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Plants from haploid tissue culture of *Larix decidua*

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Abstract. Haploid embryogenic tissue was initiated on 1/2 LM medium supplemented with 500 mg/l glutamine, 1000 mg/l casein hydrolysate, 100 mg/l inositol, 30000 mg/l sucrose, and 0.1 mg/l 2,4-D. The embryoids matured to produce plantlets. One plant from one of the two lines survived. The chromosome complement of tissue cultures, of the needle bases from the source plant, and of the plant produced in vitro were established by squashes. DNA content was assessed by DNA microdensiometry. In vitro tissues were haploid (n = 12). The plant produced was mixoploid, with a predominance of diploid cells (2n = 24).

Key words: Gynogenesis – Haploid culture – Conifers – *Larix*

Introduction

The induction of haploid cultures from male and female tissues has been increasingly successful with woody plants. However, the actual regeneration of plantlets suitable for field trials has been almost exclusively confined to angiosperms (Chen 1987), with conifers proving to be much more difficult in this respect. The general purpose for pursuing haploid culture is to create dihaploid breeding stock to capture heterosis, disease resistance, and other traits. Haploid cultures are useful for (1) the development of homozygotic breeding material, (2) the development of linkage maps, and (3) the karyotyping of the species and in situ hybridization of molecular probes (Bonga et al. 1988).

With respect to conifers, the regeneration of plants from haploid culture has been entirely unsuccessful from microspores. Even though the growth of callus and occasional organogenesis has been observed (Bonga 1974), no plants have been reported. Regeneration has been more successful when megagametophyte explants were used: the regeneration of embryoids has been recorded for *Larix* spp. (Nagmani and Bonga 1986; von Aderkas et al. 1987) and *Picea abies* (Simola and Santanen 1990). Gynogenic trees derived by organogenesis have been described for *Sequoia sempervirens* (Ball 1987).

We previously reported (von Aderkas et al. 1990) that while haploid cultures of various larch species were embryogenic, they did not produce plants that would grow beyond more than a few centimetres. This article is the first report of plants produced via in vitro embryogenesis from haploid conifer explants.

Materials and methods

Plantlets

Cell lines 2110 and 2140 originated from megagametophytes of *Larix decidua* from tree G4 on the campus of the University of New Brunswick, Fredericton, N. B. Cultures were initiated on 1/2 LM (Litvay et al. 1985) medium supplemented with 500 mg/l glutamine, 1,000 mg/l casein hydrolysate, 30,000 mg/l sucrose, and 0.1 mg/l 2,4-D. The embryogenic cultures were maintained on the same medium but with 20,000 mg/l sucrose and 100 mg/l myo-inositol. No plant growth regulators were required at this stage. Subculture was on a monthly basis. The first plantlets were produced after a year of subculture. They germinated in vitro and were planted into pots with vermiculite and peat mix and placed in a misting bed in the greenhouse. Of the five plants obtained from both cell lines only one from each line continued growing. After a year's growth ramets were struck from the one plant that had originated from line 2110 and rooted.

DNA measurement

Apices of 2110 and G4 were fixed in neutral formalin [100 ml formalin, 6.5 gm Na_2HPO_4 (anhydrous), 4.0 gm $NaH_2 \cdot PO_4 \cdot H_2O$, 900 ml distilled water] for 24 h at 4 °C, hydrolyzed in 5 *N*

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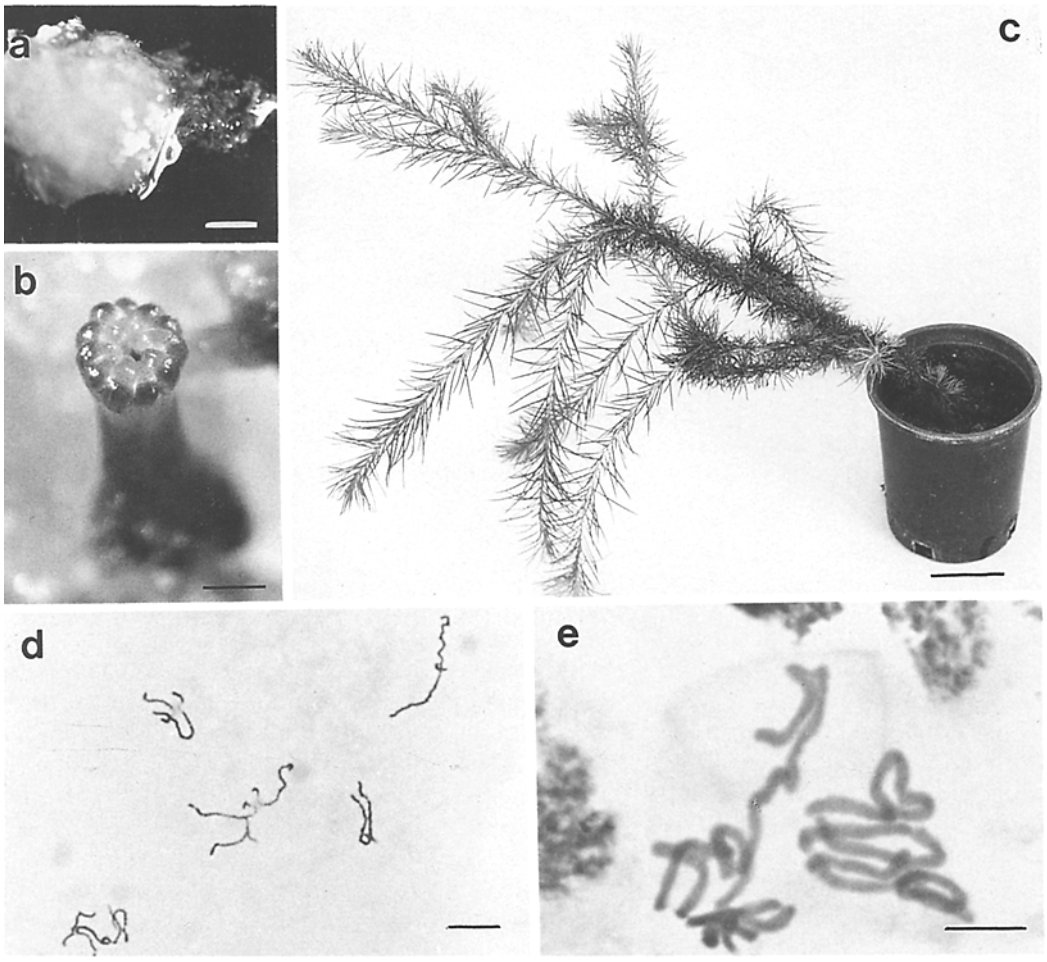

Fig. 1. **a** Dark-field micrograph of embryogenic tissue (*on right*) induced from megagametophytic tissue of *Larix decidua* (*on left*). *Bar:* 0.5 mm. **b** Embryoid of line 2110 at cotyledon-forming stage. *Bar*: 1.0 mm. **c** Plant of line 2110. Note the plagiotropic growth habit. This plant has had three growth cycles and has served as the ortet for a number of struck ramets. The tree is mixoploid, though predominantly diploid in its chromosome complement. *Bar*: 10 cm. **d** Haploid chromosome complement of line 2110 embryogenic tissue prepared by protoplast bursting. *Bar*: 10 μm. **e** Haploid count from needle bases of same plant of 2110 as in Fig. 1 c. *Bar*: 10 μm

HCl at room temperature for 50 min and then stained with Feulgen for 30 min. Three washes in sulphur water followed, each of 10 min duration. The apices were washed several times in water at 4 °C and then squashed in a drop of 45% glacial acetic acid. After the slide had been frozen on dry ice, the coverslip was removed and the slide plunged into 95% EtOH. This process is a modification of Grob's technique (1990).

Absorption of DNA was measured on a Reichert microphotometer that had been appropriately calibrated. Two hundred counts were made per sample. Prophases and anaphases were done separately to establish genome size limits for the populations. The absorbance is shown in arbitrary units.

Chromosome counts

Embryogenic tissue was processed for traditional squashing (A) or for bursting of the protoplast (B), as outlined in von Aderkas and Anderson (1993).

Flushing needles were removed from ramets of 2110 and fixed in 3:1 ethanol:acetic acid. The distal half of the needles was removed and discarded, and only individual needle bases were further processed. Hydrolysis, and all of the stages that followed, were as for traditional squash methods.

Chromosomes were counted and photographed on both a Zeiss Axioplan light microscope and a Leitz Dialux.

Results

Cultures of 2110 and 2140 were embryogenic soon after induction (Fig. 1 a). Early embryos, characterized by embryonal masses and long suspensors composed of embryonal tube cells, were evident throughout. Embryogenic cultures multiplied by cleavage polyembryony, and the spontaneous production of green embryoids (Fig. 1 b) occurred in the absence of any special protocol. These cultures have remained embryogenic since their initiation in 1988. The embryos produced cotyledons and squat

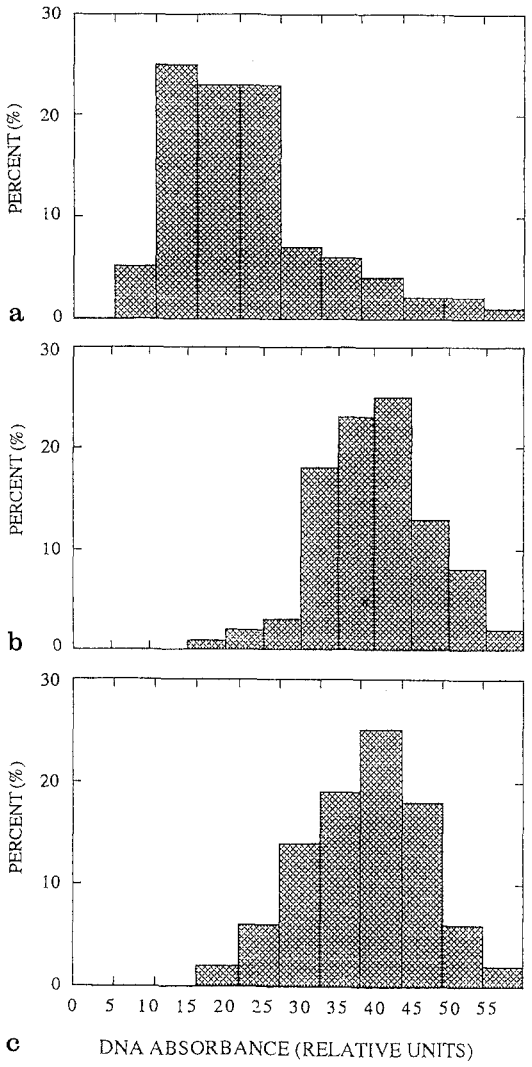

Fig. 2a–c. Histogram of DNA spectrophotometric data classed according to relative units of absorption. Prophases and anaphases were separateley counted to establish limits of haploid and diploid genome sizes. **a** Distribution of DNA measurements of haploid tissue cultures of clone 2110. **b** Distribution of DNA measurements of source tree (G4) used for the original megagametophyte culture. **c** Distribution of DNA measurements of a tree from clone 2110. DNA complement indicated on X axis

hypocotyls, but most of the embryos in culture were abnormal in morphology and germination was restricted to a low percentage. Of these, only one plant of either line took to greenhouse conditions (Fig. 1c). From its inception, the 2110 ortet, which was continuously been kept in a greenhouse, was strongly plagiotropic, as were the several ramets derived from it. After more than three growth cycles, the ortet still shows no sign of reverting to orthotropic growth. Although the plant derived from 2140 grew about 15 cm, it was killed by root weevil damage before it could be vegetatively propagated. The 2140 embryogenic line was lost in a growth chamber mishap.

Chromosome counts from the embryogenic tissue of line 2110 were overwhelmingly haploid ($n=12$) (Fig. 1d). However, counts from flushing needle bases of the plants (ortet and ramets) produced from line 2110 were predominantly diploid though haploid counts (Fig. 1e) were occasionally made; no tetraploid counts were noted. The predominantly diploid nature of the tree was also reflected in the histogram of DNA measurements (Fig. 2c). This histogram is very similar to that of the mother tree (G4, Fig. 2b) and is unlike that of haploid tissues in vitro (Fig. 2a). Anaphases and prophases were used to establish 1C, 2C and 4C genome absorbances of 6.8 ± 2.7 (1C) 35.1 ± 11.5 (2C), and 49.6 ± 12.1 (4C).

Discussion

The larch trees produced by tissue culture from a single haploid megagametophyte are mainly diploid. This observation has also been made in other haploid cultured trees, such as *Populus maximowiczii* (Stoehr and Zsuffa 1990) and *Hevea brasiliensis* (Chen 1987). Plants of megagametophyte derivation in *Sequoia sempervirens* were also diploid, but Ball (1987) thought that this was due to genetic variability in the cells of the megagametophyte explants, which ranged from 1X to 16X in ploidy level. The *Sequoia* gynogenic plantlets were derived more indirectly, having been produced from a callus that in turn produced shoots that were taken through progressive protocols to achieve elongation and rooting. However, *Larix decidua* plants were produced from embryogenic tissue, not callus. Embryos in culture developed shoot and root axes without any special induction protocols being required.

The difficulty in achieving the formation of haploid plantlets in a routine manner has yet to be overcome, but this study shows that haploid culture can be used to produce trees. Haploid embryogenesis may be difficult, but at least in some *Larix* species it is achievable. Haploid embryogenesis from conifer megagametophytes has also been achieved by Simola and Santanen (1990) in *Picea abies*. Spontaneous diploidization occurred in their cultures also (L. K. Simola personal communication). In one of our haploid lines (502) we have found extensive diploidization (von Aderkas and Anderson 1993).

The gradual change of haploid plants into diploid plants has been observed in trees such as *Populus berolinensis* (Chen 1987). The fact that the presumably small number of other mixoploid embryos never developed into plants could be due to their being in an environmentally disadvantageous position in the multiplying cell line. The possibility that the embryo that produced the tree was initially entirely haploid and that diploid cells were formed later is probably remote. The opposite situ-

ation must have arisen in the naturally mixoploid tree of *Thuja plicata* studied by Simak and coworkers (1974). Another possibility involves diploidization by compounds in the medium. Inductive agents and growth regulating substances (Liscum and Hangarter 1991) may lead to mutation within angiosperm cell cultures. It is also possible that during the brief 3-week period conifer cultures were exposed to this inductive agent; some cells may have diploidized, eventually producing plantlets – a sort of diplontic selection.

These trees will be further studied, as they will provide an ideal and, for conifers, unique opportunity for genetic studies when they flower within the next decade. Cultured megagametophytes from such a homozygous parent are central to studies of the effects that media and supplements, as well as growth conditions, may have on inducing somatic variation on in vitro material.

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