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Induction of somatic embryogenesis in excised cotyledons of *Picea glauca* and *Picea mariana*

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Somatic embryogenesis was induced in the cotyledons of *Picea mariana* (black spruce) and *Picea glauca* (white spruce). Exposure of the cotyledons to a high ratio (90 : 1) of benzyladenine (BA) to napthaleneacetic acid (NAA) for one week before subculturing on media with auxin to cytokinin ratios of 2 : 1 (2,4-dichlorophenoxyacetic acid: BA) and 50 : 1 (NAA : BA), improved embryogenic response, especially of *P. mariana*. Embryonal suspensor callus of both black and white spruce could be multiplied and plantlets regenerated from the somatic embryos. In addition to the embryogenic tissues consisting of embryonal and suspensorial cells, which are typical of conifer tissues cultured *in vitro*, direct development of somatic embryos was observed for both *P. glauca* and *P. mariana*. Also in this case plantlets could be regenerated.

Additional key words – Black spruce, white spruce.

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Résumé. *L'embryogenèse somatique a été obtenue à partir de cotylédons chez* Picea mariana (*Epinette noire*) *et* Picea glauca (*Epinette blanche*). *Le traitement des cotylédons en présence d'un rapport élevé* (90 : 1) *en benzyladénine* (*BA*) *: acide napthalène acétique* (*ANA*), *une semaine avant une mise en culture sur un milieu dont le rapport auxine : cytokinine est soit de* 2 : 1 (*acide* 2,4 *dichlorophénoxyacétique : BA*) *soit de* 50 : 1 (*ANA : BA*), *augmente la réponse embryogène des cotylédons surtout chez* P. mariana. *Les masses polyembryogènes de l'Epinette noire et de l'Epinette blanche ont été multipliées et des plantes régénérées à partir des embryons somatiques. De plus, mis à part l'obtention des tissus embryogènes formés de cellules embryogènes et de suspenseur, typiques chez les conifères, nous avons observé le développement spontané d'embryons somatiques pour* P. glauca *et* P. mariana. *Dans ce cas aussi, des plantes ont été régénérées.* Mots clés additionnels : Epinette noire, Epinette blanche.

Abbreviations. ABA, abscisic acid; BA, benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic; NAA, naphthaleneacetic acid.

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INTRODUCTION

Starting with the work of Hakman *et al.* (1985), research into somatic embryogenesis in cultured conifer tissues has undergone rapid progress during the last five years, as witnessed by the increasing number of species in which somatic embryogeny has been reported (Lelu *et al.*, 1990). With few exceptions (Krogstrup, 1986; Lelu *et al.*, 1987), most of the work to date concerning the induction of somatic embryogenesis in the Coniferales has involved, as starting material, either immature or mature zygotic embryos.

As vegetatively propagated clones are attracting increasing interest for reforestation (Bentzer *et al.*, 1989), the manipulation *in vivo* and *in vitro* of gymnospermous tissues of ever-increasing chronological age, and thus physiological maturity, assumes high priority.

Somatic embryogenesis has been reported for immature (Hakman and Fowke, 1989; Lu and Thorpe, 1987) and mature (Tremblay, 1989) embryos of *Picea glauca*. The aim of the present study was to determine whether somatic embryogenesis could be initiated in cotyledons excised from seedlings. The first indication that this was possible for *P. glauca* and *P. mariana* was reported by Lelu and Bornman (1989) and confirmed by Attree *et al.* (1990).

MATERIALS AND METHODS

Plant material. Seeds of white spruce, *Picea glauca* (Moench) Voss, and black spruce, *Picea mariana* (Mill.) Britt., were disinfested in 30% (v/v) H_2O_2 for 10 min, rinsed three times in sterile water and germinated aseptically on moist sterile filter paper in germination trays. The seeds were germinated at 23 ± 1/20 ± 1°C in a 16/8 h photoperiod. Light was provided by Osram L 36 77 W fluorescent lamps at a photon flux density of 65 μmol m^{-2} s^{-1}. After 5 days the radicles emerged and one week later the cotyledons, which were up to 5 mm in length, when excised. The number of cotyledons per seed varied from 3 to 6 for *P. mariana* and 4 to 8 for *P. glauca*. Two replications were used in all the experiments, each comprising 20 to 30 seedlings.

Callus induction. Cotyledons were excised and cut into lengths of 2 mm and 12 to 16 explants cultured on 20 ml induction medium in petri dishes. The medium was that of Murashige and Skoog (1962) supplemented with either 9.1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.5 μM benzyladenine (BA) or 21.5 μM naphthaleneacetic acid (NAA) and 0.45 μM BA. The cultures were incubated in the dark at 25 ± 1°C.

Bud induction. As one of the treatments, the cotyledons were subjected for one week to conditions known to stimulate embryo induction in *P. abies*, before being transferred onto medium for multiplication of embryonal suspensor callus. Whole, excised cotyledons were cultured on the bud induction medium reported by Lelu *et al.* (1987), supplemented with 4.5 μM BA and 0.05 μM NAA. The explants were cultured at 20 ± 1°C under continuous light of a photon flux density of 120 μmol m^{-2} s^{-1} provided by Sylvania cool-white 115 W fluorescent lamps.

Multiplication of embryonal suspensor callus. After induction, embryonal suspensor callus was transferred to the multiplication medium used by Gupta and Durzan (1986 *a*) that contained 5 μM 2,4-D and 2 μM each of BA and kinetin. The cultures were maintained in darkness at 25 ± 1°C. All media (pH 5.8) contained 90 mM sucrose and were solidified with 0.8% agar.

Statistical treatment. Chi-square statistics were used for testing the independence of variables.

RESULTS AND DISCUSSION

Callus induction

Initially, the cotyledons of both *P. glauca* and *P. mariana* enlarged in both length and width and callus initiation occurred within 2 weeks. After 4-6 weeks, both embryogenic and non-embryogenic calli could be distinguished on the same cotyledon. Non-embryogenic callus (*fig.* 1 A and B) ranged in colour from white to very light green and in texture from compact to friable. This callus eventually formed over the greater part of the cotyledon's surface.

As developing embryogenic tissue consists of a mass of both embryonal and suspensor cells, the terms embryonal suspensor mass (Gupta and Durzan, 1986 *b*) or embryonal suspensor callus seem appropriate.

Typically, cells comprising the embryonal suspensor mass of the embryogenic callus (*fig.* 1 A and B) gave the callus a translucent, sometimes snow-white, and sinuous appearance. The presence of both embryonal and suspensorial cells (*fig.* 1 C) was confirmed cytologically by staining with aceto-orcein (Lacour, 1941). Figure 1 D shows the morphology typical of a somatic embryo. Anatomically, these embryos appear similar to those described previously for various coniferous species cultured *in vitro*. The occurrence of polyembryony by the cleavage of the terminal somatic embryos was also observed occasionally, a phenomenon also noted in *Pinus lambertiana* (Gupta and Durzan, 1986 *b*).

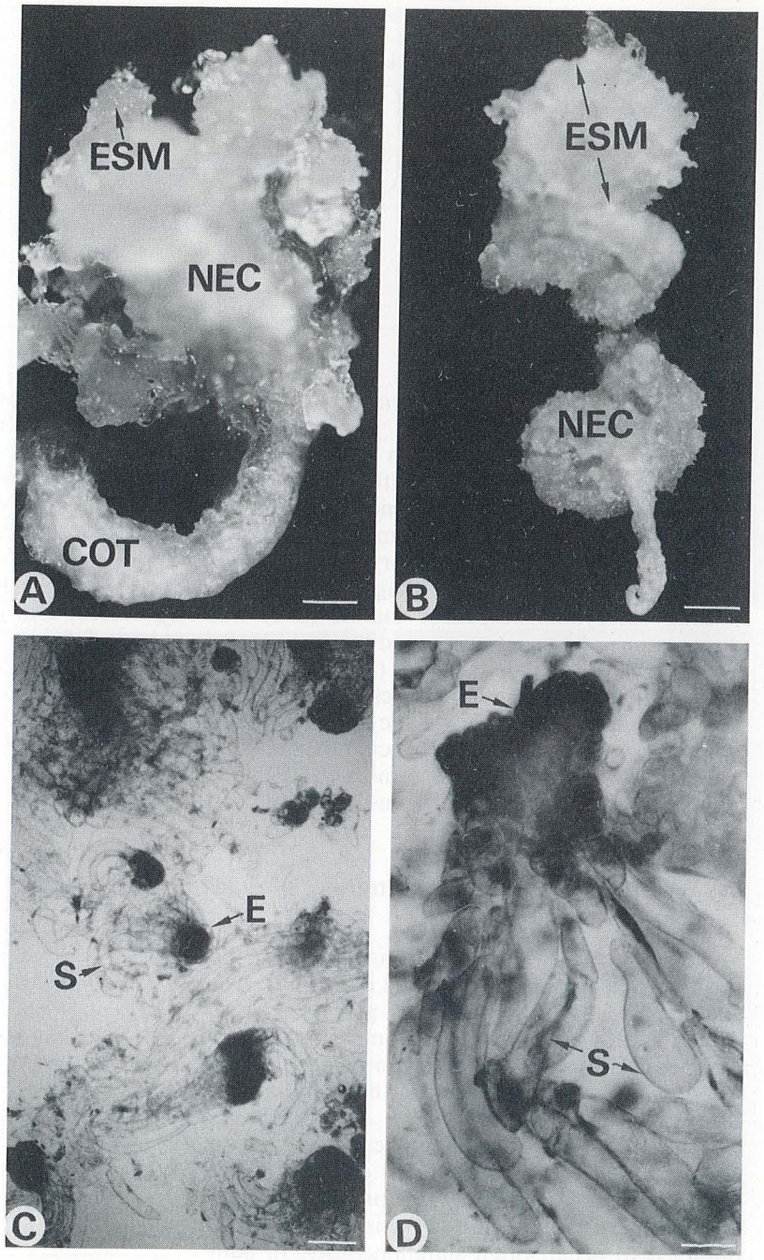

Figure 1. A, cotyledons of *Picea mariana* and **B**, *Picea glauca* with non-embryogenic callus (NEC) and developing embryonal suspensor masses (ESM) after 4 weeks on callus induction medium. COT, cotyledon. Bars represent 600 μm. **C**, somatic embryos that have differentiated in an embryonal suspensor mass of *Picea mariana*, stained with aceto-orcein to localize the chromatin-dense embryonal (E) cells. **D**, detail of *Picea mariana* embryo with component embryonal and suspensorial (S) cells. Bars represent 200 (**C**) and 50 μm (**D**), respectively.

The embryonal suspensor callus usually developed from that part of the cotyledon in contact with the medium, but could also occur at the cut surfaces. Occasionally, embryonal suspensor masses formed on the surface of the cotyledons facing away from the medium. Six to eight per

Table 1. *Morphogenic response of cotyledons excised from 7-day-old* Picea glauca *and* Picea mariana *seedlings.* Cotyledons were placed either directly on callus induction medium or subjected first to a bud-induction treatment. Observations were recorded after 5 (*P. mariana*) and 6 (*P. glauca*) weeks in callogenesis. Pg, *P. glauca;* Pm, *P. mariana;* (%).

Treatment (*week*)	Callus induction treatment (μ*M*)	Cotyledons cultured		Cotyledons with foliar structures		Cotyledons with embryonal suspensor mass	
		Pg	Pm	Pg	Pm	Pg	Pm
0	2,4-D 9.1; BA 4.5	237	244	2	4	15 (6.3)	16 (6.5)
	NAA 21.5; BA 0.45	240	246	0	1	5 (2.0)	19 (8.0)
1	2,4-D 9.1, BA 4.5	227	239	17	29	4 (2.0)	44 (18.4)
	NAA 21.5, BA 0.45	229	240	2	15	8 (3.5)	9 (4.0)

cent of the cotyledons of *P. mariana* cultured on the callus induction medium gave rise to embryonal suspensor callus with both combinations of plant growth regulators, that is 2,4-D/BA and NAA/BA (*tab.* 1), a frequency of the same order as that reported by Hakman and Fowke (1987) for immature zygotic embryos. In the case of *P. glauca*, the highest percentage of cotyledons forming embryonal suspensor masses (6%) was observed on 2,4-D-containing medium (*tab.* 1). By comparison, Lu and Thorpe (1987) reported an embryogenic response 10 times greater (67%) with respect to immature zygotic embryos of *P. glauca* dissected from cones collected in mid-July; however, when collected one month later, the frequency of embryonal suspensor callus had decreased to 9%.

Bud induction treatment prior to transfer to callus induction medium

After one week on bud induction medium, the cotyledons of *P. mariana* enlarged in width but did not elongate. When subsequently placed on callus induction medium, an opaque, compact callus was produced. Callus formation began on that part of the cotyledon in contact with the medium and eventually proceeded over the surface of the explant. After 4-6 weeks on callus induction medium, both non-embryogenic and embryonal suspensor callus formed. In these pretreated cotyledons, different pathways of histogenesis were observed to occur, often simultaneously. In addition to the embryonal suspensor mass, foliar adventitious structures were produced on the cotyledon's exposed surface and meristemoidal nodules originated from epidermal and subepidermal cells. The meristemoids later developed into adventitious buds when the cotyledons were placed under bud promoting conditions, that is, on bud induction medium lacking plant growth regulators, and under continuous light. Foliar structures were obtained more frequently in the case of pretreated cotyledons (*tab.* 1), an observation also made for the cotyledons of *P. abies* (Lelu *et al.*, 1990).

A one-week exposure to bud induction treatment significantly improved the production of embryonal suspensor callus in *P. mariana* when 2,4-D was used in the callus induction medium ($\chi^2 = 5.85^*$ and $\chi^2 = 7.43^{**}$ in the two replications). About 20% of the cotyledons gave rise to somatic embryogenic tissues. With NAA in the callus induction medium, the one-week bud induction treatment was less effective and a slight decrease in embryogenic response resulted. Only one of the replications had a significant effect ($\chi^2 = 4.92^*$ and $\chi^2 = 0.17$).

The cotyledons of *P. glauca* did not enlarge on the bud induction medium and neither did the

Table 2. *Effect of cotyledon length on embryogenic response in* Picea mariana. Cotyledons were placed either directly on callus induction medium containing 9.1 μM 2,4-D and 4.5 μM BA or subjected first to a one-week bud-induction treatment. Observations were recorded after 5 weeks culture on the callus medium.

Bud induction treatment (*week*)	Cotyledons length (*mm*)	No. of cotyledons cultured	Cotyledons with embryonal suspensor mass	
			Total	%
0	2	59	12	20.3
	3	102	1	1.0
	4	40	2	5.0
	5	43	1	2.3
1	2	80	21	26.3
	3	79	12	15.2
	4	42	11	26.2
	5	38	0	0

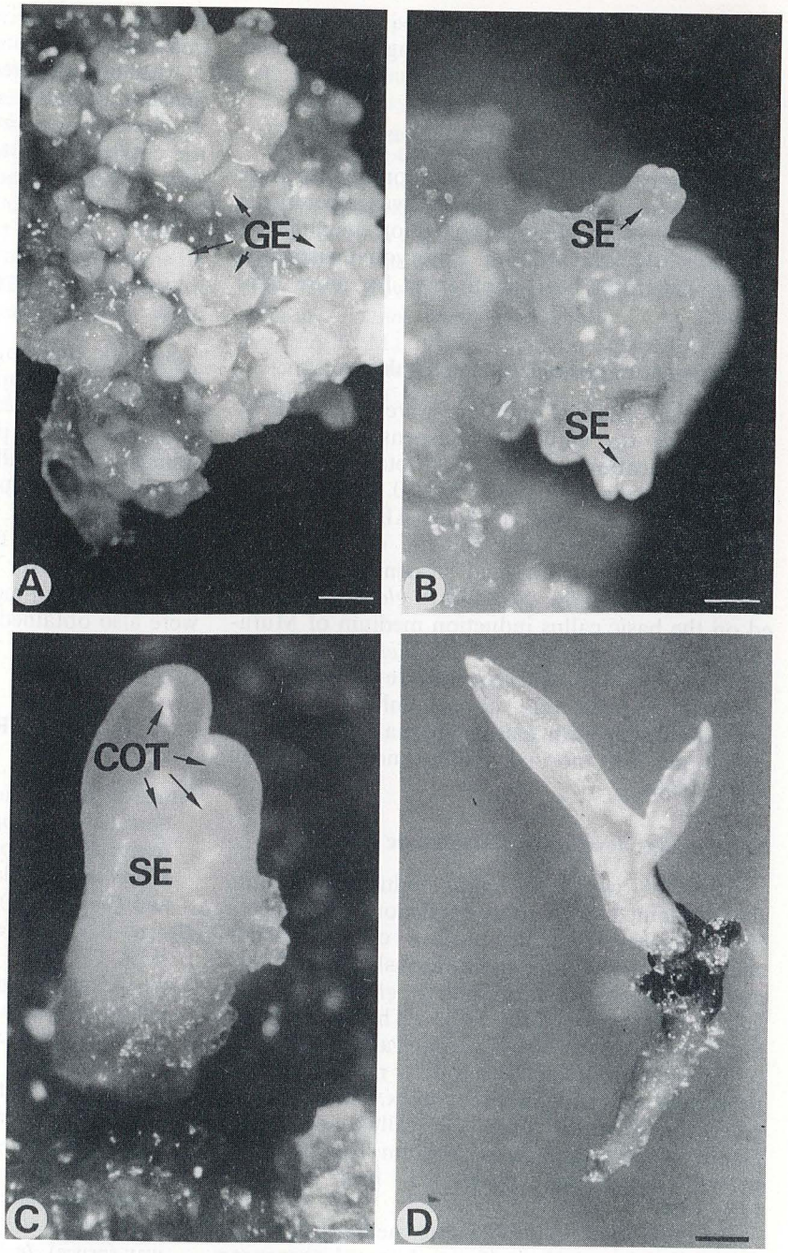

Figure 2. A, globular somatic proembryos (GE) often develop spontaneously from embryogenic callus, in this case *Picea mariana* after 5 weeks on callus induction medium. Bar = 600 μm. **B**, somatic embryos (SE) initiated directly in callus of *Picea glauca* after 6 weeks on callus induction medium. Bar = 375 μm. **C**, somatic embryo (SE) with 4 cotyledons (arrows) initiated directly in callus of *Picea mariana* after 8 weeks on callus induction medium. Bar = 250 μm. **D**, plantlet of *Picea mariana* developed from a somatic embryo similar to that shown in fig. 2 C. Bar = 800 μm.

frequency of formation of embryonal suspensor callus improve when the cotyledons were subjected to this treatment (*tab.* 1). In the case of *P. mariana*, embryogenic response on callus induction medium depended on the size of the cotyledon explant, with the smallest cotyledons (2 mm) giving the highest (20%) frequency (*tab.* 2). Following a prior bud induction treatment, 15 to 26% of cotyledons 2, 3 and 4 mm in length produced embryogenic callus (*tab.* 2). The bud induction treatment

improved overall embryogenesis in *P. mariana* cotyledons by increasing the range in size of responding explants.

Culture of embryonal suspensor callus

In order to proliferate embryonal suspensor mass after its induction, the tissue was subcultured every 10 days to the multiplication medium of Gupta and Durzan (1986 *a*). Embryonal suspensor masses have now been maintained for more than 2 years.

Development of globular somatic embryos

Globular somatic embryos were obtained by transferring the embryonal suspensor masses to the multiplication medium of Gupta and Durzan (1986 *a*) containing 1 µM 2,4-D, 0.5 µM each of BA and kinetin, and 11.4 µM ABA. However, development of globular somatic embryos were also observed in embryonal suspensor masses of both *P. mariana* (*fig.* 2 A) and *P. glauca* maintained on the basic callus induction medium of Murashige and Skoog (1962). In *P. abies*, by contrast, proliferation and subsequent development of globular embryos are always inhibited if the embryonal suspensor masses are maintained under conditions for normal callus induction (Lelu *et al.*, 1990).

Spontaneous development of somatic embryos

After 6-8 weeks on callus induction medium that contained NAA some cotyledons developed a friable callus from the surface cells of which somatic embryos arose spontaneously. This phenomenon was observed for both *P. glauca* (*fig.* 1 B) and *P. mariana* (*fig.* 1 C). These embryos displayed a typical bipolar organization consisting of hypocotyl-root axis with four or more cotyledon primordia enclosing a shoot apex. The embryos could be dissected from the callus and, when placed in the light on a medium lacking plant growth regulators, developed into plantlets (*fig.* 1 D).

As there was no visible intermediary development of the typical conifer embryonal suspensor mass, there are two possible explanations for the origin of these somatic embryos. Conceivably, the callus could have passed through a condensed embryonal suspensor mass phase, but it is more likely that a pathway exists for the spontaneous development of somatic embryos. Recently, Bourgkard and Favre (1988), reporting on the development of plantlets via somatic embryogenesis in *Sequoia sempervirens*, did not observe the formation of an embryonal suspensor mass.

Although the frequency of such spontaneously-formed somatic embryos in *P. mariana* and *P. glauca* is low, it indicates that the response of the cotyledons of these two species is different to that of *P. abies* under similar conditions.

CONCLUSION

Somatic embryogenesis can be induced in cotyledons excised from seedlings of *P. mariana* and *P. glauca*. In the case of *P. mariana*, a bud-induction treatment applied for one week prior to transfer to a callus induction medium greatly improved the frequency of formation of embryonal suspensor masses. In addition to the embryonal suspensor mass that gave rise to the normal embryo-suspensor type of conifer embryo, spontaneous development of bipolar somatic embryos were also obtained.

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