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Genotypic and media factors affecting stabilization of polyembryogenic cultures in norway spruce (*Picea abies* (L.) *Karst.*)

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ABSTRACT

Embryonal-suspensor mass lines (ESM lines) from mature embryos of three open-pollinated families of Norway spruce [*Picea abies* (L.) Karst.] were stabilized on several culture media varying in nutrient composition. A pattern of relationship between parameters of polyembryogenic culture performance and medium composition was genetically determined. Stabilized ESM lines could be classified into two phenovariants: (*i*) lines of homogenous ESM composed entirely of somatic proembryos, and (*ii*) lines of heterogeneous ESM composed of a mixture of non-embryogenic callus and aggregates of somatic proembryos. The formation of one or the other phenovariant was independent of genotype, but strongly determined by the medium composition.

Abbreviations: ABA: abscisic acid, BA: 6-benzyladenine, ESM: embryonal-suspensor mass, N: nitrogen, NAA: α-naphthyl-1-acetic acid.

INTRODUCTION

The developmental pathway of conifer somatic embryos is generally based on cleavage processes, even though the species does not normally reproduce by cleavage polyembryony (Durzan 1988). That is why conifer somatic embryos develop not from the primary undifferentiated callus, but in the highly organized morphogenetic system called the embryonal-suspensor mass (ESM).

A question of how long a time the ESM can be maintained *in vitro* in the stable differentiated state is of particular importance for research on aging as well as for development of biotechnological cloning systems. A secondary question, which needs to be answered, concerns whether the ESMs from the same species have an invariably uniform morphology and are composed entirely of cleaving proembryos.

In practice, these questions are concerned with the recovery of stabilized ESM lines. However, the factors regulating stabilization of conifer ESM lines are poorly examined (e.g., Klimaszewska 1989; Tautorus et al. 1990; Webb et al. 1989).

The present report demonstrates how genetic and non-genetic factors affect stabilization of the ESM lines in Norway spruce.

MATERIALS AND METHODS

Plant Material. Seed families (500, 501, and 502) represent progeny of three open-pollinated trees of Norway spruce [*Picea abies* (L.) Karst.] growing on the same plot near St.Petersbourg. Because the trees differ greatly in height, diameter of bole, shape of crown, type of branching, and structure of bark, it is unlikely that they are related genetically (Dolgolikov, personal communication). Cones were collected in October 1989, and stored at 3-5°C for 2 years. Mature embryos were removed from surface-sterilized seeds (25 min in a 30% (w/v) hydrogen peroxide) and placed onto culture medium.

Culture Media and Conditions. Seven basal media varying in inorganic components (Table 1) were compared for the induction of ESM and the stabilization of ESM lines. The first five media had half-strength LP-inorganics (von Arnold and Eriksson 1981), modified to include the different amounts of total N under the constant NH_4^+/NO_3^- molar ratio of 0.22 (Bozhkov et al. 1993b). The sixth and seventh media contained inorganics of L medium (Litvay et al. 1981) at half strength and BMG-1 (Krogstrup 1986), respectively (Table 1). All the media were supplemented with half-strength LP-vitamins, 3 mM L-glutamine, 0.5 mM myo-inositol, 2% (w/v) sucrose, 10 μM NAA and 5 μM BA.

Ten embryos were cultured in each 100-mL Erlenmeyer flask. ESMs were incubated in the dark at 23°C and subcultured onto fresh medium every 3 weeks. After 7 subcultures, ESMs were transferred to the same media, except for a lower concentrations of NAA (5 μM) and BA (2 μM).

Thirty embryos from each seed family were cultured on each of the media. Experiments were repeated at least twice.

Somatic embryo maturation was achieved

Table 1. Nutrient salt composition (mM) of modified LP (1-5), L (6), and BMG-1 (7) media.

Nutrient salt	Medium 1	2	3	4	5	6	7
KNO_3	0.78	14.2	21.3	26.3	42.5	9.40	23.1
NH_4NO_3	0.22	4.00	6.00	7.50	12.0	10.3	3.40
KCl	5.00	-	-	-	-	-	-
KH_2PO_4	1.25	1.25	1.25	1.25	1.25	1.25	0.63
$MgSO_4 \cdot 7H_2O$	0.75	0.75	0.75	0.75	0.75	3.75	0.75
$CaCl_2 \cdot 2H_2O$	0.60	0.60	0.60	0.60	0.60	0.07	1.50
Microsalts and FeEDTA	1/2LP	1/2LP	1/2LP	1/2LP	1/2LP	1/2L	1/2MS
Total N	1.22	22.2	33.3	41.3	66.5	30.0	29.9
NH_4/NO_3	0.22	0.22	0.22	0.22	0.22	0.52	0.13

using a protocol developed by Bozhkov et al. (1992, 1993a). Briefly, pieces of ESM, approximately 400 mg fresh weight (two to three per 100-mL Erlenmeyer flask), were placed on half-strength LP medium supplemented with 2% (w/v) sucrose, 15.2 µM ABA, and 5.0 µM BA. After 3 weeks, ESMs were subcultured several times onto the same medium without BA, until no further production of mature somatic embryos was visible. Somatic embryos developed under 16h light (30 µmol/m^2/s, cool-white fluorescent lamps) at 23°C.

All media tested were solidified with 0.7% (w/v) Serva purified agar. The pH was adjusted to 5.8 prior to autoclaving. ABA and L-glutamine were filter sterilized through a 0.22-µm Millipore filter.

Monitoring of Cultures. The formation of ESMs was recorded every month during the initial 5-month period of culture. Morphological features of the ESM were translucent appearance, a high degree of organization, and the presence of specific mucilaginous matrix (Durzan 1989). The establishment of ESM lines was recorded after 5 months of culture where at least two ESM pieces increased in size 2-4 times every 3 weeks.

Three parameters were used for the quantitative evaluation of somatic polyembryogenesis capacity (Bozhkov et al. 1993b): (*i*) the total embryogenic activity, expressed as percentage of zygotic embryos revealing ESM formation during 5 months of culture, (*ii*) the frequency of ESM line establishment, expressed as percentage of zygotic embryos produced ESM lines within 5 months of culture, and (*iii*) the transformation of ESMs into ESM lines, calculated as: (number of zygotic embryos producing ESM lines within 5 months of culture / number of zygotic embryos revealing ESM formation during 5 months of culture) x 100.

RESULTS

A pattern of relationship between the total embryogenic activity and concentration of inorganic N in the modified LP (Table 1) was genetically determined (Fig.1A). Decrease of percentage of explants inducing ESM with increase of inorganic N content was characteristic for seed families 501 and 502. Family 500 responded differently, with a maximum total embryogenic activity at 22.2 mM N. Medium 5 (66.5 mM N) suppressed induction of somatic polyembryogenesis for all seed families tested (Fig.1A).

Considering all families, modified L (medium 6) was much more effective than other ones at induction phase, with 25% total embryogenic activity (averaged across three families).

There was no clear correlation between the frequency of ESM line establishment and inorganic N content (Fig.1A,B). Moreover, the frequency of ESM line establishment was not directly proportional to the total embryogenic activity. For example, medium 2 was appropriate for the induction of the ESM

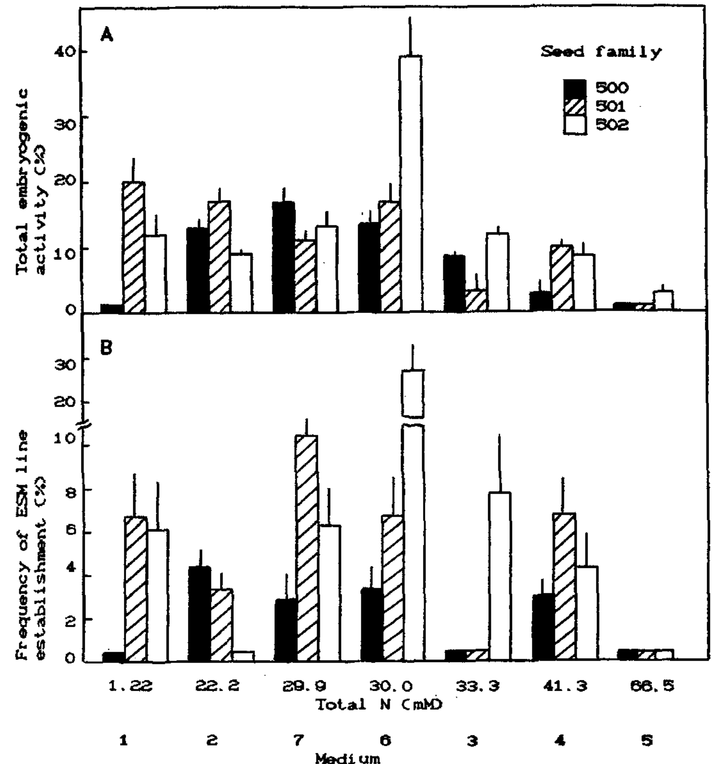


Fig.1. Influence of inorganic nitrogen concentration on induction of somatic polyembryogenesis (A) and establishment of ESM lines (B) in three open-pollinated families of Norway spruce. Bars indicate SE.

in family 502 if not supporting the ESM line stabilization (Fig.1A,B).

Using the parameter of transformation of ESMs into ESM lines allows consideration of the stabilization phase irrespective of the induction phase, and the assessment of the maintenance capacity of the culture medium. The best maintenance medium was modified LP with 41.3 mM inorganic N (medium 4). It promoted transformation of the early ESMs into the ESM lines with a 54% frequency (averaged across three families). Modified L and BMG-1 (media 6 and 7) also gave good results, with 40 and 42% transformation frequencies, respectively (data not shown).

Two-way analysis of variance showed that seed family effect contributed only 5 and 2% to the variation of the total embryogenic activity and the transformation of ESMs into ESM lines, respectively. The larger proportions (60-70%) of the observed variations were attributable to the culture medium effect and the interactive family × medium effect (Table 2).

Stabilized ESM lines could be classified into two phenovariants: (*i*) lines of homogenous ESM (designated as *hom*) composed entirely of somatic proembryos (Fig.2), and (*ii*) lines of heterogeneous ESM (designated as *het*) composed of a mixture of globular, opaque callus and aggregates of somatic proembryos (Fig.3). The formation of one or other phenovariant was independent of genotype, but strongly determined by the culture medium composition. The *hom* ESM lines generally developed on the modified LP (media 1-4). On the contrary, L-macroelements (medium 6) were required for the production of the *het* ESM lines. Modified BMG-1 (medium 7) was equally effective for stabilization of both of the phenovariants.

Two distinguishing features should be stressed about the growth patterns of *hom* and *het* ESM lines. First, the proliferation rate of the *het* ESM lines was two to four times that of the *hom* ESM lines ($P<0.001$). Secondly, the fast growing *het* ESM lines were highly susceptible to necrotic processes resulting in a tan-coloured pigmentation of the cultures (Fig.3).

The morphological characteristics of the two phenovariants of Norway spruce ESM lines were rather stable, and they have now been maintained continuously for more than two years without observable change. These are not, however, completely stable developmental states, since the *het* and the *hom* ESM lines could be fully transformed one into the other by subculturing onto appropriate media.

Under maturation conditions well-formed somatic embryos were recovered from the *hom* ESM lines (Fig.4). However, this was impossible with the *het* ESM lines which produced somatic embryos with abnormal morphology only (Fig.5). The abnormal somatic embryos had an adventitious bud-like appearance with suppressed hypocotyl development and an assymetrical arrangement of cotyledons.

Table 2. The effect of seed family (F) and culture medium (M) on the total embryogenic activity and the transformation of ESMs into ESM lines: analysis of variance.

Source	df	%SS[a]	Variance ratio	P
Total embryogenic activity				
F	2	5	4.10	0.023
M	6	32	9.88	<0.001
F×M	12	30	3.72	<0.001
Error	42	33		
Transformation of ESMs into ESM lines				
F	2	2	2.74	0.070
M	6	32	10.66	<0.001
F×M	12	36	4.71	<0.001
Error	42	30		

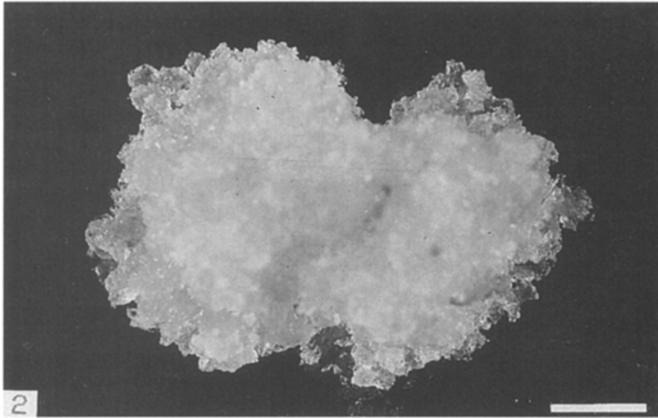
[a]Percent sums of squares

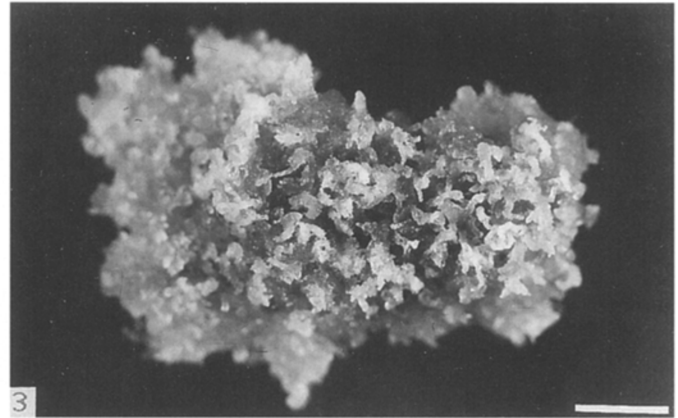
DISCUSSION

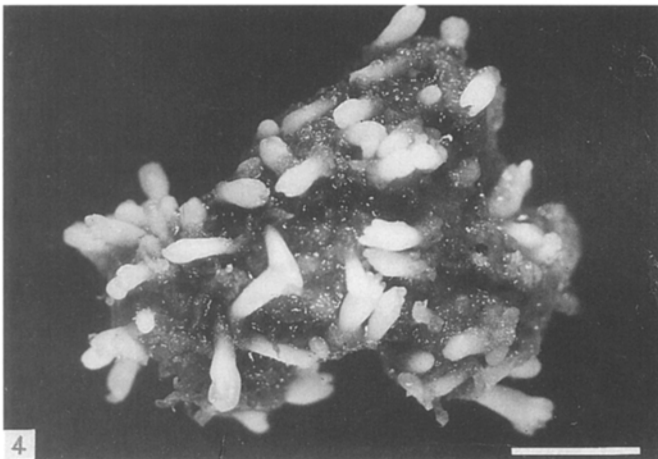
In most studies on conifer somatic polyembryogenesis, it is not assumed that the ESM induction and stabilization processes may be distinct in environmental requirements. The data obtained have shown that one and the same culture medium may be permissive for the ESM induction and suppressive for the stabilization of ESM lines (or vice versa) in mature zygotic embryos of Norway spruce. This reflects the major developmental difference between these two processes. The ESM induction process is associated with a redetermination of cells in the primary explants, whereas the stabilization of ESM lines involves proliferation of somatic proembryos by cleavage mechanisms (Durzan 1988). The genetic source of the seed explant was also a significant factor affecting polyembryogenic culture performance as well as the pattern of relationship between the quantitative parameters of polyembryogenesis and the composition of culture medium. Although the seed family effect contributed only 5 and 2% to the total variation of ESM induction and stabilization parameters, respectively, the interactive family × medium effect was much stronger, determining 30 and 36% of the total variation of respective parameters (Table 2).

A low contribution made by the seed family effect to the total variation of somatic polyembryogenesis parameters is attributable to the fact that the donor trees, chosen in the present study, grew on the same plot with the same pollen cloud. Otherwise, the genotype could be a more significant factor than the medium because of the presence of a pollinator effect. In conifers, chloroplast DNA, which has been shown to control *in vitro* embryogenesis (Sagi and Barnabas 1989), is inherited from the paternal parent (Dong et al. 1992).

The fact that the ESM lines, stabilized on the modified L medium, have a heterogeneous cellular composition and possess pronounced capacities for both proliferation and senescence, appears interesting for two reasons. First, the L-macroelements, which are routinely used for conifer somatic polyembryogenesis (Roberts et al. 1993), were not previously tested in Norway spruce. This study has shown the aberrant development of Norway spruce somatic embryos in the *het* ESM lines stabilized on the modified L medium. Second, the concurrent pattern of







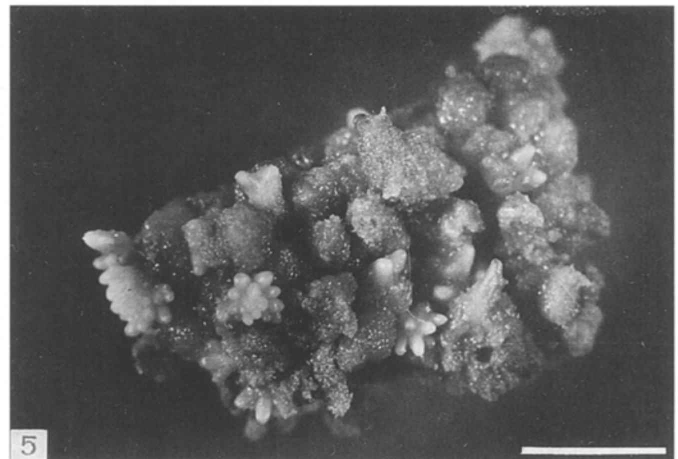


Fig.2. Phenovariant *hom* ESM stabilized on modified LP (medium 4). Bar = 5 mm.

Fig.3. Phenovariant *het* ESM stabilized on modified L (medium 6). Bar = 5 mm.

Fig.4. True-to-type development of somatic embryos in *hom* ESM line. Bar = 5 mm.

Fig.5. Aberrant development of somatic embryos in *het* ESM line. Bar = 5 mm.

cell proliferation and senescence peculiar to the *het* ESM lines contradicts the proposition that a clearly defined antagonism exists between these two processes in eucaryotic cell populations (Gelfant and Smith 1972). It is not inconceivable that the mucilage surrounding somatic proembryos of the *het* ESM contains cell division factors that promote cleavage of somatic proembryos as well as proliferation of nonembryogenic callus (Braun and Meins 1970; Durzan 1989). Non-embryogenic callus of conifers has also been shown to accumulate large amount of phenolic compounds (Wann et al. 1987). This may be responsible for the development of necrotic pigmentation of the *het* ESM lines found in the present investigation.

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