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OPTIMIZATION OF IN VITRO BUD INDUCTION AND PLANTLET FORMATION FROM MATURE EMBRYOS OF ALEPPO PINE (*PINUS HALEPENSIS* MILL.)

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SUMMARY

Studies were undertaken to optimize tissue culture conditions for micropropagation of Aleppo pine (*Pinus halepensis* Mill.) from mature embryos and various explants of the embryo. Over 90% of the embryo explants gave rise to adventitious buds within 4 wk. Intact embryos were the most suitable explants for shoot bud induction. Both isolated cotyledons and hypocotyls produced adventitious buds, but these developed slowly and failed to elongate. N^6-Benzyladenine (BA) alone at 5.0 μM was the most effective cytokinin when added to gelled von Arnold and Eriksson's (AE) medium containing 3% sucrose. Adventitious bud development was achieved on hormone-free AE medium, and shoot elongation was optimum on three quarter-strength Bornman's MCM medium, with 0.1% conifer-derived activated charcoal. Shoots were multiplied on three-quarter strength MCM medium, containing 5 μM BA. To induce adventitious roots on the elongated shoots, pulse treatment with 1 mM IBA for 6 h, followed by the transfer of the shoots to sterile peat:vermiculite (1:1) mixture, was beneficial. After acclimatization for 3 to 4 wk under mist, almost all the rooted shoots could be transplanted successfully to the greenhouse, where the plants exhibited normal growth habit. Histologic studies on the ontogeny of adventitious shoot formation from mature embryo explants revealed temporal structural changes in different parts of the explant. Induction of mitotic divisions on the shoot-forming medium resulted in the formation of meristemoids in the epidermal and subepidermal layers of the explant, located initially at both the tips of the cotyledons and the axils of adjacent cotyledons. Shoot buds arising in the axils of adjacent cotyledons were due to new cell division and not to any preexisting meristem.

Key words: Pinus halepensis; Aleppo pine; micropropagation; organogenesis; shoot formation; rhizogenesis.

INTRODUCTION

Aleppo pine (*Pinus halepensis* Mill.) is a conifer typical of the Mediterranean region, ranging from Morocco to the shores of the Black sea. Its main area of distribution is southern Europe (Spain, Italy, and Greece), and northern Africa (Algeria, Tunisia, and Morocco). In these regions, Aleppo pine is of great economic importance due to its adaptability to dry, calcareous, and poor soils. It is particularly suitable for the reforestation of marginal and submarginal areas because it is one of the most drought-resistant pines. In marine stands, Aleppo pine forests are important both for defenses against the saline winds and for landscape purposes. At present, breeding programs and field trials are being carried out in Algeria, Italy, Tunisia, Greece, and Morocco for the improvement of the tree trunk form, wood production, and gum yield (Panetsos, 1986). Hence, tissue culture techniques can prove to be very useful for the rapid clonal propagation of superior and improved genotypes over a shorter period, in comparison with the traditional methods of propagation for the achievement of genetic improvement.

Somatic embryogenesis and organogenesis via the induction of adventitious buds are the two systems of in vitro propagation of conifers (Thorpe and Biondi, 1984). The latter method of propagation essentially involves a four-step process: a) induction and development of adventitious buds on embryonic explants, b) elongation of shoot buds, c) multiplication of shoots, and d) rooting of the shoots and their transfer to in vivo conditions. After this approach, micropropagation of several conifers has been successfully worked out (Thorpe et al., 1991). Preliminary studies on adventitious bud induction and development from mature embryos of Aleppo pine have been previously reported (Lambardi et al., 1991). The aim of the present study was to characterize the best culture conditions, including the source of the explant, phytohormone quality and quantity, and the basal medium for each of the above-mentioned steps during the process of micropropagation. Histologic studies

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were carried out to determine the origin of shoots and various developmental stages in shoot ontogeny.

Materials and Methods

Seeds of Aleppo pine were collected from open pollinated trees in a natural stand in Vico del Gargano (south of Italy; lat. 41 56′, long. 3 31′ E, alt. 250 m.s.l.m.) and were stored at 4–5° C until used. Before culture, seeds were surface-sterilized in 70% ethanol for 1 min followed by 10 min in 0.1% (wt/vol) mercuric chloride, and rinsed 3 times with sterile-distilled water. The seeds were then stratified at 4° C for 4 to 5 days. The embryos were removed from the megagametophytes under sterile conditions and plated on various gelled media according to the experimental design. Only white, firm, undamaged embryos were plated horizontally on the media surface (Fig. 1 *A*). Media were adjusted to pH 5.8 before autoclaving. Explants were incubated at 27 ± 1° C under a 16-h photoperiod provided by Sylvania Gro-Lux F40712 Gro-Ws light at a photon fluence rate of 80 to 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Adventitious bud induction and development. Unless otherwise stated, entire embryos were cultured in sterile petri dishes containing mineral salts, organics, amino acids, and myo-inositol from AE medium (von Arnold and Eriksson, 1981), along with 3% sucrose and 0.8% Difco Bacto-agar. In the preliminary experiments, the influence of different cytokinins, viz., N^6-benzyladenine (BA), kinetin, 2-isopentenyl adenine (2-iP), and zeatin, on adventitious bud induction was tested. All the cytokinins were applied for 28 days at four different concentrations (1.0, 2.5, 5.0, and 10.0 μM). Next, three of these cytokinins were employed in pairs of two in every possible combination at levels of 0.5, 1.0, and 5.0 μM. The best cytokinin was subsequently tested alone for various periods (7, 14, 21, 28, 35 days) before the embryos were subcultured on a hormone-free medium. The best cytokinin treatment was then used to determine the influence of different salt formulations and gelling agents on shoot bud induction. The effect of five different media, viz., AE, DCR (Gupta and Durzan, 1985), QP (Quoirin and Le Poivre, 1977), SH (Schenk and Hildebrandt, 1972), and MCM (Bornman, 1983) was evaluated. All these media contained 3% sucrose and 0.8% Difco Bacto-agar. The best basal medium was then used to compare the effect of two gelling agents either used singly or in combination. The gelling agents included Difco Bacto-agar (0.6 and 0.8%), Gelrite (0.3 and 0.4%), and 0.3% Difco Bacto-agar with 0.2% Gelrite.

In a separate set of experiments, the best selected basal medium was used to characterize various explants, namely, intact embryos, epicotyls with cotyledons, epicotyls without cotyledons, hypocotyls, and cotyledons. Intact embryos were also cultured for different periods in hormone-free medium (0, 3, 5, 7, and 10 days) before their transfer onto cytokinin-containing medium to test the duration for which the explants retained potential to respond to the phytohormone. Unless otherwise mentioned, the explants from all the above experiments were transferred to hormone-free medium for a period of 3 wk, for the development of the adventitious buds.

Shoot elongation and multiplication. After the induction and development of adventitious buds, the explants were transferred to glass jars (100 × 80 mm), containing 100 ml of hormone-free basal medium to which 0.1% conifer-derived activated charcoal was added. The macro-salts of AE medium were tested at full strength or altered to half and three quarters of the normal concentration, in combination with 2, 3, and 4% sucrose. Three media of different nitrogen and total ionic composition (AE, DCR, MCM) were also evaluated at three quarter salt concentration for the elongation of shoots.

For multiplication, shoots with an average height of 1 cm were grown for 4 wk on cytokinin-containing three quarter strength MCM medium before their transfer to hormone-free medium. The cytokinins (BA and zeatin) at two levels (1 and 5 μM) were tested and compared with untreated shoots for their ability to produce axillary shoots.

Rooting of elongated shoots and transplantation. Shoots greater than 1 cm were used to carry out experiments on rooting on MCM medium containing a quarter macro-salts and 1% sucrose. In a first experiment, the shoots were tested for rooting after different treatments, i.e., a) pulse treatment in a sterile solution of 1 mM indolebutyric acid (IBA) at pH 4.2 for 4 h, b) culturing them for 5 days in a quarter-strength medium with 100 μM IBA, and solidified with 0.4% Gelrite at pH 5.0, and c) treating the cut ends with rooting powder containing 15 mM IBA in talcum (Hartmann et al., 1990). After each treatment, the shoots were placed in sterile glass jars containing rooting substrate (peat:vermiculite 1:1) moistened with the rooting medium, and evaluated after 4 wk for rooting. In a second experiment, the liquid pulse treatment was repeated with three different times of application of 1 mM IBA solution, i.e. 4, 5, and 6 h, after which the shoots were handled as before.

The rooted shoots were directly transferred to pots containing peat:vermiculite mixture (1:1) and irrigated with tap water. The pots were initially maintained in a growth chamber with about 70% relative humidity for 3 to 4 wk and then transferred to a greenhouse.

Data collection and statistical analysis. Each experiment was done at least twice using a minimum of four replicates with 8 to 10 U per treatment. The parameter evaluated at the end of the induction phase was the number of explants forming shoot buds per plate. After 3 wk of shoot development (i.e. 10 wk after the beginning of the experiment) the following parameters were evaluated: a) the number of well-developed shoot buds per explant, b) the bud forming capacity (BFC) index, calculated as follows:

$$BFC = (\text{average number of buds per explant}) \times (\%\ \text{explant forming buds})/100,$$

c) the percentage of shoots longer than 3 mm, and d) the shoot elongation capacity (SEC) index, calculated as follows:

$$SEC = (\text{average number of shoots} > 3\ \text{mm per explant forming buds}) \times (\%\ \text{explants forming buds})/100$$

Vitrification or hyperhydricity (Debergh et al., 1992), defined as the extent of succulence of the shoots, was a major qualitative factor evaluated.

Shoot multiplication was evaluated taking the number of axillary shoots per plantlet. After the rooting period of 4 wk, data were collected as follows: a) percentage of rooted shoots, b) average number of roots per rooted shoot, and c) length of the longest root per rooted shoot.

Statistical analysis of the non-parametric data (frequencies) 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 and McSweeney, 1977). Discrete data were subjected to analysis of variance (ANOVA), followed by the Tukey HSD test (Snedecor and Cochran, 1980) at $P \leq 0.05$ to compare means.

Histologic studies. To study the process of adventitious bud induction, embryos of Aleppo pine were placed on AE medium containing 5 μM BA, and fixed after 3, 5, 7, 9, 11, 16, 19, 22, and 25 days. At each date, embryos from hormone-free AE basal medium were sampled as control. The material were fixed in 2% formaldehyde and 2% glutaraldehyde buffered with 0.05 M phosphate buffer at pH 6.8. The tissues were then dehydrated using methyl cellosolve followed by absolute ethanol, and embedded in LKB Historesin according to the method of Yeung and Law (1987). Two-micrometer-thick sections were stained using the periodic acid-Schiff's reaction and counterstained with aniline blue black (O'Brien and McCully, 1981).

Results

Adventitious Bud Induction

When mature embryo explants of *Pinus halepensis* Mill. were cultured on shoot bud induction medium, initial swelling of the explant and slight nodular protrusions were followed by the formation of several shoot buds within 21 to 28 days of culture initiation. The initiation of shoot buds was first observed on the lower surface of the embryos, i.e. the one in contact with the induction medium (Fig. 1 *B*); subsequently, the upper surface was also involved in the regenerative process (Fig. 1 *C*). On entire embryos and excised epicotyls, well-developed shoot buds arose from the basal parts of the cotyledons, and the surrounding epicotyl/hypocotyl areas (Fig. 1 *D*). Adventitious buds occurred as well along the entire surface of cotyledons and hypocotyls; but further development, although very

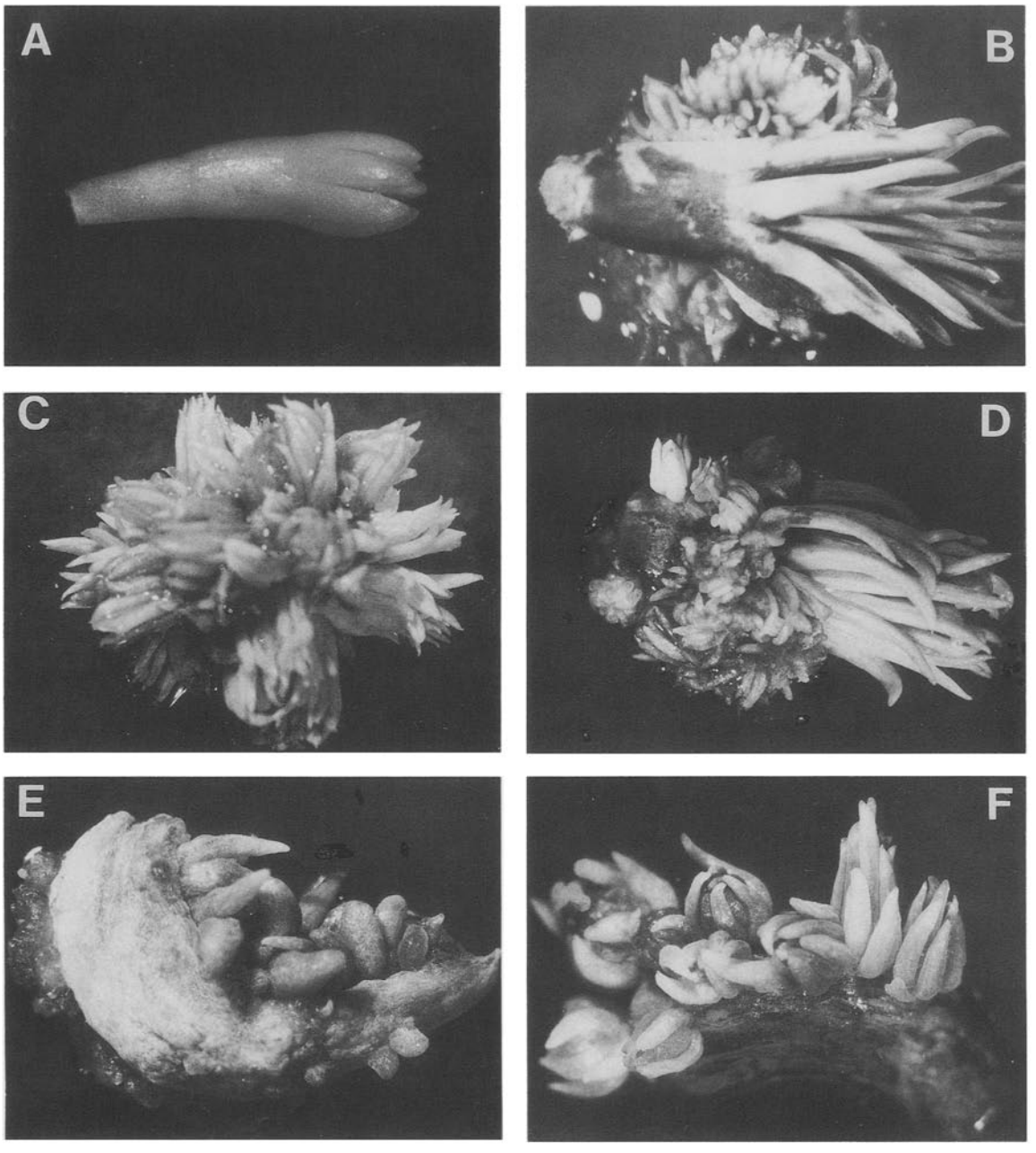

FIG. 1. *A*, embryos of Aleppo pine (*Pinus halepensis* Mill.) just after dissection. ×12. *B*, adventitious shoot buds arising from the embryo surface in contact with the medium after 14 to 21 days on AE medium containing 5 μM BA. ×12. *C*, after 28 days, the upper surface is also covered with adventitious shoot buds. ×16. *D*, shoot buds arise mainly from the basal parts of the cotyledons and adjacent zones. ×12. *E*, shoot buds arising from isolated cotyledons after 28 days on induction medium and 21 days on hormone-free AE medium. *Note* that bud development is very slow when compared to the buds from entire embryos. ×24. *F*, isolated hypocotyls after 28 days on induction medium and 21 days on hormone-free AE medium. Many adventitious shoot buds developed on hypocotyls, but only when isolated from the rest of the embryos. ×18.

slow, generally took place only when these organs were isolated from the rest of the embryos (Fig. 1 *E,F*). It should be noted that, as a rule, the first morphologic events were the formation of both cataphylls at the cotyledonary tips and adventitious shoot buds in the axils of adjacent cotyledons (Fig. 2 *A*).

Effect of cytokinins. Various cytokinins, viz., BA, kinetin, 2-iP and zeatin, were used individually at different concentrations in AE medium containing 3% sucrose. Overall, BA proved to be the best cytokinin tested, in terms of percent responding explants and number of shoot buds per explant (Table 1). Although the number of shoot buds produced per responding explant was higher with 5 and 10 μM of 2-iP, the BFC and SEC indices were higher with all the

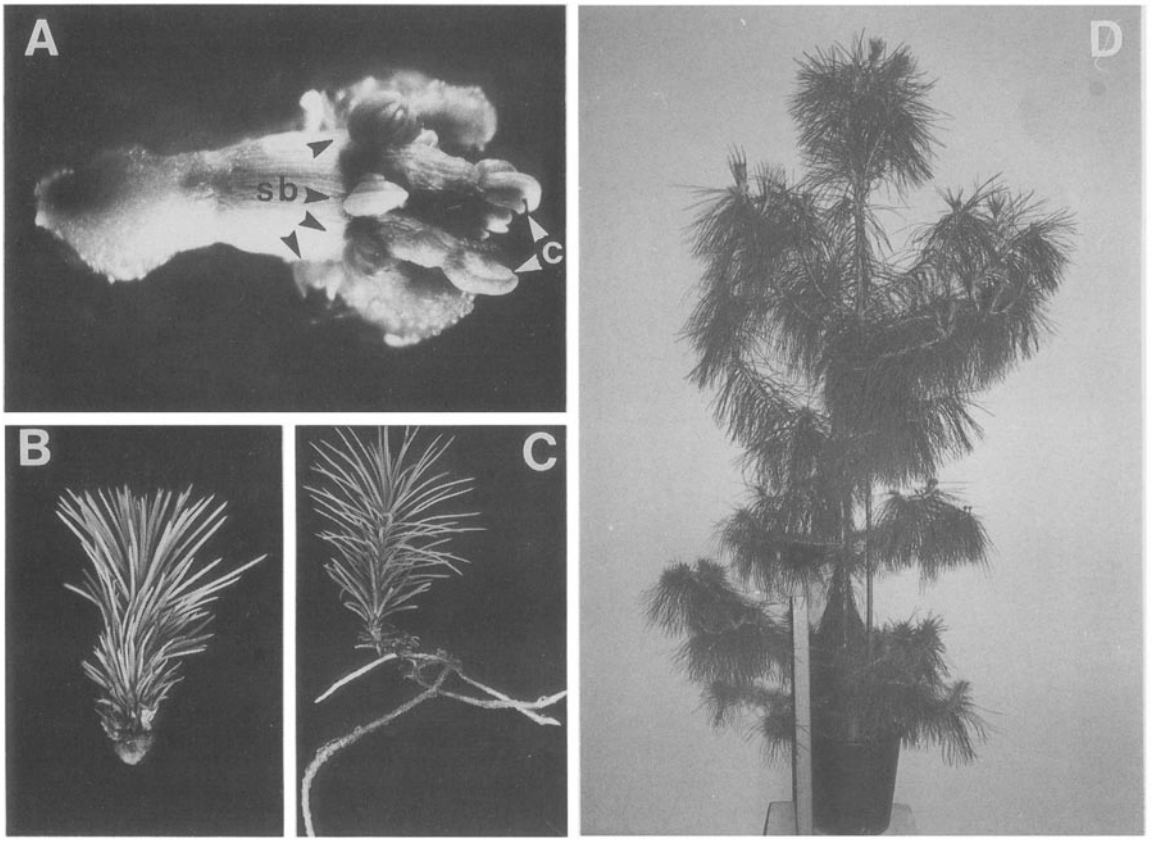

FIG. 2. *A*, Day 24. Adventitious shoot buds (*sb*) arising at the axil of cotyledons of Aleppo pine (*Pinus halepensis* Mill.). *Note* that their development is perpendicular to the embryo main axis. Here cataphylls (*c*) arose at the tip of the cotyledons. ×16. *B*, elongated shoot after 12 wk in culture. The shoot is ready to be rooted with IBA treatment or multiplied on cytokinin-containing medium. ×5. *C*, rooted shoot after 4 wk on sterile peat:vermiculite (1:1) substrate, moistened with quarter-strength MCM medium plus 1% sucrose. The shoot was previously pulse-treated with 1 m*M* IBA solution for 6 h. ×1.5. *D*, a well-developed plant of Aleppo pine from in vitro shoot bud regeneration, after 2 yr from the beginning of the culture. (*Scale* = 0.5 m).

tested concentrations of BA. Moreover, the shoots produced by 2-iP and zeatin were hyperhydric in nature. Based on these observations, BA at 5 μM concentration was selected for further experiments on induction of adventitious shoot buds from embryo explants.

To determine if BA alone was sufficient for shoot bud induction, or a combination of cytokinins was better, various cytokinins (BA, kinetin, 2-iP) were tested in combination with each other at different concentrations (Table 2). Although 5 μM BA in combination with 0.5 μM 2-iP produced the maximum number of shoots per responding explant, which was reflected in a very high BFC index (45.9), most of the shoots were stunted, thus resulting in a very low percentage of shoots longer than 3 mm and a SEC index inferior to the control. Moreover, the shoots formed in all the combinations of BA and 2-iP were hyperhydric to various extents. Other combinations too were either similar or inferior to using BA alone at 5.0 μM.

To ascertain the duration of requirement of BA in the shoot induction medium, the explants were maintained on AE medium containing 5.0 μM BA for periods from 0 to 35 days, at 7-day intervals (Table 3). After each preculture on BA-containing medium, the explants were then cultured on AE medium containing 3% sucrose until the final observations were recorded. Although the application of BA for as little as 7 days was sufficient to elicit some response, the BFC and SEC indices were very low when compared to longer periods. Exposure of the explants to BA for 14 days was sufficient to induce a high response of all the estimated parameters, and longer exposures were beneficial only in terms of the BFC index (28 days). Exposure to BA for periods longer than 28 days were harmful in terms of the SEC index because the shoots remained stunted and hyperhydric and did not elongate subsequently.

Effect of mineral salts. Five different media formulations, i.e. DCR, AE, QP, SH, and MCM, in conjunction with 5.0 μM BA, 3% sucrose and 0.8% Difco Bacto-agar, were tested for the induction of shoot buds from mature embryo explants. Except for MCM, all the other media formulations produced a comparable response in terms of the percent responding explants (Table 4). However, the BFC index was much higher (23.8) with AE medium in comparison to other formulations. The quality of shoots as represented by the SEC index was also higher for this medium (9.9), whereas it was lowest for SH medium. The percentage of shoots greater than 3 mm was very high for DCR medium, but since the number of buds per responding explant was lower, this medium exhibited lower BFC and SEC indices.

Effect of gelling agents. Two gelling media, viz., Difco Bacto-agar (0.6, 0.8%) and Gelrite (0.3, 0.4%), were tested at different

TABLE 1

EFFECTS OF DIFFERENT CYTOKININS ON THE INDUCTION AND DEVELOPMENT OF SHOOT BUDS FROM EMBRYO EXPLANTS OF *Pinus halepensis*[1]

Cytokinin Conc. & Type	Percent Explants with Buds[2]	No. of Buds per Explant[3]	BFC Index	Percent Shoots >3 mm	SEC Index	Hyperhydricity[4]
1.0 μM						
BA	70.8[a]	18.4 ± 2.5[a]	13.0	36.9	4.8	−
Kinetin	7.5[b]	1.6 ± 0.3[b]	0.1	0	0	−
2-ip	17.5[b]	16.5 ± 2.6[a]	2.9	55.5	1.6	−
Zeatin	18.7[b]	10.6 ± 0.6[a]	2.0	67.9	1.3	−
2.5 μM						
BA	74.0[a]	20.6 ± 2.4[b]	15.2	33.7	5.1	−
Kinetin	43.6[b]	3.8 ± 0.6[a]	1.6	75.9	1.3	−
2-ip	37.5[b]	16.3 ± 4.2[b]	6.1	69.3	4.2	−
Zeatin	61.5[ab]	12.4 ± 2.2[ab]	7.6	69.1	5.3	−
5.0 μM						
BA	92.0[a]	22.9 ± 2.7[a]	21.1	33.2	7.0	−
Kinetin	69.2[a]	7.3 ± 1.1[b]	5.0	70.1	3.5	−
2-ip	35.0[b]	30.0 ± 6.3[a]	10.5	12.2	1.3	+
Zeatin	70.0[a]	24.1 ± 3.8[a]	16.9	32.4	5.5	++
10.0 μM						
BA	80.4[ab]	24.0 ± 2.9[a]	19.3	37.9	7.3	+
Kinetin	90.0[a]	9.5 ± 0.8[b]	8.5	71.0	6.1	−
2-ip	62.5[b]	37.2 ± 6.4[c]	23.2	13.0	3.0	+
Zeatin	57.5[b]	−	−	−	−	+++

[1] Medium: AE + 3% sucrose + 0.8% Difco Bacto-agar. Data collected after 4 wk for the percent of explants with buds, and after 10 wk for all the other parameters.

[2] Within each concentration, percentages followed by different letters are significantly different at $P \leq 0.05$.

[3] Mean ± SE; within each concentration, means followed by different letters are significantly different at $P \leq 0.05$.

[4] Hyperhydricity was defined low (+), high (++), very high (+++), or absent (−).

TABLE 2

EFFECT OF COMBINATION OF TWO CYTOKININS ON ADVENTITIOUS BUD FORMATION AND DEVELOPMENT FROM EMBRYO EXPLANTS OF *Pinus halepensis*[1]

Cytokinin Conc, μM	Percent Explants with Buds[2]	No. of Buds per Explant[3]	BFC Index	Percent Shoots >3 mm	SEC Index	Hyperhydricity[4]
BA 5.0 (control)	92[ab]	22.9 ± 2.7[ab]	21.1	33.2	7.0	−
BA 5.0 + kinetin						
0.5	90.0[ab]	15.0 ± 1.7[a]	13.5	43.3	5.8	−
1.0	94.9[ab]	16.1 ± 1.5[a]	15.3	42.8	6.5	−
5.0	97.5[a]	16.5 ± 3.0[a]	16.1	41.5	6.7	−
BA 5.0 μM + 2-iP						
0.5	85.0[ab]	54.0 ± 9.4[c]	45.9	10.2	4.7	+++
1.0	75.0[abc]	37.6 ± 5.0[bc]	28.2	17.2	4.8	+
5.0	70.7[bcd]	51.1 ± 6.0[c]	36.1	13.1	4.7	+
Kinetin 5.0 μM + 2-iP						
1.0	37.5[d]	13.0 ± 2.4[a]	4.9	34.6	1.7	−
5.0	40.0[cd]	24.3 ± 3.7[ab]	9.7	22.4	2.2	−
10.0	65.0[bcd]	22.9 ± 3.9[ab]	14.9	15.5	2.3	

[1] Medium: AE + 5.0 μM BA + 3% sucrose + 0.8%. Data collected after 4 wk for the percent of explants with buds, and after 10 wk for all the other parameters.

[2] Percentages followed by different letters are significantly different at $P \leq 0.05$.

[3] Mean ± SE; means followed by different letters are significantly different at $P \leq 0.05$.

[4] See Table 1, *note* 4.

concentrations either alone or in combination in the shoot bud induction medium. Lower levels of Difco Bacto-agar (0.6%) and Gelrite (0.3%) produced hyperhydric shoots. Although the frequency of explants producing shoots was comparable in most cases (except for 0.6% Difco Bacto agar) the BFC and SEC indices with Gelrite were lower. Combination of 0.3% Difco Bacto-agar and 0.2% Gelrite also produced a response that was comparable to the control (0.8% Difco Bacto-agar) with relatively higher BFC (33.6 vs. 31.6) and SEC (10.4 vs. 7.8) indices, but the shoots produced were also hyperhydric. Thus 0.8% Difco Bacto-agar was used for medium gelling.

Effect of explant type. To optimize shoot bud regeneration, several types of explants from mature embryos were prepared and cultured on different BA levels (1.0, 5.0, and 10.0 μM). The explants included intact embryos, epicotyls with and without cotyledons, hypocotyls, and cotyledons. Although the intact embryo remained the most suitable explant for shoot bud induction in terms of both BFC and SEC indices, the epicotyl with cotyledons also produced a high frequency of response with comparable indices (Table 5). In contrast, the epicotyl without cotyledons produced a significantly lower response. For each BA concentration, hypocotyls and cotyledons were inferior explants in terms of all the observed parameters. It was interesting to note that epicotyls without cotyledons produced a better response at the lowest BA concentration, whereas the percentage of hypocotyls forming buds improved with increasing level of BA. However, 10 μM BA was not suitable for these explants in terms of both the BFC and SEC indices, because they callused and bud development stopped.

TABLE 3

EFFECT OF TIME OF EXPOSURE TO BA ON ADVENTITIOUS BUD FORMATION AND DEVELOPMENT FROM EMBRYO EXPLANTS OF *Pinus halepensis*[1]

Exposure, Days	Percent Explants with Buds[2]	No. of Buds per Explant[3]	BFC Index	Percent Shoots >3 mm	SEC Index	Hyperhydricity[4]
0	0	—	—	—	—	−
7	60.0[a]	6.4 ± 0.7[a]	3.8	71.4	2.7	−
14	95.0[b]	18.5 ± 2.4[bc]	17.6	74.4	13.1	−
21	97.4[b]	14.8 ± 3.5[ab]	14.4	58.3	8.4	−
28 (control)	90.6[b]	26.5 ± 3.7[c]	24.0	41.6	10.0	−
35	100.0[b]	18.2 ± 1.8[bc]	18.2	20.8	3.8	+

[1] Medium: AE + 5.0 μM BA + 3% sucrose + 0.8% Difco Bacto-agar. Data collected after 4 wk for the percent of explants with buds, and after 10 wk for all the other parameters.

[2] Percentages followed by different letters are significantly different at $P \leq 0.05$.

[3] Mean ± SE; means followed by different letters are significantly different at $P \leq 0.05$.

[4] See Table 1, *note* 4.

Effect of explant age. After the stratification treatment of the seeds, the embryos were precultured on hormone-free AE medium for different durations (0, 3, 5, 7, and 10 days) before culturing them on the bud induction medium containing 5.0 μM BA. Although the embryo explants cultured directly onto the regeneration medium produced a higher frequency of response, those precultured for 3 days produced superior response in terms of the number of shoot buds per responding explant, which resulted in the highest BFC and SEC indices (Table 6). Preculture for longer than 3 days was harmful to the explants which showed a proportional reduction in both these indices; however, the shoots were mildly hyperhydric.

Elongation and Multiplication of Shoots

Effect of medium. To optimize the strength of the AE medium for shoot elongation, different macro-salt levels (i.e. 1, 3/4, and 1/2) of the AE formulation were tested, in combination with 2, 3, and 4% sucrose. Activated charcoal (0.1%) was added to the media. Although there was no significant difference between full and three-quarter strengths on the number of shoot buds, the percentage of shoots greater than 3 mm was greatly reduced with full-strength medium. Half-strength medium was inferior in the above parameters. Sucrose concentration showed no significant effect on the number of shoot buds, whereas the higher levels produced a greater percentage of shoots more than 3 mm in length. Hence, 3% sucrose was selected for this stage of micropropagation. No interaction was found between these two factors.

Among the three different media (AE, DCR, and MCM) tested at three-quarter strength for shoot elongation, only MCM enhanced shoot growth (Table 7). Hence, MCM medium at three-quarter-macro-salt concentration was used for further work on shoot elongation and multiplication.

Effect of cytokinins. To further increase the number of shoot buds by the proliferation of axillary buds during the shoot elongation stage, two cytokinins (BA and zeatin) were included in the medium at 1 and 5 μM levels. The addition of a cytokinin in the medium increased the number of proliferating axillary buds. Zeatin at both the levels was superior to 1.0 μM BA, but was comparable to 5.0 μM BA (Table 8).

TABLE 4

EFFECT OF DIFFERENT MEDIA ON ADVENTITIOUS BUD FORMATION AND DEVELOPMENT FROM EMBRYO EXPLANTS OF *Pinus halepensis*[1]

Media	Percent Explants with Buds[2]	No. of Buds per Explant[3]	BFC Index	Percent Shoots >3 mm	SEC Index	Hyperhydricity[4]
DCR	97.5[a]	11.8 ± 1.8[a]	11.5	64.4	7.4	–
AE	90.0[a]	26.5 ± 3.7[b]	23.8	41.6	9.9	–
QP	85.0[ab]	13.1 ± 2.3[a]	11.1	41.5	4.6	–
SH	80.0[ab]	7.3 ± 0.6[a]	5.9	31.1	1.8	–
MCM	57.5[b]	9.1 ± 1.2[a]	5.2	40.6	2.1	–

[1] Media added of 5.0 μM BA + 3% sucrose + 0.8% Difco Bacto-agar. Data collected after 4 wk for the percent of explants with buds, and after 10 wk for all the other parameters.

[2] Percentages followed by different letters are significantly different at $P \leq 0.05$.

[3] Mean ± SE; means followed by different letters are significantly different at $P \leq 0.05$.

[4] See Table 1, *note* 4.

TABLE 5

EFFECT OF INTERACTION BETWEEN DIFFERENT EXPLANTS AND BA CONCENTRATIONS ON ADVENTITIOUS BUD FORMATION AND DEVELOPMENT FROM *Pinus halepensis*[1]

BA conc. (Explants)	Percent Explants with Buds[2]	No. of Buds per Explant[3]	BFC Index	Percent Shoots >3 mm	SEC Index
1.0 μM					
Embryo	76.7[a]	18.9 ± 3.1[a]	14.5	28.3	4.1
Epicotyl (+ cots)	76.7[a]	13.6 ± 2.2[ab]	10.4	34.3	3.6
Epicotyl (− cots)	66.7[ab]	17.2 ± 3.8[ab]	11.5	46.4	5.3
Hypocotyl	0	—	—	—	—
Cotyledon	37.8[b]	8.2 ± 1.2[b]	3.1	37.2	1.1
5.0 μM					
Embryo (control)	90.0[a]	22.1 ± 2.5[a]	19.9	34.6	6.9
Epicotyl (+ cots)	93.3[a]	16.3 ± 2.5[ab]	15.2	39.6	6.0
Epicotyl (− cots)	33.3[b]	20.0 ± 3.6[a]	6.7	27.1	1.8
Hypocotyl	25.0[b]	14.7 ± 5.2[ab]	3.7	13.5	0.5
Cotyledons	35.6[b]	9.7 ± 1.3[b]	3.4	5.2	0.2
10.0 μM					
Embryo	93.3[a]	23.6 ± 2.4[a]	22.0	38.5	8.4
Epicotyl (+ cots)	90.0[a]	20.7 ± 4.0[ab]	18.6	37.7	7.0
Epicotyl (− cots)	41.7[b]	—	—	—	—
Hypocotyl	41.7[b]	—	—	—	—
Cotyledons	34.4[b]	11.2 ± 1.9[b]	3.8	26.0	1.0

[1] Medium: AE + 3% sucrose + 0.8% Dicfo Bacto-agar. Data collected after 4 wk for the percent of explants with buds, and after 10 wk for all the other parameters.

[2] Within each concentration, percentages followed by different letters are significantly different at $P \leq 0.05$.

[3] Mean ± SE; means followed by different letters are significantly different at $P \leq 0.05$.

Rooting of Shoots

To induce rooting, the elongated shoots (Fig. 2 *B*) were variously treated with IBA before rooting in sterile glass jars containing rooting substrate (peat:vermiculite 1:1) and moistened with quarter strength MCM medium. The treatments were: a) liquid pulse with 1.0 mM IBA for 4 h, b) 100 mM IBA pulse in gelrite-containing medium for 5 days, and c) powder treatment of the cut ends of the shoots with talcum containing 15 mM IBA. Liquid pulse with 1.0 mM IBA for 4 h proved most satisfactory because a higher proportion of shoots produced roots that were multiple in number (Table 9 and Fig. 2 *C*). No significant differences among the treatments on root length were shown.

Inasmuch as the liquid pulse treatment with 1.0 mM IBA for 4 h was found to be beneficial for root induction, 1.0 mM IBA was applied for three different time periods, to further optimize the

TABLE 6

EFFECT OF AGE OF EMBRYO EXPLANTS AFTER STRATIFICATION ON ADVENTITIOUS BUD FORMATION AND DEVELOPMENT IN *Pinus halepensis*

Age, Days[1]	Percent Explants with Buds[2]	No. of Buds per Explant[3]	BFC Index	Percent Shoots >3 mm	SEC Index	Hyperhydricity[4]
0	92.0[a]	22.9 ± 2.7[bc]	21.1	33.2	7.0	−
3	63.1[b]	61.9 ± 8.9[a]	39.0	25.4	9.9	+
5	56.7[b]	60.7 ± 11.8[a]	34.4	22.6	7.8	+
7	64.0[b]	42.4 ± 2.7[ab]	27.1	21.2	5.8	+
10	64.0[b]	18.6 ± 2.1[c]	11.9	38.2	4.5	−

[1] Embryo explants were cultured on AE + 3% sucrose medium before culturing them on AE + 5.0 μM BA + 3% sucrose medium. Data collected after 4 wk for the percent of explants with buds, and after 10 wk for all the other parameters.

[2] Percentages followed by different letters are significantly different at $P \leq 0.05$.

[3] Mean ± SE; means followed by different letters are significantly different at $P \leq 0.05$.

[4] *See* Table 1, *note* 4.

duration of pulse treatment. The elongated shoots were treated with 1.0 mM IBA for 4, 5, or 6 h before their culture on the above-mentioned rooting medium. Treatment durations of greater than 4 h were found to be superior because almost all the shoots formed roots that had a greater root length; furthermore, 6-h treatment produced the highest number of roots per explant (Table 10). Hence, for routine applications, 6-h pulse treatment with 1.0 mM IBA was used at the rooting stage.

Transplantation of Rooted Shoots

The rooted shoots did not require any special treatment for the transplantation stage. In the growth chamber with >70% humidity, almost all the shoots survived and resumed normal growth. In the greenhouse, the plants behaved normally and produced axillary branches (Fig. 2 *D*).

Histological Studies

At Day 0, different parts of the embryo contained densely packed cells with large vacuoles. No cell divisions were observed in any part

TABLE 7

EFFECT OF DIFFERENT MEDIA ON SHOOT ELONGATION OF *Pinus halepensis*[1]

Medium	Average Number of Shoots >5 mm per Explant[2]	Average Number of Shoots >10 mm per Explant[2]
AE	1.9 ± 0.4[a]	1.0 ± 0.1[a]
DCR	1.8 ± 0.3[a]	1.0 ± 0.1[a]
MCM	2.2 ± 0.3[a]	1.5 ± 0.2[b]

[1] The shoots were elongated on three-quarter strength media (AE, DCR, MCM) for 5 wk, after 4 wk on AE + 5 μM BA medium, and 3 wk on hormone-free AE medium.

[2] Mean ± SE; within each parameter, means followed by different letters are significantly different at $P \leq 0.05$.

TABLE 8

EFFECTS OF TWO DIFFERENT CONCENTRATIONS OF ZEATIN AND BA ON *Pinus halepensis* SHOOT MULTIPLICATION[1]

Cytokinin, μM	Number of Proliferated Shoots per Explant[2]
Zeatin	
1.0	5.1 ± 0.5[a]
5.0	6.0 ± 0.6[a]
BA	
1.0	3.7 ± 0.4[b]
5.0	5.2 ± 0.5[a]
Control (no cytokinin)	3.2 ± 0.3[b]

[1] Medium: three-quarter strength MCM + 3% sucrose + 0.8% Difco Bacto-agar. Data collected after 4 wk, from shoots previously cultured for 11 wk.

[2] Mean ± SE; means followed by different letters are significantly different at $P \leq 0.05$.

of the embryo. By 3 to 5 days, random mitotic divisions could be observed in the epidermal and subepidermal layers of the embryonic tissue, especially in the epicotyl and cotyledons (Fig. 3 *A*). By 7 days the cells in different parts of the embryo enlarged and more intercellular spaces and densely staining nuclei were present. Figure 3 *B* shows mitotic divisions in the cells of the cotyledons adjacent to the stomatal complexes. By this time, cell divisions became frequent in the axillary regions of the epicotyl (Fig. 3 *C*). By 9 days the cotyledonary tip region showed more organized divisions, becoming swollen by Day 11, and having organized groups of cells, i.e. meristemoids, which themselves were densely packed (Fig. 3 *D*), with a distinct epidermal layer. At this stage, higher magnifications revealed the presence of multicellular shoot bud primordia (Fig. 3 *E*). By 19 days, cataphylls could be observed at the tips of the cotyledons (Fig. 3 *F*, and 4 *A*). Although the majority of these cotyledon tips produced shoot buds subsequently (Fig. 4 *A*), not all did so. Figure 3 *F* possibly shows such a tip which produced only a needlelike structure. Tissue below this region showed evidence of disorganization, with large intercellular spaces (Fig. 3 *F*), or re-

TABLE 9

EFFECTS OF DIFFERENT IBA TREATMENTS ON IN VITRO ADVENTITIOUS ROOT FORMATION IN *Pinus halepensis*[1]

Treatment	Percent Rooted Shoots[2]	Number of Roots per Explant[3]	Maximum Root Length, cm[3]
Liquid pulse (1.0 mM IBA × 4 h)	83.3[a]	2.6 ± 0.2[a]	2.1 ± 0.3[a]
Pulse in gelrite (100 μM IBA × 5 days)	45.4[b]	1.9 ± 0.2[b]	1.8 ± 0.5[a]
Rooting powder (15 mM IBA in talcum)	58.1[ab]	2.0 ± 0.2[b]	2.1 ± 0.3[a]

[1] Following each treatment, the shoots were transferred on quarter-strength MCM medium + 1% sucrose, and evaluated after 4 wk for rooting.

[2] Percentages followed by different letters are significantly different at $P \leq 0.05$.

[3] Mean ± SE; means followed by different letters are significantly different at $P \leq 0.05$.

TABLE 10

EFFECTS OF DIFFERENT TIMES OF IBA APPLICATION ON IN VITRO ADVENTITIOUS ROOT FORMATION IN *Pinus halepensis*[1]

Treatment Duration, h	Percent Rooted Shoots[2]	Number of Roots per Explant[3]	Maximum Root Length, cm[3]
4	66.7[a]	2.1 ± 0.2[a]	2.6 ± 0.5[a]
5	86.7[ab]	3.5 ± 0.3[b]	5.9 ± 0.7[b]
6	96.3[b]	5.0 ± 0.4[c]	5.0 ± 0.5[b]

[1] Shoots were pulsed with 1 m*M* IBA, after which they were transferred to sterile peat:vermiculite (1:1) moistened with quarter-strength MCM medium + 1% sucrose, and evaluated after 4 wk for rooting.

[2] Percentages followed by different letters are significantly different at $P \leq 0.05$.

[3] Mean ± SE; means followed by different letters are significantly different at $P \leq 0.05$.

mained intact (Fig. 4 *A*). In a transverse section of the hypocotyl at Day 22, one (Fig. 4 *B*) or many shoot buds (Fig. 4 *C*) could be seen on the periphery of the hypocotyl, between the bases of adjacent cotyledons. Subsequently, additional shoot bud promordia developed along the cotyledons (Fig. 4 *D*).

Discussion

The principles and approaches needed to bring about plantlet regeneration, via organogenesis from embryonic explants of conifers, are well known (Thorpe and Patel, 1984). As a result, today lab-scale micropropagation protocols exist for over 30 conifers (Thorpe et al., 1991). To this list *Pinus halepensis* Mill. can be added, as a result of the study reported here. This study confirms the essential empirical nature of protocol development, wherein the requirements for each stage of the regeneration process must be experimentally determined.

For bud induction in Aleppo pine, a cytokinin alone, and BA in particular, proved satisfactory. This finding is in agreement with the majority of studies carried out previously (Thorpe et al., 1991). Only occasionally (e.g., Minocha, 1980; Mott and Amerson, 1981; Chang et al., 1991) is an auxin also required for direct organogenesis in conifers. The use of the BFC and SEC indices in these studies allowed for objective quantitative measures to be used for selecting between treatments that gave apparently similar results. Thus the best explant, medium formulation and its strength, type and age of explant, and gelling agent could be determined. One interesting finding was that cotyledons and parts of the embryo explant not in contact with the medium also formed adventitious shoots. This finding is different from that observed with many conifers, e.g., *Pinus radiata* (Aitken et al., 1981), *Pinus taeda* (Mott et al., 1977), and *Pinus canariensis* (Martinez Pulido et al., 1990), in which cotyledons not in contact with the medium do not form shoots. As is found quite frequently, the transfer of buds to a cytokinin-free medium with activated charcoal enhanced shoot bud development and elongation. SEC index was particularly valuable in selecting treatments that enhanced the production of rootable shoots in the shortest period. Hyperhydricity, although common, was not a major problem, as several treatments gave good BFC and SEC indices without producing watery shoots.

Shoot multiplication through axillary bud development was possible through the use of cytokinin treatment, which gave a multiplication rate of up to fivefold in a period of 4 to 6 wk. A similar approach has been previously used by us, with *P. canariensis* (Martinez Pulido et al., 1990), and others, e.g., by Zel et al. (1988) with *P. sylvestris*, and Baxter et al. (1989) with several tropical pines. The ability to multiply the in vitro-derived shoots through repetitive axillary bud development is particularly valuable when large numbers of clones are required. To date, in vitro-derived shoots of *P. halepensis* have been maintained in culture on hormone-free medium for over 2 yr without loss of growth and multiplication potential.

Rooting of elongated shoots was induced by various methods. However, pulsing of the shoots with a sterile solution of IBA (1 m*M*) for 6 h gave the best results, i.e., nearly 100% rooting with multiple roots. Unlike the closely related *P. brutia* (Abdullah et al., 1987), a cytokinin was not required in combination with auxin for rooting. The approach by pulsing followed by ex vitro rooting is becoming standard with conifers, e.g., in *P. canariensis* (Martinez Pulido et al., 1990), and *Larix occidentalis* (Harry et al., 1991), as it produces a better quality root (Mohammed and Vidaver, 1988). Generally, rooting is the most difficult stage of the micropropagation process (Mohammed and Vidaver, 1988), but it was not a problem with Aleppo pine. Moreover, hardening was easily achieved because almost all plantlets transferred to the greenhouse survived and continued to develop. At present, several of these plants have achieved heights of over 2 m in 2 yr in the greenhouse (Fig. 2 *D*).

Histologic studies of adventitious bud induction revealed similar patterns, as reported earlier (Cheah and Cheng, 1978; Yeung et al., 1981), although some peculiarities of Aleppo pine should be noted. The earliest events related to mitotic activity were observed by Day 5, and they were restricted to the epidermal and subepidermal layers of the tissue in contact with the culture medium. The first organized mitotic divisions, leading to meristematic centers or meristemoids, were observed by Day 11, and they were located at both the tips of the cotyledons and the junctions of adjacent cotyledons. Subsequently, the former always gave rise to cataphylls and shoot bud primordia, the latter produced axillary buds, although they were not formed from preexisting meristems (Fig. 2 *A*). After these primary regenerative events, multiple shoot buds developed along the cotyledons and the entire epicotyl/hypocotyl region close to their base. It is important to note that the formation of numerous adventitious buds along the hypocotyl was observed only when they were isolated by the rest of the embryo. One can hypothesize that the first meristematic events act as strong sinks for one or more unknown substances, other than cytokinins, which are essential for further inductive events. In other systems, e.g., in *Brassica juncea* (Sharma et al., 9119), auxinlike endogenous factors have been implicated.

In summary, a protocol for the micropropagation of embryo explants of *P. halepensis* Mill. has been developed. After stratification, excised embryos were placed horizontally on AE medium, with 3% sucrose, 5 μM BA, and gelled with 0.8% Difco Bacto-agar for 28 days. After 3-wk on hormone-free AE medium for initial bud development, shoot elongation was optimized by transferring the shoot buds onto gelled three-quarter-strength MCM medium, containing 3% sucrose, 0.1% activated charcoal, followed by monthly subcultures. Shoots longer than 1 cm were pulsed in liquid IBA (1 m*M*) for

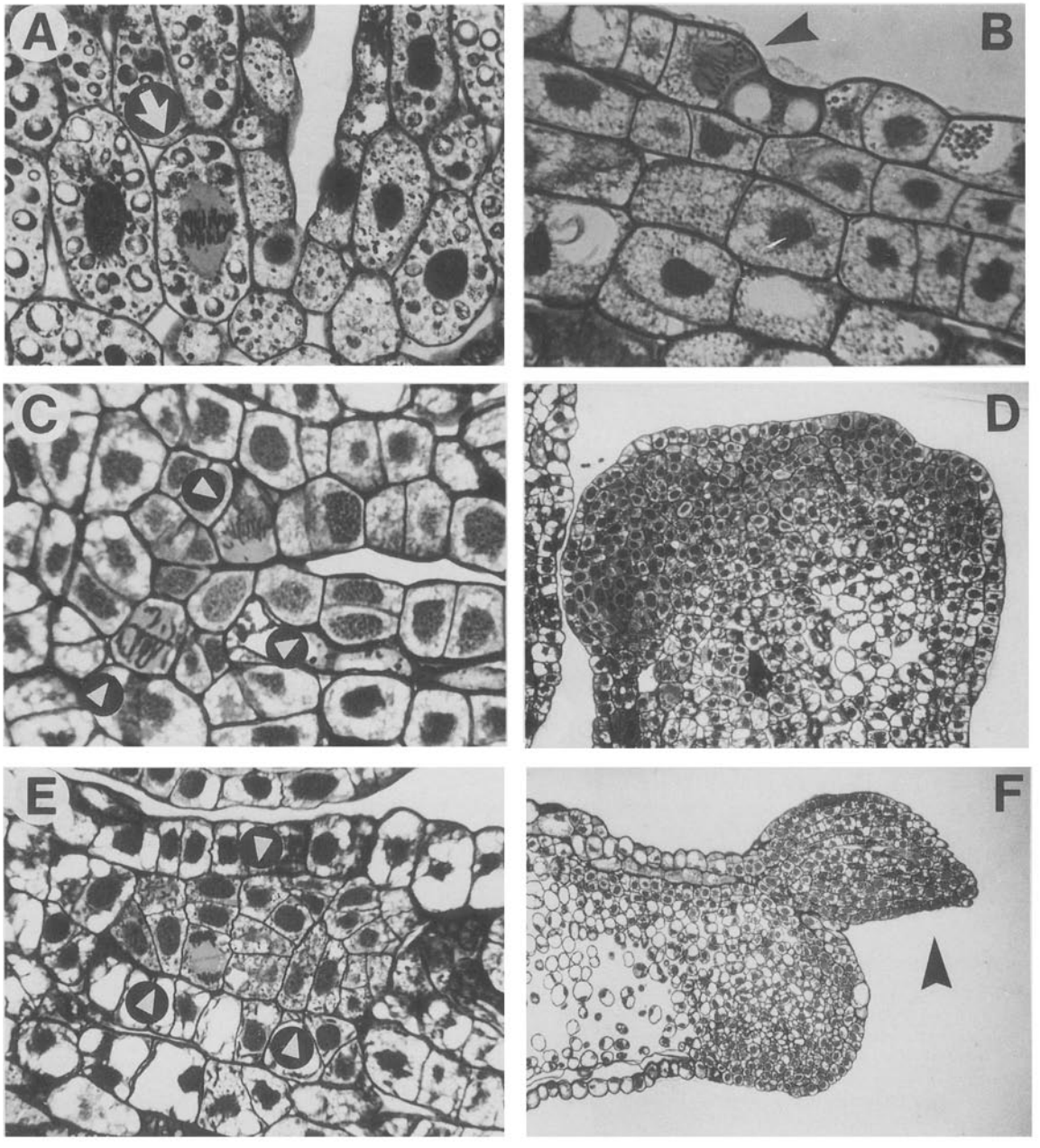

FIG. 3. Histologic studies on shoot formation in Aleppo pine. *A*, Day 3 on the induction medium. First mitotic divisions (*arrow*) in the subepidermal layer of the basal part of a cotyledon. ×384. *B*, Day 7. A dividing cell (*arrow*) of the cotyledonary epidermal layer, adjacent to a stomatal complex. ×384. *C*, Day 7. Random cell divisions (*arrows*), strictly located in the axillary regions of adjacent cotyledons. Both periclinally and anticlinally dividing mitotic figures are recognizable. The first adventitious shoot buds are in general observed in these zones (*see* Fig. 2 *A*). ×384. *D*, Day 11. Densely packed cells in the tip region of a cotyledon. *Note* the densely plasmatic cells of the mitotic zone and the distinct epidermal layer. ×200. *E*, Day 11. A 22-cell meristemoid (*arrows*) located just below the epidermal layers of a cotyledon. ×280. *F*, Day 19. A cataphyll (*arrow*) arising at the tip of a cotyledon without evidence of a shoot bud. *Note* the large intercellular spaces inside the cotyledonary tissue. ×80.

6 h, and then transferred for 4 wk in sterile peat:vermiculite (1:1), moistened with quarter-strength MCM with 1% sucrose, for rooting. Rooted shoots were finally potted in peat:vermiculite (1:1) and misted for 3 to 4 wk before transfer to the greenhouse. Using the above protocol, it is possible to produce 10 to 14 plantlets from each embryo explant during 20 to 25 wk. Furthermore, well-developed shoots can be indefinitely multiplied (ca. fivefold) by transferring them on a three-quarter-strength MCM medium, containing 5 μM BA, followed by monthly subculture of the developed shoots on hormone-free medium.

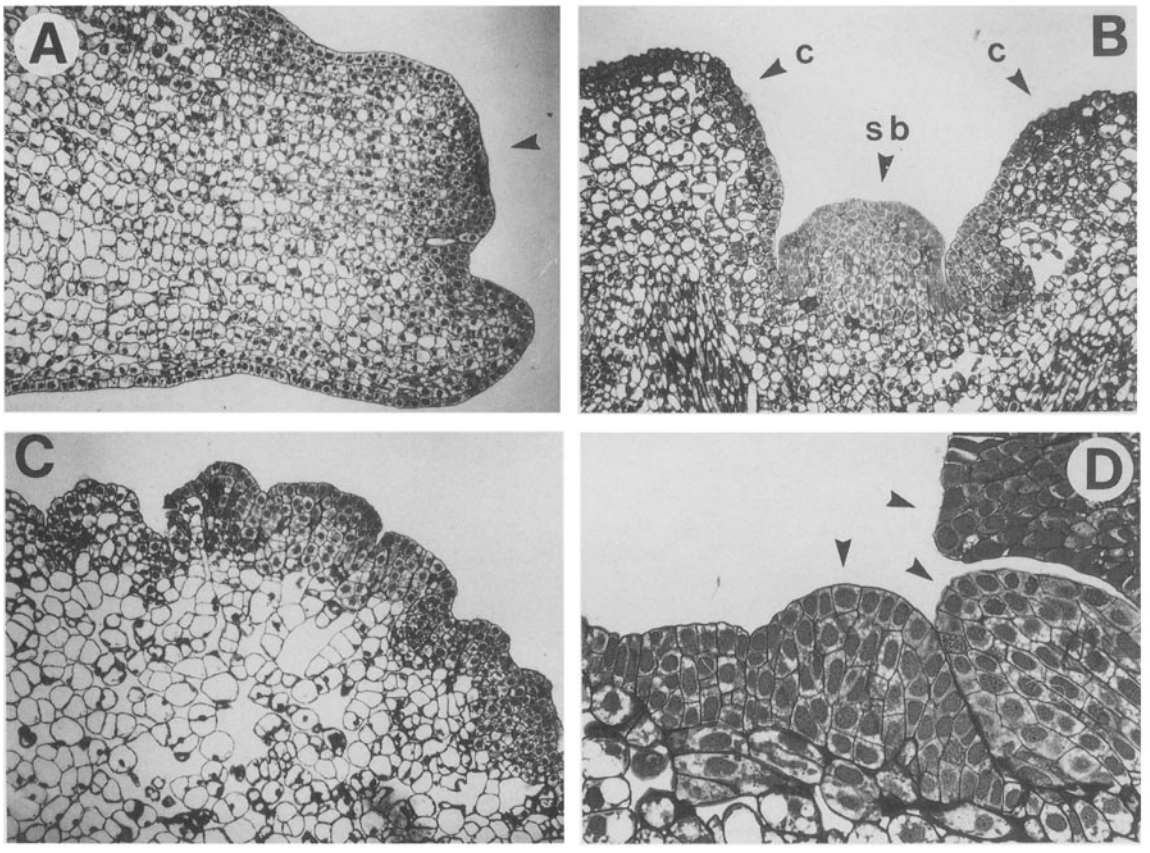

FIG. 4. Histologic studies on shoot formation in Aleppo pine. *A*, Day 19. As in Fig. 3 *F*, a cataphyll arising at the tip of a cotyledon. *Note* that a shoot bud is developing (*arrow*) and no large intercellular spaces are evident. ×80. *B*, Day 22, transverse section. A shoot bud (*sb*) emerging at the axil of two adjacent cotyledons (*c*), perpendicularly to them. ×80. *C*, Day 22, transverse section. As in Fig. 4 *B*, but here numerous adventitious shoot buds are emerging. ×80. *D*, Day 24. Adventitious shoot buds (*arrows*) emerging along two adjacent cotyledons. ×250.

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