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Somatic embryogenesis of Scots pine – advances in pine tissue culture at Metla

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Somatic embryogenesis (SE) is the vegetative propagation method providing the best multiplication factor for coniferous species. The technique has been applied in Scots pine (*Pinus sylvestris* L.) since the 1990's, but several steps of the method still need improvement. Research efforts at Metla's Punkaharju Research Unit have been focused, firstly, on enhancement of seed-embryo derived SE in Scots pine, and secondly, on cloning of mature Scots pine trees through SE. Recent advances and on-going work for developing somatic embryogenesis in Scots pine is described.

Keywords: zygotic embryo explant, mature tree explant, *Pinus sylvestris*, vegetative propagation

Introduction

World-wide, clonal or varietal forestry is advancing fast at the moment, because of evident gains achieved by multiplication of the very best individuals for silvicultural purposes. The biggest problem counteracting this development is the recalcitrance of mature conifers for vegetative propagation: there has been no way to clone well-known, selected adult trees having superior growth and wood qualities.

Scots pine (*Pinus sylvestris* L.) has been involved in forest tree breeding since its beginning, but enhanced clonal testing and silviculture options have been unattainable due to recalcitrance of the species in vegetative propagation. Rooted cuttings could provide an option for clonal testing in breeding programmes, but the most potential method for producing large amounts of clonal material is tissue culture through somatic embryogenesis (SE).

The SE techniques developed and used for coniferous species start generally from young explants, and in the case of Scots pine, from immature seed embryos. Research and development efforts for seed-embryo derived somatic embryogenesis in Scots pine has been going on at Finnish Forest Research Institute (Metla), Punkaharju Research Unit, since 1990's.

Initiation and establishment of embryogenic cultures from vegetative shoot apices of mature trees of conifers has been attempted in a few species. Recently, Dr. Ravindra Malabadi and his

co-workers have published reports on successful regeneration of SE plants starting from mature tree explants e.g. in *Pinus patula* (Malabadi & van Staden 2005), *P. roxburghii* (Malabadi & Nataraja 2006), *P. wallichiana* (Malabadi & Nataraja 2007), and *P. kesiya* (Malabadi et al. 2004). In collaboration with Dr. Malabadi, an attempt to apply this technique to Scots pine was initiated in 2006 at Metla's Punkaharju Research Unit.

Seed-embryo derived somatic embryogenesis

The method for somatic embryogenesis of Scots pine developed at Metla (Häggman et al. 1999) uses immature zygotic embryos taken from immature cones and surrounded by megagametophyte as explants (Fig. 1A). In the recent years, research efforts have been focused to study the possibilities to improve the different steps of Scots pine somatic embryogenesis, i.e. initiation frequency, proliferation rate, production of mature somatic embryos, and conversion of embryos into plants. Special attention has been paid on embryo quality and root formation and their connections to preceding treatments. Also performance of the produced somatic embryo plants in greenhouse has been observed, and the plants will be further tested in field experiments starting in 2009.

The results achieved for enhancement of seed-embryo derived SE in Scots pine are described in detail by Aronen et al. (2009) and shortly summarised here. Genetic background has significant effect on initiation of somatic embryogenesis: Initiation frequencies of 20-30 % can be achieved, but there are also families with very low or no initiation. Proliferation of the established SE cultures is better with tissues suspended on filters than when they are grown as calli. Proliferation method also has an effect on the abundance of proembryos in the cultures, as well as on the number and quality of the mature somatic embryos later on. The most important factor for embryo production is, however, maturation technique used. Specific attention should be paid on quality of the somatic embryos produced, slim-type embryos having the best germination and greenhouse survival.



Fig. 1. Somatic embryogenesis in Scots pine: **A)** Induction of embryogenic tissue from immature seed embryo explant. **B)** Mature somatic embryos ready for germination. **C)** Somatic embryo plants on left and seedlings on right. (Photos: Jouko Lehto and Tuija Aronen)

To conclude, in order to get high-quality somatic embryos (Fig. 1B) that also perform well as emblings in greenhouse (Fig 1C), the SE cultures should be proliferated and matured suspended on filter paper, with high abscisic acid concentration applied during maturation. Mature somatic embryos should be picked up for germination only for a limited period, and their germination performed *in vitro* on tissue culture media. With optimised conditions and quality control of the embryos, over 90% of the somatic embryos germinate and develop into well-growing emblings.

On-going studies with shoot explants

In the last couple of years, research efforts have been focused on application of the Malabadi's published SE method (Malabadi & van Staden 2005) using explants from mature Scots pine trees. Tissue culture initiations using shoot apex explants from 10- and 15-year-old Scots pines were done through growing season to find right timing for SE induction. During a period in spring the explants seem to be responsive, and embryogenic cultures have been raised.

For initiation of somatic embryogenesis in shoot explants, distinguishing and separation of SE tissue from explant is of crucial importance. The explants need to be examined with stereomicroscope to find potential SE-tissue. Furthermore, acetocarmine staining should be used to evaluate embryogenic potential of induced tissue, and to avoid proliferation of non-embryogenic callus (Fig. 2A), often mixed with embryogenic one (Fig. 2B).

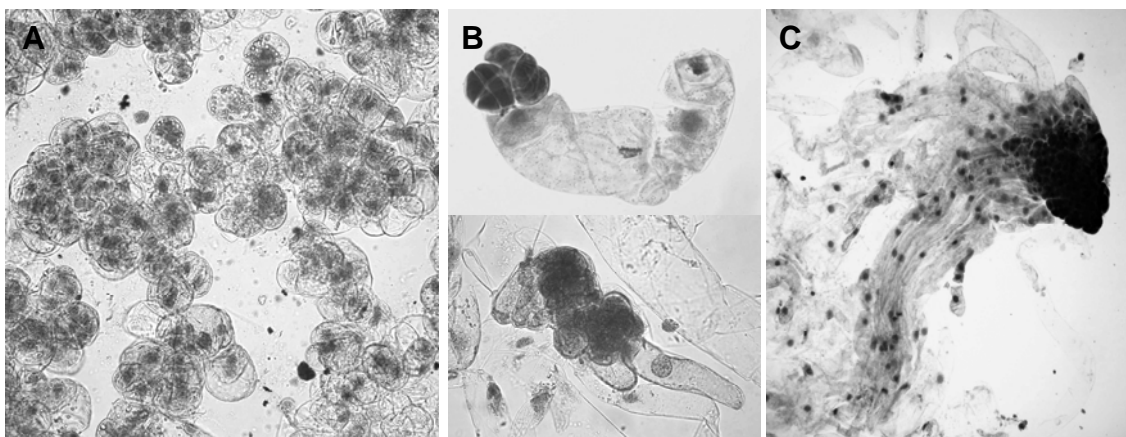


Fig. 2. Acetocarmine staining of tissues induced in shoot explants. **A)** Non-embryogenic cells with large cytoplasm. **B)** Early somatic embryos with strongly stained head cells and a few transparent suspensors. **C)** Well-formed embryo in proliferating SE-culture. (Photos: Ravindra Malabadi, Tuija Aronen and Leena Ryyänen.)

The majority of established SE lines proliferate well, and their recovery from cryopreservation has been 100% using the protocol developed at Metla (Häggman et al. 1998). According to microscopical observations, the proembryo formation in the most of the cultures is abundant, and the embryos are well-formed with complete head and prolonged suspensors (Fig. 2C).

Maturation and germination of somatic embryos in the cultures originating in mature trees has, however, been found to differ from the SE-lines of seed embryo origin. The first maturation experiments done according to the Malabadi's published protocols (Malabadi & van Staden 2005) resulted in very slow production of a few embryos, although the same method works for the Scots pine SE lines of seed-embryo origin (Aronen et al. 2009). Moreover, the quality of the embryos produced from the SE-lines of mature tree origin was not satisfactory (Fig. 3A), and they failed to germinate. Optimisation of maturation and germination steps is currently performed in collaboration with Dr. Marie-Anne Lelu from INRA, France. In the latest experiments, somatic embryos of good quality have been produced (Fig. 3B). In addition, marker analyses are performed to assess the genetic fidelity of the SE cultures originating in the mature trees.

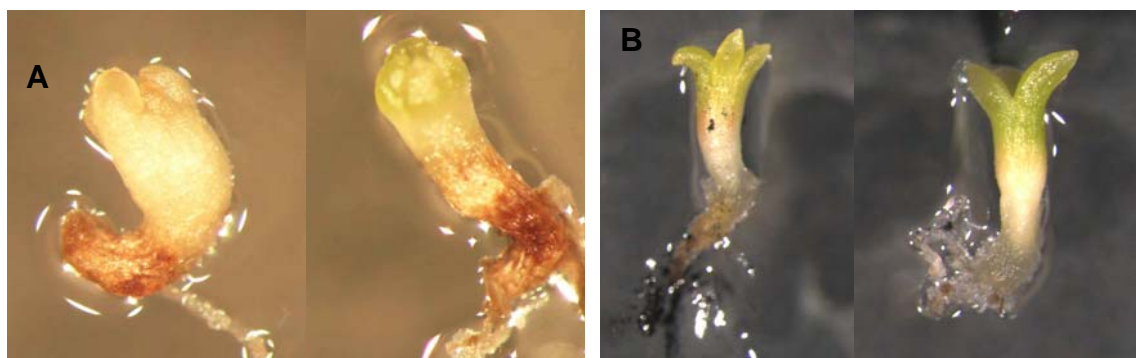


Fig. 3. Somatic embryos produced from SE lines originating in mature tree explants: **A)** Bad-quality embryos, and **B)** good-looking embryos. (Photos: Tuija Aronen)

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