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Trisomy associated with loss of maturation capacity in a long-term embryogenic culture of *Abies alba*

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Abstract Karyological studies were made on a 6-year-old embryogenic cell line of *Abies alba*. Embryogenic cells were obtained from a mature zygotic embryo cultivated on modified MCM-medium and subcultured every 3 weeks. Three years after induction, part of the cell line was transferred to media supplemented with 500 or 1000 mg l^{-1} caseine hydrolysate and 500 mg l^{-1} L-glutamine. Approximately 3 years after addition of the organic nitrogen to the medium, morphological changes such as malformation of the suspensor cells and a loss of maturation capacity occurred. Chromosome counts revealed that all cells cultivated on medium with organic nitrogen were trisomic. Fluorescent-banding methods and comparison with an euploid cell line showed that the additional chromosome belonged to the group of long, metacentric chromosomes of *Abies alba* without secondary constriction. Those cells cultured on medium not supplemented with caseine hydrolysate and L-glutamine retained a stable chromosome number of $2n = 24$. Both normal and deformed suspensor cells were observed. The maturation frequency was very low. The emergence of aneuploidy within one cell line could be the consequence of high selection pressure caused by the different culture conditions.

Key words *Abies alba* · Organic nitrogen · Somatic embryogenesis · Somaclonal variation · Trisomic karyotype

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Introduction

During the last decade, somatic embryogenesis has become the most important method for the in vitro multiplication of conifers. The successful production of plantlets by somatic embryogenesis has been reported for several coniferous species (Gupta and Grob 1995). The great advantage of somatic embryogenesis over conventional multiplication methods is the possibility of large-scale propagation of superior genotypes. While this is of special interest for the preservation of endangered genotypes, it is also a tool for studying the process of embryogenesis, and not least it is of commercial interest.

For a successful mass cloning it is necessary to avoid tissue culture instability and thus somaclonal variation (Larkin and Scowcroft 1981). In vitro cultures of conifers were originally considered to be genetically stable (Mo et al. 1989; Eastman et al. 1991; Isabel et al. 1993), and it has only been in the last few years that some cases of somaclonal variation have been reported for embryogenic cultures of *Larix* sp. (DeVerno et al. 1994), *Picea abies* (Fourré 1985) and *Picea glauca* (Isabel et al. 1995). But until now, there was no evidence for tissue culture instability in *Abies* (Schuller et al. 1989; Gajdošová and Vooková 1991; Gajdošová et al. 1995; Libiaková and Gajdošová 1993; Libiaková et al. 1995).

In the investigation described here morphological as well as karyological studies were made on a 6-year-old embryogenic cell line of *Abies alba* cultivated on media containing different levels of organic nitrogen.

Materials and methods

Plant material

The experiments were carried out with the embryogenic cell line 2/56, which was established in 1989 from a mature zygotic embryo collected at Innsbruck, Austria (Grahsl et al. 1991). The cell line was

maintained on modified MCM-medium (NO, without organic nitrogen; Bornmann and Jansson 1981; Hristoforoglu et al. 1995) at 24° ± 1°C in the dark and subcultured every 3 weeks. Three years after induction, part of the cell line was transferred to media N1 and N2 that had been supplemented with different levels of organic nitrogen (see Table 1). At least three petri dishes per treatment were subcultured independently.

The embryogenic cell line R39/1 was induced in 1995 from an immature zygotic embryo collected on the mountain Rax, Austria, and propagated on a modified BLG-medium (J. Norgaard, personal communication).

For the maturation of somatic embryos, the cultures were transferred onto a medium containing 20 µ*M* ABA (abscisic acid).

Zygotic embryos of *Abies alba* were collected on the mountain Rax, Austria, in the summer of 1995, isolated from the megagametophytes aseptically and stored at − 20°C until further utilization.

Chromosome counts

Metaphase chromosome spreads were obtained from embryogenic cell masses. The material was treated with 0.05% colchicine at 4°C for 18 h and fixed in methanol : acetic acid (3 : 1) overnight. Feulgen staining was carried out according to standard procedures (Greilhuber and Ebert 1994). At least 50 metaphase chromosome spreads of the cell line 2/56 on three different media (N0, N1, N2) and of the cell line R39/1 were evaluated.

Fluorescent chromosome banding and silver impregnation of nucleoli

Embryogenic cell masses were treated with 0.05% colchicine for 18 h at 4°C and fixed in ethanol : chloroform : acetic acid (2 : 1 : 1) (Hizume et al. 1991) at 4°C overnight. After washing in a 0.01 *M* citrate buffer the fixed material was macerated in an enzyme mixture (2% cellulase and 20% pectinase in 0.01 *M* citrate buffer, pH = 4.8) for 60 min at 37°C. The material was washed in citrate buffer again and squashed in a drop of 45% acetic acid on a slide. After freezing the coverslip was removed.

For fluorescent banding the fluorochromes chromomycin A_3 (CMA), which specifically binds to GC-rich DNA, and 4,6-diamidino-2-phenylindole (DAPI), specific for AT-rich DNA, were used. The staining procedures followed Schweizer (1976) with slight modifications. The preparations were treated with 0.5 mg ml^{-1} CMA in McIlvaine buffer containing 5 m*M* $MgCl_2$ for 30 min in the dark and rinsed with distilled water. After drying, they were stained with 2 µg DAPI per milliliter McIlvaine buffer for 20 min in the dark. The preparations were rinsed with distilled water again, dried and mounted in glycerine : McIlvaine (1 : 1) supplemented with 5 m*M* $MgCl_2$.

Fluorescent banding was carried out with the subclone of cell line 2/56 cultivated on N2 and with cell line R39/1. Per experiment, at least 20 fluorescent-banded metaphase chromosome spreads were evaluated. The fluorescent-banded chromosomes were observed under an epifluorescence microscope (Zeiss) equipped with appropriate filter combinations.

The silver-staining of the nucleoli followed the methods of Bloom and Goodpasture (1976) and Kodama et al. (1980). The embryogenic cell masses of R39/1 and of 2/56 on N2 were used.

Flow cytometric analysis

The isolation of nuclei followed the method of Galbraith et al. (1983). About 10 mg of plant material was chopped with a razor blade in 0.6 ml of 0.1 *M* citric acid containing 0.5% Triton X-100 (De Laat et al. 1987). The suspension of nuclei was filtered through a 48-µm

Table 1 Differences in the three subclones of cell line 2/56 cultivated on media with (N1, N2) or without (N0) organic nitrogen supplements

Medium	Morphology of embryogenic cell masses	Organization of stage-1 somatic embryos	Proliferation rate (days required for doubling of fresh weight)	Maturation frequency: somatic embryos per gram fresh weight		Chromosome number (2n)
				Stage 2	Stage 3	
Modified MCM without organic nitrogen (N0)	Brownish, with spontaneous embryos	Poorly organized, with both long and round suspensors	8	213	6	24
Modified MCM + 500 mg l^{-1} L-glutamine + 500 mg l^{-1} caseine hydrolysate (N1)	White, translucent, with spontaneous embryos	Well organized, with long suspensors similar to zygotic embryos	3	126	0	24
Modified MCM + 500 mg l^{-1} L-glutamine + 1000 mg l^{-1} caseine hydrolysate (N2)	White, without spontaneous embryos	Poorly organized, with round suspensors	7	245	1	25

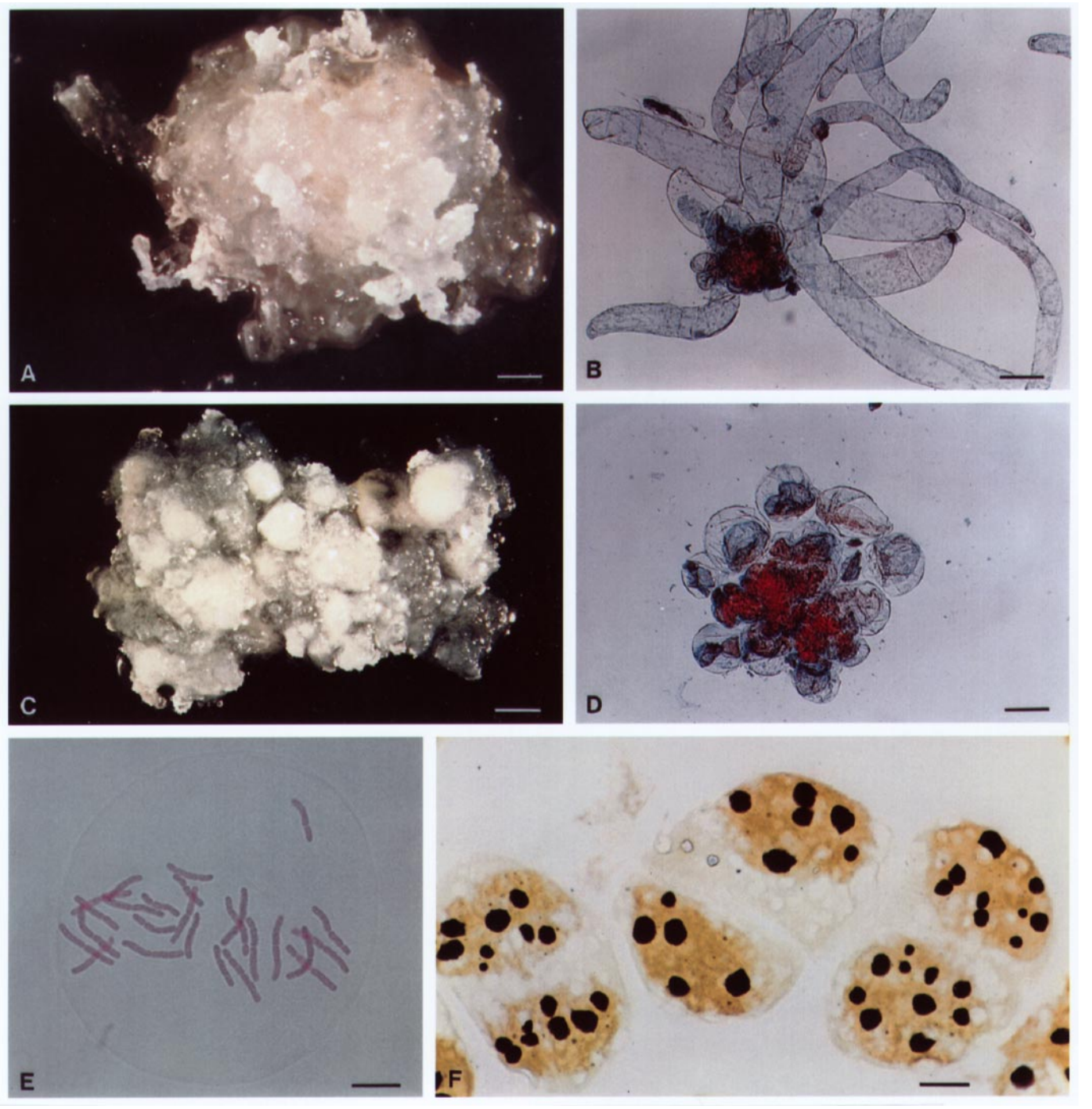


Fig. 1A–F Embryogenic cell line 2/56. **A** Embryogenic cell line 2/56 cultivated on modified MCM supplemented with N1. *Bar*: 1 mm. **B** Proembryos of cell line 2/56 cultivated on modified MCM supplemented with N1, stained with carmine acetic acid and Evan's Blue. *Bar*: 0.25 mm. **C** Embryogenic cell line 2/56 cultivated on modified MCM supplemented with N2. *Bar*: 1 mm. **D** Proembryos of cell line 2/56 cultivated on modified MCM supplemented with N2, stained with carmine acetic acid and Evan's Blue. *Bar*: 0.25 mm. **E** Metaphase chromosome spread of the trisomic subclone 2/56 on modified MCM supplemented with N2; Feulgen stained. *Bar*: 10 µm. **F** Silver-stained telophase nuclei of the trisomic subclone 2/56 showing a maximum of ten nucleoli. *Bar*: 10 µm

nylon net and stained with 5 μg ml^{-1} DAPI and 3 μg ml^{-1} sulforhodamine in 0.4 *M* sodium hydrogen phosphate (Ulrich and Ulrich 1986).

For a direct comparison of relative DNA content, similar amounts of mature zygotic embryos and stage-3 somatic embryos of cell line 2/56 matured on medium with N2 and 20 μM ABA were chopped simultaneously. In addition, both zygotic and somatic embryos were chopped together with the internal standard *Pisum sativum* 'Kleine Rheinländerin'. Five runs per preparation were carried out. Relative DNA content was measured on a Partec CA II flow cytometer equipped with a 100 W high-pressure mercury lamp.

Feulgen densitometry

Feulgen densitometry was carried out according to the protocols of Greilhuber and Ebert (1994). *Pisum sativum* 'Kleine Rheinländerin' was used as reference value with a known 1C content of 4.42 pg DNA (Baranyi and Greilhuber 1996).

Results

Approximately 3 years after addition of organic nitrogen (0, N1, N2) to the proliferation medium, morphological changes and a loss of maturation capacity occurred (Table 1). The three subclones of 2/56 were found to vary greatly in the following parameters: morphology of the embryogenic cell mass, maturation capacity, growth rate and form of suspensor cells (Fig. 1A–D). The changes were especially striking on modified MCM supplemented with N2: the stage-1 somatic embryos were poorly organized and surrounded with completely round, suspensorlike cells. After ABA treatment, the production of stage-2 somatic embryos was still high (245 per gram fresh weight), whereas the maturation of stage-3 somatic embryos was totally suppressed.

Chromosome counts revealed that all cells cultivated on medium with N2 were aneuploid (2n = 25, Fig. 1E). However, the subclones cultivated on medium without organic nitrogen or supplied with N1 showed a stable chromosome number of 2n = 24.

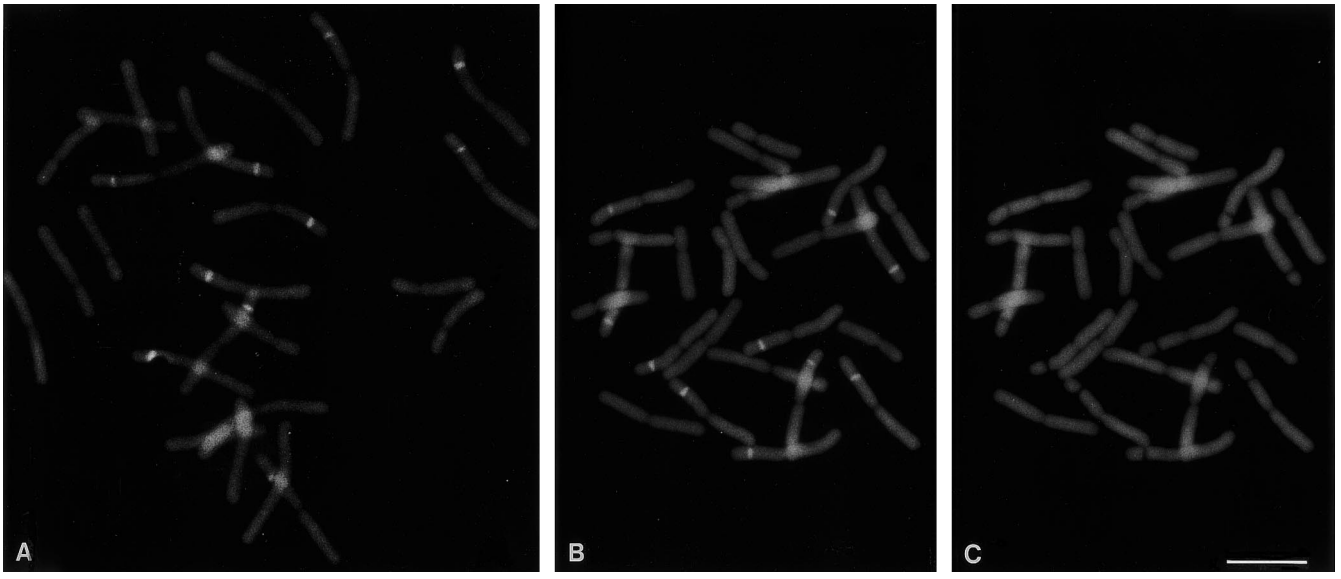
After CMA/DAPI staining, brightly fluorescent CMA-bands appeared clearly in some chromosomes with the CMA filters (Fig. 2A, B). With DAPl filters no bright bands could be observed; negative DAPI-bands appeared in the bright CMA-banded regions (Fig. 2C).

The diploid karyotype of the euploid cell line R39/1 showed 10 long, metacentric chromosomes with positive CMA-bands at the secondary constrictions. In addition, 4 long, metacentric chromosomes without CMA-bands and 10 shorter, subtelocentric chromosomes could be found (Fig. 3A).

A comparison of a fluorescent-banded karyotype of the subclone of cell line 2/56 with 2n = 25 (Fig. 3B) with the euploid cell line R 39/1 (Fig. 3A) showed that in every metaphase analyzed the additional chromosome belonged to the group of long, metacentric chromosomes without a nucleolus organizing region (NOR). This group of chromosomes consists of two pairs of chromosomes in the normal karyotype. This result was also confirmed by the silver staining of nucleoli: a maximum of ten nucleoli in the telophase nuclei of both cell lines was observed (Fig. 1F).

The aneuploidy was also detected by DAPI-flow cytometry. The separate measurements, each type of sample combined with the internal standard *Pisum*

Fig. 2A–C Fluorescent-banded metaphase chromosome spreads of embryogenic cell lines of *Abies alba*. *Bar*: 10 μm. **A** Chromosomes of cell line R39/1; CMA banded. **B** Chromosomes of the trisomic subclone of cell line 2/56; CMA banded. **C** Chromosomes of the trisomic subclone of cell line 2/56; DAPI banded



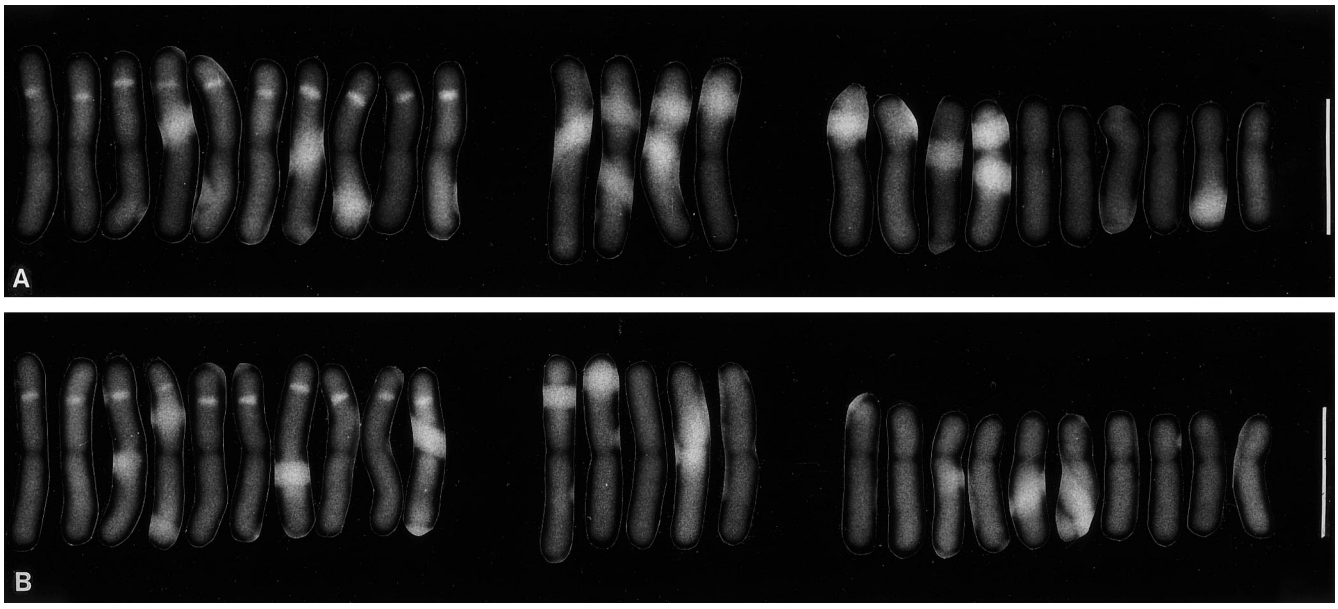


Fig. 3A, B CMA-banded karyotypes of embryogenic cell lines of *Abies alba*. *Bars*: 10 µm. **A** Euploid cell line R39/1, **B** Trisomic subclone of cell line 2/56. Note the additional chromosome belonging to the group of long, metacentric chromosomes without CMA-positive band

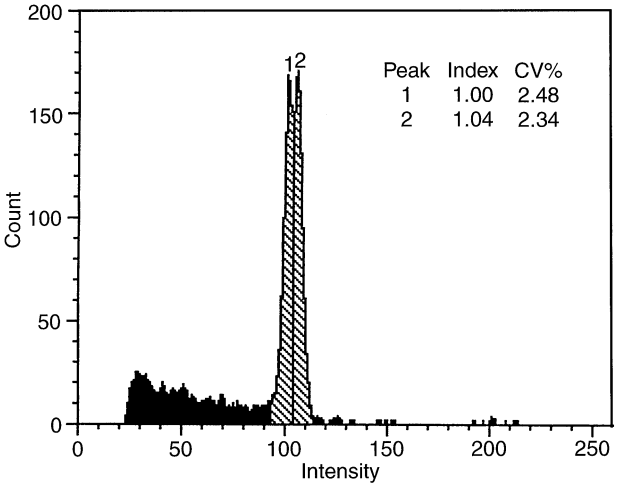


Fig. 4 Flow cytometric histogram (DAPI) obtained after a combined measurement of nuclei of mature zygotic embryos and stage-3 somatic embryos of cell line 2/56 on medium supplemented with N2

sativum 'Kleine Rheinländerin', gave a highly significant difference of 4.06% ($P < 0.001$) between the trisomic cell line and the zygotic embryos, with the higher relative DNA content corresponding to the trisomic cell line. Also, the histogram obtained after a combined measurement of nuclei of the trisomic cell line and zygotic embryos showed two peaks close together, corresponding to the 2C nuclei of the respective cell lines (Fig. 4).

On the basis of Feulgen densitometry measurements, the DNA content corresponding to 1C could be estimated at 16.55 ± 0.48 pg for zygotic embryos and 17.25 ± 0.28 pg for somatic embryos of the trisomic subclone (25 late-telophase or early-prophase nuclei per cell line were measured). The difference of 4.05% was significant ($P < 0.01$) and very close to the values obtained by flow cytometry.

Discussion

Trisomy in a cell line of *Abies alba*, as analyzed here, has not been described previously. Completely trisomic embryogenic cell masses of conifers were also reported for *Picea abies*, based on chromosome counts (Fourré 1995) and *Pinus* sp. with flow cytometry and chromosome counts (O'Brien et al. 1996).

Aneuploidy may have its origin in aberrations of spindle function and/or chromosome movement, such as non-congression at metaphase or non-disjunction of anaphase. Such aberrations may result in the loss or gain of single chromosomes (Gill et al. 1986).

It is not clear how the subclone on modified MCM supplemented with N2 has become completely trisomic. Either the same mutation occurred many times in the cultures, or the chromosome doubling happened only once and the resulting trisomic cell had a selective advantage over all other cells. In both cases, the complete mutation would be consequence of very high selection pressure, probably caused by the different culture conditions.

On the other hand, aneuploidy is regarded to be the most detrimental numerical chromosome variation because it results in genic imbalance (Karp and Bright 1985). It is obvious that the observed trisomy is one

reason for the loss of maturation capacity of the subclone cultivated on medium supplemented with N2. Nevertheless, it cannot be the only reason for the degenerative morphological changes in cell line 2/56 because the malformation of suspensor cells and grossly decreased maturation of somatic embryos also occurred on medium without organic nitrogen where the cells are still euploid. Probably this was caused by one or even more mutations at the DNA level which cannot be detected by the methods used in this investigation.

Genetic instability is known to increase with prolonged in vitro culture. Several reports have described similar cases for angiosperms (Bayliss 1980), but until recently conifers have been considered to be genetically stable. Only in the last few years an increased number of reports about genetic instability or somaclonal variation in in vitro cultures of conifers has appeared. As it is the aim of somatic embryogenesis to produce phenotypically and genetically uniform plants, the occurrence of somaclonal variation should be minimized. Therefore, the time span for the production of regenerated plantlets after the initial induction of somatic embryogenesis should be kept as short as possible, and alternatives to subculturing, such as cryopreservation, should be established.

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