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Abstract. Somatic embryogenesis (SE) is the biotechnology of propagation that enables to put multi-varietal forestry (MVF) into practice. MVF involves the use of genetically tested varieties in plantation forestry, balancing genetic gain with diversity. Embryogenic lines can be cryopreserved in liquid nitrogen while corresponding trees are tested in the field, and those showing genetic superiority can be recovered for mass production of elite plants. The SE process in pines involves the initial outgrowth of embryo-suspensor masses (ESM) from immature zygotic embryos, the establishment of embryogenic cultures capable of further differentiation into somatic embryos, and their germination as somatic seedlings. We reported plant regeneration by SE in stone pine, and further research is in progress to improve the efficiency of the several steps. Induction was obtained at very low frequency (0.4%), and the best embryogenic line and maturation condition yielded 220 mature embryos per gram of ESM. Germination was achieved at 70%, and 35 % of somatic embryos were converted into plants. Suspension cultures offer advantages over semisolid cultures for mass propagation. The establishment of ESMs of stone pine in liquid medium is reported, and the recovery of mature embryos from tissues grown in liquid culture is also described. Current main bottlenecks of SE for implementing MFV in stone pine are the low induction rates and the cessation of growth of converted somatic seedlings.

Keywords. Biotechnology – High yielding varieties – *In vitro* culture – Tree breeding – Vegetative propagation.

Clonage de pin pignon (*Pinus pinea *L.) par embryogenèse somatique

Résumé. *L'embryogenèse somatique (ES) est la biotechnologie de propagation qui permet de mettre la culture forestière multi-variétale (FMV) en pratique. La FMV implique l'utilisation de variétés génétiquement testées en plantation en exploitant au mieux l'équilibre entre le gain génétique et la diversité. Les lignées embryogènes peuvent être cryoconservées dans l'azote liquide, tandis que les arbres correspondants sont testés sur le terrain. Ceux qui montrent une supériorité génétique sont sélectionnés pour la production en masse de plants d'élite. Le processus d'ES du pin implique la croissance initiale des masses d'embryons et de suspenseurs (ESM) à partir d'embryons zygotiques immatures, l'établissement de cultures embryogènes capables de différenciation en embryons somatiques matures, et la germination de ceux-ci. Nous présentons la régénération des plantes de pin pignon par ES et les recherches qui sont en cours pour améliorer l'efficacité des différentes étapes. L'induction a été obtenue à très basse fréquence (0,4%). La meilleure lignée et le meilleur traitement de maturation ont fourni 220 embryons matures par gramme d'ESM. La germination a atteint 70%, et 35% des embryons germés se sont convertis en plantes. Les cultures en suspension offrent des avantages par rapport aux cultures sur milieux semi-solides pour la propagation en masse. L'établissement d'ESM en milieu liquide et l'obtention d'embryons matures de pin pignon à partir de ces cultures sont décrits. Les principales étapes limitantes de l'ES pour mettre actuellement en application la FMV pour le pin pignon sont les faibles taux d'induction et l'arrêt de croissance des plantules somatiques.*

Mots-clés. *Amélioration des arbres – Biotechnologie – Culture* in vitro *– Multiplication végétative – Variétés à rendement élevé.*

I – Introduction

1. Clonal forestry and multi-varietal forestry

The demand of forest products is constantly growing. The forecasts for timber requirements at the half of this century are six billions of cubic meters (Sutton, 2000). Both the recycling of paper and the establishment of forest plantations that can respond to the growing needs of pulp, timber and other products, are considered the best defence of native forests threatened by deforestation (Sutton, 1999). Forest plantations with intensive production may alleviate the great pressure on natural ecosystems. In Brazil, for example, high-yield *Eucalyptus* plantations, which account for only 2% of the country's entire crop, are responsible for 60% of their nation's production. In Chile and New Zealand plantations of radiate pine covering about one fifth of the total area are responsible of more than 90% of the total production (Sedjo, 1999). Actually, at present forest plantations constitute only five per cent of global forest cover but already supply up to 35 per cent of global round wood (Carle *et al*., 2005).

Cultural and genetic improvement practises have to be applied to increase yield in intensively managed forest plantations. Plant propagation is one of the major issues in tree breeding and genetic resource conservation. The more powerful tool that has been used in genetic improvement of many woody crops such as wines, fruit trees and olive trees is vegetative propagation. Cloning plays also an important role in breeding forests species (Zobel and Talbert, 1984). In fact, species of the genus *Populus*, *Salix*, *Cryptomeria* and *Eucalyptus* that are amenable of vegetative propagation are intensively managed using this form of propagation.

However many forest species are recalcitrant to vegetative propagation. Currently, plant biotechnology may solve problems linked to the obtaining vegetative propagules at very high amount and reduced cost. But forest biotechnology must be coupled with classical breeding schemes in order to obtain their full advantages. The strategy of multi-varietal forestry (MVF) (Park, 2004) which is based on the former clonal forestry (Park, 2002) and is defined as the use of genetically tested tree varieties in plantation forestry, balancing genetic gain with diversity, begins to be implemented due to the development of embryogenic systems. Plant regeneration by somatic embryogenesis (SE), combined with cryopreservation, offers an opportunity to develop highly valuable clonal varieties. Embryogenic lines can be cryopreserved in liquid nitrogen maintaining their regeneration potential while corresponding trees are tested in the field. Clonal lines that have shown genetic superiority can be recovered after thawing for mass production of elite plants. Thus clonal propagation by SE can quickly capture the benefits of breeding and can be implemented in a flexible way, so it is expected to play an important role in increasing productivity, sustainability and uniformity of many forest plantations.

2. The embryogenic system in conifers and its commercial application

The embryogenic system as a way of plant regeneration is the basis of several applied biotechnologies (clonal propagation, cryopreservation, genetic transformation) that are used for the conservation and improvement of genetic resources. The implementation of MVF for *Pinus* species requires a SE system with high initiation and plant conversion rates, maintaining the genetic integrity; hence the development of optimized protocols is needed to bring this biotechnology to its full potential. After the first reports in conifers (*Picea abies*, *Larix decidua*) in 1985, SE has been achieved in many pine species as *Pinus pinaster*, *P. radiata*, *P. strobus* and *P. taeda*. In this last species SE is currently being used at the operational practice by private companies. The SE process in pines involves the initial outgrowth and continuous growth of embryo-suspensor masses (ESM) from immature zygotic embryos, allowing the establishment of embryogenic cultures capable of further differentiation into somatic embryos. Then somatic embryos germinate giving somatic seedlings. Growth rates of ESM are usually fairly high ensuring a high multiplication potential. However maturation and quality of somatic embryos are limiting factor to conversion into plants.

The protocols for regeneration of conifer species by SE have been considerably improved in the last years (Klimaszewska *et al*., 2007). These improvements have generated a strong commercial activity of production of clonal plants mainly in the species *Pinus taeda*, *Pinus radiata* and *Pseutsuga menziesii*. In fact private biotechnological companies are making alliances with classical forest corporations to apply the recent developments in SE. For instance the US company ArborGen [http://www.arborgen.com/], whose motto is *more wood, less land*, signed joining agreements with International Paper, MeadWestvaco, and Weyerhaeuser to produce and test improved loblolly pine varieties. The Canadian company Cellfor [www.cellfor.com] is producing and trading more than 27 million plants of loblolly pine per year (Park, 2007). Several other companies are using SE to establish clonal tests and starting commercialisation: BioForest and GenFor (Chile), Carter-Holt Harvey, Rubicon and Rayonier (New Zealand) and JD Irving (Canada) (Cyr and Klimaszewska, 2002). In Europe practical applications are less developed but recently a company has been created in Sweden, SweTree Technologies [http://www.swetree.com/index.html]. In collaboration with traditional forest companies are developing industrial methods to produce elite embryogenic lines of Norway spruce at large scale. They plan to have by 2012 a pilot production system which will have a capacity of producing millions of plants per year.

An attribute of increasing interest in the last years is the epigenetic control on the phenotype. It has been observed that the temperature during maternal reproduction affects adaptive traits: independent tests in several conifer tree species have shown that growth, bud phenology and frost hardiness of progenies are influenced by the climatic conditions during sexual reproduction, and the memory effects seem to endure for many years in the filial generation. Elevated temperature during female flowering revealed that the period from embryogenesis through seed maturation is the most likely sensitive period (Johnsen *et al*., 2005). Epigenetic changes in gene expression that can be transmitted from one generation to the next are supposed to be behind this effect. This “epigenetic memory”, which is expressed as a long-lasting effect on height growth and bud phenology in the progenies, has also been evidenced using somatic plants produced by SE under different temperature regimes (Kvaalen and Johnsen, 2008). This aspect can be of great interest in the case of stone pine, a species that is claimed to have virtually no neutral genetic variation, but show enough phenotypic plasticity for spreading over a very large and environmentally diverse area (Mutke *et al*., 2010). Somatic plants can be very useful to study this phenomenon.

II – The embryogenic system in stone pine

Taking into account the economic importance of the pine nut production, breeding programs of stone pine have begun, aiming at improved cone production (Mutke *et al*., 2005). These programs are based on the selection of elite trees for grafting and planting in orchards designed for the production of its edible megagametophyte (Mutke *et al.*, 2007). Therefore the management of stone pine in plantation forestry for fruit production might require the development of rootstock varieties adapted to different environmental conditions.

Clonal rootstocks are widely used in the cultivation of many woody crops (Castle, 1995**;** Webster, A.D. 1997; Kester *et al*., 2002). They can exert considerable effects over the performance of the scion and, consequently, over the tree growth, flowering and fruit size. In addition clonal rootstocks provide a uniform orchard that facilitates management. In forest species marked rootstock effects on growth and reproduction have been observed, and even some crown characters and nutrient content of scions can be manipulated by using specific rootstocks (Jayawickrama *et al*., 1991). In Norway spruce clonal rootstocks reliably increased the number of flowering grafts. Hence the development of early, abundant and continuous flower-stimulating rootstocks was envisaged (Melchior, 1987). Consequently the development of vegetative propagation techniques for *Pinus pinea* should be desirable.

Attempts for cloning stone pine by micropropagation have been successfully carried out using

the organogenic pathway of regeneration (Alonso et al., 2006; Cuesta *et al*., 2008; Cortizo *et al*., 2009). However the procedures are very laborious and rooting percentages are not high enough to consider this option for profitable propagation. At present, only SE is consider the option of choice for mass vegetative propagation of difficult-to-root forest species.

1. Initiation, proliferation and cryopreservation of embryogenic lines

We reported plant regeneration by SE in stone pine (Carneros *et al*., 2009). Embryogenic lines were obtained from developing zygotic embryos by culturing immature seeds without coats (Fig. 1A). Different induction media containing several concentrations of an auxin (2,4-dichlorophenoxyacetic acid) and a cytokinin (6-benzylaminopurine) were tested to induce SE. Overall it was obtained at a very low frequency (0.4%). However differences related to the year of collection and half-sib family were found, with some families doubling the general mean. Within a year, the most responsible collection date was at the beginning of July, when zygotic embryos were at the post-cleavage state of development. By this time, although the extrusion of tissue from the micropilar end of the megagametophytes occurred on average at almost 10% of the explanted seeds, only a tenth of these proliferations became initiated as embryogenic lines. Some families had *temporal windows of response* broader than others. Also they showed different initiation frequencies, some of them reaching 2.5% of established embryogenic lines.

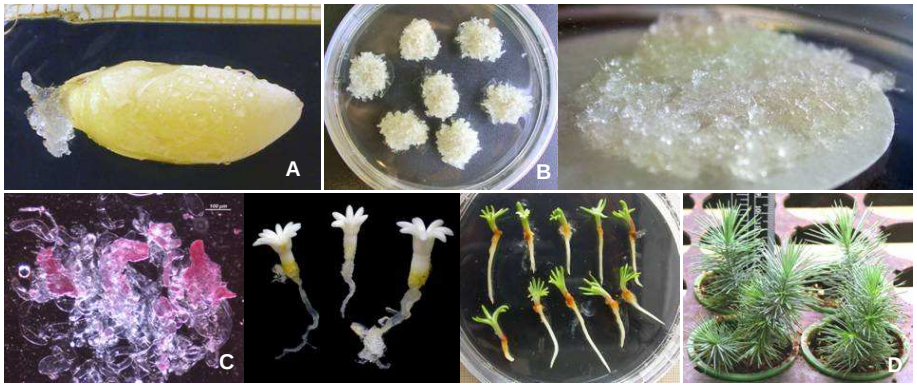

Fig. 1. Several steps of plant regeneration by somatic embryogenesis in *Pinus pinea*. A. Induction: outgrowth of embryogenic tissue from megagametophyte; B: Proliferation: growth of embryo suspensor masses on semisolid medium, as clumps (left) or dispersed on filter paper (right); C: Differentiation, maturation and germination of somatic embryos (from left to right); D: somatic seedling of stone pine in forest containers.

Proliferation of embryogenic lines takes place in conifers by cleavage polyembryony that sets a recurrent process of multiplication, forming masses of embryonal heads and suspensor cells. As the growth of these ESM is usually very active, this process is very productive. We tested with stone pine two procedures for the proliferation of this embryogenic tissue: growing as clumps or dispersed on filter paper (Fig. 1B). The second procedure was much more productive (Carneros *et al*., 2009). Overall fresh weight of tissue growing as clumps was multiplied by 30 after three biweekly subcultures while it was multiplied by 70 when the dispersal procedure was used. In some genotypes this difference was even higher, reaching more than 10-fold.

A cryopreservation protocol for embryogenic lines of stone pine was developed (Carneros, 2009), that allows their long term conservation without loss of vigour and regeneration potential. Cryoprotective agents such as sorbitol and DMSO showed some toxicity that reverted after several subcultures. A pretreatment with these substances followed by a slow-freezing step

previous to immersion in liquid nitrogen were used. All cryopreserved lines recovered growth after thawing, but after a long lag phase. Mature embryos with germination ability could be obtained from two out of three tested lines.

2. Maturation of somatic embryos and conversion into plants

Differentiation and maturation of somatic embryos from ESM are needed to obtain clonal plants (Fig. 1C). First results showed a relatively low number of mature embryos, up to 36 embryos per gram of embryogenic tissue (Carneros *et al*., 2009). This figure is in the range of the ones published in *Pinus nigra* (Salajova and Salaj, 2005) and *Pseudotsuga menziesii* (Gupta and Timmis, 2005). Then different factors were evaluated for improving embryo maturation and conversion. It was observed that the effect of ABA concentration was highly dependent on genotype. Medium with 121 or 161 µM ABA produced the highest number of mature embryos depending on the embryogenic line tested. The treatment that produced the most dramatic effect was the periodic subculture onto maturation medium, compared to continuous culture in this medium: repeated subculturing resulted in a 13-fold increase in mature embryos in two embryogenic lines. Tissue desiccation also enhanced maturation: a period of 2-4 hours under laminar flow conditions that caused a 7,5-17% relative water loss, increased the number of mature embryos up to almost double in one embryogenic line and 5-fold in another. The best embryogenic line and maturation condition yielded between 170 and 220 mature embryos per gram of ESM, which is in the range published in *Pinus taeda* (Pullman *et al*., 2003) and *Pinus pinaster* (Lelu-Walter *et al*., 2006). However, the inclusion of PCIB, an auxin inhibitor, in the maturation medium did not improve and even reduced the number of mature embryos as regards the control.

In average 70% of somatic germinated within four weeks. Conversion was enhanced (35%) when germinated embryos were transferred for further growth to vent culture boxes filled with SH medium (Carneros, 2009; Toribio *et al.*, 2011). Some somatic seedlings were transferred to forest containers (Fig. 1D).

3. Establishment of cultures in liquid media

Most protocols developed for cloning forest trees by SE are based on *in vitro* culture techniques using semisolid media. These protocols are useful to produce the reduced number of plants required to establish clonal tests necessary for validating selected varieties. However, for profitable mass production of improved planting stocks the development of culture in liquid medium is required. Liquid cultures offer technical advantages over semisolid cultures as a faster rate of growth based on the rapid uptake of nutrients by cells. In addition, the suspension culture system allows the study of physiological characteristic such as growth parameters, nutrient uptake and maturation capacity that are important for large scale production of somatic embryos in bioreactors. Furthermore, culture in liquid medium is required for the automation of cultures, lowering the high labour costs associated with tissue culture techniques. Culture in liquid medium and bioreactors has been developed for several conifer species (Gupta and Timmis, 2005)

We initiated suspension cultures from ESM of several embryogenic lines that are currently being maintained on semi-solid medium for more than five years retaining the embryogenic ability (Celestino *et al*., 2011). Pieces of tissue were suspended in the same maintenance medium without gelling agent, and cultured in Erlenmeyer flasks on rotary shakers. The initial inoculum density was determinant for establishing the suspension, as was observed in other *Pinus* species (Salaj *et al*. 2007). Cultures evolved showing an initial lag phase followed by an exponential growth phase up to a maximum after which no further growth was detected, a typical pattern of suspension cell cultures (Azevedo *et al*., 2008). Qualitative differences were observed in the growth of embryogenic masses depending on the degree of agitation. Higher orbiting speed caused more disaggregated and homogeneous proliferations (Fig. 2) that

produced more packed suspensions and reduced the settle cell volume recordings (Fig. 3). Embryo-suspensor masses showed more structured somatic embryos when were grown on solid medium than in liquid medium. However, mature embryos could be obtained from tissues grown in suspension cultures, though differentiation and maturation required plating the embryo-suspensor masses from liquid to semi-solid medium.

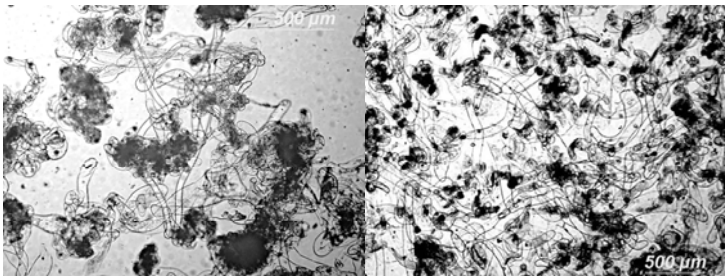

Fig. 2. Qualitative differences of embryogenic tissues grown at 50 rpm (left) and 100 rpm (right). Higher orbiting speed caused more disaggregated and homogeneous proliferations.

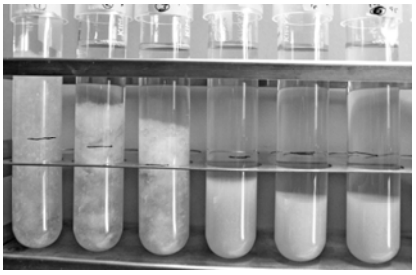

Fig. 3. Settle cell volumes recorded in embryogenic cultures growing at 50 rpm (left) and 100 rpm (right) that show much more packed suspensions.

III – Conclusions

Although methods for cloning stone pine by somatic embryogenesis have been defined, further research is needed to improve the efficiency of the several steps of the process. At present the main bottleneck is the very low induction rate. Genetic improvement programs needs to work with as much as possible genetic variability to avoid the risk of genetic erosion. Overall, one of the main drawbacks of the SE technology in forest species is the low frequency of initiation, which reduces the number of genotypes captured for breeding, and the recalcitrance of some high-value crosses to initiate embryogenic tissue. This might be solved on one hand by improving the present methods. Frequencies of induction described in the first studies in other species were quite low, contrasting with the frequencies reported several years later using optimised protocols. For instance, in Douglas-fir first data were in the range of only 1-2% proliferating ESMs after six months in culture (Durzan and Gupta, 1987). Nowadays with improved protocols initiation frequencies and establishment of embryogenic lines in Douglas-fir are in the range of 40-57% (Pullman *et al*., 2009). A relevant characteristic of the induction

process in stone pine is that extrusion rates are much higher than the initiation ones. Therefore a technique such as liquid overlay could be useful for enhancing initiation of embryogenic lines (Pullman and Skryabina, 2007). On the other hand, it is known that the different phases of plant regeneration by SE are genetically controlled (Park *et al*., 1993; Park *et al*., 1994). Thus this fact opens the possibility of breeding for higher induction rates, looking for families with the highest induction frequencies and using the mother trees as female parents in controlled crosses (MacKay *et al*., 2006).

Germination of mature embryos and conversion into somatic seedlings are limiting steps of SE in some species. In the case of stone pine, reasonable high percentages of germination (growth of both root and plumule) are obtained. However, after germination a high percentage of these plants stop growing. This current bottleneck reduces the efficiency of the SE process. Some studies on the effect of light quality on these steps have shown that subsequent seedling growth was promoted by red light (Merkle *et al*., 2005). This could be useful to improve current methods in the stone pine.

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