-day pre-culture on a hormone-free MS (Murashige and Skoog 1962) medium with the sucrose concentration increasing each day. For osmo-protection, a loading solution and plant vitrification solution 2 (PVS2) were applied to the embryogenic masses and these were immersed in liquid nitrogen. Following rapid thawing in a water bath at 40°C and before placing the tissues in recovery conditions, the PVS2 solution was replaced with a washing solution. The EM were then transferred to a regeneration medium and recovery was determined by monitoring the weight of the samples monthly over four months. The average growth rate of cryopreserved EMs reached about 0.5 g per month for all lines (50% less than control EMs). A 50% decrease in the germination rate was observed for cryopreserved EMs when compared to the control. The results so far obtained have shown that PVS2 vitrification is a viable method to maintain embryogenic masses of tamarillo; however further optimization of the procedure may still improve the results.

Keywords: cryopreservation, embryogenic masses, germplasm storage, vitrification, woody species

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Maruyama TE et al.

Somatic embryogenesis and plant regeneration in Japanese pines and cypresses

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Abstract

Japanese pines (*Pinus thunbergii, P. densiflora, P. armandii* var. *amamiana, P. luchuensis*) and cypresses (*Chamaecyparis obtusa, C. pisifera*) are important in Japan for reforestation and landscaping. However, these species are affected by various biological problems and need urgent measures for their propagation.

Somatic embryogenesis is the most promising technique for mass propagation of clones, and for plant regeneration in genetic transformation protocols used in basic studies and in tree improvement programs. In this presentation, the current status of protocol development for somatic embryogenesis in Japanese pines and cypresses is described with special emphasis on germination and plant conversion rates after the embryo maturation process.

Somatic embryogenesis was initiated from excised megagametophytes containing immature zygotic embryos. Embryogenic cultures were maintained and proliferated in a medium supplemented with 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine, sucrose, and glutamine. The somatic embryo maturation experiments were performed in darkness at 25°C. Embryogenic tissues were cultured on maturation media containing maltose, activated charcoal, abscisic acid, and polyethylene glycol (PEG). The addition of PEG to the medium dramatically stimulated embryo maturation and resulted in an enhanced yield of mature embryos as the PEG concentration is increased. Although the cotyledonary embryo production varied according to the species, supplementation of medium with 100-150 g l^{-1} PEG was found to be suitable for high-quality embryo production in Japanese pines and cypresses.

Mature somatic embryos germinated and then converted into plantlets after their transfer to plant growth regulator-free medium. However, for the pine species, desiccation of somatic embryos after PEG-maturation was found to be essential for achieving both high germination and high conversion rates. Desiccation of somatic embryos at high relative humidity resulted not only in a marked increment in germination frequency but also, subsequently, in an improved plant conversion rate. In addition, this treatment resulted in a considerable improvement of the synchronization of the germination period, compared to that of the untreated control. In contrast, when somatic embryos of Japanese pines were matured on medium without PEG but containing a high concentration of gellan gum, somatic embryos readily germinated without any post-maturation treatments.

Although the improved protocol represents a promising perspective for efficient mass propagation of these species, more efforts are required to establish an optimal protocol for the commercial production of somatic plants with high field performance. Actually, the scale-up production of somatic embryos in liquid medium was also examined in order to improve the propagation rate.

Keywords: *Chamaecyparis* spp., embryo culture, gellan gum, *Pinus* spp., polyethylene glycol, somatic embryo

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Reeves C et al.

A hybrid tissue culture protocol that combines conifer somatic embryogenesis with organogenesis as an alternative propagation platform for specialist applications

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Abstract

Current somatic embryogenesis (SE) protocols for many pine species remain suboptimal for the purpose of reforestation. Some of the bottlenecks include loss of lines through the SE process due to lack of continued proliferation, poor embryo maturation or poor conversion of somatic embryos to *ex vitro* conditions. There often is significant variation among genotypes with respect to productivity and SE protocol improvements do not always benefit all genotypes.

There often is the desire to propagate specific cell lines, for example from top-ranked families. Selected lines may show desirable characteristics such as disease resistance or a high ability to be genetically engineered. There is also a need to propagate cell lines from species that are in general recalcitrant to somatic embryogenesis (e.g., *Pinus densiflora*). Cell lines from hybrid crosses, where there can be incompatibility issues between parent species, also present several challenges. In particular there are no protocols available for these new hybrid crosses which are often less responsive to all steps in the SE process.

An example of this recalcitrance could be a cell line which is highly transformable but only produces a limited number of good quality mature somatic embryos. This makes direct planting of somatic embryos to *ex vitro* conditions an unviable option. If the desired cell line will not respond positively to modification of the standard SE protocols or media for the species, then the options for bulking up this material are limited. A hybrid tissue culture system combining germinated somatic embryos with organogenesis allows the flexibility to work with these desirable cell lines and allows the best aspects of the component propagation methods to be exploited.

In conclusion, we have a propagation protocol that takes advantage of the benefits of SE, which include cryopreservation to ensure retention of juvenility while field testing takes place and the potential for genetic engineering. These features combined with organogenesis provide the advantage of increased multiplication, uniformity of shoots and rooting and conversion rates high enough to generate planting stock for stoolbeds. This is a winning strategy for some conifer species.

Keywords: conversion, recalcitrance, bottlenecks, flexibility

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Högberg KA

Selection effects of somatic embryogenesis in Norway spruce

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Abstract

A substantial loss of genotypes is a typical result when propagating Norway spruce via somatic embryogenesis (SE). Does this selection for propagation ability affect important traits? To investigate this question, a project is running where SE plants and seedlings of the same families are compared.

Embryos from 50 half-sib families of Norway spruce were put on initiation medium in 2011, followed by proliferation and maturation of somatic embryos during 2012. Embryos germinated and plantlets were acclimatised in 2013, and cultivated until the autumn of 2014. Seedlings from the same families were grown in parallel and both plant types were planted in field trials in the spring of 2015. Prior to winter hardening, cuttings were excised from both SE plants and seedlings and put in a rooting environment in the late summer of 2014.

When SE reached the acclimation step, 26% of the embryos had been successfully propagated and one family was lost. The number of clones per family varied considerably, as well as the number of plants per clone. Seedlings were taller than SE plants at the end of plant production while SE plants had larger stem diameter in relation to plant height in two families. The cutting propagation resulted in low rooting percentages, lowest when SE plants were donors.

In one field trial, time for flushing was assessed in the spring of 2016 and height was measured in the autumn of 2016, i.e., after two years in the field. No difference in flushing time was found between SE plants and seedlings. Seedlings were taller than SE plants after two years in the field but the differences had decreased compared with height at planting time, both in absolute and relative terms.

The seedlings could be divided into two types, i) those from which cuttings had been harvested and ii) those that had not provided cuttings. Surprisingly, these differed significantly in height and flushing time, with seedlings that had not provided cuttings flushing earlier than ones that had provided cuttings and at about the same time as SE plants. Both types of seedlings exceeded SE plants in height, but seedlings that had provided cuttings were significantly taller than both SE plants and seedlings that had not provided cuttings. The deviation in flushing time and height exhibited by seedlings that had not provided cuttings is puzzling and difficult to explain.

The overall performance of the plant types in height growth and flushing time does not indicate any apparent selection effects due to losses of genotypes during SE propagation.

Keywords: cuttings, field trials, time for flushing, vegetative propagation

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Find JI

Results from the first full rotation of growth in clonal field trials of nordmanns fir (*Abies nordmanniana*)

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†*Jens I. Find suddenly passed out in early December 2016*

Abstract

Nordmanns fir (*Abies nordmanniana*) is grown in Danish forestry as an ornamental for the production of Christmas trees. The production only covers 10 % of the forest area, but economically it is the most important tree species in Danish forestry, where it amounts to approximately 90 % of the total income. The Danish production is 12 million trees per year and the European market is 70 million trees each year.

There is great economical interest from conventional growers in uniform plant material selected on basis of form and growth. The presentation reports from test of larger scale production. Larger scale production of Christmas trees of nordmanns fir, based on organic principles, is at present not a realistic possibility. The area of organic production only amount for 1.5 % of the total production area in Denmark. The reason is mainly due to two problems: 1) a general reduced growth and faint coloration due to reduced application of nitrogen in fertilizers accepted in organic farming, and 2) severe damage on needles and shoots by the aphid *dreyfusia nordmanniana.* In conventional production, the aphid is mitigated by application of pesticides, which are not allowed in organic production. It is expected that plants selected for traits such as insect resistance and improved nutrient uptake may allow for organic production in larger scale. The presentation will report on results from the first clonal field trial that was established in 2007 (**Fig. 1**), and will furthermore report on preliminary results from larger field trials with 500 clones established in 2014 and in 2015. Field trials established at different locations to test for genotype and environmental effects on specific traits, and on the possibility of clonal selection for insect- and fungi resistance and for improved efficiency in nitrogen uptake.

Keywords: somatic embryogenesis, clonal field trials, selection, traits

Figure 1. Clonal field trial of *A. nordmanniana* established in 2007 to test growth, performance and phenotypic uniformity of SE plants

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The Fourth International Conference of the IUFRO Unit 2.09.02

"Somatic Embryogenesis and Other Vegetative Propagation Technologies"

Development and Application of Vegetative Propagation Technologies in Plantation Forestry to Cope with a Changing Climate and Environment

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La Plata (Argentina) and the Conference Venue (Council of Economic Sciences)

Introductory Workshop at FCAyF-UNLP (Sept. 19, 2016)

"Innovation and challenges in the forestry sector"

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Opening Ceremony at the Council of Economic Sciences (Sept. 19, 2016)

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Icebreaker reception (Sept. 19, 2016)

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Icebreaker Reception (continued)

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During the sessions (Sept. 20-23, 2016)

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City tour and free evening! (Sept. 20, 2016)

Satellite Workshop (Sept. 21, 2016) at the Council of Economic Sciences

"*Current status and prospects of vegetative propagation technologies in Argentinean Patagonia*"

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Gala Dinner (Sept. 21, 2016), Central Hall of the UNLP Presidency building

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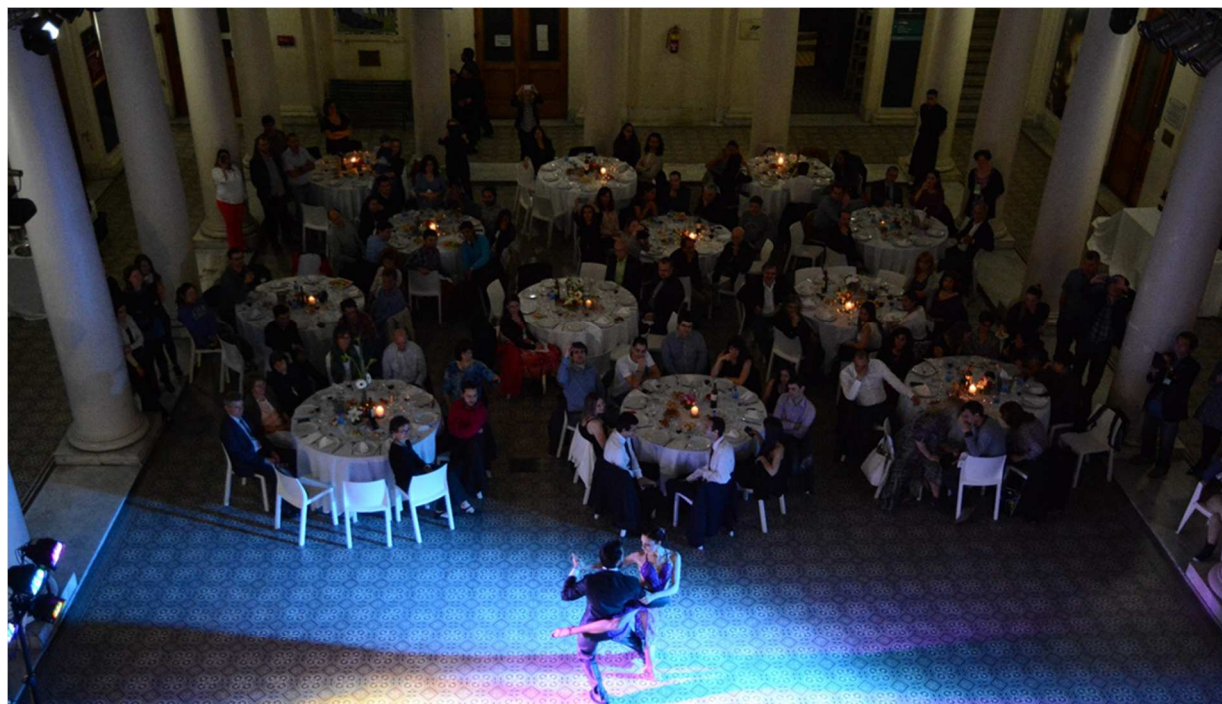
Gala Dinner: at the tables

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Gala Dinner: Tango & Salsa exhibition

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**During the Awards Ceremony (Sept. 21, 2016) of the
Second Biennial IUFRO 2.09.02 Student's Scientific Competition**

*"Advances in vegetative propagation technologies and
application of somatic embryogenesis in tree breeding and biotechnology"*

The Winner of the competition, **João Filipe da Silva Martins** (Portugal) has been invited with full support to make an oral presentation during the conference.

All 5 runners-up were invited to contribute to the scientific program:

Kanagaraj Suganthi (India) - Oral presentation (IUFRO SPDC full support)
Anna Maria Wójcik (Poland) - Oral presentation (partial support)
Giovanna Campos Mamede Weiss de Carvalho (Brazil) - Poster presentation
Evelyn Raquel Duarte (Argentina) – Poster presentation
Taiane Pires de Freitas de Oliveira (Brazil) – Poster presentation

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During the Recognition Ceremony of famous colleagues (Sept. 21, 2016)

Drs. **M. Raj. Ahuja** (USA), **Scott A. Merkle** (USA), and **Marguerite Quoirin** (Brazil) during the conference. Although they could not join us in La Plata, recognition was also expressed during the conference to Drs **Gale H. McGranahan** (USA), **Jenny Aitken** (New Zealand), **William J. Libby** (USA), **Gerald S. Pullman** (USA), and **Mariano Toribio** (Spain).

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Photo gallery

**Visits to the astronomy observatory "Planetario" (Sept. 22, 2016) and
nursery "Charles Darwin" (Sept. 23, 2016)**

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Typical Argentinean barbecue & Folklore dance (Sept. 23, 2016)

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Photo gallery

The last day was the opportunity to visit the « Republica de los Ninos » (Sept. 23, 2016)
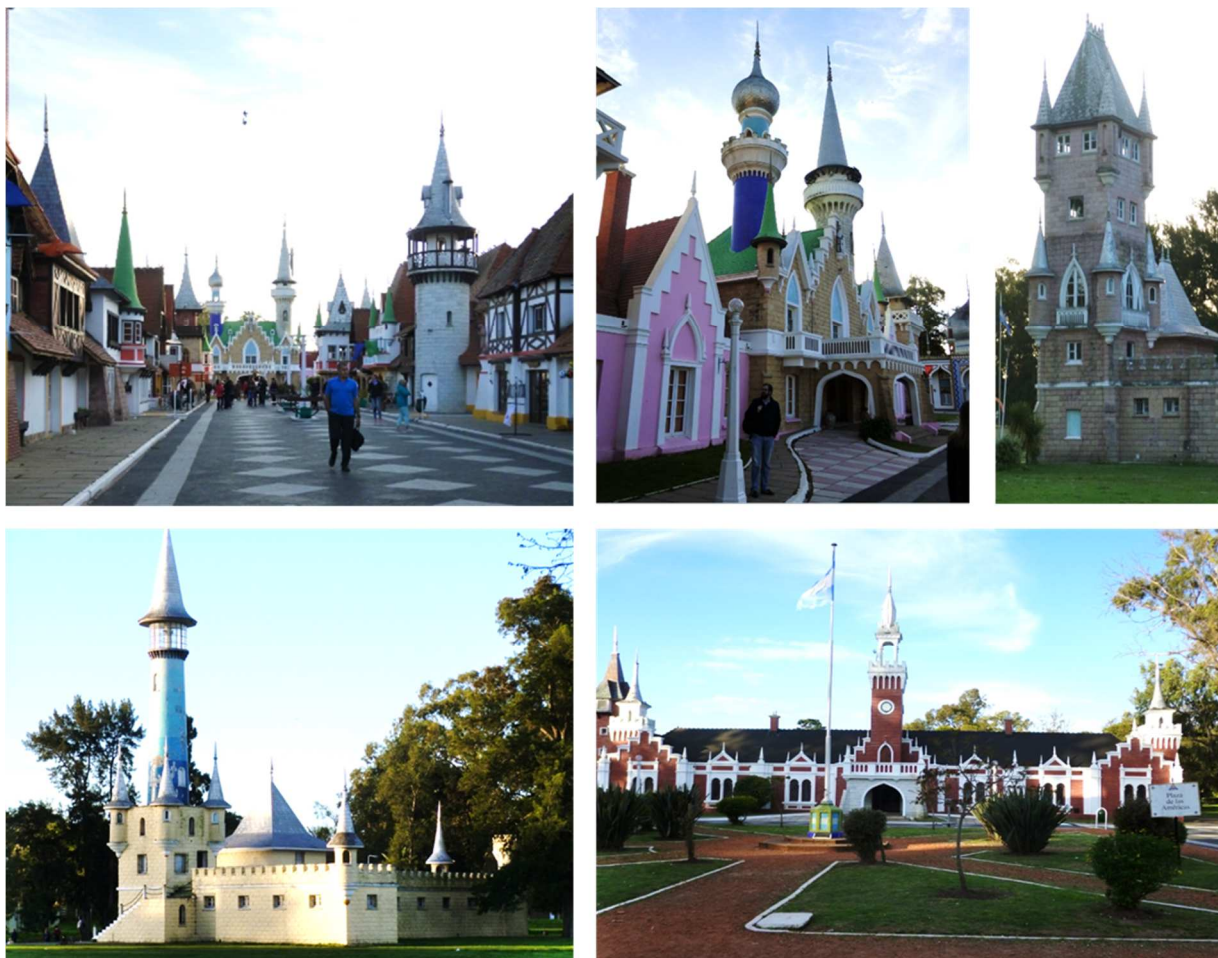
More downloadable photos are available from:

https://drive.google.com/drive/folders/0B4zGrr4qaTOibEtHbk5lVEdaa28?usp=sharing

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Photo gallery

Our Very Special Thanks to the Fantastic Hosting Team in La Plata!

Thanks!

Sandra
Claudia
Maite
Corina
Elina
Patricia
María Laura
Manuela
Elizabeth
Julieta
Tatiana

Thanks!

Sebastián
Javier
Fernando
Pedro
Julián
Juan Ignacio

Thank you!

Mr. Raúl Aníbal Perdomo
President of UNLP

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Jorge Canhoto

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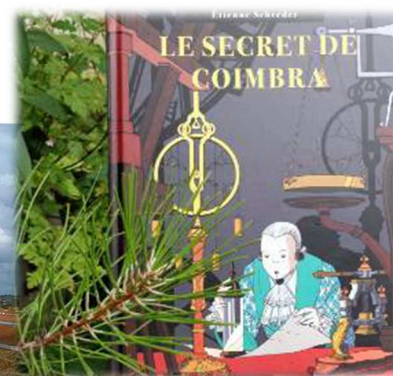
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An affectionate thought from IUFRO 2.09.02 members

Our IUFRO 2.09.02 Unit founder and friend Yill-Sung Park could not attend La Plata 2016 and lost his lovely wife Kumok a few months after the conference. Many of us had the opportunity to meet with Kumok Park during our previous IUFRO 2.09.02 conferences.

Yill-Sung, this photo was a big thought to you during the conference in La Plata. On behalf of all IUFRO 2.09.02 members, we would like here to simply express to you and your family all our profound sympathy and strong support in these very sad circumstances.

Bordeaux, France, July 25, 2017

Jean-François Trontin
IUFRO 2.09.02 Coordinator

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Summary report

Summary report of La Plata 2016

*Extended abstract (across **oral** and **poster** presentations)*

The importance of plantation forestry (currently 7 % of the world's forested area) to cope with a changing climate was highlighted (**S. Galarco**, opening lecture). Range shifts, pole-ward migration, shifts in phenology, threat to biodiversity, extinction risks, increase in drought conditions, changes in precipitations, increase in sea level and increase in pest populations are main impacts of climate change (**M.R. Ahuja**, closing lecture). In this context, easy/accessible, flexible and popular vegetative propagation (VP) methods with reduced number of steps are needed worldwide (**G. Salvatierra, A. Masson, M.R. Ahuja**). VP is a requirement not only for forestry; i.e. to make easier and more efficient the transfer of genetic gains to commercial plantations and by-pass sexual limitations (**P.A. González**/*P. Taeda;* **T. Benneckenstein**/hybrid larch; **C.D Vera Bravo**/*P. taeda* and hybrids**),** but also for landscaping engineering towards healthy and aesthetic environment as the world is becoming more urban than rural (**L. Roussy**).

There is a need for more short term, application-oriented research to efficiently propagate selected genotype/varieties at the cheapest cost (**O. Monteuuis, G. Salvatierra, A. Masson, P.A. González, S. Suharyanto**) and with fulfillment of national guidelines and prevailing laws (e.g. regarding the marketability, **T. Benneckenstein**). Depending on both the species and end-uses, the best propagation strategy (i.e. seeds vs. VP technologies) must be considered in close collaboration with selectors/breeders. "Real breeding without breeding" can be achieved through the careful selection and propagation of natural resources (**S. Merkle/**white ash**, P. Rojas Vergara/**eucalypts).

VP of elite trees (i.e. with added value) expressing natural or induced defense/adaptation response to biotic/abiotic stresses is a key issue for increasing resilience of planted forests (**S. Galarco**, **I. Arrillaga**/oak, pine, **I. Trujillo**/banana; **K. Suganthi**/mangroves, **P. Rojas Vergara**/eucalypt; **J. González**/eucalypts). Micropropagation through axillary/adventitious budding or somatic embryogenesis (SE), more conventional VP methods (grafts, cuttings) or a judicious combination of both may help for efficient production at the cheapest cost of improved planting stocks (**O. Monteuuis, C. Hargreaves, C. Reeves, P. Rojas Vergara, K.A. Högberg, D. Breton, P. Gupta, M.E. Aguilar**). The management of mini clonal gardens (production of cuttings from micropropagated mother plants) in nursery is one current large-scale technology for the cost-effective production of improved varieties of commercial interest (**R. Penchel**, eucalypts; **J. Schapovaloff**, *Pinus taeda*; **V. Rudoy**, blueberry, sugar cane). In radiata pine, hybrid tissue culture systems combining germinated somatic embryos with organogenesis (adventitious shoot formation) and/or cuttings production allowed the best aspects of the component propagation methods to be exploited (**C. Reeves**) for variety deployment, up to 2 million plants per year at approximate $900/1000 plants (**C. Hargreaves**). In *Hevea*, remarkably, the self-rooted cuttings production from *in vitro*-issued stock plants in nursery conditions is cost-effective and allowed to by-pass current limitations of micropropagation and grafting for deployment of industrial clones (**A. Masson**). Genotype-specific protocol development is required in species of commercial interest (**P. Gupta**/*Pinus taeda*, Douglas-fir; **D. Breton**/*Coffea*; **I. Trujillo**/banana; **N. González Cabrero**/Stone pine; **A. Masson**/*Hevea*; **J. Raschke**/larch, Douglas-fir, Nordmanns fir). In the frequent case of selected varieties that are recalcitrant to *in vitro* VP, systematic approaches based on the mineral nutrition status of young plant tissues may help to design genotype-specific fine-tuned basal media (**J. Oberschelp**/eucalypt).

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Climate change is affecting the reproductive system of many species (flowering, seed development). Micropropagated plants, both through organogenesis (axillary/adventitious budding and adventitious rooting) and SE, can be used to study the development of selected genotype in specific environmental conditions (biotic/abiotic stress) and to identify candidate genes (**J.F. Martins, M.A. Lelu-Walter, I. Arrillaga, C. Sánchez, I.A. Montalbán**). SE has been demonstrated (molecular physiology) to be a good *in vitro* model (controlled conditions) of early zygotic embryo (ZE) development stages (**M.A. Lelu-Walter**/conifers). The identification of genes and gene networks (and related expression profiles) playing key roles during acquisition of embryogenic competence and embryo development (both SE and ZE) in different environmental conditions may help to develop seed-based propagation strategies and to refine SE protocols for producing high-quality SE plants similar to the zygotic reference. Various genes and proteins involved in key processes (auxin-mediated pathways, stress-related genes, putative epigenetic regulators such as miRNA, cell-to-cell trafficking, *etc*.) have been highlighted (**M.A Lelu-Walter**/larch, pine, **J. Canhoto**/tamarillo, radiata pine, **S. Correia**/tamarillo, **C. Miguel**/maritime pine; **B. Navarro**/*Araucaria*, **A. Wójcik**/*Arabidopsis*) after genomic profiling of tissue with different embryogenic potential. Fluorescence-activated cell sorting (FACS) is useful for transcriptome profiling of differentially expressed genes in very specific and localized cells (**S. Correia**/tamarillo). Interestingly most miRNA are themselves targeting other key regulators such as transcription factors (**C. Miguel**/maritime pine; **A. Wójcik**/*Arabidopsis*). Specific studies of some epigenetic marks (DNA methylation) confirmed that methylation-sensitive markers could be polymorphic in relation to culture conditions (temperature, water availability) during SE proliferation (**N. González-Cabrero**/stone pine). Temperature and/or water availability were shown to affect initiation rate and embryo development in radiata, halepo and maritime pines (**I. Montalbán, M.A. Lelu-Walter, I. Arrillaga**). Similar pathways such as the auxin-mediated regulatory pathways are equally important during adventitious rooting and some genes are good candidates for a role in establishing the required auxin gradient for inducing the root initiation process (**C. Sanchez**/chestnut).

In most species, somatic embryogenesis is only obtained from juvenile explants and protocol refinements are needed to increase initiation rate (**J. Degenhardt-Goldbach**/*Pinus caribaea*; **P. Boeri**/*Melia azedarach*, *Prosopis alpatac*), maintain embryogenic potential (**F. Gautier**/Douglas-fir) and achieve high-quality plants with zygotic-like features. Some current issues are to reduce the frequency of abnormal embryos (**M. Quoirin**/palm tree; **B. Navarro**/Araucaria; **L.F.D. Oliveira**/*Araucaria*), to increase embryo maturity (**M.A. Lelu-Walter**/maritime pine; **P. Gupta**/*Pinus taeda*), to improve light spectrum and reduce associated stresses (**K. Eliášová**/ Norway spruce, UV-B; **S. Varis**/Norway spruce, LED light system; **S. Merkle**/chestnut, hemlock, red light) and to develop adapted support/plugs and controlled growth conditions (including fertilization) for conversion to *ex vitro* conditions (**YW Kim**/larch; **D. Breton**/*Coffea*; **F. Avilés**/*Pinus radiata*).

SE initiated from mature, selected trees would be a paradigm shift for clonal forestry especially to reduce the development cost of new varieties. Significant achievements were obtained in hardwoods/angiosperms (**I. Trujillo**/banana; **D. Breton**/coffea; **A. Masson**/Hevea; **V. Cano**/holm oak; **J.A. Kim**/*Prunus serrulata*) but conifer species are still highly recalcitrant (**S.P. Rocha**/*Pinus taeda*). Interestingly, SE induction protocol successfully applied in primordial shoots of white spruce somatic plants up to 10 years old (Klimaszewska et al. 2011, Planta 233: 635–647) was successfully used to initiate embryogenic lines in Norway spruce SE plants that are 3-4 years old (**S. Varis**).

When routine protocols are available, strong selection effects are still observed during the whole SE process, i.e. lines are lost at every steps until acclimatization (**K.A. Högberg**/Norway spruce; **C. Reeves**/*Pinus* spp.). When established in field conditions, initial growth is consistently reduced

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compared to control seedlings (**K.A. Högberg**/Norway spruce; **T. Aronen**/Scots pine; **M.-A Lelu-Walter**/maritime pine) and may be linked with partial embryo maturity at the time of harvesting (**M.A. Lelu-Walter**/maritime pine). However it is difficult to synchronize the development of somatic seedlings and control seedlings and more appropriate controls may be developed such as standard lots of somatic seedlings (**T. Aronen**). No selection effect was observed for flushing (**K.A. Högberg**/Norway spruce) and yearly growth of SE could be similar to that of seedlings (**T. Aronen**/Scots pine). Moreover, good phenotypic conformity of SE plants was observed in large field trials established at different locations (**J. Find**[†]/*Abies nordmanniana*; **P. Gupta**/*Pinus taeda*). Interestingly, clonal selection for resistance to insects or diseases may be possible from such field experiments (**J. Find**[†]/Abies; **T. Aronen**/Scots pine). Registration process of SE lines for commercial forest plantation is being to be implemented in Finland (**M. Tikkinen**/Norway spruce). Registration is possible for both untested material (SE lines from high value parent trees for bulk propagation) or as qualified/tested material (combination of 4-11 clones with proven breeding values).

In other well-established VP systems such as production of cuttings/minicuttings from stoolbeds in poplar (**C. Graciano**), fertilization (N or P) affects relative development of roots and sprouts as well as wood density but not early vigor of cuttings. Fertilization can reduce tolerance to drought (N) or temporary flooding (P). Nutritional requirements were equally important in *Toona ciliata* (**T.P.F. Oliveira**). Hormone concentration in young apical leaves has been associated with appropriate adventitious rooting (**P. Reyes Torres**/*A. perutilis*). *Ex vitro* rooting could be more efficient and cheaper than *in vitro* rooting **C. Hargreaves**/*P. radiata*; **S. Suharyanto**/*Acacia crassicarpa*). When VP technologies are sufficiently refined to deploy selected clones in the field (**R. Aggangan**/native species/cuttings; **J.F. Trontin**/eucalypt hybrid/cuttings from micropropagated plants through axillary budding), productivity was shown as for conventional varieties to be affected by major pedo-climatic conditions such as soil water availability and fertility, as well as rainfall.

There are rapid increases in the number and scale of menaces to forest trees in many parts of the world (**S. Merkle, M.P. Guerra**). Low-cost biotechnological approaches are developed to assist domestication and conservation strategies of both exotic and native, rare ornamental form or multipurpose tree species (**F. Niella, M.P. Guerra, M.A. Basiglio, P. Boeri, H. Mattes, N. González-Cabrero, A.I. Putri, P. González; L. Koch, L.E. Taccari, J.C. Araujo Vieira de Souza, G. Campos Mamede Weis de Carvalho, E. Duarte, T. Nikkanen, I. Trujillo, R. Aggangan**). Forest are becoming increasingly fragmented (loss of habitat) and vulnerable (loss of genetic diversity, insect pests, pathogens, abiotic stresses). Conservation issues are critical in Latin America, especially Brazil, the richest megadiverse country in the world (**M.P. Guerra**). Native, indigenous forest tree species can have high potential but are usually difficult to propagate through seeds outside their natural habitat and *in vitro* VP/SE are therefore needed (e.g. **S. Werbrouck**, *Melia volkensii* in Kenya, eastern Africa, a semi-arid region highly affected by climate change). Conservation of genetic resources and selection/breeding of resistant/tolerant varieties could be greatly enhanced by employing *in vitro* VP systems. SE, in particular, is well-suited for this purpose, due to the high multiplication rates and the amenability of embryogenic cultures to cryostorage (**S. Merkle, M.R. Ahuja, S. Correia**). Various cryopreserved collections have been successfully established using SE in threatened species such as chestnuts (blight), hemlocks (woolly adelgid), ashes (emerald ash borer), and Atlantic white cedar (overcutting) (**S. Merkle**).

For commercially important species, a general trend to achieve cost-effective, rapid deployment of clonal varieties (i.e. at similar cost than reference seedlings) is to develop efficient technologies for scaling-up

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(production of million micropropagated plants/year) such as RITA® and other temporary immersion systems (**M.E. Aguilar**,/coffea, *Gmelina*, teak; **M.P. Guerra**/Peach palm; **R. Penchel**/eucalypts; **S. Merkle**/chestnut; **V. Rudoy**/sugar cane; **P. Gupta**/Douglas-fir; **G. Salvatierra/**eucalypts; **E. Tapia**/cherry; **T. Aronen**/Norway spruce), and/or liquid phase in disposable bioreactors (**D. Breton**/coffea; **P. Gupta**/*Pinus taeda*; **T. Maruyama**/*Pinus thunbergii*). Such systems are amenable to photoautotrophic conditions to produce healthier and better-adapted plantlets to acclimatization (**R. Penchel/**eucalypt; **N. Vidal**/chestnut). Main risks to address for successful scaling-up include malformation and contaminations, alteration of bioreactor structure after multiple manipulations, limitation of robotics for harvesting, sorting/quality assessment, planting/transplanting of million plants, the need for customized plant biofactory (indoor vertical high-throughput clonal gardens) and/or greenhouse facilities and, may be more importantly, business expectations. Typically, a facility to produce 4 million plants/year is estimated to be a 2 million $ investment (**R. Penchel**/eucalypts).

When both high-quality embryos/plants and production scaling-up is achieved with high efficiency for the SE technology, manufactured, artificial seeds appeared to be the ultimate, versatile technology (fit well existing systems for managing seeds and seedlings) for a high degree of automation and reduced cost/plant for the production of new varieties (**J. Find**[†]/*Abies*, **P. Gupta**/*Pinus taeda*).

Increased resilience of selected varieties can also be obtained more directly through genetic transformation or induced defense/adaptation through genetic/epigenetic mechanisms (resistance genes) or inoculation with natural symbionts (**I. Arrillaga, M.R. Ahuja**).

Natural symbiont microorganisms (endophytes, bacteria or fungus) could be identified and successfully used to confer protection against pathogens after inoculation during micropropagation (**J. Martins**/chestnut/ink disease). Similarly arbuscular mycorrhizal fungi inoculated just before acclimatization greatly improve survival and growth of SE-derived plantlets (**N. Aggangan**/*Kalopanax*, *Liliodendron*). The *in vitro* induction of epigenetic changes to elicit defense response is another ongoing strategy to increase plant resistance to specific pathogen. Various elicitors are currently tested in holm oak SE lines (e.g. paraaminobenzoic acid) to tentatively obtain resistance to *Phytophtora cinnamomi*/oak decline syndrome (**N. González-Cabrero, I. Arrillaga**).

Genes can be introduced by direct genetic modification in somatic embryos of selected genotypes with the final objective to produce transgenic plants with improved properties such as resistance to environmental stress (**M.R. Ahuja**/poplar; **I. Arrillaga**/oaks, pines, **P. Rojas Vergara**/*Eucalyptus globulus*; **C. Hargreaves**/radiata pine, **J.F. Trontin**/maritime pine; **T. Maruyama**/Japanese pines & cypresses, **M. Blasco**/*Pinus pinea*). Clonally replicated genetically-modified (GM) trees can be easily tested in different field conditions through assisted migration to pole-ward location (frost tolerance) or in drier climate (drought tolerance) (**M.R. Ahuja**). Pest-resistant GM poplar have been released in China, frost-tolerant eucalyptus and *Pinus taeda* engineered for increased wood density are expected to be released in USA (**M.R. Ahuja**). Overexpression of genes encoding pathogenesis-related (PR) proteins such as thaumatin-like proteins (antifungal properties) is a strategy tested in cork and holm oaks that are decimated by the oak decline syndrome (**V. Cano**).

More generally SE can be efficiently used as an enabling technology for reverse genetics (**J.F. Trontin**/maritime pine) through *Agrobacterium*-mediated genetic transformation for up/down regulation of candidate genes in whole plants. Genome profiling (transcriptomics/proteomics) is however required to identify suitable transgenic lines for further phenotypic analysis. Using such first-generation strategies for genetic modification, transgene instability/silencing and off-target, pleiotropic effects could be detected owing to variable transgene position in different lines. As a result it is required to screen multiple lines and

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to produce multiple plants in order to detect putative target effects of transgene expression. Next generation tree biotechnologies and particularly genome editing (such as CRISPR/Cas9) may allow site-directed mutagenesis at reduced cost (**M.R. Ahuja, J.F. Trontin, T. Maruyama, S. Werbrouck**). Proliferating embryogenic cells may be particularly amenable to genome editing in whole plants as single embryogenic cells or and/or very immature embryos are usually targeted in such embryogenic cultures (**J.F. Trontin, M.R. Ahuja**).

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Any inquiry/idea about vegetative propagation of trees? Please, contact us!

Development and Application of Vegetative Propagation Technologies in Plantation Forestry to Cope with a Changing Climate and Environment

The importance of plantation forestry was highlighted as forests are becoming increasingly fragmented and vulnerable in the context of both anthropic- and climate-related pressure. Flexible and popular, low-cost vegetative propagation methods are considered critical and well-suited (especially somatic embryogenesis) for both sustainable plantation forestry and conservation of genetic resources (breeding and wild populations from marketable or endangered, multipurpose native species). Application-oriented research efforts in close collaboration with breeders may allow the careful selection and cost-effective, rapid deployment (and turn-over) of elite varieties expressing natural or induced adaptation response (endophytes, mycorrhizal fungus, elicitors, genetic modification) to environmental stress.

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