What is the best way to maintain embryogenic capacity of embryogenic lines initiated from Douglas-fir immature embryos?

Gautier F[1,2], Eliášová K[3], Reeves C[4], Sanchez L[1], Teyssier C[1], Trontin J-F[5], Le Metté C[1], Vágner M[3], Costa G[2], Hargreaves C[4], Lelu-Walter M-A[1]*

[1]INRA, UR 588, Unité Amélioration, Génétique et Physiologie Forestières,
2163 Av. de la Pomme de pin, CS 4001 Ardon, 45075 Orléans cedex 2, France.
[2]Université de Limoges, Laboratoire de Chimie des Substances Naturelles,
123 avenue Albert Thomas, 87000 Limoges, France.
[3]Institute of Experimental Botany CAS, Rozvojová 263. Praha 6-Lysolaje 165 02, Czech Republic.
[4]Scion, Private Bag 3020, Rotorua, New Zealand.
[5]FCBA, Biotechnology and advanced Forestry Department, 71, Route d'Arcachon, Pierroton, 33610 Cestas, France.

*Corresponding author: marie-anne.lelu-walter@inra.fr

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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

(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 shows 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**): "pure lines" (D1, D2), i.e., white-translucent EMs with immature somatic embryos (SE) as typically described in conifer species, or 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).

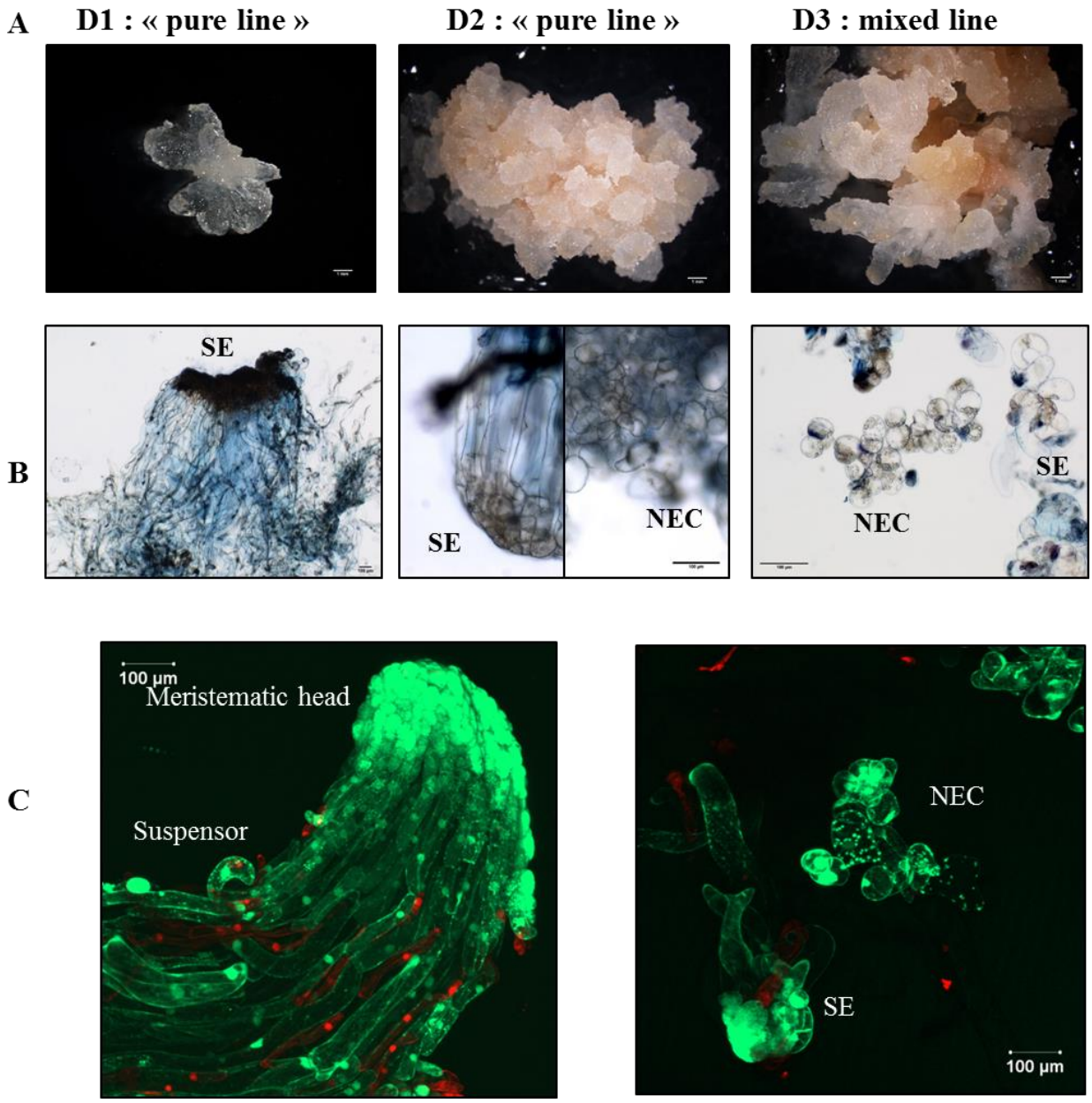

Figure 1. Observation of three Douglas-fir embryogenic lines (D1, D2, D3).

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.

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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