FACTORS AFFECTING *IN VITRO* ADVENTITIOUS BUD INDUCTION FROM EXCISED EMBRYOS OF SWISS STONE PINE (*PINUS CEMBRA* L.)

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ABSTRACT

Several culture and pre-culture factors affecting *in vitro* adventitious bud induction on excised embryos of Swiss stone pine (*Pinus cembra* L.) were investigated. Embryos gave rise to adventitious buds within 4 weeks of culture in a cytokinin-containing medium. Among the tested cytokinins, N^6-Benzyladenine (BA) proved to be the most effective, especially when 2.5, or 5 μM were added to the von Arnold and Eriksson's (AE) medium. All the tested pre-induction treatments (4, 8, or 12 week seed chilling, and embryo pre-culture in a hormone-free medium, prior culturing them on the bud induction medium) negligibly affected adventitious budding. Whole embryos were the most suitable explants for bud induction, as poor regeneration was achieved when isolated hypocotyls and epicotyls were cultured. A 21-day exposure of the unchilled explants to BA induced 100% regenerating embryos, and more than 16 buds per explant. Less adventitious shoots were obtained with a 7-day treatment, but shoot growth was moderately accelerated. Bud development was achieved on a hormone-free AE medium (3 weeks), while shoot elongation was optimized on an activated charcoal-containing (0.1%) medium (4 weeks). 15 % of the elongated shoots produced adventitious roots, after they were pulsed in liquid 1-mM IBA for 5 hours, prior to being transferrred to a hormone-free medium.

Keywords: *Pinus cembra*, micropropagation, tissue culture, conifers, organogenesis

INTRODUCTION

Swiss stone pine (*Pinus cembra* L.) is a typical species of the high mountain regions of the Alps and of the Carpathians, where it grows at 1,300 to 2,000 m altitudes. In the subalpine zone it occurs in stands as a pioneer species, and in solitary above the tree line. A peculiar aspect of Swiss stone pine is its slow growth: in natural stands it attains an average height of 2–3 m after 50 years. It is widely used as ornamental tree as well, but even at lower altitudes the trees do not grow much faster (VIDAKOVIĆ 1991). The species starts to bear cones after 40–50 years, and seed production is abundant only every 5–10 years. At the time of seed collection, the embryos still retain different levels of endodormancy (HEUMADER 1992), and their germinability, even if high, can drop quickly (SUSMEL 1954). In spite of this, seed propagation currently represents the only way to reproduce the species, as traditional methods of vegetative propagation (grafting, cutting) remain uncommon. As a result, tissue culture can be an important tool for both the preservation and the spreading of the species. The opportunity to realize the clonal multiplication of young material from genetically high-quality control-pollinated seed is of high value in forest genetics, in which breeding programmes are time-consuming, and often leads to a limited supply of superior genotypes that, if required for afforestation, would need first to be propagated vegetatively.

Significant progress has been recorded recently in the *in vitro* propagation of conifer species (THORPE *et al.* 1991). Organogenesis of several *Pinus* species was achieved by culturing whole embryos or embryonic explants (AITKEN *et al.* 1981; VON ARNOLD & ERIKSSON 1981; PATEL & THORPE 1984; ABDULLAH *et al.* 1985; CHESICK *et al.* 1991; MARTINEZ PULIDO *et al.*; 1992. LAMBARDI *et al.* 1993; CAPUANA & GIANNINI 1995), and the importance of culture factors has been shown to be effective in the adventitious bud induction of conifers (see BONGA & VON ADERKAS 1992).

The possibility of inducing adventitious budding on mature embryos of Swiss stone pine has been previously reported (CAPUANA *et al.* 1994). The aim of this study was to investigate the influence of culture and pre-culture factors (*i.e.*, different combinations of cytokinins, basal medium formulations, seed and embryo pre-treatment, explant selection, duration of hormone application) in the induction of adventitious

buds from excised embryos. Preliminary studies on elongation and rooting of the *de-novo* formed shoots were also carried out.

MATERIAL AND METHODS

Plant material and culture methods

Seeds of *Pinus cembra* L. were collected from open pollinated trees in a natural stand, located in the Italian Province of Cuneo (Northern Italy; latitude 44°36', longitude 7°21', altitude 2,000 m). Seeds were surface-sterilized in ethanol (15 min), soaked with 7 % NaOCl for 15 min, and then thoroughly rinsed with sterile distilled water. Prior to embryo excision, the seeds were stored in sterile water at 4 °C for one week. The embryos (3–6 mm length) were then removed from the megagametophytes under sterile conditions and horizontally plated on different media, in accordance with the experimental design. Explants were cultured in Petri plates (ϕ 90 mm) containing 25 ml of medium. All the media were added of 3% sucrose, and gelled with Difco-Bacto® agar at 0.8% (w/v). The pH was adjusted to 5.8 before autoclaving. Unless otherwise stated, explants were cultured for 28 days at 23±1 °C, under a 16L/8D photoperiod, at a photon fluence rate of 80 $\mu E.m^{-2}.s^{-1}$. Best culture conditions and pre-culture treatments for adventitious bud induction and shoot development were characterized in sequential experiments, as described below. Each experiment was performed using at least five plates (replicates) per treatment, with 10 explants per plate.

Cytokinins and basal medium formulations

In order to choose the best cytokinin/basal medium combination, two exploratory experiments were conducted. In the first, whole embryos were cultured on medium containing mineral salts, organics, aminoacids, and myo-inositol from the AE medium (VON ARNOLD & ERIKSSON 1981), along with N^6-Benzyladenine (BA), kinetin, or 2-Isopentenyl adenine (2-iP), indivi-dually added as growth regulators. All the cytokinins were applied at 1.0, and 5.0 µM. In the second experiment, the most effective cytokinin in inducing adventitious buds (namely, BA) was tested at a concentration of 5 µM with the following medium formulations, applied at full strength: AE, DCR (GUPTA & DURZAN 1985), QP (QUOIRIN & LE POIVRE 1977), and SH (SCHENK & HILDEBRANDT 1972). The media were chosen for their consistent differences in total ionic, ammonium and nitrogen concentrations.

Following these preliminary experiments, the AE formulation was utilized as the basal medium in all the later trials, and tested with either increasing BA concentrations (1, 2.5, 5.0, and 10 µM), or with the combination of two cytokinins (*i.e.*, BA plus 2-iP, and BA plus kinetin), both at 5 µM concentration.

Seed selection and pre-induction treatments

Due to the lack of uniformity in the size of the seeds, in part correlated to the embryo and megagametophyte dimensions, a specific experiment was carried out, in which whole embryos were isolated from seeds of two size categories, *i.e.* (1) below or equal to 1 cm, and (2) over 1 cm. Subsequently, in order to test the influence on adventitious budding of embryo endodormancy (that, in *Pinus cembra*, persists even at the moment of cone opening), a combination of pre-induction treat-ments was applied, before placing the embryos on the bud induction medium. The treatments were arranged as follows: (1) seeds were chilled at 4 °C for 4, 8, 12 weeks, or not chilled (control), before embryo excision; (2) embryos from chilled or unchilled seeds were pre-cultured for different periods (*viz.*, 0, 2, 6, and 8 days) on a hormone-free AE medium to stimulate the surmounting of endodormancy, before their transfer onto cytokinin-containing medium.

In both experiments, the bud induction medium was the AE formulation with 5µM BA.

Explant selection and duration of hormone application

In a separate set of experiments, the best medium selected (AE, plus 5µM BA) was used to determine the aptitude of various explants (namely, whole embryos, epicotyles with cotyledons, and hypocotyles) to produce adventitious buds. Embryos were isolated from both chilled (8 weeks) and unchilled seeds, and plated on the bud induction medium either intact or divided in two portions, cutting them just below the cotyledons. Chilled and unchilled whole embryos were also plated for increasing periods (*i.e.*, 7, 14, 21, 28, and 35 days) on a cytokinin-containing medium before being transferred onto a hormone-free medium, in order to identify the best time of exposure to BA for inducing adventitious buds.

Adventitous bud development, shoot elongation and rooting

Following the bud induction period (4 weeks, with the exception of the specific experiment on the duration of hormone application), the explants from all the above experiments were transferred to a hormone-free AE medium for a period of 3 weeks to promote the

development of the adventitious buds. To stimulate shoot elongation, the explants were then transferred and monthly subcultured in glass jars (ϕ 55 x h 60 mm), containing 25 ml 1/2-strength AE medium to which 0.1 % conifer-derived activated charcoal was added. In order to evaluate the rooting aptitude of the elongated shoots, 100 shoots (length 8 mm at least) were pulsed in liquid 1-mM indolebutyric acid (IBA) for 5 hours, prior to being transferred to hormone-free medium to promote the development of root primordia.

Data collection and statistical analysis

At the end of the induction phase (4 weeks) the percentage of explants forming buds was calculated. Only structures which had defined whorls of needles were counted as buds. After an additional 3-week period of bud development, plus 4 weeks of shoot elongation (*i.e.*, 11 weeks after the beginning of the experiments) the evaluated parameters were (i) the number of well-developed shoot buds per explant, (ii) the bud forming capacity index (BFC), calculated as: [(average number of buds per explant) × (% explant forming buds) / 100], and (iii) the percentage of shoots longer than 3 mm. Rooting was evaluated as percent of rooted shoots.

Statistical analysis of the percent data was carried out by the test for homogeneity of proportions, and significant treatment differences selected by a non-parametric statistical test, the Post Hoc Multiple Comparisons Test (MARASCUILO & MCSWEENEY 1977). Standard error (SE) was calculated for discrete data.

Slide preparation for histologic observations

Whole embryos plated on AE medium with 5 μM BA were sampled at days 0, 2, 4, 7, 10, 15, 20, 25, and 30, and fixed in a 4% solution of formaldehyde and glutaraldehyde (1:1) buffered with 0.05 M phosphate buffer at pH 6.8. The tissues were then dehydrated and embedded in LKB Historesin®, according to the method of YEUNG and LAW (1987). 3-μm-thick sections were then stained with the periodic acid-Schiff's reaction and counterstained with aniline blue black.

RESULTS

Adventitious bud induction and shoot development

Within the first week on a BA-containing AE medium, cultured embryos elongated slightly and turned red in the basal portion of the hypocotyl. In place of root development, the proliferation of a small amount of white-translucent callus was noticed just below the hypocotyl. Histologic observations showed that, after 4 days of culture, the 3–4 subepidermal layers of the embryonic tissue were made up of densely-packed, isodiametric cells, with enlarged and densely-stained nuclei. The cytoplasm, too, appeared dense and stained blue (Fig. 1, A). Various mitotic divisions were observed, spreading out in this area. During the 2nd week of culture, embryos started to turn green, while their cotyledonary and upper-hypocotyl part began to enlarge and to swell, due to the high proliferative activity of the subepidermal layers of cells. The first, isolated promeristemoids (*i.e.*, organized groups of 10–15 cells, anticlinally and periclinally dividing, precursors of adventitious buds) were observed after about 20 days of culture (Fig. 1, B). The prome-ristemoids gave rise quickly to meristematic domes (Fig. 1, C), and subsequently to adventitious buds (Fig. 1, D). The first well-developed adventitious buds were generally localized at the axils of adjacent cotyledons (Fig. 1, E.); then they spread out over the cotyledonary surface, especially the one in contact with the induction medium. When the regenerating embryos were transferred to the hormone-free AE medium for the following three weeks of culture, adventitious buds started to develop (Fig. 1, F) and to originate shoots. Further elongation of the *de-novo* formed shoots was stimulated by their transfer onto a 0.1%-activated charcoal-containing medium.

Effects of different cytokinins and medium formulations

Among the cytokinins tested in the preliminary experiment (viz., BA, kinetin and 2-iP), BA proved to be the best in terms of the percentage of responding explants after 4 weeks on the induction medium, particularly at the lower tested dose (Fig. 2, *top*). No significant difference between the two BA concentrations was observed. However, because of the better appearance of the explants cultured on the 5 μM-BA medium, this concentration was chosen to compare the four basal medium formulations. Among the tested media, a comparable response was observed; nevertheless, a peak of 94% regenerating explants was reached when embryos were cultured on AE medium (Fig. 2, *bottom*).

The diverse concentrations of BA influenced bud induction and shoot development differently (Table 1). While the percentages of explants forming buds did not show any statistical difference among the tested BA concentrations, both the average number of buds per explant and the BFC index were consistently higher when the 2.5 μM dose was apllied. However, the relative shoots showed stunted growth, even after being transferred to an activated charcoal-added medium. In the same condition, almost 24% of the shoots from embryos cultured on a 5 μM-BA medium were longer than 3 mm. No consistent improvement of adventitious budding was observed when BA was applied in combination with 2-iP or kinetin (5μM).

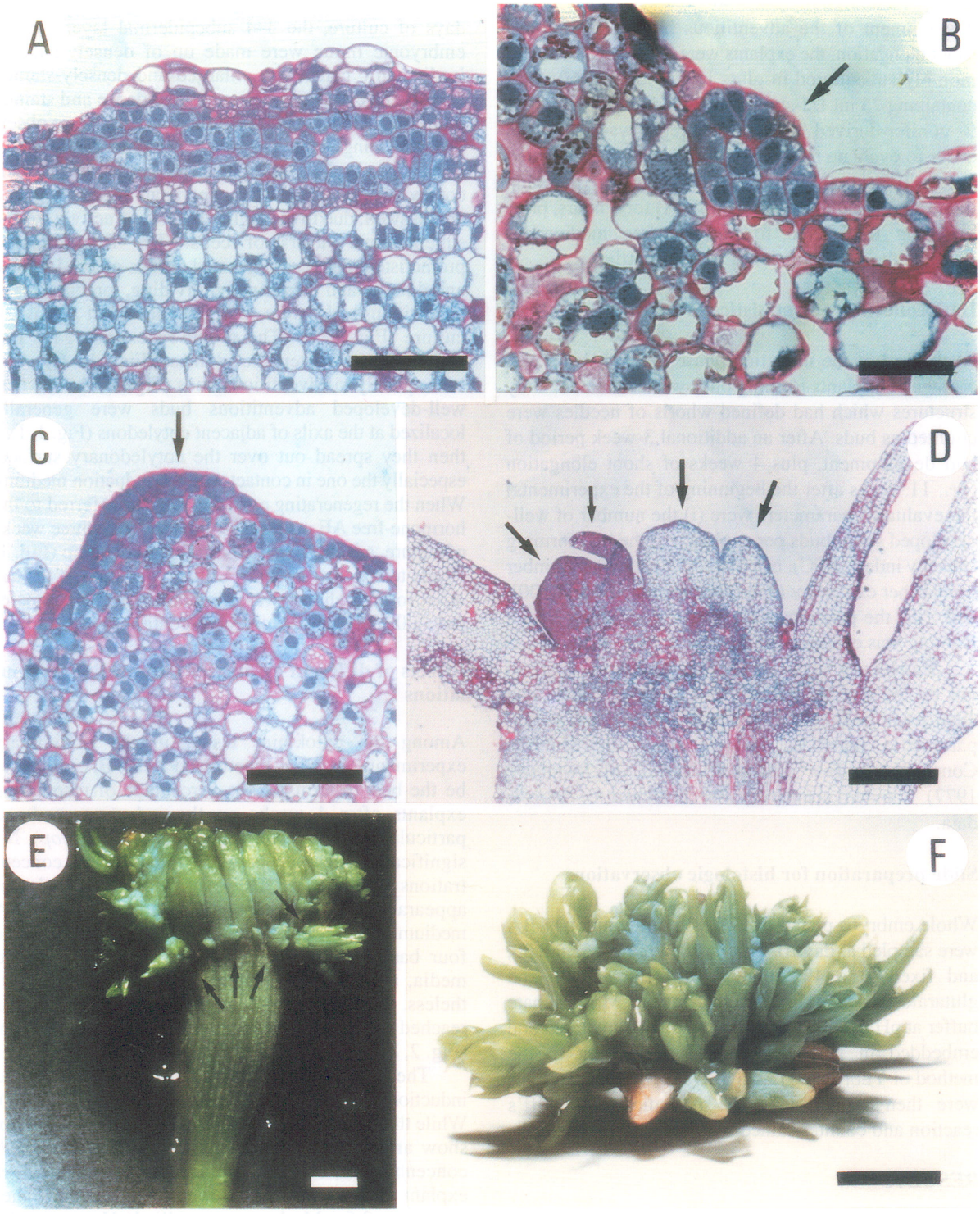

Figure 1 A – day 4 on the induction medium. The cells of the 3–4 subepidermal layers of the embryonic tissue show densely-stained cytoplasm and enlarged nuclei. Anticlinally and periclinally dividing mitotic figures are recognizable in this area (bar – 0.1 mm). **B** – day 20. A 15-cell meristemoid (*arrow*) located at the base of a cotyledon (bar – 0.05 mm). **C** – day 25. Meristemic dome of a developing adventitious bud (*arrow*) , showing a distinct epidermal layer (bar – 0.1 mm). **D** – day 30. Multiple adventitious buds (*big arrows*) arising in the axillary region of adjacent cotyledons. A cataphyll (*small arrow*) is developing from the basal part of a bud (bar – 0.5 mm). **E** – the first adventitious buds arise generally at the axil of cotyledons (*arrows*), perpendicularly to the embryo main axis (bar – 1 mm). **F** – abundant adventitious bud proliferation from a portion of embryo explant (bar – 1 mm)

Table 1 Effects of different concentrations and combinations of BA on the induction and development of adventitious buds from embryo explants of *Pinus cembra*

Induction treatments [1]	Explants with buds (%) [2]	Buds per explant ($\bar{x}$ ± SE) [3]	BFC index [4]	Shoots > 3 mm (%) [2]
BA alone				
1.0 µM	67.6[a]	7.4 ± 1.7	5.0	22.1[a]
2.5 µM	75.0[a]	16.3 ± 5.2	12.2	9.3[b]
5.0 µM	80.2[a]	8.3 ± 1.6	6.6	23.9[a]
10.0 µM	61.2[a]	14.1 ± 2.3	8.6	15.8[a]
BA in combination				
BA + 2-iP (5µM)	35.4[a]	13.8 ± 3.2	4.9	22.8[a]
BA + Kinetin (5µM)	58.8[b]	18.6 ± 2.3	10.9	22.2[a]

[1] Basal medium: AE + 3 % sucrose + 0.8 % Difco Bacto-agar. Data collected after 4 weeks for the percentage of explants with buds, and after 11 weeks for all the other parameters.
[2] Within each box, percentages followed by different letters are significantly different at $P \leq 0.05$ (Post Hoc Multiple Comparisons Test).
[3] SE = standard error.
[4] BFC index = [(average N° of buds per explant) × (% explants forming buds)/100].

Table 2 Adventitious bud induction on different embryonic explants of *Pinus cembra*, excised from 8-week chilled, and unchilled seeds

Seed chilling & explants [1]	Explants with buds (%) [2]	Buds per explant ($\bar{x}$ ± SE) [3]	BFC index [4]	Shoots > 3 mm (%) [2]
Chilling 0				
Whole embryo	64.0[a]	25.7 ± 4.9	16.4	14.4
Epicotyl	0.0[b]	—	—	—
Hypocotyl	0.0[b]	–	—	—
Chilling 8 wk				
Whole embryo	56.5[a]	12.7 ± 1.1	7.2	27.4[a]
Epicotyl	6.6[b]	15.5 ± 0.5	1.0	19.3[a]
Hypocotyl	4.5[b]	3.0 ± 0.1	0.1	66.6[a]

[1] Seeds were chilled for 8 weeks, or unchilled, afterwhich whole embryos, epicotyls, hypocotyls were cultured onto the induction medium (AE + 5 µM BA). Data collected after 4 weeks for the percentage of explants with buds, and after 11 weeks for all the other parameters.
[2] Within each box, percentages followed by different letters are significantly different at $P \leq 0.05$ (Post Hoc Multiple Comparisons Test).
[3] SE = standard error.
[4] BFC index = [(average N° of buds per explant) × (% explants forming buds)/100].

In view of the above results, the AE formulation, supplemented with 5 µM BA, was chosen as bud inducing medium in all the subsequent experiments.

Influence of seed size and pre-induction treatments

Embryos from seeds longer than 1 cm, cultured on AE medium containing 5 µM BA, regenerated better than those from seed under 1 cm (Fig. 3). Indeed, both evaluated parameters (percent of explants forming buds, and the average number of buds per explant) increased significantly when embryos from longer seeds were utilized (respectively, 41% vs 16%, and 10 vs 6). As a consequence, the BFC index was consistently higher as well (4.3 vs 1.1).

Chilling the seeds for increasing time, prior to culturing the excised embryos, induced contrasting effects (Table 3). While explants from unchilled seeds gave the highest percentage of regeneration (59%), the average number of buds per explant and the BFC index

Table 3 Effects of combination of pre-induction treatments on adventitious budding from embryo explants of *Pinus cembra*

Pre-induction treatments [1]	Explants with buds (%) [2]	Buds per explant ($\bar{x}$ ± SE) [3]	BFC index [4]	Shoots > 3 mm (%)[2]
Chilling 0 ($\bar{x}$)	**59.3^{A}**	**10.2 ± 0.9**	**6.0**	**52.9^{A}**
Days 0	81.8^{a}	14.1 ± 1.1	11.5	44.0^{a}
" 2	36.3^{b}	11.5 ± 2.2	4.1	47.8^{ab}
" 6	60.8^{ab}	8.9 ± 1.7	5.4	63.0^{b}
" 8	58.3^{ab}	6.3 ± 1.1	3.6	68.2^{b}
Chilling 4 wk ($\bar{x}$)	**35.7^{B}**	**9.3 ± 1.3**	**3.5**	**48.2^{A}**
Days 0	47.8^{a}	15.5 ± 3.4	7.4	30.9^{a}
" 2	26.1^{a}	8.7 ± 1.9	2.3	65.5^{b}
" 6	37.5^{a}	7.8 ± 2.1	2.9	56.4^{b}
" 8	31.8^{a}	5.0 ± 1.3	1.6	62.8^{b}
Chilling 8 wk ($\bar{x}$)	**47.0^{A}**	**14.0 ± 2.0**	**6.4**	**31.2^{B}**
Days 0	70.0^{a}	12.3 ± 2.3	8.6	49.6^{a}
" 2	41.2^{a}	16.2 ± 3.1	6.7	21.0^{b}
" 6	31.2^{a}	10.2 ± 4.0	3.2	18.0^{b}
" 8	40.0^{a}	17.6 ± 6.8	7.0	29.5^{b}
Chilling 12 wk ($\bar{x}$)	**47.1^{A}**	**17.1 ± 2.7**	**8.0**	**25.8^{B}**
Days 0	70.0^{a}	13.3 ± 4.1	9.3	55.0^{a}
" 2	40.0^{a}	25.4 ± 6.5	10.2	11.2^{c}
" 6	53.8^{a}	16.6 ± 2.4	8.9	34.5^{b}
" 8	33.3^{a}	13.4 ± 6.7	4.5	18.1^{c}

(1) Seeds were chilled for 4, 8, 12 weeks, or unchilled, and excised embryos were pre-cultured for 0, 2, 6, and 8 days on hormone-free medium, before their transfer onto the induction medium (AE + 5 µM BA). Data collected after 4 weeks for the percentage of explants with buds, and after 11 weeks for all the other parameters;

(2) Within each chilling treatment, percentages followed by different lower-case letters are significantly different at $P \leq 0.05$. Upper-case letters refer to the separation of the chilling treatment total percentages (Post Hoc Multiple Comparisons Test);

(3) SE = standard error;

(4) BFC index = [(average N° of buds per explant) × (% explants forming buds)/100].

were positively affected by pre-treating the seeds at low temperature for at least 8 weeks. However, after 11 week of culture, the percentage of shoots longer than 3 mm was significantly higher when the shoots originated from unchilled or 4-week chilled embryos.

After each chilling treatment, embryos were pre-cultured on a hormone-free AE medium for different periods of time (0, 2, 6, or 8 days) before culturing them on the bud induction medium. Although the explants cultured directly onto the regeneration me-dium (indicated in Table 3 as '0') always produced a higher frequency of response, those excised from the 12-week chilled seeds and pre-cultured for 2 days produced the maximum response in terms of the number of shoot buds per responding explant (25.4). However, non-precultured embryos from unchilled seeds (*i.e.*, embryos of the '0/0' combination) gave the highest value of BFC (11.5). Data on the percentages of shoots longer than 3 mm did not support any specific relation with the embryo pre-culture treatments.

Effect of explant type, and time of hormone application

Intact embryos proved to be the most suitable explant for adventitious bud induction, when compared with both epicotyl, and hypocotyl explants (Table 2). Indeed, there was no regeneration from epicotyls and hypocotyls of unchilled embryos, while poor adventitious budding was observed when the same explants came from 8-week chilled seeds.

To ascertain the duration of BA requirement in the bud induction medium, for a total period of 35 days intact embryos were transferred weekly from the BA-containing medium to the hormone-free medium, and evaluated for adventitious budding (Table 4). Although the application of BA for as little as 7 days was suffi-

Table 4 Effects of time of exposure to BA on adventitious bud induction from embryo explants of *Pinus cembra*, excised from 8-week chilled, and unchilled seeds

Seed chilling & explants [1]	Explants with buds (%) [2]	Buds per explant ($\bar{x}$ ± SE) [3]	BFC index [4]	Shoots > 3 mm (%)[2]
Chilling 0				
7 days	53.3^{a}	9.0 ± 1.3	4.8	53.3^{a}
14 "	95.6^{bc}	11.4 ± 1.6	10.9	36.0^{ab}
21 "	100.0^{b}	16.4 ± 2.8	16.4	25.0^{b}
28 "	81.8^{ac}	13.1 ± 2.2	10.7	30.5^{b}
35 "	87.5^{ab}	13.0 ± 1.2	11.4	32.3^{b}
Chilling 8 wk				
7 days	60.0^{a}	7.2 ± 2.0	4.3	50.0^{a}
14 "	63.6^{a}	15.8 ± 3.6	10.0	35.8^{a}
21 "	66.6^{a}	9.2 ± 2.6	6.1	26.1^{ab}
28 "	71.4^{a}	13.7 ± 2.4	9.9	13.8^{bc}
35 "	100.0^{a}	11.5 ± 1.5	11.5	6.5^{c}

(1) Embryos from 8-week chilled, and unchilled seeds were cultured onto the induction medium (AE + 5 µM BA) for 7, 14, 21, 28, or 35 days, prior to be transferred to the hormone-free AE medium. Data collected after 4 weeks for the percentage of explants with buds, and after 11 weeks for all the other parameters.

(2) Within each box, percentages followed by different letters are significantly different at $P \leq 0.05$ (Post Hoc Multiple Comparisons Test).

(3) SE – standard error.

(4) BFC index – [(average N° of buds per explant) × (% explants forming buds)/100].

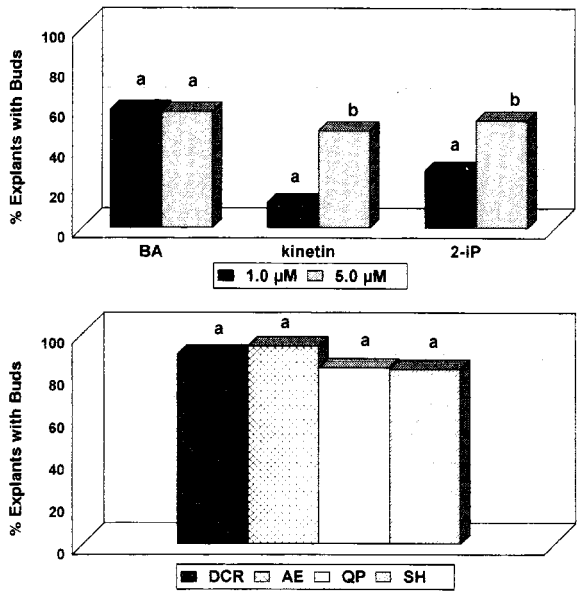

Figure 2 Effects of different cytokinins (*top*) and medium formulations (*bottom*) on adventitious bud induction from embryo explants of *Pinus cembra* (percentage labelled with different letters significantly different at P ≤ 0.05 by Post Hoc Multiple Comparisons Test)

cient to elicit some response in the embryos from both unchilled and 8-week chilled seeds, the number of buds per explant and the BFC index were both very low. However, exposure of the unchilled explants to BA for 21 days was sufficient to induce 100% regenerating embryos, and the highest values of buds per explant and BFC index. Shoot growth was statistically lower only to that obtained with the 7-day exposure. With 8-week chilled seeds, 100% regeneration was reached when the embryos were cultured for 35 days on the BA-containing medium, but all the other parameters were consistently lower than those of the 21-day cultured embryos from unchilled seeds.

Shoot elongation and rooting

Cutting the regeneration explants into 2–3 pieces, prior to putting them in contact with an activated charcoal-added medium, induced a slow elongation of the *de-novo* formed shoots. Indeed, after three monthly subcultures, shoots were on average 5–10 mm high. The rooting process, too, was very slow in Swiss stone pine. Only 15% of the IBA pulsed shoots produced adventitious roots after 8 weeks of culture, while remaining shoots callused profusely at their base and failed to root.

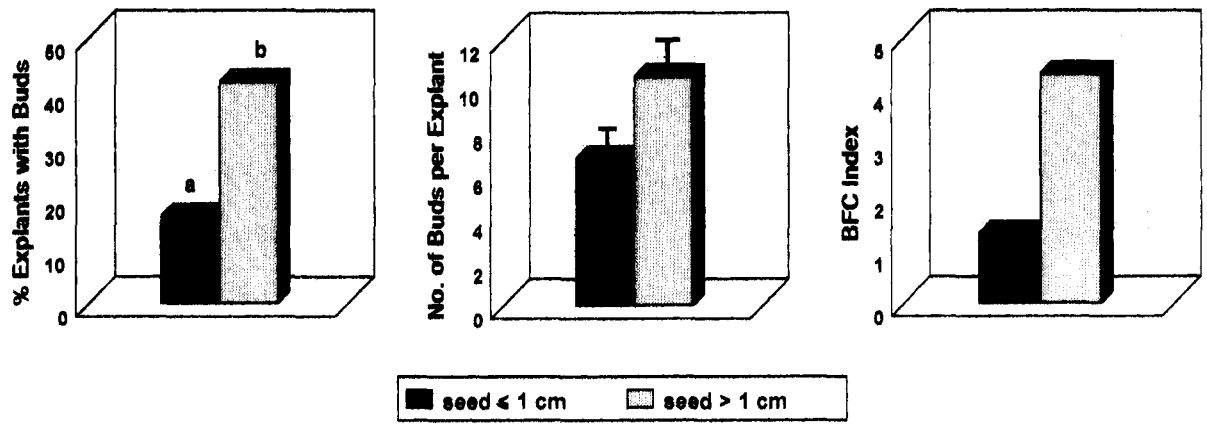

Figure 3 Adventitious bud induction and development on embryonic explants of *Pinus cembra*, excised from seeds of different length (percentages labelled with different letters are significantly different at $p \leq 0.05$ by Post Hoc Multiple Comparison tests; bars represent the standard error).

DISCUSSION

This study confirms that the organogenic capacity of *Pinus* sp. pl. embryonic explants is strongly influenced by several factors, among which those relating to the culture conditions during bud induction play a major role. In conifers, adventitious buds arise from embryonic tissue generally when the medium is supplemented with exogenous cytokinins. Among these, BA repeatedly proved to be the most effective (MARTINES PULIDO *et al.* 1990; LAMBARDI *et al.* 1993; HARRY *et al.* 1994, CAPUANA & GIANNINI 1995). In Swiss stone pine, BA produced a higher percentage of regenerating embryos in comparison to kinetin and 2-iP, especially at the dose of 1μM. Upon comparing increasing concentrations of the hormone, better results were observed, in terms of buds per explant and BFC index, when BA was applied at 2.5 μM, but the subsequent development of the shoots was insufficient. On the contrary, adventitious shoots appeared more vigorous, and they elongated faster when BA was used at 5μM. The combination of two cytokinins, which at times improved adventitious shoot elongation, *e.g.* in *Pinus caribaea* (WEBB & DIAZ SANTIAGO 1983), did not have a similar effect in *Pinus cembra.* These results are consistent with previous observations in *Pinus canariensis* (MARTINEZ PULIDO *et al.* 1990), and *Pinus halepensis* (LAMBARDI *et al.* 1993).

It has been repeatedly reported that a greater amount of adventitious buds can be induced when isolated parts of the embryo (viz. cotyledons, epicotyl, hypocotyl) are cultured in a cytokinin-containing medium, rather than the whole embryo. This was the case, for example, with *Pinus monticola* (MOTT & AMERSON 1981), *Pinus canariensis* (MARTINEZ PULIDO *et al.* 1990), *Pinus pinea* (CAPUANA & GIANNINI 1995). As with *Pinus resinosa* (NOH *et al.* 1988), and *Pinus halepensis* (LAMBARDI *et al.* 1993), in *Pinus cembra* it was difficult to separate individual cotyledons from relatively small embryos without damaging tissue, and poor adventitious budding was obtained when isolated hypocotyls and epicotyls were cultured. It has been reported that as little as one day (NOH *et al.* 1988) or a three day exposure to BA (BIONDI & THORPE 1982) can be sufficient to elicit the production of adventitious buds. However, some weeks are generally required to achieve best results in terms of number of well-developed shoots per explant. Up to 8 weeks of exposure to cytokinin-containing medium were necessary for *Pinus caribaea* (WEBB & DIAZ SANTIAGO 1983), and *Pinus ponderosa* (ELLIS & BILDERBACK 1984). However, with the majority of *Pinus* species, explants need to stay 2–4 weeks in contact with cytokinins to produce many adventitious buds that will subsequently develop satisfactorily (*e.g.* MARTINEZ PULIDO *et al.* 1990; BRONSON & DIXON 1991; LAMBARDI *et al.* 1993). Indeed, a 21-day exposure to 5μM BA was effective in inducing 100% regenerating explants and the highest number of buds per explant on unchilled embryos of Swiss stone pine. As previously observed in *Pinus halepensis* (LAMBARDI *et al.* 1993), a shorter exposure time (7–14 days) produced less adventitious shoots, but their growth was moderately accelerated.

An interesting finding of this study was that, unlike the culture factors, the tested pre-induction treatments (seed chilling, and embryo pre-culture in hormone-free medium) only slightly affected adventitious budding. One can hypothesize that the proliferative events stimulated on the embryo surface by the presence of cytokinins in the medium are practically unconnected with the level of embryo maturity and endodormancy.

In conclusion, this study showed that the induction of adventitious buds on *in vitro* cultured embryos of Swiss stone pine can be succesfully achieved, and that the phenomenon is strongly modulated by several culture factors. However, as reported for numerous other conifers (see MOHAMMED & VIDAVER 1988), rooting can represent a major problem that needs to be better investigated, before a complete micropropagation procedure can be formulated.

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