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Somatic embryogenesis and somaclonal variation in Norway spruce: morphogenetic, cytogenetic and molecular approaches

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Abstract Four embryogenic clones of Norway spruce have been subcultivated and observed over several years to determine the evolution of production of mature embryos and to assess the quality of the embryos produced. A wide range of intraclonal quantitative and qualitative variability has been observed within this production. Certain morphologic deviations appeared at the immature stage and after maturation, such as immature embryos with a diffuse organization, complete or part albino mature embryos or acclimated somatic seedlings comparable to dwarf mutants. All of these phenotypic variations could be the result of a modification of the genome itself or of only the expression of the genome. Two approaches, chromosome counting and RAPD (random amplified polymorphic DNA), were chosen for their capacity to detect genotypic variations: respectively, genomic and chromosomic or genic mutations. The cytogenetic approach revealed, for the first time in this species, three cases of mutated acclimated somatic plants: one totally trisomic and two chimeras with trisomic buds and diploid roots. Other cases of 5-year-old trisomic, double trisomic, tetraploid or mixoploid embryogenic masses were also detected. The molecular approach (RAPD) revealed no somaclonal variation despite the large sample of DNA and primers used and the important interclonal variation observed.

Key words Somaclonal variation · Somatic embryogenesis · Conifers · RAPD · Trisomy · Chimerism

Introduction

Aside from the already widely applied traditional propagation by cuttings, somatic embryogenesis is often considered to be a very promising method for the vegetative multiplication of conifers. Moreover, this method is already used as a regeneration model for genetic transformation experiments. Amongst conifers, species of the *Picea* genus, such as Norway spruce (*Picea abies* (L.) Karst.), have provided the best results as far as the induction of somatic embryogenesis and regeneration of viable plantlets is concerned (Tautorus et al. 1991). However, in the years since the first reports of Chalupa (1985) and Hakman et al. (1985) the number of acclimated somatic seedlings of Norway spruce has remained rather small.

Plantlets derived from in vitro culture might exhibit somaclonal variation (Larkin and Scowcroft 1981), which is often heritable. Due to the very long life cycle of conifers this phenomenon constitutes a drawback for the micropropagation since the occasional mutation can sometimes only be noticed very late, during tree development stages or even in its offspring. It would be important to be able to detect the eventual variation early. For this purpose, several levels of approach and methodologies have been proposed. The variations can be analysed at the level of the phenotype or the genotype. Phenotypic variations, analysed with morphologic or protein markers, can be the result of a modification of the genome itself or only of the expression of the genome. Genotypic variations can be genomic, chromosomic or genic. Genomic mutations affect the number of chromosomes (the ploidy) and can be detected by cytometry or chromosome counting. Contrary to cytometry, the time-consuming chromosome counting enables an unfailing detection of all, even the smallest, modifications in chromosome number. Chromosomic mutations like inversion, deletion or translocation and genic mutations could be detected by genetic molecular markers like RFLPs (restriction fragment length polymorphisms) and RAPDs (random amplified polymorphic DNA) that can detect DNA sequence modifications. It is also important to remember that mutations can occur on nuclear as well as on mitochondrial (mt) or chloroplast (cp) DNA.

Lelu (1988) found 3.7% tetraploid cells (10/272) in immature (globular) Norway spruce somatic embryos. The 10 tetraploid cells were recorded in 2 embryos, 1 being chi-

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meric (having other diploid cells). Salajova and Salaj (1992) observed 2.8% polyploid cells in embryogenic masses of *Pinus nigra*. Nkongolo and Klimaszewska (1995) and O'Brien et al. (1996) detected, respectively, two *Larix*×*eurolepis* and one *Pinus radiata* trisomic cell lines, but it is not known if the trisomy arose during culture or in the particular embryo from which the culture was derived. Contrary to the results found using chromosome counting, no examples of genetic deviation have been detected in conifer somatic embryogenesis using cytometry (Mo et al. 1989; Gajdosova et al. 1995). Except for some mtDNA modifications observed by De Verno et al. (1994) using RFLPs, no other molecular markers (RAPDs or isozymes) have been found to reveal conifer somaclonal variation (Heinze 1993; Isabel et al. 1993; Eastman 1991).

In gametic embryogenesis (initiated from megagametophyte tissue) of *Larix decidua* and *Picea sitchensis*, von Aderkas and Anderson (1993), Pattanavibool et al. (1995) and Baldursson et al. (1993) observed, within initially haploids lines, a tendency to diploidization or aneuploidization, or some cases of tetraploidy and mitotic irregularities.

Some other cases of genomic mutations, more often tetraploidy, have been detected during callus culture, adventitious budding or micropropagation in conifers (Risser 1964; Isikawa et al. 1963; Salmia 1975; Mott et al. 1977 cited by Berlyn et al. 1986; Partanen 1963; Mehra and Anand 1979, 1983; Papes et al. 1983; Noh et al. 1988; Gajdosova and Vookova 1991). The rate of mutation was, nevertheless, always limited (maximum 14% of the analysed cells, Salmia 1975); there were no examples of a general tendency to polyploidization.

All of the other karyological analyses concerning conifer somatic embryogenesis (Baldursson et al. 1993; Nkongolo and Klimaszewska 1994; Schuller et al. 1989), adventitious budding (Wochok et al. 1980; von Arnold and Eriksson 1986) or callogenesis (Manandhar and Gresshoff 1980; Tominaga and Oga 1970; Konar and Nagmani 1972; Partanen 1963; Venketeswaran and Huhtinen 1978) have indicated a perfect stability of the chromosome number. However, we can assume that these lasts results were the consequence of a too small sampling.

The purpose of the study presented here was to analyse (1) the intraclonal morphologic and developmental deviations, and (2) the genomic mutations that occurred amongst four embryogenic clones capable of regenerating viable somatic seedlings and (3) to inquire about the applicability of RAPDs as an alternative method for assessing the genetic conformity of embryogenic tissues and somatic seedlings.

Materials and methods

Plant material

Four embryogenic mass clones of Norway spruce (*Picea abies* (L.) Karst.), initiated from mature zygotic embryos, were selected out of several hundred clones for their ability to produce healthy mature somatic embryos. The A, B and C clones were initiated on July 3, 1989 (from seeds collected in 1984 at seed stand "Cedrogne 031A", Belgium), and the V clone was initiated on March 5, 1990 (from a seed collected in 1984 at seed stand "Jubilé St Jean", Belgium). Seeds were soaked in water for 24 h, surface-sterilized for several seconds in 70% ethanol and aseptically dissected in a laminar flow cabinet. Embryos were placed in contact with the initiation medium (1M1) of Gupta and Durzan (1986) modified as follows: 10 g/l sucrose, 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 2 μM kinetin (KIN), 2 μM 6-benzylaminopurine (BAP) and solidified with 3 g/l Gelrite. After initiation, the embryogenic masses were subcultivated every week on the multiplication medium (1M2), which was identical to the initiation medium except that the concentrations of the growth regulators were ten times lower in the former. Each week embryo maturation was obtained by transferring a part of the embryogenic mass to a multiplication medium deprived of auxin and cytokinin and containing 30 g/l sucrose. After autoclaving, abscisic acid (ABA) was added to this medium (medium 1M3). During these successive stages, the petri dishes remained in darkness at a temperature of 22°C.

The somatic embryos were germinated under a 16-h photoperiod in petri dishes, on modified Murashige and Skoog medium (Misson 1988) containing a quarter of the NH_4NO_3 concentration, 20 g/l sucrose, 4 g/l activated charcoal and solidified with 3 g/l Gelrite. Plantlets were acclimated in a growth chamber under a constant light using a hydroponic culture system. They were lodged within the holes of a 2-mm-thick perforated expanded polystyrene plate placed in a plastic basin containing the constantly ventilated B+C nutrient solution of Ingestad (1971). The surviving individuals were planted in containers filled with a mixture of compost and Perlite in a proportion of 2:1.

Culture system

Since January or June, 1990, a standardized system has been used to record the evolution of the multiplication and maturation rates. For each clone, six pieces of embryogenic mass numbered 1–6 were subcultivated and regenerated independently in the same petri dish. Each week, one-half of each piece of embryogenic mass was subcultivated on 1M2, whereas the other half was transfered to 1M3 and took the pathway to the production of acclimated plants.

The measurement of the fresh weight of the in total six embryogenic masses placed respectively on medium 1M2 and 1M3, as well as the counting of the mature somatic embryos appearing on the part of the embryogenic masses that had been placed on a maturation medium, made it possible to determine a weekly multiplication rate (ratio of the eventual mass to the initial mass) as well as a maturation rate (expressed by the number of mature embryos produced by 1 g of embryogenic mass placed on 1M3).

Histological staining

For microscopic morphological examination of the immature embryos, fresh embryogenic mass samples were squashed in 2% acetocarmin. Precise morphological analyses were done in early 1994; after some 4 years of culture.

Chromosome squashes

Metaphase chromosome spreads were obtained from root tips and/or sprouting buds of acclimated somatic seedlings. The samples were collected when the mitotic index was higher: in the morning when there was no late frost for the root tips, and in the afternoon for the sprouting buds. The samples were pretreated in a saturated solution of 1-bromonaphthalen at 0°C. Adequate condensation of chromosomes required a 24-h pretreatment. The material was then successively fixed in a 80% ethanol and glacial acetic acid solution (3:1) at 4°C for 24 h, hydrolysed in a 5 *N* HCl solution at room temperature for 8 min, and rinsed with water. The samples were stained by the Feulgen method for 1 h. The meristematic tissues were picked up and placed onto glass slides using a needle with a flattened tip

and squashed with the coverglass in a drop of alun-hematoxylin. When the chromosome preparation was judged to be satisfactory, it was sealed with the VALAP (⅓ vaseline, ⅓ lanolin, ⅓ paraffin).

Metaphase chromosome spreads of embryogenic masses were obtained according to the procedure of Nkongolo and Klimaszewska (1994).

DNA isolation

DNA was extracted from flushing bud needles, embryogenic tissues and somatic embryos according to Doyle and Doyle (1990). Approximately 50 mg of tissue was ground using a 1.5-ml Eppendorf tube and a plastic pestle in 450 μl of 2×CTAB buffer. The extraction buffer consisted of 2% (w/v) CTAB (cetyl trimetylammonium bromide), 1.4 *M* NaCl, 20 m*M* EDTA (ethylenediaminetetraacetic acid), 100 m*M* TRIS pH 8, 1% Poly(1-vinyl-2-pyrollidone) (PM: 25000) and 0.2% (v/v) β-mercaptoethanol. The homogenate was incubated at 60°C for 30 min, extracted with 800 μl of chloroform:isoamylalcohol (25:1), and then centrifuged at 13000 *g* for 5 min. The chloroform:isoamylalcohol extraction was repeated twice more. DNA was precipitated from the aqueous phase by mixing with 350 μl isopropanol (–20°C). After centrifugation at 13000 *g* for 15 min at 4°C, the DNA pellet was rinsed with 70% ethanol, vacuum-dried, and resuspended in 40 μl distilled water. After fluorimetric measurement of the DNA concentration, the sample was diluted with water at 50 ng/l.

DNA amplification

Primers ten nucleotides long were used for polymerase chain reaction (PCR) amplification following a protocol previously described by Williams et al. (1990). Amplification reactions were performed in volumes of 25 μl containing 50, 100 or 200 ng of genomic DNA, 100 μ*M* of each dNTP, 0.6 or 1 unit of thermostable DNA polymerase (Dynazyme from *Thermus brockianus*, Finnzymes or *Taq* DNA polymerase from *Thermus aquaticus*, Boehringer), 2.5 μl of 10× reaction buffer supplied with the DNA polymerase and 0.4 μ*M* of primer (Operon Technologies). The mixture was covered with a drop of mineral oil. For the DNA amplification, a Perkin Elmer (DNA Thermal Cycler 480) or a Prem (LEP scientific) Thermal Cycler was programmed for 35 cycles, each cycle consisting of a denaturation step of 1 min at 94°C, followed by an annealing step of 1 min at 37°C and an extension step of 3 min at 72°C. The first cycle was preceded by 3 min at 94°C, 1 min at 37°C and 3 min at 72°C, and the last cycle was followed by 10 min at 72°C. After amplification was completed, the samples were loaded and electrophoresed on 1.2% agarose gels, followed by staining with ethidium bromide. In all cases lambda phage DNA digested with *Eco*RI and *Hin*dIII (Pharmacia) was used as the size marker.

We only compared RAPD profiles obtained with DNA samples of the same clone tested with the same primer, DNA polymerase, thermal cycler, and reaction mixture (same primer, DNA, DNA polymerase concentrations). Three experiments, using respectively 3, 10 and 29 primers, were done.

Results

Morphology of immature embryos

Under the microscope, the morphology of the immature embryos that constituted the embryogenic masses was well-organized (type A), poorly organized (type B) or intermediate. The A and B types corresponded to those described by Jalonen and von Arnold (1991). The intermediate type showed the A-type well-developped embryo head with the B-type poorly-developped suspensor.

The three types of immature embryos were observed in the four clones. The most frequent occurrence was that a piece of embryogenic mass was composed of more than one type of immature embryo, in variable proportions. The six pieces of embryogenic mass of the same clone in the same petri dish often had different immature embryo compositions.

Behaviour on maturation medium

Four main types of behaviour were recorded on maturation medium. The embryogenic mass either (1) died rapidly, (2) continued to proliferate without maturation, (3) produced nodules that did not further differentiate or (4) produced mature embryos. In fact, a multitude of other intermediate behaviours were also observed.

From time to time, new, strong and healthy embryogenic masses appeared within embryogenic masses that had declined for several months, without any subculture, on maturation medium (1M3); these new embryogenic masses (-called proliferation substrains) continued to proliferate on this maturation medium, sometimes producing isolated high-quality mature embryos. This proliferation substrain phenomenon was observed among embryogenic masses arising from the A, C and V clones, never from the B clone.

Some of the embryos produced were abnormally swollen, elongated or tiny instead of growing to become well-shaped. Morphologically the somatic embryos differed from zygotic ones by the fact that in the former the cotyledons were unfolded. The number of cotyledons per somatic embryo varied from 1 to 13 within one clone.

The comparison of plant distribution as a function of the number of cotyledons for two somatic seedling clones (B and V) and for zygotic seedlings that were a mixture of various genotypes revealed no important differences. The minimum and the maximum number of cotyledons was in each case 5 and 11, respectively, and the more frequent numbers of cotyledons were 7 and 8 (Fig. 1).

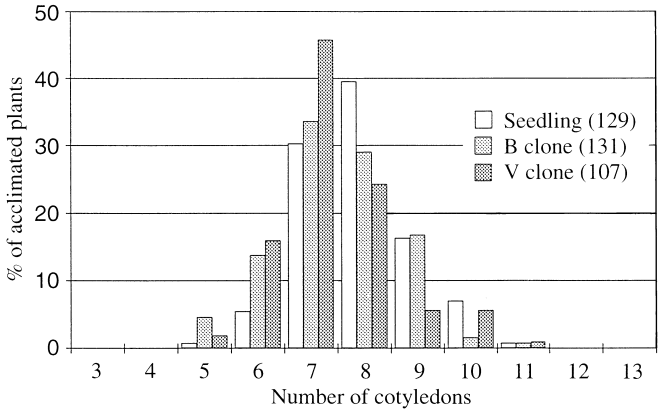

Fig. 1 Repartition of the 131 and 107 somatic seedlings from the B and V clones, respectively, acclimated from 9 January to 18 July, 1991, and of 129 zygotic seedlings, as a function of the number of cotyledons

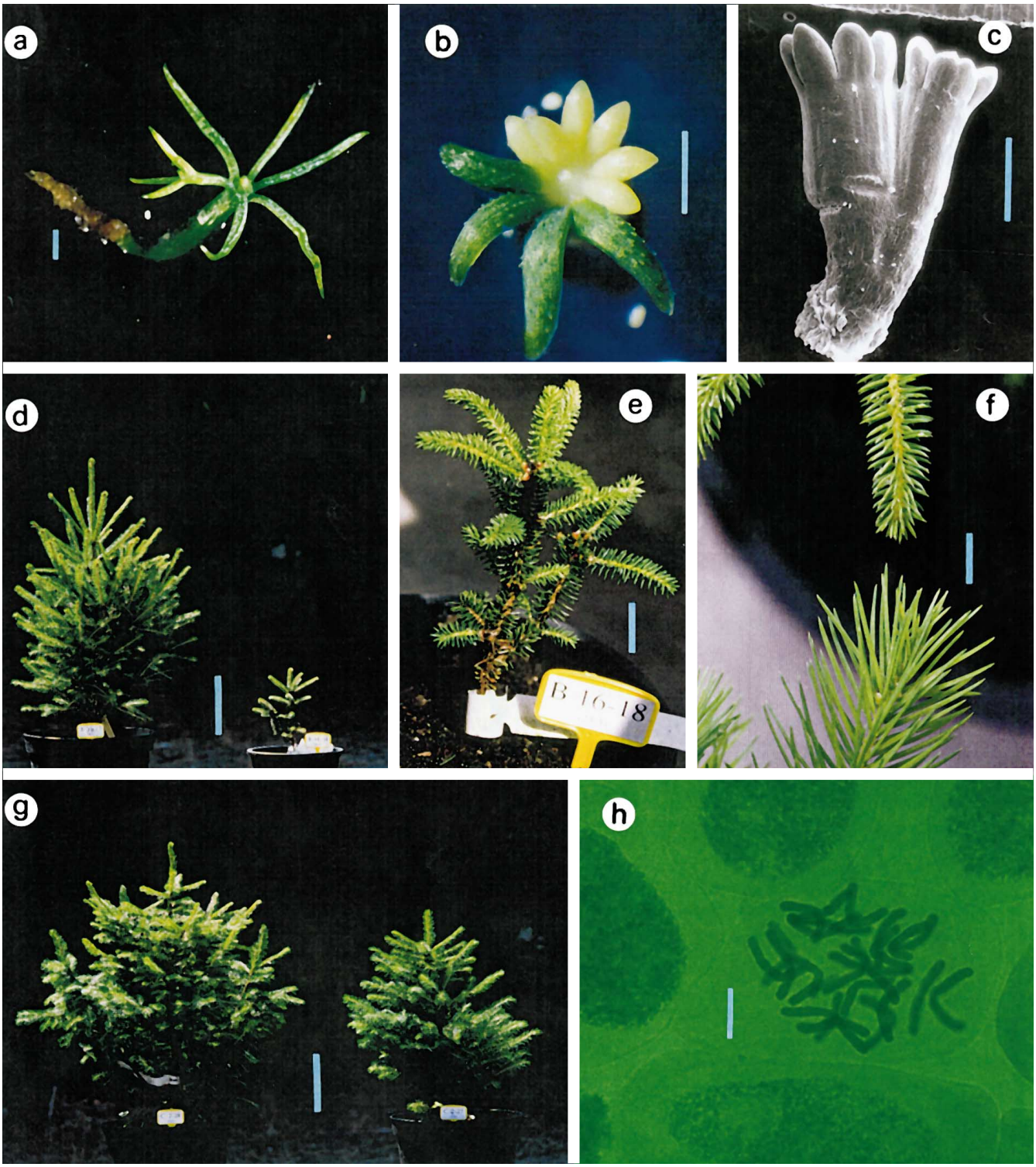

Fig. 2 g–h Examples of morphologic and genomic variations detected among regenerated Norway spruce somatic embryos or seedlings. **a** Young V clone somatic seedling with a cotyledon dividing into 5 parts (*bar*: 1 mm). **b** partly-albino C-clone somatic embryo, after 15 days of germination (*bar*: 1 mm), **c** Siamese embryos of the A clone observed by scanning microscopy (*bar*: 1 mm). **d** comparison between 4-year old diploid (B 10–17) and trisomic chimera (B 16–18) B-clone somatic plants (*bar*: 10 cm). **e** close-up view of the B clone trisomic chimera somatic seedling (B 16–18) showing a typical dwarf morphology (*bar*: 2 cm), **f** comparison of needle morphology between the diploid and the trisomic chimera B-clone somatic plants (*bar*: 1 cm). **g** comparison between 5-year-old diploid (C 2–28) and trisomic (C 2–27) C-clone somatic plants (*bar*: 10 cm), **h** metaphasic plate of the C-clone trisomic plant (C 2–27) (*bar*: 10 μm)

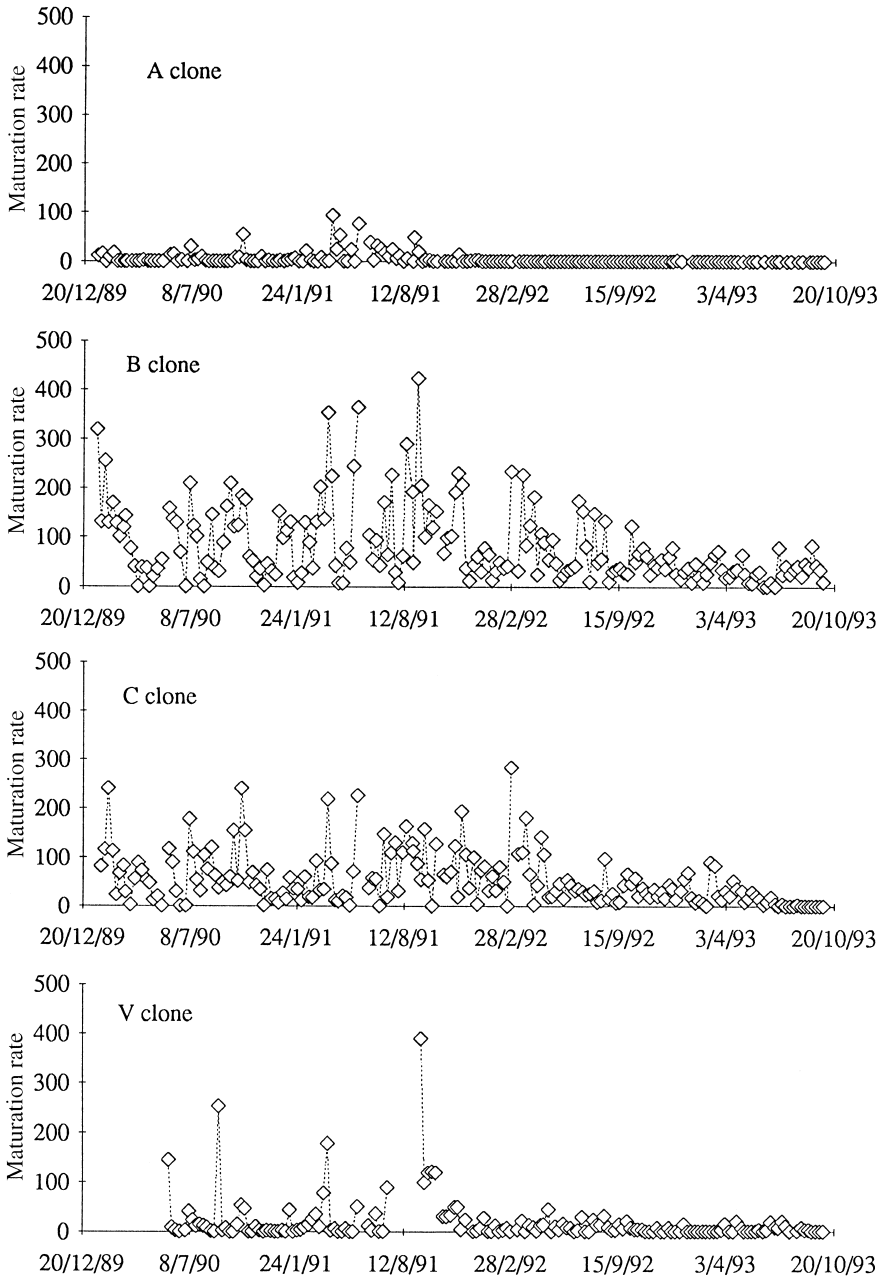

Fig. 3 Weekly evolution of the global maturation rate (expressed by the number of mature embryos produced by 1 g of embryogenic mass placed on the maturation medium 1M3) of the *A*, *B*, *C* and *V* clones multiplied on medium 1M2

As Lelu (1988) we regularly observed embryos with merging cotyledons and even noticed a plantlet with a cotyledon dividing into five parts (Fig. 2a). Our four clones occasionally produced Siamese embryos which were able to germinate and could be acclimated (Fig. 2c). It also occurred that certain embryos missed shoot and/or root meristems.

An embryogenic mass exclusively composed of B type immature embryos seemed to be unable to produce mature embryos, but the presence of even a small percentage of intermediate immature embryos could sometimes enable the production of mature embryos. In contrast, there was no absolute correlation between the presence of the A-type immature embryo and the capacity to mature.

Maturation rates

The weekly multiplication rate of the four clones always roughly reached two, which seems particularly high for an embryogenic mass subcultivated in a solidified medium. Maturation rates showed a very high variability from week to week as well as amongst the different pieces of embryogenic mass of the same clone.

Week after week, high variations in the global maturation rate of the six embryogenic masses of a single clone were noticed with a succession of high and almost nil production phases (Fig. 3). Clone A, which reached a very high production rate prior to the use of the standardized system (up to 1000 embryos per gram of embryogenic

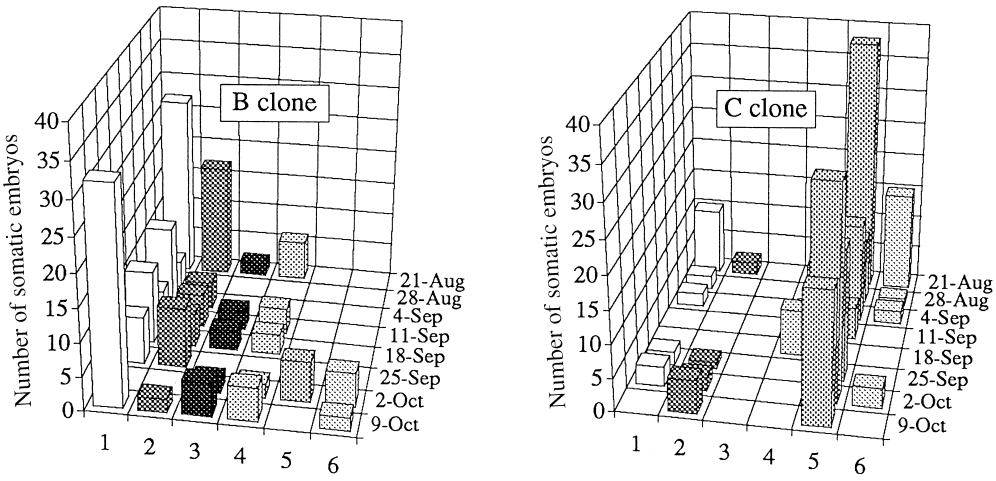

Fig. 4 Individual production (absolute number of mature embryos produced on maturation medium 1M3) of the six pieces of embryogenic mass (noted *1* to *6*) of the B and C clones multiplied on 1M2, scored between August 21 and October 9, 1992

mass), exhibited thereafter a very low rate (practically zero, since Sept. 1991). Clone B, which also reached 1000 embryos per gram of embryogenic mass shortly after its initiation, maintained a highly variable maturation rate later on. However, this rate never exceeded 100 following the end of 1992. Clone C, which was initially observed to produce up to 350 embryos per gram of embryogenic mass (prior to standardized system), showed a very similar behaviour to that of clone B. Clone V has also shown a highly variable maturation rate in the course of the subcultures, with a degradation since 1992.

Totally different maturation rates could be observed amongst the six pieces of embryogenic mass of a same clone within the same petri dish as revealed by the individual production (absolute number of mature embryos produced) of the six pieces of embryogenic mass of the B and C clones multiplied on 1M2 between August 21, 1992 and October 9, 1992 (Fig. 4).

Behaviour of the somatic seedlings

Somatic seedlings presented shorter hypocotyl and cotyledons than zygotic ones. There were some clonal characteristics with respect to the developmental pattern of the somatic seedlings, but some important intraclonal variation were also noted. The difference in quality between in vitro plantlets produced by one embryogenic mass or another was striking, as was the difference from one maturation experiment to the other. Within each clone and particularly for the C clone, it occurred that part of the regenerated plantlets vitrified or that the rootpart of the plantlets did not develop. About a year and a half after their initiation, the A and C clones occasionally produced partly or entirely albino somatic seedlings (Fig. 2b). The albino zones were well-marked during the first weeks of germination; they eventually turned green, but their growth rate was poorer.

The acclimated somatic seedlings were germinated between 3.8 and 28.5 months after initiation of the embryogenic masses. After the end of 1991; after 2½ years of subculture, a significant alteration in the embryo behaviour of the four clones was observed during germination that prevented a successful acclimation. The small number of acclimated somatic seedlings was caused by a reduced maturation rate in the A clone and by a low acclimation rate of the C clone. Amidst the thousands of acclimated somatic seedlings, 1 plant from the B clone (Fig. 2d, e, f) and another from the C clone, regenerated after 2 years of subcultures, showed a typical dwarf morphology comparable to that described by Illies (1953).

Chromosome counting

Chromosome counting of seven substrains of 5-year-old embryogenic masses (Table 1) revealed important genomic mutations such as monoploidy ($2n$-1=23), trisomy ($2n$+1=25), double trisomy ($2n$+2=26) and tetraploidy ($4n$=48). Some masses seemed to be completely mutated (one trisomic, one double trisomic and one tetraploid) while others were normal (diploid $2n$=24) or presented different ploidy levels: monosomy, trisomy and diploidy. The completely mutated embryogenic masses (Vno. 1,Vno. 2 and C1) and two genomically normal ones (A1 and A2) were unable to regenerate mature embryos. In contrast, the mixoploid substrains were able to produce either mature embryos (B) or nodules (C).

A sample of 240 acclimated plants (from a total of 1000) regenerated during hundreds of weekly subcultures (3.8–28.5 months after initiation) were analysed by chromosome counting: 218 for root-tip cells only, 13 for bud cells only and 9 for both types of cells (Table 2). One plant with a quite normal phenotype was totally trisomic (Fig. 2 g, h). Another phenotypically normal plant was a chimera, with trisomic buds and diploid roots. A third one, with a dwarf phenotype, was also a chimera with the same genomic anomaly (Fig. 2d, e, f). The 3 mutants were regenerated from three different clones 12.9, 13.2 and 23.3 months, respectively, after initiation.

Table 1 Chromosome counting of seven substrains of 5-year-old embryogenic masses. Distribution of the analysed metaphasic plates according to the chromosome number observed

Substrain	Behaviour on 1M3[a]	Chromosome number observed							Σ	Percentage of anomalies
		23	23+f	24	24+f[b]	25	26	±48		
B	mse	4	1	21	2	2	–	–	30	30
C	nod	3	–	24	–	3	–	–	30	20
V no. 1	nec	–	–	–	–	–	10	–	10	100
V no. 2	nec	–	–	–	–	8	–	–	8	100
A1	prol	–	–	3	–	–	–	–	3	0
A2	prol	–	–	9	–	–	–	–	9	0
C1	prol	–	–	–	–	–	–	5	5	100

[a] 1M3, Maturation medium; mse, production of mature somatic embryos; mod, production of nodules; nec, necrosis; prol, proliferation of the immature embryos
[b] f, presence of a chromosome fragment

Table 2 Chromosome counting of acclimated somatic seedlings. Distribution of the plants on the basis of the ploidy level [diploidy (d) or trisomy (t)] observed in the roots and/or in the buds. Globally, 634 plant metaphasic plates were analysed: 580 from the roots and 54 from the buds. The mean number of metaphasic plates per plant was 2.6.Thirteen metaphasis plates (3 from roots+10 from buds) were scored for the A-clone genomic chimera, 14 (8+6) for the B-clone genomic chimera and 31 (25+6) for the trisomic C-clone plant

Clone	Root[a] (d,?)	Root and bud			Bud (?,d)	Σ
		(d,d)	(d,t)	(t,t)		
A	19	–	1	–	–	20
B	112	–	1		7	120
C	8	–	–	1	–	9
V	79	6	–	–	6	91
Σ	218	6	2	1	13	240

[a] t, trisomy; d, diploidy; ?, not analysed

Genetic molecular markers

RAPDs were used to assess the genetic conformity of our in vitro production. A total of 2154 RAPD profiles were scored, 50 resulting from a combination of DNA extracted from the roots of 5 somatic plants and 10 primers (revealing 133 bands), 1691 resulting from a combination of DNA extracted from the buds of 443 somatic plants and 3, 29 or 32 primers or amplification conditions (revealing 77, 285 or 362 bands) and 413 resulting from a combination of 56 DNA samples (extracted from embryogenic masses, mature somatic embryos or somatic seedlings) and 3, 10 or 29 primers (revealing 77, 133 or 285 bands).

The analysed somatic plants were regenerated 3.8–27.1 months after initiation. The samples of embryogenic masses, mature embryos and somatic seedlings were collected 27–57 months after initiation. Some of the embryogenic masses, mature embryos or somatic seedlings were

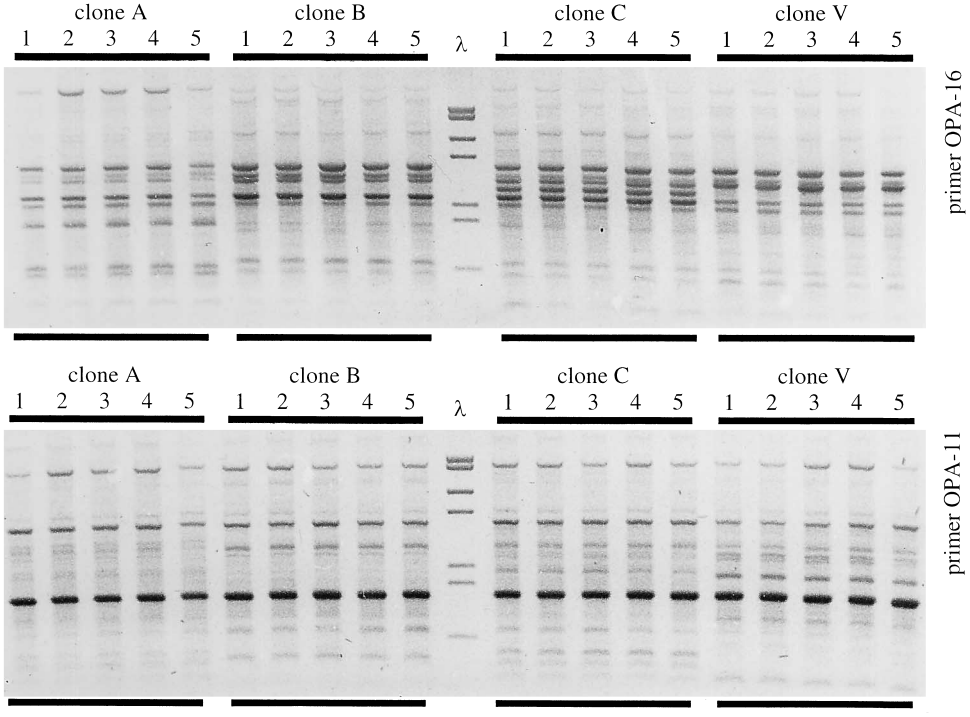

Fig. 5 Example of RAPD profiles. Gel electrophoresis of RAPD fragments obtained with primer OPA-11 or OPA-16 (Operon Technologies). Lanes λ are size marker λ digested with *Eco*RI and *Hin*dIII. For the four somaclones (*A*, *B*, *C* and *V*), *lanes 1–5* show RAPD products of 5 different individuals

abnormal (unable to maturate, swollen or hyperhydric). Buds of the genomic chimeras arising from the A and B clones were analysed respectively with 3 and 29 primers, respectively. Roots and buds of the trisomic plant regenerated from the C clone were analysed with 10 and 32 primers, respectively.

No intraclonal variation was observed despite the large sample of DNA and primers used, but for many primers we were able to differentiate the four clones (Fig. 5).

Discussion

Morphogenetic approach

Embryogenic mass

Multiplying, independently, pieces of embryogenic mass on a solidified medium over a long time period, but under identical conditions, has made it possible to stress development deviations between the various pieces of embryogenic mass of one single clone. A very high variability of mature embryo production from week to week, as well as its long term alteration were also observed. It probably would have been possible to slow down this reduction in yield by selecting embryogenic masses of the clone keeping the best regeneration capacities. Unfortunately, embryogenic masses with a high or a poor yield cannot be discriminated using morphological criteria during multiplication. At variance with the findings of various authors (Jalonen and von Arnold 1991; Bellarosa et al. 1992; Egertsdotter and von Arnold 1993; Egertsdotter et al. 1993), the morphology of the immature embryos of our study seemed to be insufficient to confidently predict the maturation capacities of an embryogenic mass.

The behaviour deviations of embryogenic masses of any one clone multiplied in the same petri dishes under the same conditions are a major impediment to large-scale production: they add complexity to the study of the influence of culture medium composition on the behaviour of embryogenic lines.

Reduction in the maturation rate of some embryogenic masses could be the consequence of either genetic deviations, or a progressive change in the embryo organisation, or a physiological evolution resulting in a diminished capacity in the synthesis of endogenous components required for embryo maturation. The occurrence of embryogenic mass proliferation substrains on masses declining on their maturation medium also illustrates behaviour variation, the origin of which remains unclear.

In the literature pertaining to the somatic embryogenesis of conifers, divergent interclonal behaviours are regularly described, especially in the maturation stage, but intraclonal deviations have only been reported by Ruaud (1993) and Dunstan et al. (1993). In contrast, Eastman et al. (1991) consider that interior spruce embryogenic lines have a stable behaviour on maturation medium even after reinitiation.

Somatic seedling

A number of morphologic variations were observed. The welding of cotelydons, the fasciation or the absence of shoot meristems between cotyledons are recurrent but limited phenomena that could be due to the influence of the phytohormones contained in the culture media and not necessarily imply genetic deviations. On the other hand, the occurrence of completely or partly albino embryos could have been the consequence of chloroplastic mutations induced in vitro. As far as we know, however, this is the first time that such deviations are reported for the in vitro culture of conifers. Albinism and other types of chlorophyllic deficiencies are often encountered amidst conifers propagated by seeds (Squillace and Kraus 1963) and have been frequently reported in other species such as cereals (Karp 1993).

The typical dwarf morphology of 1 of the acclimated somatic seedlings was correlated with a genomic mutation: trisomy. Conifer morphologic mutants of a similar type are regularly encountered in nature, or can be produced at the seedling or budding stage after treatment with colchicine. In some cases they were shown to be due to genomic mutations (Kiellander 1950; Illies 1958; Owens 1967; Johnson and Saylor 1973; Rehfeldt et al. 1983). Dwarfness could also be the consequence of a chromosomic or genic mutation that cannot be detected by chromosomic counting.

Cytogenetic approach

Embryogenic mass

Chromosome counting of the embryogenic masses revealed important and indisputable cases of somaclonal variation which could sometimes be related to behaviour deviations on the maturation medium. Some cases of trisomy, double trisomy and tetraploidy seem to be related to an incapacity to maturate, although this incapacity is not necessarily caused by a genomic mutation as found in substrains A1 and A2. Genomic mutations were caused by mitotic anomalies: chromosomes or chromatids which cannot be separated, which separated belatedly, or an irregular or incomplete spindle.

Somatic seedling

The only type of genomic mutation observed amongst our sample of acclimated somatic seedlings was trisomy. The other mutations (double trisomy, monoploidy and tetraploidy) observed in the immature embryos perhaps prevent the maturation of embryos or diminish drastically the vitality of regenerated mature embryos or seedlings.

The three somatic seedlings appeared singly and quite early during the subcultures of the A, B and C clones: between 12.9 to 23.3 months after initiaton. These are the first cases of trisomic Norway spruces recorded (Fourré 1992; 1995). Unexpectedly, we also observed some cases

of chimerism of ploidy, i.e. “diploid roots and trisomic buds”, which seemed to be more frequent than the complete trisomy. Once again it is the first time that such a deviation is reported. With a larger sample, other trisomic cases would possibly have appeared in the buds. Globally, at least 1.25% of the 240 analysed acclimated plants were genotypically abnormal. The B-clone dwarf plant was a ploidy chimera, and curiously, the 2 other trisomic plants seemed to be phenotypically normal. These results demonstrate that somatic embryogenesis can enable the regeneration of plantlets that do not conform to the initial genotype or phenotype; and moreover, it can imply the risk that these variations might display later during tree development or in its offspring.

With respect to the zygotic seedlings, the literature mentions very small percentages (0.002–0.008%) of genomic anomalies, mainly polyploidy, with a preponderance of tetraploidy, which were established on karyological analyses of the only phenotypical abnormal plants (Kiellander 1950; Winton 1964). Some rare cases of trisomy were detected amongst seedlings of *Pinus radiata* (Pederick 1967; Johnson and Saylor 1973) and *Pseudotsuga menziesii* (Owens 1967; Ching and Doerksen 1971) that were morphologically abnormal. These cases detected in nature were probably caused by meiotic anomalies, whereas our observations were caused by mitotic anomalies.

Molecular approach

Chromosome counting revealed important genomic mutations, but this time-consuming method is ineffective for detecting chromosomic or genic mutations that could appear during in vitro culture.

Genetic molecular markers made it possible to observe genetic variability in the DNA sequences. Amongst the different methods, RAPD has the advantages of requiring only a small amount of material, of being quick and highly polymorphic and of requiring no detailed knowledge of the genome.

This method made it possible to easily recognize the four different clones at each step of the production. It was an ideal tool to detect handling mistakes and to certify the origin of a somatic seedling. But, despite the high interclonal polymorphism observed and despite the large number of samples tested, some being cultivated in vitro for very different time periods, and sometimes showing morphogenetic or genomic modification, no RAPD intraclonal variation was detected.

In a similar way, several authors using RAPD or RFLP failed to observe intraclonal variations in various species including Norway spruce, *Picea mariana*, *Festuca pratensis*, *Saccharum*, *Pennisetum purpureum* or *Liliodendron tulipifera* (Heinze 1993; Isabel et al. 1993; Vallés et al. 1993; Chowdhury and Vasil 1993; Shenoy and Vasil 1992; Merkle et al. 1988) or were not able to distinguish sports of apple tree (Nybom 1990; Mulcahy et al. 1993; Harada et al. 1993). The same result was obtained using isozymes (Eastman et al. 1991).

In contrast, De Verno et al. (1994), using RFLP, observed some mtDNA modifications occurring in somatic embryogenesis of *Larix* species, Brown et al. (1993) found RAPD variations amongst plants of the same cultivar of *Triticum aestivum* or *Triticum tauschii* regenerated by somatic embryogenesis. Similarly, Rani et al. (1995) found RAPD variations amongst 23 micropropagated *Populus deltoides* plants originating from the same clone and morphologically similar. Bouman et al. (1992) and Bouman and Kuijpers (1994) also found intraclonal RAPD polymorphism amongst micropropagated *Begonia* but at a lower frequency than phenotypic variations and without any correlation with the phenotype. Yang and Schmidt (1994) differentiated an X-ray-induced cherry tree dwarf mutant with RAPDs

All these results demonstrate that RAPD or other molecular markers could miss important variations like genomic mutations or drastic morphologic mutations like dwarfism. The absence of intraclonal RAPD polymorphism certainly can not guarantee genetic stability. In the meantime, RAPD could detect some anodyne variations, i. e. mutations in parts of the genome that do not influence the phenotype.

Somaclonal variation is a very complex problem that needs several approaches to be correctly appreciated. Obviously, the only use of molecular markers like RAPDs, RFLPs or isozymes to assess the genetic stability of an in vitro production system is insufficient, and the morphological and cytogenetical approach appears to be a valuable complementary tool.

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