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Enhancement of somatic embryogenesis in Norway spruce (*Picea abies* L.)

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Summary. Embryogenic callus developed in 55% of the mature embryo explants of Norway spruce (*Picea abies* L.) growing on a LP medium minus the amino acids and sugars (except sucrose). This is the highest reported yield of embryogenic callus from mature embryos of *P. abies* that has ever been reported. Callus induction from either the middle or the end of the hypocotyl of the embryos began after 2–3 weeks. Three types of calli were recovered: (a) globular, (b) light green-compact, (c) white mucilaginous. Only the white mucilaginous calli were embryogenic. The globular and light green-compact calli never become embryogenic, even after several subcultures. The development of somatic embryos was accomplished on half-strength macro-elements of NSIII medium containing 1 μM α-naphthaleneacetic acid, 1 μM abscisic acid, and 3% sucrose. The addition of $10^{-7} M$ buthionine sulfoximine to the medium increased the development of somatic embryos by three fold. These results suggest that there is a great potential for increasing the frequency and development of somatic embryos in *P. abies*. Careful selection of the genotype and modification of the culture medium is required.

Key words: Norway spruce – *Picea abies* – Modified LP medium – Somatic embryogenesis – DL-Buthionine sulfoximine

Introduction

The regeneration and clonal propagation of conifer trees by tissue culture has been extensively reported (Jain et al. 1988 a; von Arnold and Tillberg 1987; Abdullah et al. 1985; Kim et al. 1985; Ahuja 1983, 1984; Biondi and Thorpe 1981; Bonga 1981; Palta-Mehra et al. 1978). The prospects for their use in tree improvement has become more feasible due to recent reported successes of plant regeneration via somatic embryogenesis in *Picea abies* (Hakman and von Arnold 1985; von Arnold 1987; von Arnold and Hakman 1986; Hakman et al. 1985), *P. taeda* (Gupta and Durzan 1987), *P. glauca* (Lu and Thorpe 1987), and *Larix decidua* (Nagmani and Bonga 1985). However, plant regeneration from somatic embryos of forest trees is still a complex task.

Cultured immature embryos have given rise to embryogenic callus in gymnosperms (Becwar et al. 1987 a; Hakman and von Arnold 1985; Lu and Thorpe 1987; Gupta and Durzan 1987; Norstog and Rhamstine 1967) and in angiosperms (Rangaswamy 1986; Vasil 1987). Furthermore, somatic embryogenesis has also been initiated from mature zygotic embryos in *P. abies* (von Arnold 1987) and *P. lambertiana* (Gupta and Durzan 1986). However, in conifers, the efficiency of somatic embryoggenesis from mature embryos has not been as high as that derived from immature embryos. Recently, von Arnold (1987) reported the induction of embryogenic callus in 50% of the *P. abies* mature embryo explants: 15% of these calli produced plantlets. Recent research indicated that the develoment of embryogenic callus may be enhanced by varying culture methods and media (Rangaswamy 1986; Lazzeri et al. 1987 a, b).

The objective of this study was to enhance the frequency of somatic embryogenesis from cultured mature embryos of Norway spruce (*P. abies* L.).

Materials and methods

Plant material

Seeds of Norway spruce (*Picea abies* L.) were stored in plastic bags at 4 °C for 1 year. They (60–70) were then soaked in a small

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volume of sterile distilled water for 24 h in darkness at 4 °C, surface sterilized in 10% clorox for 15 min, and washed 3 times in sterile distilled water. Mature embryos were aseptically dissected from the seeds under a binocular microscope and placed horizontally on a solidified culture medium in 100 × 20 mm Petri dishes.

Media and culture conditions

Amino acids and sugars (except sucrose) were omitted from the LP medium (von Arnold and Eriksson 1981) to give the modified medium NSIII (Table 1). For embryogenic callus initiation from mature embryo explants 10 μ*M* 2,4-D (2,4-dichlorophenoxyacetic acid), 5 μ*M* BAP (N^6-benzyladenine), and 1% sucrose were supplemented to the NSIII medium. Different strengths of NSIII basal medium were tested for their capacity to enhance the frequency of embryogenic callus formation. The effect of auxins on embryogenic callus formation was tested by adding 10 μ*M* NAA or IAA (indoleacetic acid), or 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) to medium containing 5 μ*M* BAP and 1% sucrose. Somatic embryos were devloped on half-strength macro-elements of NSIII medium containing 1 μ*M* NAA (α-naphthaleneacetic acid), 1 μ*M* ABA (abscisic acid), and 3% sucrose. Several concentrations (10^{-7}–10^{-4} M of DL-buthionine sulfoximine (BSO) were added to the culture medium used for the development and maturation of the somatic embryos. The pH of the medium was adjusted to 6.2 with 1 N KOH or HCl and solidified with 0.4% gelrite.

Non-embryogenic callus was cultured either on a sterile filter paper moistened with NSIII liquid medium or directly in 10 ml NSIII liquid medium in a 100 × 20 mm Petri dish. Fresh liquid medium was added after 2 weeks. The filter paper was moistened with 1–2 ml liquid medium once a week.

Fifteen mature embryos were cultured in 100 × 20 mm Petri dishes, and at least 50–60 embryos per treatment were used. The Petri dishes were sealed with parafilm and incubated at 25 °C in the dark. The tissues were subcultured once a month.

Histological staining

Callus was stained with 2% acetocarmine according to the procedure of Gupta and Durzan (1987). Cells were examined under the light microscope and photographed.

Results and Discussion

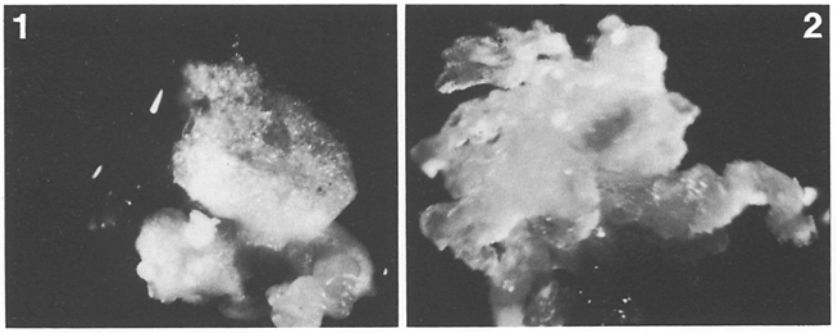
After 2 weeks, callus formation was initiated from either the middle or the end of the hypocotyl of the mature embryo explants growing on NSIII medium. In four separate experiments, three types of calli were recovered: (a) globular, (b) light green-compact, (c) white mucilaginous. Globular and light green-compact calli (Figs. 1 and 2) developed from 40% and 46% of the mature embryo explants, respectively (Table 2); white mucilaginous callus was initiated from 32% of the embryos (Table 2). Staining with acetocarmine showed that the globular and light green-compact calli were non-embryogenic. White-mucilaginous calli stained red with acetocarmine and embryo heads and suspenor cells were clearly visible (Fig. 7). The formation of three types of calli under identical culture conditions indicate the presence of different cell populations which respond differentially to exoge-

Table 1. Composition of modified LP medium (NSIII)

Constituent	LP concentration (mg/l)	NSIII concentration (mg/l)
Inorganic macroelements		
KH_2PO_4	340	340
KNO_3	1900	1900
NH_4NO_3	1200	1200
$MgSO_4 \cdot 7H_2O$	370	370
$CaCl_2 \cdot H_2O$	180	180
Inorganic microelements		
$MnSO_4 \cdot H_2O$	1.69	1.69
H_3BO_3	0.63	0.63
Zn-EDTA	4.05	–
$FeSO_4 \cdot 7H_2O$	14.0	14.0
EDTA	19.0	19.0
KI	0.75	0.75
$Na_2MoO_4 \cdot 2H_2O$	$0.25 \cdot 10^{-1}$	$0.25 \cdot 10^{-1}$
$CuSO_4 \cdot 5H_2O$	$0.25 \cdot 10^{-2}$	$0.25 \cdot 10^{-2}$
$CoCl_2 \cdot 6H_2O$	$0.25 \cdot 10^{-2}$	$0.25 \cdot 10^{-2}$
Amino Acids		
L-Glutamine	0.40	–
L-Alanine	0.05	–
L-Cysteine HCl	0.02	–
L-Arginine	0.01	–
L-Leucine	0.01	–
L-Phenylalanine	0.01	–
L-Tyrosine	0.01	–
Glycine	2.0	–
Sugars		
D-Sucrose	34200	10000
D-Glucose	180	–
D-Xylose	150	–
L-Arabinose	150	–
Vitamins		
Nicotinic acid	2.0	2.0
Pyridoxine HCl	1.0	1.0
Thiamine HCl	5.0	5.0
Meso-inositol	100	100

Table 2. Three types of callus that developed in cultured mature Norway spruce embryos on NSIII medium containing 10 μ*M* 2,4-*D*, 5 μ*M* BAP, and 1% sucrose after 8 weeks in the dark. Numbers in parenthesis are percentages

Expt. no.	No. of mature embryos cultured	Type of callus		
		Globular (a)	Light green-compact (b)	White mucilage (c)
1	50	18 (36)	23 (46)	9 (18)
2	50	18 (36)	18 (36)	14 (28)
3	50	20 (40)	14 (28)	16 (32)
4	50	16 (32)	20 (40)	14 (28)



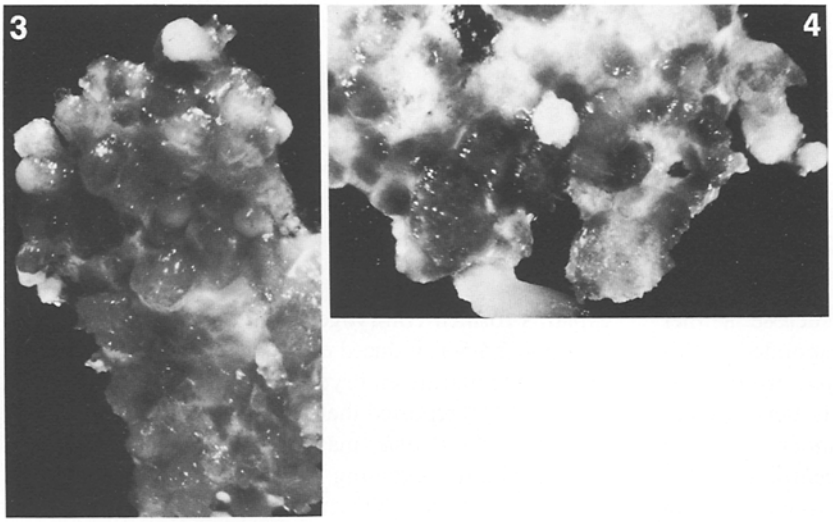


Fig. 1. Globular nonembryogenic callus from mature zygotic embryos after 45 days

Fig. 2. White mucilaginous embryogenic callus developing from mature embryos after 45 days

Fig. 3. Development of white embryogenic nodules in embryogenic callus

Fig. 4. Embryonic nodular structures developing into somatic embryos

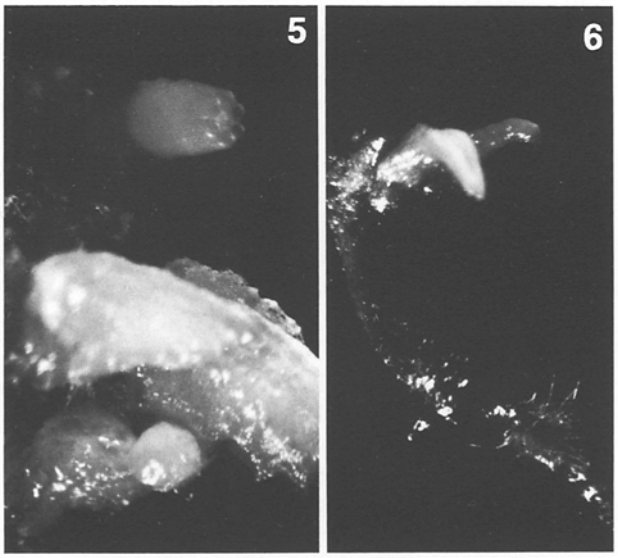


Fig. 5. A well-developed somatic embryo with hypocotyl and cotyledons

Fig. 6. Root development in a somatic embryo

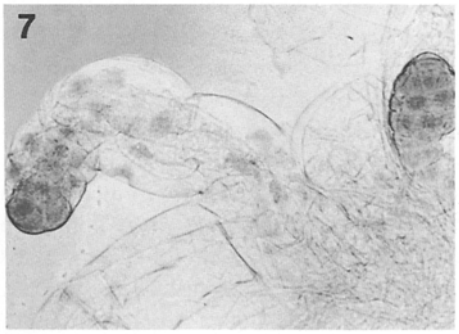


Fig. 7. Histochemical staining with acetocarmine indicates suspensor cells and embryo heads

nous plant growth regulators (Bhaskaran and Smith 1988). Embryogenic cells were long and vacuolated, while non-embryogenic cells were round and small. These calli were subcultured either on NSIII liquid medium or on a sterile filter paper moistened with liquid medium. After 6 subcultures, well-developed calli were transferred to NSIII solidified medium supplemented

Table 3. Frequency of embryogenic callus formation in mature embryo explants on different strengths of NSIII basal medium supplemented with 10 μ*M* 2,4-D and 5 μ*M* BAP after 8 weeks in the dark

Strength of medium	Expt. no.	No. of mature embryos cultured	No. of mature embryos forming embryogenic callus	(%)
1/2 basal	1	60	29	48
	2	60	28	47
	3	60	24	40
	4	60	28	47
1/2 macro-elements	1	60	21	35
	2	60	24	40
	3	60	22	37
	4	60	25	42
Full	1	60	17	28
	2	60	21	35
	3	60	19	32
	4	60	23	38

Table 4. Frequency of embryogenic callus formation in cultured mature embryos on half-strength of NSIII basal medium containing 10 μ*M* auxins, 5 μ*M* BAP, and 1% sucrose after 8 weeks in the dark

Auxin	Expt. no.	No. of mature embryos cultured	No. of mature embryos forming embryogenic callus	(%)
NAA	1	60	28	47
	2	60	31	52
	3	60	27	45
	4	60	23	55
IAA	1	60	20	33
	2	60	22	37
	3	60	17	28
	4	60	21	35
2,4,5-T	1	60	15	25
	2	60	21	35
	3	60	18	30
	4	60	23	38

with 10 μ*M* 2,4-D, 5 μ*M* BAP, and 3% sucrose: neither globular nor light green-compact calli responded to this osmotic shock treatment. In *Brassica napus* protoplasts, the induction of embryogenic callus and the development of somatic embryos is due to osmotic shock (Jain and Newton 1988). An optimal sucrose concentration (1%) was effective in the induction of embryogenic callus in *P. abies* mature embryo explants (von Arnold and Hakman 1986), but on a half-strength basal medium, 3% sucrose was beneficial in inducing somatic embryogenesis (von Arnold 1987).

The frequency of embryogenic callus formation was affected by the strength of the basal medium. Half-strength basal medium was the most effective in promoting embryogenic callus formation, while 40% and 48% of the explants formed calli on half-strength macro-elements and basal medium, respectively (Table 3). Gupta and Durzan (1987) induced formation of white mucilaginous callus in 9%–10% of *P. taeda* immature embryo explants on a half-strength modified MS medium. In addition, the yield of embryogenic callus from *P. abies* mature embryos was higher on half-strength than on full strength LP basal medium (von Arnold 1987). Similar results have been observed in mango *Mangifera indica* (Litz 1984 a), *Eugenia jambos,* and *E. malaccensis* (Litz 1984 b) on half-strength modified MS medium.

The effects of the different auxins (NAA, IAA, and 2,4,5-T) indicated that NAA was superior in inducing somatic embryogenesis. The highest frequency of embryogenic callus formation occurred on half-strength NSIII medium containing 10 μ*M* NAA, 5 μ*M* BAP, and 18% sucrose (Table 4) where 55% of the mature embryo explants formed embryogenic callus. The other auxins, IAA and 2,4,5-T, induced embryogenic callus in 37% and 38% of the mature embryos, respectively (Table 4). Von Arnold (1987) reported the formation of embryogenic callus from 50% *P. abies* mature embryos on half-strength basal medium containing 20 μ*M* 2,4-D, 5 μ*M* BA, 1% sucrose, 15 μ*M* NH_4NO_3, and 0.4% gelrite. In *P. glauca,* 67% of the embryo explants formed embryogenic callus on a picloram-containing medium (Lu and Thorpe 1987). An intense somatic embryogenesis was reported in *Santalum album* in MS medium containing 2.5 mg/l 2,4-D and 1 mg/l gibberellic acid (Lakshmi Sita et al. 1980). The addition of several different kinds of cytokinins (1–10 μ*M*) to the culture medium had no pronounced effect on embryogenic callus induction in mature embryo explants of *P. abies* (Hakman and von Arnold 1985). Therefore, 5 μ*M* BAP was used according to the procedure of Hakman and von Arnold (1985). However, the stimulation of a higher yield of embryogenic callus in mature embryo explants of *P. abies* was due to both NAA and 2,4-D (von Arnold 1987).

Bhaskaran and Smith (1988) reported that shoot tip cultures from 2–3 day-old seedlings of *Sorghum bicolor* (L.) on MS medium supplemented with 2.5 mg/l 2,4-D and 0.05 mg/l Kinetin developed highly embryogenic callus. However, our results indicate only a slight increase in embryogenic callus formation over earlier reported results (von Arnold 1987). To determine the effect of seed batch on embryogenesis, we have tried a fresh lot of *P. abies* seeds of a different genotype, harvested during 1987, for the induction of embryogenic callus in mature embryos on NSIII and LP media. After 45 days, in two experiments, all the developing callus was non-embryo-

genic. It appears that genotype, controlled pollination, and pre-conditioning of the explants are of tremendous importance for the initiation and enhancement of embryogenesis. The evaluation of different genotypes as well as the effect of the growth conditions is important for the induction of embryogenic callus (Hakman and Fowke 1987). Amino acid and sugar supplements, with the exception of sucrose, were not essential for inducing embryogenic callus in mature embryos of *P. abies*.

Mathias and Simpson (1986) assessed the relative contributions made by media ingredients and genotype in vitro and suggested that the genotype may be a more significant factor than the medium in affecting culture behavior. Mathias and Fukui (1986) showed that the substitution of chromosome 4B of wheat var. 'Capelle-Desprez' into the 'Chinese Spring' variety improves the performance of calli in vitro. They concluded that factors which stimulate the growth, morphogenesis, and regeneration of wheat callus are located on chromosome 4B. Higgins and Mathias (1987) demonstrated that the substitution of different 4B chromosomes into 'Chinese Spring' wheat cells significantly increases morphogenesis and shoot regeneration from wheat callus, and suggested that one or only a few genes are involved in the determination of culture response. It is possible, therefore, that a similar small number of genes may be affecting somatic embryogenesis in *Pinus*. Genes that control culture characteristics for somatic embryogenesis and their location within the genome have not yet been identified.

Formation of somatic embryos

To enhance the development of somatic embryos from embryogenic callus, 1 μ*M* ABA was added to NSIII medium with half-strength macro-elements and 1 μ*M* NAA and 3% sucrose. After 3–4 weeks, 12% of the embryogenic callus cultures developed somatic embryos with cotyledons and the hypocotyls (Fig. 5). Decreasing the sucrose concentrations to 1% resulted in a reduction in the formation of somatic embryos. *Picea glauca* (Lu and Thorpe 1987) and *P. abies* (von Arnold 1987) somatic embryos continued to develop when transferred to a medium containing higher sucrose levels, but the addition of ABA did not enhance the development of *P. glauca* somatic embryos (Lu and Thorpe 1987). Similar results were obtained for *P. abies* (Hakman and von Arnold 1985). Due to the fact that ABA is known to suppress abnormal somatic embryo formation in carrot (Ammirato 1983), ABA was also added to basal medium used for the development of *P. abies* somatic embryos (Becwar et al. 1987 b). After the transfer of embryogenic callus to a higher sucrose (3%) medium, the embryonal mass of cells developed white and dense nodules (Fig. 3), from which somatic embryos subsequently developed (Fig. 4). This was fallowed by the elongation of the embryos and initiation of cotyledons and the hypocotyl (Fig. 4). These results indicate that development of somatic embryos on a medium having a higher sucrose concentration may be partly due to osmatic effects. Similar observations were recorded in *P. glauca* (Lu and Thorpe 1987) and *P. abies* (von Arnold 1987).

Table 5. Effect of buthionine sulfoximine (BSO) on the development of somatic embryos on half-strength macro-elements of NSIII medium containing 1 μ*M* NAA, 1 μ*M* ABA, and 3% sucrose after 4 weeks in the dark

Concentration in molar	No. of embryogenic calli cultured	Average no. of embryogenic calli developing somatic embryos	(%)
0	50	5	10
10^{-7}	50	15	30
10^{-6}	50	5	10
10^{-5}	50	2	4
10^{-4}	50	0	0

Several concentrations (10^{-7}–10^{-4} M) of BSO were added in the culture medium for the maturation of somatic embryos (Table 5). Buthionine sulfoximine is an inhibitor of reducing agents such as phenolics (Wan et al. 1986). The maturation of somatic embryos tripled on a medium containing 10^{-7} M BSO. Higher concentrations appeared to have an inhibitory effect on the maturation of somatic embryos. Becwar et al. (1987 b) reported that BSO doubled the maturation frequency in lines NSI No. 8 of *P. abies*.

Germination of somatic embryos

Well-developed somatic embryos with cotyledons and hypocotyls (Fig. 5) were cultured on half-strength macro-elements and half-strength N SIII basal medium without hormones to promote germination. After 3 weeks, 11%–15% of the somatic embryos formed roots in the dark (Fig. 6). Embryos kept in the light turned dark brown, and in some cases, further growth of shoots was arrested. Our efforts are now being directed towards improving conditions for the germination of somatic embryos and their regenerations into large numbers of plantlets. We are also studying the regeneration of transgenic plants via somatic embryogenesis. It has been suggested that if one can microinject isolated DNA directly into immature barley embryos and induce somatic embryogenesis from selected transformed calli, then subsequent plant regeneration will result with less variation (Jain et al. 1988 b).

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