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In vitro regeneration of plantlets from mature embryos of *Pinus ayacahuite*

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Summary A plantlet regeneration protocol was developed for *Pinus ayacahuite* var. *ayacahuite* (Ehrenb.). Embryos from mature seeds from ten provenances were cultured in a 16-h photoperiod for 3 days on a medium containing 30 mM sucrose and 0.7% agar. Cotyledons from these embryos were subcultured onto MCM medium (Bornman 1983) supplemented with 50 μM N^6-benzyladenine and 90 mM sucrose for 2 weeks. Bud development and shoot elongation were maximized by subculturing the explants on half strength AE medium (von Arnold and Ericksson 1981), supplemented with 60 mM sucrose and 0.05% activated charcoal every 30 days. Seed source had a significant effect on the responses of the embryos to the bud induction protocol. For the provenance with the best response to bud induction, about 79% of the cultured cotyledons formed buds, and each cotyledon formed a mean of 9.1 buds, so that about 70 shoots could be induced from each seed. The best rooting response (40% rooting) was obtained by treating the shoots for 8 h with 100 μM naphthalene acetic acid.

Keywords: conifer, explant, hyperhydricity, micropropagation, organogenesis, pine, provenances.

Introduction

Pinus ayacahuite var. *ayacahuite* Ehrenb. (Mexican white pine) is found at elevations of 1800 to 3200 m above sea level in El Salvador, Guatemala, Honduras and Mexico. The species, which grows on fertile alfisols and andisols where soil water is seldom limiting (Dvorak and Donahue 1992), is becoming endangered because its wood is used extensively for rustic furniture, roofing shingles, wood carvings and firewood and because expansion of crop industries and cattle grazing has led to deforestation (Donahue et al. 1991).

Pinus ayacahuite is propagated from seeds; however, the seedlings need a long nursery period before they can be transferred to the field, because they abruptly enter a resting stage at a shoot height of approximately 100 mm. In addition, when planted in the field, the seedlings remain in this pseudo-grass stage for 3 to 4 years before exhibiting the rapid shoot growth spurt that typifies seedlings of most pine species (W.S. Dvorak, unpublished results). Difficulties in the cultivation of this species resulting from the long seedling phase are compounded by the difficulties in achieving genetic improvement by conventional tree breeding methods, which are generally long-term and laborious (Mohammed et al. 1986, Thorpe and Patel 1986). As an alternative, vegetative propagation of mature trees is also difficult and has a low success rate (Thorpe and Harry 1990).

In vitro techniques may bypass the problems associated with irregular seed cone production, long life cycles and vegetative propagation. *In vitro* clonal propagation can be used to produce plantlets of desirable genotypes, thereby accelerating the selection, breeding and testing cycle (Thorpe et al. 1991). It also complements field conservation banks and seed banks. Finally, emerging methods of recombinant DNA technology in combination with clonal propagation could create opportunities to modify the conifer genome, e.g., for disease resistance (Attree et al. 1991).

Plantlet regeneration in conifers occurs by organogenesis and somatic embryogenesis, with the former being the predominant route (Thorpe et al. 1991). Plantlet formation by organogenesis is a multistaged process consisting of several distinct steps that include: (1) establishment or bud induction, or both; (2) bud and shoot development and multiplication; (3) rooting of developed shoots; and (4) hardening of plantlets. The objective of this study was to develop a protocol for plantlet regeneration of *P. ayacahuite* by the organogenic route, based on the empirical approach outlined by Thorpe and Patel (1984), in which the various factors known to be involved in *in vitro* organogenesis are systematically examined.

Materials and methods

Plant material

Seeds of *Pinus ayacahuite* (Mexican white pine) were used as the source of mature embryos for all of the experiments. Seeds

were obtained through the Central America and Mexico Coniferous Resources Cooperative (CAMCORE), from 10 locations in Honduras, Guatemala and Mexico that extend over an area of more than 5° in latitude and a wide range of altitudes to almost 1000 m (Table 1). The seeds also varied in shape, color and size. Because only a small sample of seeds from each provenance was available, seeds were pooled, based on their geographic location, into three groups by mixing an equal amount of each provenance in its respective group (Table 1). Only seeds of Group 1 were used for the experiments, but the response of each provenance to the protocol developed was tested at the end of the study.

Establishment of sterile culture and culture conditions

The most efficient procedure for sterilizing Mexican white pine seeds was to hydrate the seeds for 48 h under cold running tap water and then sterilize them with a solution of 30% commercial bleach (6% NaOCl) and four drops of Tween per 100 ml for 15 min. The seeds were then rinsed three times with sterile water and stratified in sealed sterile petri dishes for 7 days at 4 °C. Stratified seeds were sterilized for 15 min followed by three rinses with sterile water. The seed coats were removed with forceps under sterile conditions in a laminar flow hood, and the megagametophytes were resterilized for 15 min. Following three rinses with sterile water, the embryos were placed on a sucrose medium consisting of 30 mM sucrose and 0.7% agar (Difco Bacto-agar) for 3 days, after which the cotyledons were removed and placed on bud induction medium + benzyladenine (BA). Because germination was not synchronous, it was necessary to culture the dissected embryos on sucrose medium to obtain enough embryos at a similar stage (Muriithi et al. 1993). Only hard, well-hydrated embryos were used for the experiments.

Except where noted otherwise, the pH of the medium used was adjusted to 5.7 ± 0.1 before autoclaving at 121 °C and 20 to 25 ml of medium was poured into each sterile plastic petri plate (90 × 15 mm). After inoculation, petri dishes were sealed with household plastic wrap and maintained at 25 ± 1 °C in a 16-h photoperiod provided by Sylvania Gro-Lux F40T12 Gro-WS lights at a photon flux of 60–80 μmol m^{-2} s^{-1}.

Table 1. Classification of seeds of *Pinus ayacahuite* according to their geographical distribution.

	Provenances	Latitude /longitude range	Geographical zone
Group 1	Chuipachec Palestina Las Trancas Plan del Rancho Buenavista El Porvenir	14°07′ to 16°44′ N and 87°49′ to 92°36′ W	Southern Mexico Guatemala Honduras
Group 2	Ixtlán Nundaco Concepción	17°09′ to 17°50′ N and 96°21′ to 97°38′ W	Central Mexico Oaxaca
Group 3	Los Cubes	20°23′ N, 98°24′ W	Northern Mexico Hidalgo

Bud induction

The effects of conditions known to affect bud induction (e.g., hormone concentration, exposure time to the hormones, age of explant, and salt formulation) were analyzed in separate experiments, following published procedures (Thorpe and Patel 1984).

Unless otherwise specified, the following conditions were used for all experiments. For bud induction, the cotyledons were cultured for 15 days on bud induction medium consisting of MCM (Bornman 1983) supplemented with 10 μM N^6-benzyladenine (BA) and 90 mM sucrose. The explants were then transferred to bud development medium consisting of MCM medium containing 60 mM sucrose but no hormones for 30 days. For bud growth and shoot elongation, the explants were subcultured every 30 days on half strength MCM (half strength of the major salts) supplemented with 60 mM sucrose and 0.05% activated charcoal (Sigma No. C4386). Day 0 refers to the time when the explants were transferred to bud induction conditions.

Explant selection In preliminary experiments, sterile megagametophytes were placed on sucrose medium in a 16-h photoperiod for 0, 1, 4, or 7 days. Subsequently, whole embryos were cultured on bud induction medium for 15 days and then transferred to bud development medium for 30 days. Ten embryos were used per treatment, and they were evaluated at Day 45. In a parallel experiment, cotyledons from embryos growing for 3 days on sucrose medium were cultured under bud induction and development conditions and evaluated at Day 45.

Effect of stratification Sterile, hydrated seeds from Los Cubes provenance were stored for 0, 1, 9 or 16 days at 4 °C. After stratification, the embryos were treated as specified above. Cotyledons were evaluated at Days 45 and 75.

Medium Four basal media were tested at both full and half strength of their macronutrient composition: SH (Schenk and Hildebrandt 1972, as modified by Reilly and Washer 1977), MCM, QP (Quoirin and LePoivre 1977), and AE (von Arnold and Ericksson 1981). After bud induction, at Day 15, each treatment was transferred to the corresponding modified bud development medium containing half strength of the major salts and cultured for 30 days. Subsequent transfers were made every 30 days to the same medium containing 0.05% of activated charcoal. Explants were evaluated at Days 45 and 75.

Effect of BA concentration Cotyledons of 3-day-old cultured embryos were treated with BA at 0, 1, 5, 10, 50, 100, 500 or 750 μM for 15 days. Cotyledons were then transferred to bud development medium for 30 days. Thereafter, the explants were subcultured every 30 days on AE medium at half strength of the major salts, instead of MCM medium. Cotyledons were evaluated at Days 45, 75 and 105.

Effect of BA exposure time Cotyledons from 3-day-old embryos were cultured on bud induction medium supplemented with 50 μM of BA for 0, 7, 14, 21 or 28 days. Cotyledons were then cultured as specified for the BA concentration experiment. Cotyledons were evaluated at Days 45, 75 and 105.

Effect of explant age To evaluate the effect of explant age on bud induction, cotyledons from embryos growing on sucrose medium for 0, 1, 2, 3, 4, 6, 8 or 12 days were compared. Fifty μM BA was used for bud induction. Cotyledons were then cultured as specified for the BA concentration experiment, and evaluated at Days 45, 75 and 105.

Provenances Seeds from each of the provenances were compared for their response to bud induction and development. Bud induction medium was MCM at full strength supplemented with 50 μM BA for 15 days. Cotyledons were then cultured as specified for the BA concentration experiment and evaluated at Day 105.

Bud growth

Medium The bud growth medium refers to the medium used from Day 45 until the shoots reached 0.5 cm and were separated from the cotyledons. The same media used for bud induction were tested, but only at half strength of the major salts, and in the presence of 0.05% activated charcoal and 60 mM sucrose. Explants were evaluated at Days 75 and 105, i.e., after 30 and 60 days of culture on bud growth medium, respectively.

Sucrose concentration Cotyledons were cultured for 15 days on bud induction medium (MCM medium, supplemented with 90 mM sucrose and 10 μM BA), and then transferred to bud development medium consisting of MCM medium supplemented with 30, 60 or 90 mM sucrose for the following 30 days. Thereafter, the cotyledons from each treatment were transferred to half strength AE supplemented with 0.05% activated charcoal and 30, 60 or 90 mM sucrose for the following 60 days, with subculture to fresh medium after 30 days.

Shoot elongation

Medium For shoot elongation, four basal media (AE, QP, SH and MCM) were tested at half strength of the major salts. Each medium was supplemented with 60 mM sucrose and 0.05% activated charcoal, and the explants were subcultured every 30 days. Shoots were planted in sterile glass storage jars (100 × 80 mm) containing 75 ml of agar-solidified medium. During subculture, the base of the stem was cut to remove any callus formation. The shoots used were those that were already separated from the cotyledon and were 2 cm tall. Shoot growth and physical appearance were evaluated 30, 60, 90 and 120 days after initiation of the experiment.

Hyperhydricity We examined the effect of using 0.5% Gelrite® (Scott Labs., Carson, CA) in place of 0.7% agar on the development of shoot hyperhydricity. Two-cm shoots were cultured on half strength MCM supplemented with 60 mM sucrose.

Rooting

Gelrite treatment For root induction, 2 cm shoots were cultured on one-quarter strength of MCM medium (1/4 MCM, pH 5.0) containing 100 μM of indolebutyric acid (IBA), 30 mM sucrose and 0.4% Gelrite for 0 to 12 days and then transferred to peat:vermiculite (1:1). In another experiment, shoots were cultured for 9 days under the above conditions, and then transferred to medium solidified with 0.4% Gelrite. In each case, treatments were replicated four times, and each replicate consisted of six plants.

Liquid treatment of shoots A filter-sterilized solution (pH 5.0) of 1 mM IBA, naphthalene acetic acid (NAA) or indoleacetic acid (IAA) was used to immerse the bases of the shoots for 0, 4, 8, 12 or 24 h. The shoots were then placed in half strength AE medium (pH 5.0) supplemented with 60 mM sucrose and solidified with 0.4% Gelrite.

Shoots were also incubated in a solution (pH 4.5) containing 100 μM NAA or 1 mM IBA for 0, 2, 4 or 8 h, and then transferred to half strength GD medium (Gresshoff and Doy 1972) supplemented with 30 mM sucrose, 0.05% activated charcoal, and 1% agar.

Incubation of shoots with auxins Shoots were incubated for 15 days with either IBA at 4, 20, 100, or 500 μM or NAA at 0.8, 4, 20 or 100 μM, or with a combination of both hormones, 20 μM IBA plus 0.8 or 4.0 μM NAA for 15 days. The root induction medium was half strength GD medium containing 60 mM sucrose solidified with 0.7% agar. For root development and elongation, the treated shoots were transferred to half strength GD medium containing 60 mM sucrose, 0.05% activated charcoal and 1% agar, but no hormones. The pH of the medium for both stages was 4.5 before autoclaving. Each treatment was replicated twice and each replicate consisted of five shoots. All rooting treatments were evaluated after a total of 60 days.

Data analysis

All data were subjected to an analysis of variance (ANOVA) based on a complete randomized experimental design. Means were separated by LSD for an equal number of replications as described by Little and Hills (1978).

The bud induction results were evaluated based on the percentage of responding explants and the mean number of buds per explant at Days 45, 75, 105 or 195 of culture. Although our aim was to increase both the number of explants responding and the mean number of buds per explant, in many instances the two parameters were negatively correlated and decisions based on one parameter were often inaccurate. For this reason the bud formation capacity (BFC) index and the shoot elongation capacity (SEC) index were used. These indices incorporate both parameters and often were used to determine the best treatment. Standard errors of the means are presented in each Table.

$$\text{BFC index} = (\text{Mean no. of buds per cotyledon forming buds}) (\%\ \text{cotyledons forming buds})/100.$$

SEC index = (Mean no. of buds of a specific size per cotyledon forming buds)(% cotyledons forming buds)/100.

All experiments, which were repeated at least twice, consisted of at least three replications (plates or jars). Each replication had nine explants. When cotyledons were used as explants, the eight or nine cotyledons of each embryo were distributed randomly among the treatments, giving one cotyledon per treatment.

Results and discussion

Aseptic culture establishment

No contamination was observed if intact seeds were treated with 10 to 50% commercial bleach for 15 min before being cultured on sucrose medium. If the seed coat was removed and the megagametophytes were cultured, some bacterial contamination was observed; however, if the megagametophytes were also sterilized, 100% sterile embryos were obtained. Therefore, the double sterilization procedure—30% commercial bleach for 15 min, followed by three rinses with sterile water—was adopted for the remaining experiments.

Adventitious bud induction

Explant selection When explants of cotyledons and embryos were cultured in the presence of 10 μM BA for 15 days, bud formation always occurred on the side of the explant in contact with the medium. However, as noted for *Pinus radiata* D. Don (Aitken et al. 1981), cotyledon explants had several advantages over embryo explants. First, because buds formed only at the surface of the explant in contact with the medium, and because a larger surface area is exposed when cotyledons are dissected from the embryo, more shoots were obtained per seed when cotyledons rather than entire embryos were used (means of 40 versus 25). Second, most seeds had at least eight cotyledons, which could be distributed among the various treatments. This reduces variability, because most cotyledons from the same embryo behave alike when cultured under the bud induction conditions (data not shown). Therefore, we used cotyledons as explants for the following experiments.

Medium selection The MCM at full strength produced the highest mean number of buds per cotyledon, and it also yielded the highest percentage of cotyledons forming buds (Table 2). Shoot elongation was also highest at Day 45 with this medium, as reflected by the SEC index. However, although full strength MCM was the best medium for bud induction, it was probably not the best medium for bud elongation. For example, at Day 75, half strength SH had the highest SEC index, and at Days 45 and 75 the mean number of buds per explant was almost the same as for MCM (Table 2).

Explant age Lu et al. (1991) reported that explant age affects bud induction. Embryos transferred directly to bud induction medium or left on sucrose medium for 6 or more days before being transferred to bud induction medium showed a significant decline in the number of buds produced per cotyledon compared with embryos cultured for 2 to 4 days on sucrose medium before being transferred to bud induction medium (Table 3). The percentage of cotyledons forming buds and the BFC showed similar patterns.

The cotyledons elongated with time, and cotyledons from 1.24 to 1.44 mm in length produced the most buds. These cotyledons corresponded to embryos maintained on sucrose medium for 2 to 4 days. Cotyledons from embryos cultured for 3 days on sucrose medium produced the most buds, about eight buds per cotyledon by Day 105 (Table 3). Although some

Table 2. Effect of full and half strength (1/2) salt concentration on induction and elongation of buds on cotyledons of *P. ayacahuite*. Cotyledons were harvested from 3-day-old embryos (Day 0) growing on a sucrose (30 mM) and agar (0.7%) medium, and cultured for 15 days on the medium specified (treatments) supplemented with 10 μM BA and 90 mM sucrose. At Day 15 each treatment was transferred to the same medium formulation for 30 days, but using only half strength of the major salts, without BA and with 60 mM sucrose. Subsequent transfers were made every 30 days, onto the latter medium, supplemented with 0.05% of activated charcoal. Explants were evaluated at Day 45 and Day 75. Values are means ± SE ($n = 81$). Means followed by the same letters within columns are not significantly different at $P \leq 0.01$ according to the Least Significant Difference Test.

Media[1]	% CFB[2]	No. of buds per cotyledon Day 45[3]	BFC index Day 45	SEC index > 0.3 cm Day 45	No. of buds per cotyledon Day 75[3]	SEC index > 0.5 cm 75 days
MCM	66.6	3.5 ± 0.9 a	2.28	0.38	3.9 ± 0.8 a	0.53
1/2 MCM	61.1	2.2 ± 0.6 b	1.34	0.18	3.5 ± 1.0 ab	0.41
AE	55.5	1.8 ± 0.7 b	1.06	0.24	3.3 ± 1.3 ab	0.36
1/2 AE	51.8	1.8 ± 0.7 b	1.02	0.05	3.7 ± 0.9 ab	0.21
SH	42.6	1.8 ± 0.6 b	0.85	0.04	2.5 ± 1.1 b	0.24
1/2 SH	57.4	2.4 ± 0.6 b	1.38	0.02	3.4 ± 0.8 ab	0.58
QP	25.9	0.9 ± 0.3 cd	0.26	0.02	1.0 ± 0.5 c	0.05
1/2 QP	22.9	0.6 ± 0.2 d	0.13	0.00	1.0 ± 0.2 c	0.00

[1] MCM: Bornman 1983; AE: von Arnold and Ericksson 1981; SH: Schenk and Hildebrandt 1972; and QP: Quoirin and LePoivre 1977.
[2] % CFB = Percentage of cotyledons forming buds.
[3] Excludes cotyledons without buds.

Table 3. Effect of embryo age on the bud forming capacity of cotyledons of *P. ayacahuite*. Embryos were cultured for various durations in a sucrose and agar medium. After each period, the cotyledons were removed and cultured under bud induction conditions: MCM medium supplemented with 90 mM sucrose and 10 μM BA for 15 days. The embryos were then transferred to the same medium but with 60 mM sucrose and without BA for 30 days. Subsequent transfers were made every 30 days to AE supplemented with 60 mM sucrose and 0.05% activated charcoal. Cotyledons were evaluated for bud formation and bud elongation at Day 75 and Day 105 of culture.Values are means ± SE (n = 81). Means followed by the same letters within columns are not significantly different at $P \leq 0.01$ according to the Least Significant Difference Test.

Days cultured in sucrose	Initial cotyledon size (mm)	% CFB[1]	No. of buds per cotyledon[2]	BFC index	SEC index 0.1–0.5 cm
0	1.21	23.7	2.0 ± 0.5 de	0.65 ± 0.19	0.27
1	1.24	59.3	6.4 ± 2.2 ab	4.25 ± 1.01	2.11
2	1.26	68.2	7.5 ± 3.2 ab	5.79 ± 1.85	3.45
3	1.40	78.2	7.9 ± 1.2 a	6.22 ± 1.04	3.67
4	1.44	65.2	5.9 ± 2.7 ab	4.71 ± 1.62	2.46
6	1.68	58.5	5.3 ± 1.8 bc	3.47 ± 0.88	1.94
8	1.94	45.2	3.3 ± 1.1 cd	1.88 ± 0.85	1.19
12	2.63	11.1	0.6 ± 0.2 e	0.14 ± 0.08	0.07

[1] % CFB = Percentage of cotyledons forming buds.
[2] Excludes cotyledons without buds.

cotyledons from embryos cultured for 0 to 12 days on sucrose medium produced buds, most cotyledons died when they were obtained from embryos cultured for 0, 8 or 12 days on sucrose medium (Table 3).

Yeung et al. (1981) proposed that the high bud-forming capacity of cotyledons of *Pinus radiata* was related to the undifferentiated state of cotyledonary cells at the time of culture (cf. Aitken-Christie et al. 1985). It is possible that most cotyledonary cells of embryos kept in culture for six or more days lose some ability to dedifferentiate and therefore are unable to respond to the bud induction conditions. We observed that older cotyledons produced shoots only from their apical region, which is the growing point of the cotyledons and where mitotic activity continues longest.

Effect of BA concentration The concentration of N^6-benzyladenine (BA) significantly affected the number of buds produced per cotyledon (Table 4). Highest values were obtained with 50 μM BA, with a mean of about three buds being obtained at Day 75. The BFC and SEC indices were also highest in cotyledon explants treated with 50 μM BA. Concentrations of BA of 100 μM caused callus formation, whereas higher concentrations (≥ 500 μM) caused death of the cotyledons. Concentrations of BA of less than 50 μM allowed the formation of buds, but to a smaller extent.

Shoot elongation also seemed to be affected by the concentration of BA; however, this effect was difficult to assess because of the experimental design used. When BA was not present, cotyledons developed as if they were attached to the plant.

Effect of BA exposure time The time of exposure to BA affected bud induction and bud development. Among the exposure times examined, bud elongation at Day 105 was greatest when the cotyledons were exposed to BA for 7 days, but fewer buds were observed in this treatment (data not shown).

Table 4. Effect of N^6-benzyladenine (BA) concentration on the ability of cotyledons of *P. ayacahuite* to produce and elongate buds. Cotyledons from 3-day-old embryos were cultured for 15 days with different concentrations of BA (treatments) on MCM medium supplemented with 90 mM sucrose. The cotyledons were then transferred to the same medium, for 30 days, but with 60 mM sucrose and without BA. Subsequent transfers were made every 30 days to half strength AE medium supplemented with 60 mM sucrose and 0.05% activated charcoal. Cotyledons were evaluated for bud formation at Day 75 of culture. Values are means ± SE (n = 54). Means followed by the same letters within columns are not significantly different at $P \leq 0.01$ according to the Least Significant Difference Test.

BA (μM)	% CFB[1]	No. of buds per cotyledon	BFC index Day 75	SEC index 0.1–0.5 cm
0	0	0 c	0	0
1	28.3	1.0 ± 0.5 c	0.32 ± 0.25	0.16
5	45.5	1.3 ± 0.5 c	0.67 ± 0.35	0.30
10	46.0	1.8 ± 0.6 bc	0.86 ± 0.36	0.41
50	51.0	3.2 ± 0.9 a	1.78 ± 0.92	1.05
100	40.7	2.5 ± 1.1 ab	1.30 ± 0.82	0.79
500	0	0 c	0	0
750	0	0 c	0	0

[1] % CFB = Percentage of cotyledons forming buds.

At Day 195 (Table 5), the highest number of buds corresponded to the longest exposure to BA (28 days), however, callus formation occurred under these conditions. As the exposure time was reduced, a decrease in the number of buds was observed, but no callus was detected. Exposing cotyledons to BA for 21 days did not increase the number of buds produced per cotyledon or change the BFC or SEC over the values obtained after a 14-day exposure to BA, suggesting that it is better to culture shoots in the presence of BA for 14 days than for 21 days, because the culture time was reduced by 1 week,

Table 5. Effect of bud induction culture time on bud production and elongation of cotyledons of *P. ayacahuite*. Cotyledons from 3-day-old embryos were cultured for different periods of time (treatments) on MCM medium supplemented with 50 μM BA and 90 mM sucrose. The cotyledons were then transferred to the same medium for 30 days but with 60 mM sucrose and without BA. Subsequent transfers were made every 30 days to a half strength AE medium supplemented with 60 mM sucrose and 0.05% activated charcoal. Cotyledons were evaluated for bud formation at Day 195 of culture. Values are means ± SE (n = 81). Means followed by the same letters within columns are not significantly different at $P \leq 0.01$ according to the Least Significant Difference Test.

Days on BA	% CFB[1]	Presence of callus	No. of buds after Day 195	BFC index	SEC index > 0.5 cm
0	0.0	no	0.00 c	0.00	0.00
7	51.8	no	4.2 ± 1.7 b	2.50 ± 1.14	1.31 ± 0.62
14	55.5	no	6.4 ± 2.0 ab	3.90 ± 1.81	1.64 ± 0.77
21	46.3	no	5.5 ± 2.2 ab	3.02 ± 1.34	1.04 ± 0.57
28	46.3	yes	7.5 ± 1.9 a	3.54 ± 1.16	1.18 ± 0.40

[1] % CFB = Percentage of cotyledons forming buds.

and long exposures to cytokinin have been associated with the development of hyperhydric shoots. We conclude that exposure to cytokinin is essential for bud formation, but that the time of BA removal is important both to allow bud development and to prevent callus formation.

Explant pre-treatments Stratification of seeds is known to affect bud formation of embryo explants. Although the mechanism underlying this response is not known, stratification is a common practice in conifer micropropagation, e.g., *Pinus brutia* Ten. (Abdullah et al. 1985), *P. contorta* Dougl. ex Loud. (Patel and Thorpe 1984), *Picea engelmannii* Parry ex Engelm. (Patel and Thorpe 1986), and *Larix occidentalis* Nutt. (Harry et al. 1991). When hydrated seeds of *Pinus ayacahuite* were stratified for 9 or more days at 4 °C, the number of embryos that produced bud-forming cotyledons increased from 63 to 83%, but stratification had no effect on the number of buds produced per explant, although bud elongation seemed to increase with stratification time (Table 6).

Bud growth and shoot elongation

For bud growth (Day 45 until the shoots are separated from the explant) and shoot elongation (0.5 cm shoots until they reach rootable height), the same four media used for bud induction were tested at half strength, because a low ionic strength has been shown to enhance bud and shoot elongation (Kulchetscki et al. 1995). Although MCM, AE and SH were suitable for bud elongation (data not shown), half strength AE was chosen because it produced significantly more rootable shoots (> 1.0 cm) at Day 75. The best media for shoot elongation were half strength AE and SH (data not shown). Both media gave significantly greater shoot lengths than half strength MCM or half strength QP. Half strength AE was selected because shoots grown on this medium had a more vigorous appearance than shoots grown on the other media. Thus, for both bud and shoot elongation, half strength AE proved superior. The high total nitrogen concentration of the AE medium may have favored bud growth and shoot elongation.

All of the media tested had a similar total ionic strength, ranging from 58.1 to 78.5 mM (full strength) and from 29.0 to 30.5 mM (half strength). Ionic strength has been associated with the growth response of plants *in vitro* (McCown and Sellmer 1987) and the lower ionic strength of QP medium may be associated with the poor response of explants on this medium. However, MCM medium at half strength has a lower ionic strength than QP at full strength, and still gave a significantly higher response in culture than QP. The differential response may be associated with the concentration of specific elements, the ratio between different components of the me-

Table 6. Effect of stratification of seeds on the ability of cotyledons of *P. ayacahuite* to produce and elongate buds. Sterile, imbibed seeds were kept at 4 °C for different periods before culturing on sucrose–agar for 3 days. Excised cotyledons were transferred to bud induction conditions (MCM medium with 90 mM sucrose and 10 μM BA for 15 days). Cotyledons were then transferred to the same medium with 60 mM sucrose and evaluated for bud formation and bud elongation at Day 45 of culture. Values are means ± SE (n = 54). Means followed by the same letters within columns are not significantly different at $P \leq 0.01$ according to the Least Significant Difference Test.

Days at 4 °C	% CFB[1]	No. of buds	BFC index	SEC index > 0.5 cm	% Responding embryos[2]
0	91.4	3.9 ± 0.3 a	3.60 ± 0.33	0.23	63.2
1	97.7	3.3 ± 0.4 a	3.28 ± 0.46	0.31	56.3
9	98.8	4.2 ± 0.4 a	4.23 ± 0.33	0.79	83.3
16	96.4	4.5 ± 0.4 a	4.33 ± 0.45	0.55	n/a[3]

[1] % CFB = Percentage of cotyledons forming buds.
[2] Percentage of the total number of embryos cultured that yield bud forming cotyledons.
[3] n/a = Data not available.

dium or the organic components of each medium. The response also seems to depend on the species, because QP and MCM were better media for bud induction of *Picea rubens* Sarg. and *Larix decidua* Mill. than SH and AE (Harry et al. 1991, Lu et al. 1991).

Sucrose Sucrose has a major role in the response of explants to bud induction conditions. For *Thuja occidentalis* L. (Harry et al. 1987) and *Tsuga heterophylla* (Raf.) Sarg. (Harry et al. 1994), 60 mM sucrose gave the best response, whereas for *Picea rubens* (Lu et al. 1991) 90 mM was optimum. The best response for *Pinus ayacahuite* was obtained when the sucrose concentration was maintained at 90 mM during the whole period of culture, or when it was reduced to 60 mM after Day 45, as demonstrated by the number of buds per cotyledon, the BFC and the SEC indices (Table 7). Organogenesis is a high energy requiring process (Thorpe 1980), and the effect of the reduction in sucrose concentration immediately after the bud induction period coincides with this requirement. Although the appearance of hyperhydric shoots was associated with high concentrations of sucrose, it was generally observed that reducing the concentration of sucrose to 30 mM caused a significant reduction in number of buds produced per cotyledon.

Hyperhydricity Hyperhydric shoots were observed as early as Day 30 or 40 of culture. These shoots were dark green, and the stems had a watery appearance. Hyperhydric shoots maintained in medium containing 0.7% agar initially grew more than normal shoots, but eventually their leaves turned brown and only a few of these shoots were still alive after 60 days of culture.

Because the type of gelling agent has been associated with the appearance of hyperhydricity, we compared the response of shoots growing on agar (0.7%) and Gelrite (0.5%). Hyperhydricity was lost in most shoots growing in Gelrite in less than 30 days, whereas 56% of the hyperhydric shoots maintained in agar died, and 10% remained vitreous after 60 days of culture. Nairn et al. (1995) reported the isolation of chemical components from Difco-Bacto agar responsible for hydric control in *P. radiata*. A preliminary characterization of the compound(s) indicated a cold-water-soluble, heterogeneous agaroid-type xylogalactan bearing pyruvate and sulfate substituents.

Hyperhydric shoots of *P. ayacahuite* seemed to originate during the early stages of bud formation from buds that were in contact with the medium. Normal shoots maintained in media solidified with agar did not become hyperhydric during culture. Therefore, it seems that, in addition to the chemical component described by Nairn et al. (1995), there are other conditions that affect the hydric control of shoots, e.g., injury of shoots at excision, and position of the buds with respect to the medium during the initial stages of bud development. Gelrite allowed hyperhydric shoots of *P. ayacahuite* and *P. canariensis* Sweet ex K. Spreng. (Martinez Pulido et al. 1990), but not *P. radiata* (Nairn et al. 1995), to revert to a normal hydric state, indicating that hydric control may be species dependent.

Provenances There were significant differences in the responses of explants from the different provenances to the developed protocol (Table 8). Effects of provenance included differences in % CFB, the mean number of buds per cotyledons and the percentage of hyperhydric shoots present. These differences probably have a genetic base, because we observed variations in seed shape and in the number of cotyledons per embryo both within a provenance and between provenances. Seed collection and storage could also have affected the response, because seed from Concepción could not be sterilized properly and sterile cultures could not be established. Shoot formation was observed in explants from eight of the 10 prove-

Table 7. Effect of sucrose concentration on bud elongation of *Pinus ayacahuite* cotyledonary explants. Cotyledons were harvested from 3-day-old embryos growing on a sucrose (30 mM) and agar (0.7%) medium, and cultured for 15 days on MCM medium supplemented with BA (10 μM) and 90 mM sucrose (day 0 to day 15). Cotyledons were then transferred to the same medium but with 30, 60 or 90 mM sucrose and without BA for 30 days (day 15 to day 45). Subsequently these cotyledons were transferred to half strength AE with 30, 60 or 90 mM sucrose and 0.05% activated charcoal for the next 30 days (Day 45 to Day 75). Finally, the cotyledons were transferred to the half strength AE with 0.05% activated charcoal and the same concentration of sucrose used in the previous medium (Day 75 to Day 105). Shoots were evaluated at Day 105 of culture for bud induction and bud elongation. Values are means ± SE (n = 54). Means followed by the same letters within columns are not significantly different at $P \leq 0.05$ according to the Least Significant Difference Test.

Treatment sucrose[1] (mM)	% CFB[2]	No. of buds per cotyledon	BFC index	% Hyperhydric shoots	SEC index 0.5–1.0 cm
90^{w}-30^{x}-30^{y}-30^{z}	51.9	2.9 ± 0.7 b	1.58 ± 0.70	50.2 ± 9.3	0.27
90-60-30-30	63.0	3.6 ± 1.4 b	2.29 ± 1.00	63.1 ± 13.0	0.24
90-90-30-30	77.8	5.0 ± 0.3 ab	3.93 ± 0.60	79.3 ± 8.9	0.33
90-30-60-60	66.7	6.3 ± 1.4 a	4.29 ± 1.40	48.7 ± 6.5	1.16
90-60-60-60	59.3	3.9 ± 1.1 b	2.48 ± 1.10	49.0 ± 8.2	0.58
90-90-60-60	70.4	6.6 ± 0.3 a	4.65 ± 0.60	68.9 ± 5.7	1.31
90-30-90-90	48.2	3.8 ± 0.8 b	1.89 ± 0.60	42.1 ± 3.9	0.61
90-60-90-90	66.7	4.4 ± 1.1 ab	3.11 ± 1.40	60.4 ± 12.9	0.53
90-90-90-90	81.0	6.5 ± 0.6 a	5.26 ± 0.70	84.1 ± 7.1	1.45

[1] ^{w,x,y,z} = Sucrose concentration (mM) at, w: Day 0 to Day 15; x: Day 15 to Day 45; y: Day 45 to Day 75; and z: Day 75 to Day 105 of culture.
[2] % CFB = Percentage of cotyledons forming buds.

nances examined, and explants from five of the 10 provenances produced more than five buds per cotyledon. The mean number of buds per cotyledon was highest for the Los Cubes and Las Trancas provenances, both producing more than eight buds per cotyledon after 105 days of culture, whereas Chuipachec and Palestina only produced one and two buds per cotyledon, respectively. We conclude that the method developed for this species can be applied to the various provenances, but further optimization is required for provenances exhibiting low responses.

Rooting

Rooting in conifers is generally difficult, and is considered a major obstacle to successful micropropagation of conifers (Mohammed and Vidaver 1988, Thorpe and Harry 1990). Up to 20% rooting was achieved when shoots were treated for 2 weeks on a medium with 500 μM of IBA, and up to 30% rooting was achieved when shoots were treated with a combination of 0.8 or 4 μM NAA and 20 μM IBA. In all of these treatments, the roots obtained appeared normal, and no callus formation was observed.

No rooting was observed when shoot bases were incubated in 1 mM IBA, NAA or IAA for 0–8 h, followed by *ex vitro* or *in vitro* culture, but callus developed in all cases, and was accentuated with longer exposure to hormones. However, when 100 μM NAA was added for 8 h, followed by *in vitro* culture on half strength GD medium with activated charcoal (0.05%), sucrose (30mM) and agar (1%), callus formation was reduced, and up to 40% of the shoots rooted. Although these rooting percentages were similar to those obtained with several other pines, they could severely limit the usefulness of the protocol developed. The plantlets survived transfer to greenhouse conditions.

Conclusions

A protocol for *in vitro* plantlet regeneration of *Pinus ayacahuite* was developed following the sequential stepwise approach outlined by Thorpe and Patel (1984). Bud induction was obtained from embryos or cotyledons of mature embryos. The age of the explant, the sucrose and cytokinin concentrations and the exposure time to cytokinin all had critical effects on bud induction. The best results were obtained under the following conditions. After stratification of seeds for 9 days at 4 °C, embryos were germinated on sucrose–agar for 3 days. Cotyledonary explants were cultured on MCM medium with 90 mM sucrose and 50 μM BA for 14 days. Bud growth and development (Days 15–45) and elongation of separated shoots (after Day 45) were best on half strength AE medium (macroelements) supplemented with 60 mM sucrose and 0.05% activated charcoal, with 0.5% Gelrite being used in the later subcultures. For rooting, shoot bases were incubated in 100 μM NAA for 8 h, followed by culture on half strength GD medium with 30 mM sucrose, 0.05% activated charcoal and 1% agar. For the Los Cubes provenance, up to 9.1 ± 1.7 buds were obtained from each cotyledon, with 78.9% of the cotyledons forming buds with a mean of 9.8 cotyledons per seed, it was possible to obtain about 70 rootable shoots per seed after 15 weeks in culture. Up to 40% of rooting was obtained, and therefore a yield of about 28 plantlets per seed was possible. The mean response for all the responding provenances was about 24.6 shoots in 15 weeks and 9.8 plantlets per seed in about 23 weeks.

Table 8. Comparison of 10 different provenances of *Pinus ayacahuite* seeds to micropropagation *in vitro*. One hundred and twenty seeds from each provenance were hydrated for 48 h under running tap water, sterilized and stratified for 7 days at 4 °C. Then, the embryos were cultured on an agar and sucrose medium for 3 days. Cotyledons from the growing embryos were placed on the bud induction medium for 15 days (MCM, 90 mM sucrose, 50 μM BA). The cotyledons were then transferred to MCM medium with 60 mM sucrose for 30 days, and subsequent transfers were made to half strength AE medium with 60 mM sucrose and 0.05% activated charcoal, every 30 days. Cotyledons were evaluated for bud induction and elongation at Day 105. Values are means ± SE (n = 54). Means followed by the same letters within columns are not significantly different at $P \leq 0.01$ according to the Least Significant Difference Test.

Provenance	% CFB[1]	% Responding embryos[2]	No. of cotyledons per embryo (range)	No. of buds per cotyledon	BFC index	SEC index 0.1–0.5 cm	% Hyperhydric shoots
Palestina*[3]	18.9	22.5	7.3 (6–8)	2.1 ± 1.1 d	1.54 ± 0.84 ef	0.38 d	18.0
Ixtlán	74.5	63.3	9.2 (6–12)	7.3 ± 1.3 ab	6.61 ± 1.40 abc	2.01 b	62.5
Porvenir*	40.0	47.5	8.7 (6–11)	5.5 ± 2.0 bc	4.80 ± 1.82 cd	1.75 bc	40.1
Nundaco	44.4	35.8	8.7 (6–11)	3.8 ± 1.4 cd	3.67 ± 1.40 de	0.79 cd	37.7
Los Cubes	78.9	83.3	9.8 (9–13)	9.1 ± 1.7 a	8.76 ± 1.72 a	2.22 b	69.1
Plan del Rancho	35.6	60.8	9.3 (8–11)	1.2 ± 0.4 d	0.94 ± 0.38 f	0.25 d	40.9
Chuipachec*	45.6	37.5	7.6 (6–10)	5.8 ± 1.9 bc	5.07 ± 1.80 bcd	1.73 bc	36.9
Las Trancas*	76.7	66.7	9.5 (8–12)	8.6 ± 1.3 a	7.75 ± 1.4 ab	3.53 a	68.6
Buenavista*	n/a[4]	0.01	n/a	n/a	n/a	n/a	n/a
Conception	n/a	58.3	n/a	n/a	n/a	n/a	n/a

[1] % CFB = Percentage of cotyledons forming buds.
[2] Percentage calculated from the number of embryos growing after 3 days of culture of the 120 seeds used for each treatment.
[3] * = Seeds from Group 1 (see Table 1).
[4] n/a = Data not available.

Although it is difficult to compare our results with those of other studies, because of the differences in the minimum size of shoots measured and the time in culture, nevertheless, our findings agree with those obtained with similar pines. For example, *Pinus brutia* produced 55 shoots per seed after 24 weeks (Abdullah et al. 1985), *P. canariensis* yielded 24 shoots per seed after 15 weeks (Martinez Pulido et al. 1990), and *P. roxburghii* Sarg. gave about 25 shoots per seed after 15 weeks (Muriithi et al. 1993). In contrast, *P. radiata*, probably the most prolific shoot-forming system in conifers, produced over 180 rootable shoots per seed after 13 weeks (Aitken et al. 1981). However, it must be recognized that shoot multiplication, which was not thoroughly evaluated in this study, and rooting ability vary greatly with species, and therefore the ultimate number of plantlets formed will differ correspondingly.

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