Somatic embryogenesis in cotyledons of Picea abies is enhanced by and adventitious bud-inducing treatment

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Somatic embryogenesis in cotyledons of *Picea abies* is enhanced by an adventitious bud-inducing treatment

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Application. Somatic embryogenesis in the Coniferales is regarded as a potential tool in the micropropagation of forest trees. This study reports on somatic embryogeny in *Picea abies*, induced in excised cotyledons. It represents a possible basis for the establishment of the technology that will be required to manipulate tissues of superior trees.

Abstract. Somatic embryos were obtained from young, isolated cotyledons of *Picea abies*. A bud-inductive treatment for one week before transfer to a callus induction medium, resulted in a tenfold increase in the percentage of cotyledons in which embryonic tissues could be induced. The degree of expression of somatic embryogeny depends on whether cotyledons are first subjected to a callus-inducing medium or first exposed to a cytokinin treatment followed by a callus-inducing medium. It is hypothesized that two populations of cells exist, both with the inherent potential of becoming embryogenic after treatment with plant growth regulators.

Introduction

Somatic embryogenesis, first reported in *Picea abies* (L. Karst.) by Hakman et al. (1985), has since been induced in several coniferous species (Table 1). Until now, the tissue source has been either immature zygotic embryos or mature zygotic embryos prior to germination. However, more recently embryogenesis was obtained also from cotyledons excised from newly-germinated seeds of *Picea abies* (Lelu et al. 1987).

In attempting to induce embryogenesis most conifer experimentalists hitherto have subjected their explants to conditions that promote callogenesis. However, Krogstrup (1986) first pretreated them for 3 weeks with

Table 1. Induction of *somatic* embryos in the Coniferales.

Species	Developmental stage of embryo explant (*, via suspension culture)	Whole plant regeneration reported	Reference
Pinaceae			
Albies alba	Immature	No	Schuller et al. (1989)
Larix x eurolepis	Immature	Yes	Klimaszewska (1989)
Picea abies	Immature	No	Hakman et al. (1985)
	Immature	Yes	Hakman and von Arnold (1985), Becwar et al. (1987)
	Mature	No	von Arnold and Hakman (1986), Krogstrup (1986)
	Mature	Yes	Gupta and Durzan (1986b), von Arnold (1987)
	* Mature	Yes	Boulay et al. (1988)
Picea glauca	Immature	No	Hakman and Fowke (1987)
	Immature	Yes	Lu and Thorpe (1987)
	* Immature	Yes	Hakman and von Arnold (1988)
Picea mariana	Immature	No	Hakman and Fowke (1987)
Picea sitchensis	Immature	Yes	Krogstrup et al. (1988)
	Mature	No	von Arnold and Woodward (1988)
Pinus lambertiana	Mature	No	Gupta and Durzan (1986a)
Pinus taeda	Immature	Yes	Gupta and Durzan (1987)
Pseudotsuga menziesii	* Immature	Yes	Durzan and Gupta (1987)
Taxodiaceae			
Sequoia sempervirens	Mature	Yes	Bourgkard and Favre (1988)

benzyladenine (BA) and Lelu et al. (1987) observed that pre-culturing on an adventitious bud-induction medium promoted subsequent embryogenesis. The goals of this study were to:

- use napthaleneacetic acid (NAA) rather than 2,4-dichlorophenoxyacetic acid (2,4-D) (Lelu et al. 1987),
- reconfirm the prior observation of the beneficial effect of an adventitious bud-inducing medium containing NAA on somatic embryogenesis, and
- develop a better understanding of the process of embryo induction.

Material and methods

Seed

Seeds of *Picea abies* were collected in 1984—1985 from trees in the Gerardmer area of France. They were stored at 4 °C in darkness for 2 years. After immersion in a surfactant (1% v/v Teepol), the seeds were surface-sterilized in 15% (v/v) H_2O_2 for 10 min and rinsed twice in sterile distilled water. The seeds were planted in sterile, moist vermiculite and germinated aseptically at 25/20 °C ± 1 °C in a 16/8 h day/night. After one week, the radicles emerged and 3 to 5 days later the 2- to 5-mm-long cotyledons were excised. It had been determined earlier (Lelu et al. 1987) that a length of 2—5 mm was optimal for the induction of somatic embryogenesis.

Culture media and culture conditions

Table 2 summarizes the culture media and conditions used. All cultures were incubated in 90-mm diameter petri dishes containing 20 ml medium; pH of the medium was adjusted to 5.8 ± 0.1 prior to sterilization by autoclaving at 100 kPa and 120 °C for 20 min. Where used, light was applied at a photon flux density of 70 μmol m^{-2} s^{-1} supplied by 40 W fluorescent lamps (Sylvania Grolux). Whole excised cotyledons were cultured on bud-induction medium (Table 2) for 1, 2 or 3 weeks. After this treatment, half the number of cotyledons were transferred to a bud-development medium (Table 2) and well-formed buds were scored 2 months later (since other neoformations were also visible, Bornman 1987). The remaining cotyledons were cut into 2 mm explants, which were placed on callus-induction medium (Table 2). As control, pieces of excised cotyledons were also placed directly on callus-induction medium.

Table 2. Culture media and culture conditions.

Constituents and conditions	Media for		
	Bud induction	Bud development	Callus induction
Macronutrients elements	CD	CD (half-strength)	MS
Micronutrients elements	MS (half-strength)	MS (half-strength)	MS
Sucrose, mM	90	45	90
Myoinositol, mM	2.7	0.55	0.55
Thiamine.HCl, μM	15	1.2	30
BA, μM	4.5	0	0 or 0.45
NAA, μM	0.05	0	5.4 or 10.7
Agar (Bacto-Difco), gl^{-1}	8	8	8
Light (photoperiod, h)	continuous	16/8	darkness
Temperature, °C ± 1	20	25/20	25

CD, Campbell and Durzan (1975); MS, Murashige and Skoog (1962).

The presence of embryogenic callus was determined after 4 weeks on the latter medium by cytological examination.

Cytological and histological examination

For callus, squashes were prepared in aceto-orcein (Lacour 1941) as this dye neither stains the cytoplasm or nucleoli, nor overstains the chromosomes. Callus was considered as embryogenic when multiple embryonal segments and masses of cells as well as suspensor-like cells could be identified. Morphogenesis of the cotyledon during the culture phases on bud- and callus-induction media was followed by examining 2 μm-thick sections of glutaraldehyde-fixed and glycol methacrylate-embedded tissue stained with toluidine blue and basic fuchsin (Feder and O'Brien 1968).

Results and discussion

When subjected directly to callus induction without a bud-induction pretreatment, some cotyledons developed embryogenic callus after 4 weeks. The embryogenic nature of the culture was confirmed cytologically and found to correspond with the observations made previously in respect of *P. abies* by Hakman et al. (1985), Gupta and Durzan (1987), and Lelu

et al. (1987). Table 3 shows that using only callus-induction medium, the percentage of plated seedling cotyledons that developed embryogenic callus was low, approximately 2%. By comparison, Hakman and von Arnold (1985) reported a 50% yield in embryogenic callus from immature embryos and von Arnold (1987) a similar frequency from mature zygotic embryos. We believe this difference may be accounted for by explant age, that is loss of juvenility.

However, when we applied a bud-inducing treatment prior to the transfer of the explants to the callogenic medium, the response varied according to the duration of the culture on the bud-inducing medium, but a one-week exposure to the highest auxin concentration (10.7 μM NAA in combination with 0.45 μM BA) resulted in an increase from 2 to 20% of cotyledons with embryogenic callus. The concentrations of the plant growth regulators used in the callus-inducing medium (Table 2) were actually lower than and the combinations different from those previously used, i.e. 2,4-D 9.05/BA 4.5 μM or NAA 21.5/BA 0.45 μM (Lelu et al. 1987). Somatic embryogenesis from excised cotyledons of *Picea abies* was also induced with auxin alone (NAA 5.4 μM), an observation that differs

Table 3. Effect of duration of bud induction treatment on formation of embryogenic callus from excised cotyledons of *Picea abies* (2—5 mm length) subjected to different callus induction treatments. The data, obtained after 4 weeks on callus induction medium, were pooled from 3 separate experiments.

Bud induction pretreatment (BA 4.5, NAA 0.05 μM) Week	Callus induction treatment: concentration of plant growth regulators, μM		Number cotyledons cultured	No. cotyledons with embryogenic callus per 100 plated cotyledons
	BA	NAA		
	0	5.4	112	2.6
0	0.45	5.4	118	2.5
	0.45	10.7	128	2.3
	0	5.4	102	8.0
1	0.45	5.4	111	2.0
	0.45	10.7	103	20.3
	0	5.4	107	10.3
2	0.45	5.4	101	2.0
	0.45	10.7	111	3.6
	0	5.4	109	0
3	0.45	5.4	110	4.5
	0.45	10.7	101	3

from that of von Arnold (1987) who found that the obtention of embryogenic callus from mature zygotic embryos of *Picea abies* was both auxin- and cytokinin-dependent.

Increasing the duration on the bud-inducing medium beyond one week decreased the embryogenic response (Table 3). The small increase from 2 to 4.5% observed with NAA (5.4 μM) and BA (0.45 μM) after a 3-week exposure could be considered as experimental variation.

With bud-inducing treatment, in addition to the embryogenic and non-embryogenic calli, newly-formed adventitious, meristematic and foliar structures occurred on the cotyledon's surface (Fig. 1) and in the dark most of these subsequently developed into etiolated buds on the callus-induction medium. The total number of cotyledons responding to the bud-induction treatment did not increase markedly between weeks 2 and 3, but the number of adventitious buds doubled over the same period (Table 4), a phenomenon also observed previously (Bornman and Jansson 1981; Bornman 1983). With exposure to bud-induction treatment, in contrast to induction of embryogenic callus, the number of reacting explants doubled between weeks 1 and 2 and slightly later (43-83-89% for weeks 1-2-3, respectively, Table 4), whereas the number of newly-formed adventitious buds continued to increase (87-319-627 for weeks 1-2-3, respectively).

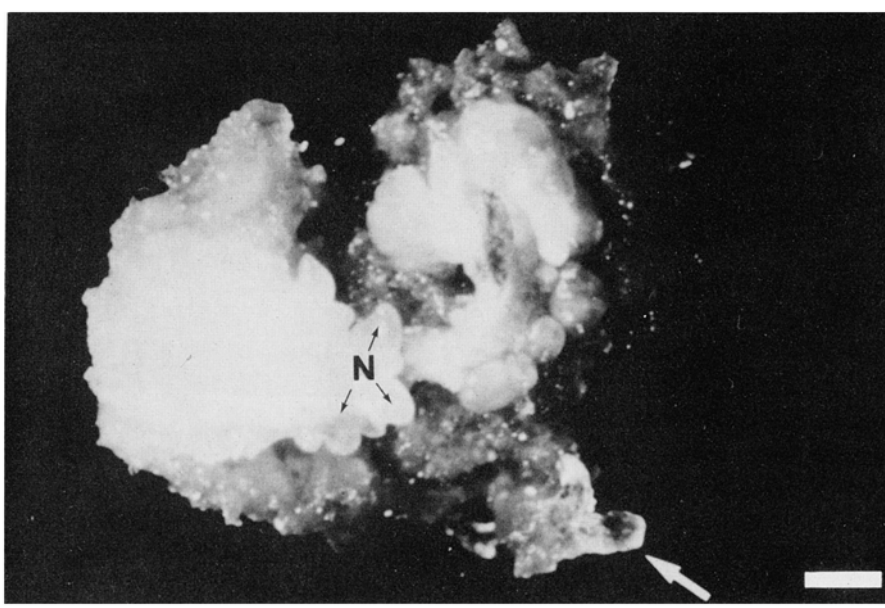

Fig. 1. Cotyledon of *Picea abies* exposed to a one-week bud-inducing treatment followed by 4 weeks on a callus-inducing medium. Embryogenic callus (arrow) and neoformed adventitious structures (N) can be distinguished. Bar represents 1 mm.

Table 4. Effect of duration of bud induction treatment on formation of adventitious buds from excised cotyledons of *Picea abies* (2—5 mm length). The data, obtained after 8 weeks in culture on bud development medium, were pooled from 2 separate experiments.

Bud induction pretreatment (BA 4.5, NAA 0.05 µM) Week	No. cotyledons cultured	Per cent cotyledons with adventitious buds, %	No. adventitious buds per 100 plated cotyledons
0	111	0	0
1	76	43	87
2	87	83	319
3	66	89	627

Histological examination of cotyledons of *Picea abies* showed that the conditions for callusing led to an enlargement of especially the mesophyll cells (Fig. 2), resulting in the subsequent disorganization of the tissues they comprised in relation to form and orientation. This was in marked contrast to the bud-inducing treatment, which resulted in the differentiation of

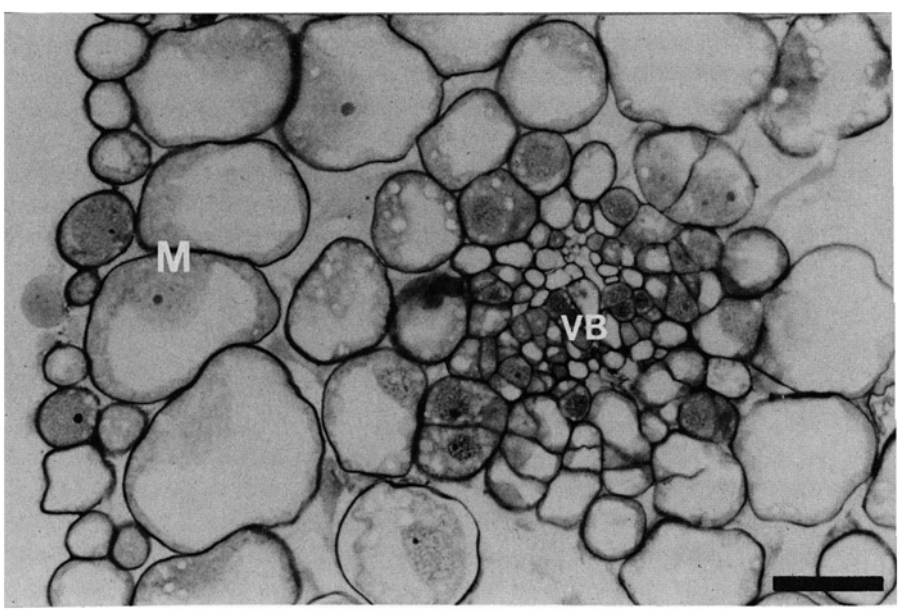

Fig. 2. Transverse section of midpart of cotyledon subjected for one week to callus-inducing medium only. As compared to the initial tissue, cells of the mesophyll (M) are hypertrophied and, except for the parenchyma associated with the vascular bundle (VB), show virtually no meristematic activity. Bar represents 50 µm.

promeristemoids in epidermal and sub-epidermal cells after 1 week (Fig. 3), the reactivity becoming very pronounced when a 3-week treatment was applied (Lelu et al. 1987). Sub-epidermal layers have been demonstrated to be active in the formation of adventitious buds in isolated cotyledonary explants of various coniferous species (Yeung et al. 1981; Jansson and Bornman 1981; Bornman 1983; Villalobos et al. 1985; Flinn et al. 1988).

After 3 weeks in callogenesis, the presence of somatic proembryos were found to have originated from cells of the epidermis and sub-epidermis (Fig. 4).

The results obtained in our study lead us to hypothesize the existence of two cell populations. One cell population could be triggered directly by auxin only, or by a high ratio of auxin to cytokinin (NAA:BA = 12:1 or 24:1), giving rise to embryogenic cultures (such as those in Table 3 corresponding to 2% of the cotyledons that reacted directly to the callus induction treatment). The other cell population responds more efficiently to auxin treatment when first pretreated with cytokinin. The highest percentage of embryogenic cultures were obtained after 1 week on a bud-induction medium in which the ratio of cytokinin to auxin was 90:1. In this

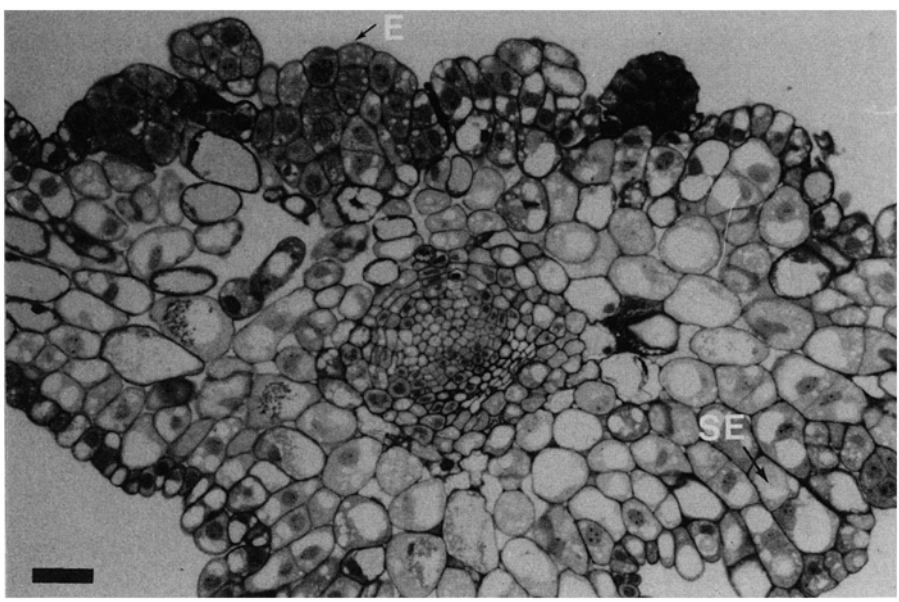

Fig. 3. Transverse section of midpart of cotyledon exposed to callus-inducing medium for one week, but pretreated for one week on a bud-inducing medium. In addition to cell divisions in the mesophyll, extensive meristematic activity can be observed in epidermal (E) and sub-epidermal (SE) cell layers, as well as in the parenchyma of the vascular bundle. Bar represents 50 μm.

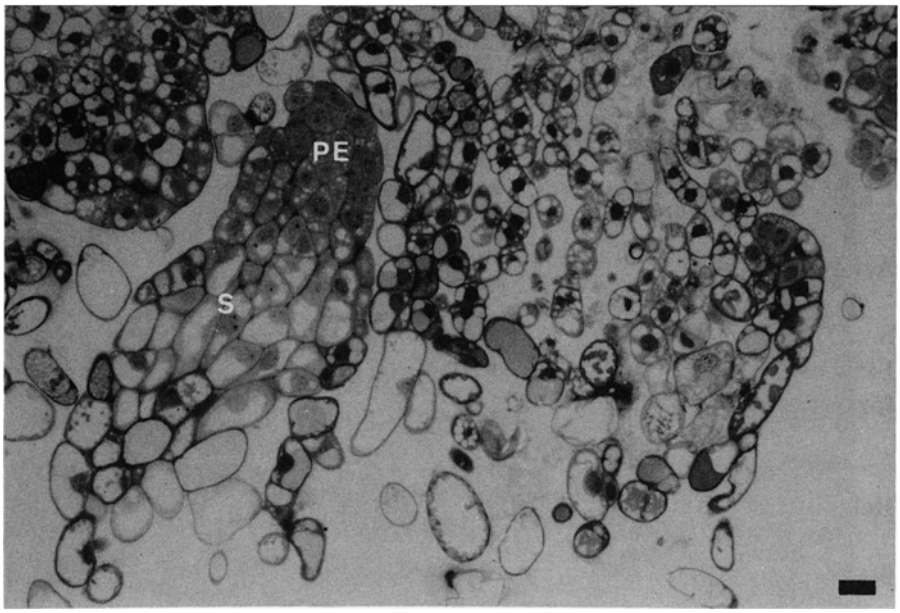

Fig. 4. Longitudinal section of midpart of cotyledon exposed to a one-week bud-inducing treatment followed by 3 weeks on a callus-inducing medium. Developing and well-developed somatic pro-embryos are now presented. Somatic proembryos are characterized by proembryonal (PE) and suspensorial (S) parts. Bar represents 50 μm.

case, the duration of the cytokinin treatment appears to be critical for the dedifferentiation of epidermal and sub-epidermal cells into either embryogenic precursor cells (treatment of relatively short duration, 1 week) or into adventitious buds (treatment of relatively long duration, 2—3 weeks).

Embryogenic calli thus obtained can be multiplied using the medium described by Gupta and Durzan (1986b). Entire spruce plants have also been regenerated.

Conclusion

In our experiments, a cytokinin bud-induction pretreatment has been useful in increasing the embryogenic capacity of isolated cotyledons of *P. abies*. Krogstrup (1986) pretreated his explants with BA, but gave no evidence of its beneficial effect and was unable to obtain regeneration of whole plants. There is a further interest in using the system described here, namely its possible application to tissues of mature trees. Vegetative dormant buds and needles and even needle primordia, have been shown to react in a manner similar to cotyledons as far as adventitious bud

induction is concerned (Jansson and Bornman 1983; von Arnold and Eriksson 1979a, b). Such buds are now being used as primary explants for induction of embryogenic cultures from young and maturing trees. The first experiments using such dormant and flushing buds have shown interesting morphological responses.

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