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Diploidization in megagametophyte-derived cultures of the gymnosperm *Larix decidua*

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Abstract Tested haploid embryogenic lines (n=12) of *Larix dedicua* Mill. initiated from megagametophyte tissue were maintained on half-strength LM medium without growth regulators. The cultures were analyzed for ploidy level after 1–9 years. All lines tested were found to have doubled (2n=24) their chromosome number at the end of the experiment, though there were a few lines that still gave occasional haploid counts. Flow cytometric data of embryogenic tissue confirmed these results. Protoplasts were stained in ethidium bromide, and cultured human leucocytes and chicken erythrocytes were used as internal standards. Haploid megagametophytes from immature seeds of *L. decidua* and known diploid culture lines of a related hybrid (*L.* × *eurolepis*) were also analyzed by flow cytometry. Haploid reference material had 12.3–13.6 pg DNA per cell, whereas formerly haploid callus lines had an average of 25.0 pg DNA per cell. The one exception was a known, genetically unstable line of *L. decidua* (34.8 pg DNA per cell). The diploid cell line of *L.* × *eurolepis* had 27.6 pg DNA per cell. The results show that spontaneous diploidization of megagametophyte lines is relatively rapid and that both haploid and dihaploid lines are embryogenic in larch.

Key words Flow cytometry · DNA · Diploidization
Haploidy · *Larix* · Megagametophyte

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Introduction

A goal of many plant improvement programs is to create homozygous lines for breeding stock. Homozygous lines can be used for controlled hybridization or as background for transferring single genes. Achieving this in conifers by inbreeding is difficult because of the long period of time required to produce reproductive stock. However, in vitro methods may be used in which haploid reproductive tissues serve as explants. In angiosperms, pollen has been the most successfully exploited source. Unfortunately, in conifers, pollen has proven fairly intractable to date. Limited organogenesis has been recorded, such as root regeneration from callus obtained from pollen of *Picea abies* (Simola and Huhtinen 1986). It is megagametophytes that have lent themselves to this type of culture. In megagametophyte cultures of *Picea abies*, Simola and Santanen (1990) achieved embryo formation, and in *Larix* Bonga and coworkers (Nagmani and Bonga 1985; von Aderkas et al. 1990) were able to get embryos to mature and occasionally germinate. Plantlets have been regenerated via organogenesis in *Sequoia sempervirens* (Ball 1987) and via embryogenesis in *Larix decidua* (von Aderkas and Bonga 1993). In other gymosperms such as *Ephedra foliata* (Singh et al. 1981) organogenesis from megagametophytes has led to plantlet formation, and protoplast cultures of megagametophytic cells of *Gingko biloba* produced somatic embryos (Laurain et al. 1993)

Plantlets produced from such cultures are valuable if the genetic stability of the cultures is known. A particular culture line of *L. decidua* resulted in mosaicism of ploidy level in the larch plantlet produced (von Aderkas and Bonga 1993). Part of the plantlet is diploidized and homozygous. Once the homozygous parts produce fertile sexual cones, these would be clearly of value in breeding. The diploid parts could also be vegetatively propagated to build up stock.

The purpose of this investigation was to test how long haploid cultures would remain stably haploid in embryogenic cultures of *L. decidua* derived from explanted meg-

agametophytes. All of these had been verified as haploid (n=12) after induction. An important additional element to this study was to develop reliable methods to test for ploidy level.

Materials and methods

Plant material

Megagametophyte tissue lines of two *L. decidua* trees (G4 and G5, Fredericton, Canada) were initiated (Nagmani and Bonga 1985, von Aderkas et al. 1987, 1990) and maintained as described in von Aderkas and Anderson (1993). All lines were screened using two different approaches – chromosome squashes and flow cytometry.

Larix × *eurolepis* embryogenic material was initiated from cultured diploid embryos in 1990 (von Aderkas et al. 1990).

Chromosome squashes

Initially a modified squash method described in von Aderkas and Anderson (1993) was used. However, it was later found that the method published by De Carvalho and Saraiva (1993), which involves air-drying the chromosomes was easier. All cultured material was assessed using renumbered, coded samples to avoid bias in the investigation. Chromosomes were counted using a 100 Planapochromat lens on a Zeiss Axioplan microscope. Photographs on the same instrument were done through a no. 15 Wrattan filter (Kodak).

Flow cytometry

Preparation and staining of protoplasts. Megagametophyte callus cultures of *L. decidua* (cell lines 501, 502, 624, 1105, 2036) and *L.* × *eurolepis* (cell line 2086) were grown on half-strength LM medium (Litvay et al. 1985) and transferred at 4-week intervals.

For flow cytometric DNA analyses callus pieces (1–1.5 g) were incubated for 16–18 h at 25 °C in 7 ml of a solution containing: 0.5% Macerozyme "Onozuka R-10": (Yakult, Honsha), 1% cellulase "Onozuka R-10" (Yakult Honsha), 1% cellulysin (Calbiochem), 0.02% pectolyase (Calbiochem), 0.5 *M* sucrose, and 5.0 m*M* $CaCl_2$, at pH 5.8.

Table 1 Nuclear DNA levels in *Larix decidua*(*) culture lines of megagametophyte origin and of *Larix* × *eurolepis* zygotic embryo origin (-) line, as well as megagametophyte tissue

Flow Cytometry	
Source	pg DNA/cell
* 501	25.2 ± 0.2
* 502	34.8 ± 1.60
* 624	24.8 ± 0.95
*1105	23.5 ± 0.91
*2036	26.6 ± 0.14
-2086	27.6 ± 0.95
L. decidua megagametophyte (Can)	12.3 ± 0.06
L. decidua megagametophyte (Fin)	13.6
Biochemical analysis	
L. decidua megagametophyte (Can)	14.1
L. × *eurolepis* line 2086	23.0

The protoplast suspension in the enzyme solution was passed through a nylon sieve (50 µm), and the filtrate was centrifuged for 7 min (200 *g*). The protoplasts were rinsed with W5 solution (Menczel et al. 1981) and centrifuged for 5 min (200 *g*). A staining buffer solution (1–1.5 ml) containing 10 m*M* TRIS-HCl, 3 m*M* $MgCl_2$, 10 m*M* NaCl, 0.5% Non-Idet P40 and 50 µg/ml ethidium bromide, at pH 7.4 was added to the protoplast pellet (Larsen et al. 1980). The samples were stained for 3–4 h on ice, and the vials were shaken a couple of times. Before flow cytometric analysis the samples were sieved through 30-µm nylon tissue.

Measurement of DNA

A Becton Dickinson Facscan flow cytometer was used in DNA estimations (Department of Pathology, University of Helsinki). The excitation wavelength was 488 nm. In most samples about 10 000 nuclei were analyzed (minimum 2,000 nuclei). Each sample was assayed in triplicate. Human leucocytes (HL, cultured cell line) with 5.8–6.0 pg DNA per cell and chicken erythrocytes (CRB) having 2.3 pg DNA per cell were used as references. These cells were stained as described for *Larix* protoplasts. The suitability of animal cells as references for plant DNA levels was checked using protoplasts of *Arabidopsis thaliana*. On the basis of comparison with the animal cells, the *Arabidopsis* cells contained 0.2 pg DNA per cell, which is the same as the genome size reported for this species (Bennett et al. 1982).

In order to estimate the haploid genome size of *Larix*, megagametophyte tissues were dissected from a mature seed sample (G4) and from an immature sample (Finland) of *Larix* preparation and the DNA/cell measurements were as described above.

DNA per cell – biochemical method

A known weight of tissue, between 50–130 mg fresh weight, was processed by two methods – one for biochemical isolation of DNA, the other to assess the cell number. *L. decidua* gametophytes from trees G4 and G5 were used, as well as cultures of zygotic embryo origin of *L.* × *eurolepis* (line no. 2086).

To isolate DNA, tissue was ground in liquid nitrogen in a ball mill for 30 s. Immediately afterward one volume of the powder was brought into suspension in an equal volume of 2CTAB buffer [2% CTAB, 100 m*M* TRIS (pH 8.0), 20 m*M* EDTA (pH 8.0), 1.4 m*M* NaCl, 1% PVP 40 000] following a modification of the method described by Kim et al. (1990). This solution was pipetted into an Eppendorf tube and heated to 65°C, at which point a 1/10 volume of 2% sarcosyl was added. The mixture was kept at 65°C for 15–20 min. To this was added an equal volume of chloroform:isoamyl alcohol (24:1) at room temperature. This was mixed thoroughly to form an emulsion. The solutions were centrifuged at 14 800 *g* for 3 min. The top aqueous layer was removed to a clean tube, and two volumes of ethanol were added. The DNA precipitate was either hooked out (if abundant) or spun down again at 14 800 *g* for 5 min. Once the supernatant was removed, the pellet was dried using a Savant Speed Vac. TE buffer (50 µl) was added to the DNA to bring it into suspension. The samples were stored at –20°C until needed. Analysis was on a UV spectrophotometer at 280/260 (Pye Unicam UV absorbance spectrophotometer).

To establish cell number, six replicates were digested in CrO_3 as described in Berlyn and Miksche (1976). This was done for cultures as well as for megagametophytes. Once the cell number was known, the pg amoung of DNA per cell could be calculated (Table 1).

Results

Each of the megagametophyte lines had been counted since initiation at 12- to 18-month intervals and found to be initially haploid. One of the lines (2110) began to exhibit a

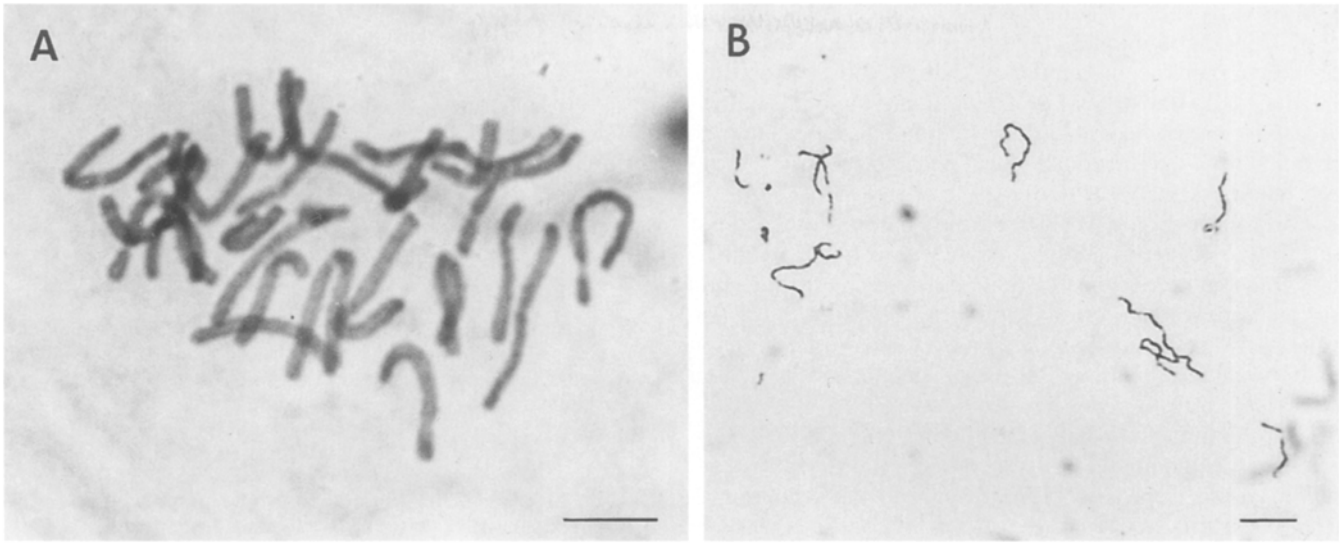

Fig. 1A, B **A** Chromosome squash of cells of line 502, showing 24 chromosomes. *Bar*: 10 m. **B** Chromosome squash of protoplast of line 2159, showing 12 chromosomes. *Bar*: 10 m

Table 2 Chromosome counts from *L. decidua* embryogenic culture lines derived from megagametophytes

Line	Numbers of years haploid
501	9
502	6
624	9
1105	7
2036	2
2110	2

low proportion of diploid counts by 2 years post-initiation, it and was almost exclusively diploid by 2 years later. The other lines were generally slower to diploidize, with two of the five taking 9 years before diploid counts were found (Table 2). Subsequent chromosome counts of these lines were stably diploid (Fig. 1A). Occasional haploid counts were found in one line, 2159 (Fig. 1B).

Recent flow cytometric results, presented in Table 1, show a general diploidization in originally haploid lines 501, 502, 624, 1105, and 2036. Line 2086 is a diploid line derived from a zygotic embryo of *L.* × *eurolepis* (a hybrid of *L. leptolepis* and *L. decidua*), put in as a control, and G4 megagametophyte material collected from the tree at the time of fertilization in early June represents the haploid maternal complement (lines 624 and 1105 were derived from this tree, all the rest were from G5). Megagametophytes collected in late August from a Finnish tree of *L. decidua* gave similar results. Levels of DNA measured by flow cytometry for lines 501, 502, 1105 and 2036 were generally similar, but differed markedly from line 502. The average amount of DNA per cell was found to be 22.5 pg per cell for *L.* × *eurolepis* line 2086.

Discussion

The conclusion from the present chromosome and flow cytometric study is that haploid lines of *Larix* will diploidize if kept in culture for many years. While diploidization happens slowly in *L. decidua*, it is more rapid in *Pinus lambertiana* (Borchert 1968). Flow cytometric data from megagametophyte maternal tissues show levels about half of those found in the cultures derived from megagametophytes of these trees and subcultured for several years. Megagametophytes had a uniform ploidy level, which is in keeping with other studies of megagametophytes [*P. abies* by Hakman and coworkers (1984) and *L.* × *eurolepis* by Wyman and coworkers (1993)]. In contrast, Ball (1987), working on in vitro organogenesis of *Sequoia sempervirens* from megagametophyte-derived cultures, found ploidy variability ranging from 1 to 16. Mixoploid and diploid callus lines from megagametophytes of *P. abies* (Simola and Honkanen 1983) and *P. sitchensis* (Baldursson et al. 1993) have been reported. In *Larix*, a mosaic plantlet of arose from partially diploidized cultures of an originally haploid culture (von Aderkas and Bonga 1993). In this case, it was concluded that age of the culture had an influence on the chromosome level, increasing age causing increases in ploidy. This was further borne out by a study of *L. decidua* in which a line was found to have aneuploid and tetraploid cells (von Aderkas and Anderson 1993).

The higher levels of DNA per cell are fairly uniform amongst *L. decidua* lines, ranging from 23.5 to 26.6 pg DNA/nucleus. This is above the published level for *L. dedicua* roots (19.7 pg per cell) (Dhillon 1987). An exceptionally high level is found in line 502, which has 34.8 pg DNA per nucleus, but this is a line that is known to be genetically unstable (von Aderkas and Anderson 1993), exhibiting aneuploidy, diploidy, and tetraploidy. The level for our diploid *L.* × *eurolepis* (27.6 0.95) is close to that published by Wyman et al. 1993 (32.5 ± 4.0 or 32.0 ± 6.1 pg DNA per nucleus depending on the standard used for dip-

loid in vitro shoot tissue of organogenic origin). Wyman et al. (1993) comment that their nuclear isolation methods provide a more accurate method than protoplast isolation. Earlier, Teoh and Rees (1976) similarly claimed that increased accuracy was afforded by using internal standards. In our study, *L.* × *eurolepis* line 2086 has higher DNA levels than the *L. decidua* lines.

The chromosome work confirmed the interpretation of the flow cytometric study. *Larix* chromosomes are difficult to separate because of their large size. Getting good counts depends on getting enough mitotic figures, which is helped significantly by the fact that the mitotic index in embryonal mass cells of the embryogenic tissue is high (10–13%) (von Aderkas and Anderson 1993).

Duplication of chromosome number is desirable in haploid lines as the material, if regenerated to the plant stage, will be able to reproduce. It represents a significant reduction in the time required to produce inbred lines suitable for breeding purposes (Bonga et al. 1988). The cause of duplication is unknown in these cultures, though long-term culture can be a cause in *Chimonanthus* (Radojevic et al. 1988). Ploidy changes also occur because of the effects of auxins and cytokinins on the cell cycle (Liscum and Hangarter 1991). All of our *L. decidua* cultures have also diploidized, although it has taken as long as 9 years in some cases, and as little as 2 years in others. These results argue strongly for using methods of cryopreservation for keeping physiologically unique cell lines genetically intact.

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