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Aneuploidy and polyploidization in haploid tissue cultures of *Larix decidua*

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Haploid embryogenic lines of *Larix decidua* which had been maintained on medium free of plant growth regulators were screened for chromosomal abnormalities. Chromosome squashes were prepared from fast-growing suspension culture. Alternatively, a method using burst protoplasts was devised for improving chromosome spreads. One line showed polyploidization with chromosome numbers ranging from haploid to tetraploid. This line is presently predominantly diploid. Aneuploidization also occurred. The most effective method for counting chromosomes was from burst protoplasts. It is recommended that long-term cultures of conifer tissues be checked for genetic stability.

Key words – Aneuploidy, conifers, gynogenesis, larch, *Larix*, polyploidy.

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Introduction

Cultured tissues of plants are used for a wide variety of purposes ranging from the provision of novel breeding material to micropropagation of selected genotypes. In general, it is desirable that the plants derived from such a process are genetically stable and uniform.

Genetic instability has been frequently recorded from callus-derived plants, as well as from cultures which have had prolonged exposure to plant growth regulating substances with known mutagenic properties, such as 2,4-dichlorophenoxyacetic acid (2,4-D) (Bayliss 1980).

Another source of variation resides in the parent tissues. The few reports of chromosome variation in situ imply that conifers are generally uniform and stable. However, chromosome irregularities have been found in *Pinus sylvestris* (Butorina et al. 1980). Furthermore, embryos with a mosaic of aneuploid cells are known in *Picea abies* (Illies 1964). Trees of mixed ploidy level are known from *Thuja plicata* (Simak et al. 1974). Mixoploidy is also known in artificially polyploidized larches (Illies 1966a,b). There are a number of reports of genetic instability in somatic embryogenesis of angiosperm trees (Tulecke 1987). Berljak et al. (1987) showed that aneuploidy occurred in bud cultures of *Pinus nigra,* but that these cells were generally eliminated after a longer period of culture. Hakman et al. (1984) showed that nuclear DNA content was similar in both adventitious bud cultures and buds of field-grown trees. The chromosome number of *Pseudotsuga menziesii* plantlets produced from cultured cotyledons was stable (Wochok et al. 1980). In contrast, higher DNA levels were found in cultured adventitious shoots of *Pinus coulteri* (Patel and Berlyn 1982). *Picea glauca* tumour cells cultured in vitro showed heterogeneity in chromosome number (de Torok 1968). In few studies of somatic embryogenesis in conifers, no somaclonal variation has been observed so far (Mo et al. 1989, Eastman et al. 1991).

Haploid megagametophytes have been induced to form embryonic tissue in *Larix* (Nagmani and Bonga 1985) and *Picea* (Simola and Santanen 1990). Haploid megagametophyte-derived cultures of various larch species have been grown for a number of years without change in ploidy level (von Aderkas et al. 1987, von Aderkas and Bonga 1988). However, we have one clone which did not follow that pattern.

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This study reports the occurrence of aneuploidy and polyploidization in haploid embryogenic cultures of *Larix decidua*.

Materials and methods

Cultures

Larch (*Larix decidua*) embryogenic cultures used in this study were all derived from excised megagametophytes. These cultures had all been verified as haploid by chromosome squashes carried out soon after the embryogenic cultures were induced. The cultures were from induction experiments spanning a number of different growing seasons (Nagmani and Bonga 1985, von Aderkas et al. 1987, 1990). Lines used were 130, 501, 502, 624, 1105, 2036, 2110. Cultures were maintained on 1/2 strength LM (Litvay et al. 1981) medium supplemented with 2% (w/v) sucrose, 3 m*M* glutamine, 0.55 m*M* *myo*-inositol. Growth regulating substances were restricted to the initial 3-week induction period. Maintenance was, therefore, on medium without auxin or cytokinin.

Chromosome counting

Chromosomes of larch were spread by either a squash method, or by bursting of protoplasts. For squashing, embryogenic tissue was fixed in 3:1 ethanol (95%): glacial acetic acid, washed, hydrolyzed for 15–20 min in 5 *M* HCl at room temperature, and squashed under a coverslip in a drop of 45% acetic acid. After quick freezing on dry ice, following by removal of the frozen coverslip with a single-edged razor blade, the still frozen slide with the frozen squash was plunged into 95% ethanol. After 2 min the slide was removed and air dried overnight. The slide was then stained for 8 min in 10% dilution of Giemsa stock, washed 3 times with double-distilled water for 3 min each and air-dried again before mounting in Histoclad mounting medium (Clay Adams, Parsippany, NJ, USA).

The other preparation method, protoplast-bursting, involved a modification of the splash method for mitotic chromosomes in which cells are dropped onto a slide (Yang and Zhang 1988). Embryonic tissue was prepared as follows: (1) Protoplasts were isolated from cells in a solution of 2% (w/v) cellulase (Sigma) and 0.5% (w/v) Macerozyme-R10 (Yakult Honsha, Japan) in 9% (w/v) mannitol, 5 m*M* 2-[N-morpholino]ethanesulfonate (MES) (Sigma) and 5 m*M* $CaCl_2$ for at least 5 h at room temperature. (2) The volume of protoplasts in suspension was diluted by adding an equal volume of a solution of ice-cold methanol and glacial acetic acid (3:1). (3) The tubes were kept on ice for 5 min, and the protoplasts centrifuged at 150 *g* for 5 min. (4) The pellet was resuspended in ice-cold methanol and glacial acetic acid (3:1) and centrifuged twice more. (5) The final pellet was resuspended and a frozen Pasteur pipette used to drop the solution (1 drop per slide) from a height of 14 cm onto frozen slides, which were immediately flamed. (6) Staining was in 10% Giemsa for 8 min. (7) The fixed protoplasts were finally washed in tap water, air-dried overnight, and mounted in Histoclad mounting medium. Chromosomes were counted and photographed with a 100 × objective lens.

Results

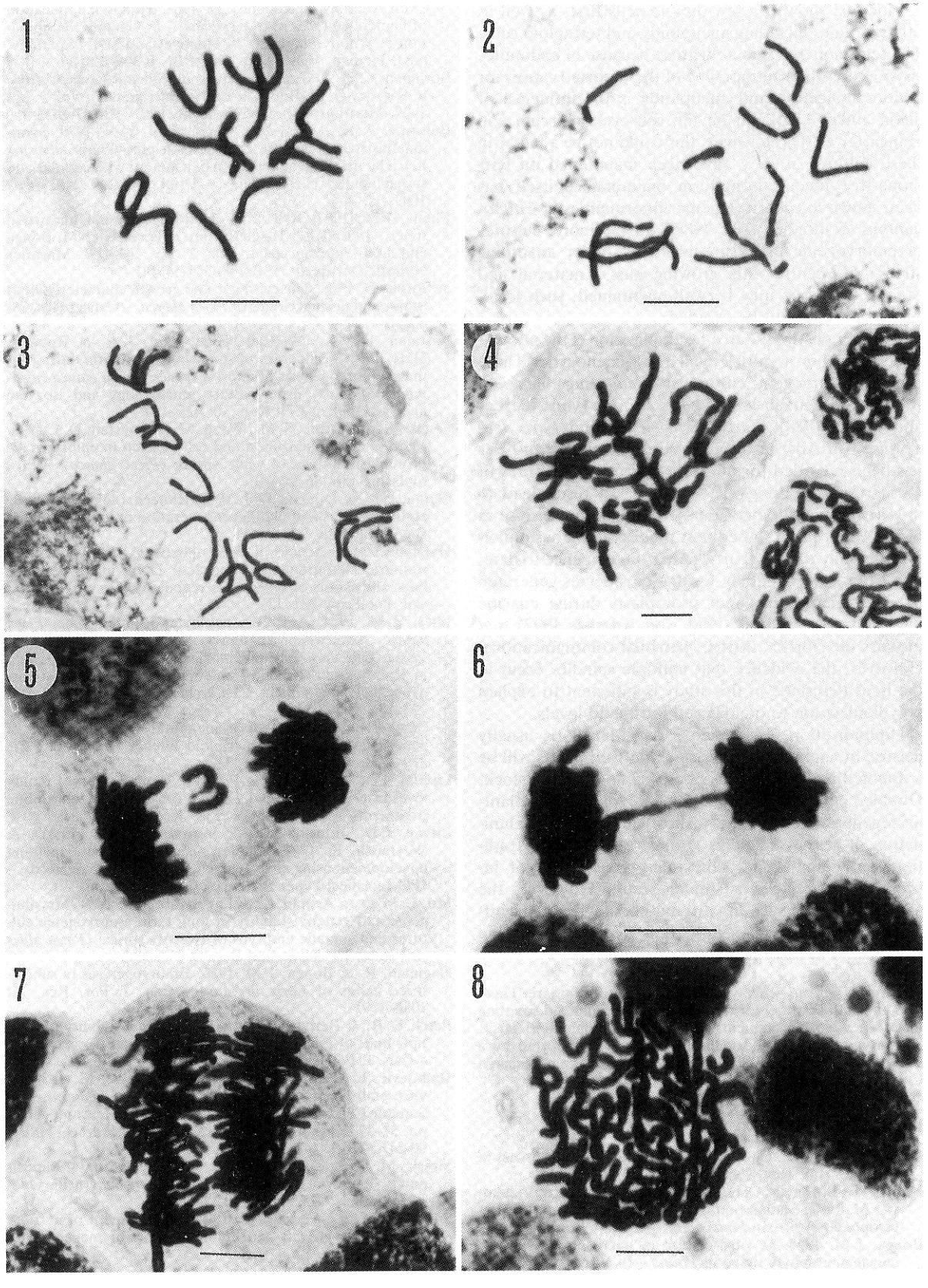
Generally, chromosome counts gave a haploid number, namely n = 12 (Fig. 1). This was found to be the usual result over the years. Lines 2036 and 2110 occasionally also gave a diploid count, but the cultures remained haploid. Embryogenic capacity fluctuated but was not lost in any of the lines. However, line 502, one of the original lines derived by Nagmani and Bonga (1985), differed from the other lines. Until 1989 it was consistently haploid. However, at this point aneuploid cells appeared within the general population. For example, cells with chromosome complements of n = 11 (Fig. 2) and n = 14 (Fig. 3) were recorded. In 1990, the occasional diploid count was recorded (Fig. 4). In addition, mitotic irregularities such as lagging chromosomes (Fig. 5) and anaphase bridges (Fig. 6) were noted. Polyploid cells in division (Fig. 7) as well as tetraploid chromosome complements (Fig. 8) were occasionally recorded.

In 1991, counts (total of 160) were mostly diploid (71%) with haploids (2%) and tetraploids (2%) occurring rarely. Aneuploid chromosome numbers were n = 10 (1), n = 19 (3), n = 20 (2), n = 21 (4), n = 22 (12), n = 23 (5), n = 25 (2), n = 26 (2), and n = 27 (4).

Discussion

Most of the lines of haploid cultures had consistently haploid counts of 12 chromosomes. This was the case for lines that are presently in their 7th year. All of these lines produce green embryoids with cotyledons. All lines have shown fluctuations in embryo production. Although initially line 130 produced embryos (Nagmani and Bonga 1985), it lost this ability within a year of initiation. Despite numerous experiments to reinvigorate this line no success was achieved (P. von Aderkas, unpublished results). However, last year, without any treatment beyond routine subculture, this line began producing embryos anew. Line 130 showed no change in chromosome number.

Figs 1–8. (1) Haploid number (n = 12) of chromosomes, prepared from protoplasts of cells of line 2110. Bar = 10 μm. (2) Aneuploidy (n = 11) in cells of line 502. The preparation is from protoplasts. Bar = 10 μm. (3) Fourteen chromosomes in a cell of line 502 which was prepared from protoplasts. Bar = 10 μm. (4) Diploid number (2n = 2x = 24) of chromosomes from cells of line 502. Bar = 10 μm. (5) Lagging chromosomes in a cell squash of line 502. Bar = 10 μm. (6) An anaphase bridge in a squash of a cell of line 502. Bar = 10 μm. (7) Polyploid cell in anaphase in a squash of a cell of line 502. Bar = 10 μm. (8) Tetraploid number (2n = 4x = 48) of chromosomes from a cell squash of line 502. Bar = 10 μm.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Line 502 showed a few diploid cells first, as well as other ploidies including aneuploidy and tetraploid cells. The tetraploids presumably arose because of endomitosis or endoreduplication. One of the reported causes for endoreduplication and aneuploidy is the influence of auxin and/or cytokinin on the cell cycle (Liscum and Hangarter 1991). However, this could not be a factor in these cultures as they have been maintained on hormone-free media. Long-term maintenance itself has been shown to cause cytogenic abnormalities in *Chimonanthus* (Radojevic et al. 1988). Furthermore, cultures of embryogenic tissue are probably under anaerobic stress in the centre of the growing mass of material, and though the tissue may be well-maintained, such localized stresses cannot be avoided.

Another possible source for duplication is the population of cells in a culture which are coenocytic. These occur in embryogenic cultures of *Pseudotsuga menziesii* (Gupta and Durzan 1986), *Larix decidua* (von Aderkas and Bonga 1988), *Larix* × *eurolepis,* and *Larix leptolepis* (von Aderkas et al. 1990). Nuclei may fuse, as has been recorded for nuclei in microspore cultures of *Picea resinosa* (Bonga 1974), and as a result lead to mosaicism in embryogenic tissue. Should nuclei fuse in bi-nucleate cells in these lines, then spontaneous diploidization may easily occur. There is no evidence to date, e.g. from experimentally-formed coenocytes generated by spontaneous fusion of protoplasts during enzyme digestion (Fowke et al. 1990, von Aderkas 1992) that mosaics develop (S. Attree, personal communication). However, the evidence that multiple spindles occur in the lines described in this study is sufficient to explain polyploidization to diploid and tetraploid levels.

Diploidization, spontaneous or induced, is usually desired in haploid cultures as any derived plants will be a doubled haploid and may be used as breeding stock. Doubled cultures of conifer material followed by plantlet regeneration represent a short cut to massive elimination of recessive lethals (Bonga et al. 1988). Traditional breeding would take many generations of inbreeding to achieve a similar result. However, the advantages from such in vitro methodologies are significantly diminished if aneuploidy is frequent within the culture and in field-grown material.

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