Biol Res 32: 19-28, 1999

Optimization of *in vitro* culture conditions for *Pinus radiata* embryos and histological characterization of regenerated shoots

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ABSTRACT

Different *in vitro* culture conditions were tested on *Pinus radiata* organogenic embryos. Optimum shoot induction occurred at 26.1°C, whereas the best elongation resulted at 21.4°C. Supplements of 2.5 mg/l or 5 mg/l of BAP added to the induction media produced a similar number of regenerated shoots, which differed statistically from 1.0 mg/l of BAP and 0.025 mg/l TDZ. Addition of 10 mg/l $MnSO_4$ to $LP_{1/2}$ medium significantly increased the number and quality of *in vitro* regenerated shoots. The removal the apical region of shoots cultured in LP 2.5 mg/l of BAP increased the number of *de novo* generated shoots by 23%, compared to a control group with intact shoots. Approximately 70% of the *in vitro* shoots of *P. radiata* were of wet phenotype (hyperhydrated appearance); the rest were waxy in appearance. Histological cuts did not produce any differences in phenotypes, but scanning electronic microscopy of needles gave evidence of differences in epicuticular wax deposits.
Abbreviations: LP: Quoirin and LePoivre basal medium, without plant growth regulators; LP_1: LP medium + 1 mg/l BAP; $LP_{2.5}$: LP medium+ 2.5 mg/l BAP; LP_5 : LP medium + 5 mg/l BAP; $LP_{1/2}$: LP basal medium at half strength of macroelements, 2% commercial sugar, ammonium nitrate 100 mg/l, calcium nitrate 564.5 mg/l, hydroxyquinoleine 1.25 mg/l, MS vitamins and without plant growth regulators; $LPT_{0.025}$: LP medium + 0.025 mg/l TDZ; BAP: N-6 benzylaminopurine; TDZ: Thidiazuron.

Key words: *P. radiata*; BAP, TDZ, *in vitro* culture; wet and waxy phenotype

INTRODUCTION

Pinus radiata is Chile's most important forest species for wood and cellulose production. Selection of elite phenotypes and controlled pollination are currently used for its genetic improvement. However, this is an expensive and laborious method that produces a small number of seeds with high genetic and commercial value. With the aim of increasing the number of selected individuals obtained by traditional methods, *in vitro* micropropagation from the embryos of controlled pollinated seeds has been implemented. Given the multiplying effect of the process, it is essential to ensure the conservation of the elite genotypes throughout the successive stages of propagation.

Micropropagation of *P. radiata* is based on the induction of zygotic embryos by organogenesis (1). The success of the method depends upon the number and quality of the adventitious shoots produced per embryo. Our results have shown high heterogeneity in the regenerative capacity of the embryos and in the quality of the shoots produced. A significantly high proportion of regenerated shoots show dark green and clustered needles that appear to be hyperhydrated. This phenotype, which is also difficult to acclimatize, was described as "wet" by Aitken-Christie *et al* (2). High quality shoots that can be acclimatized suc-

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Received 4.4.99. In revised form 12.5.99. Accepted 24.5.99

cessfully were designated "waxy" (2) due to their appearance.

Some authors propose that the shoot generation ability of *in vitro* cultures of *P. radiata* has a genetic component (4, 17). It is not clear to date whether the wet and waxy phenotypes had a genetic origin, if they were determined by nutritional and environmental factors, or if they were somaclonal variants induced by *in vitro* conditions. Aitken-Christie *et al* (2), suggest that the wet phenotype corresponds to an intermediate state of hyperhydricity between the vitrified (translucid) and the waxy shoot and that there might be culture medium factors that trigger this response.

Cytokinins, micronutrients and microenvironmental conditions have been documented to play an important role in the morphogenic response of several forest species (2, 4, 5, 22, 25, 26). In our current work, we have studied the effect of different culture conditions on the number and quality of shoots produced through embryo organogenesis. Temperature effect, different BAP concentrations and manganese addition to embryo cultures were specifically tested. The induction of lateral buds through apex removal and needle pruning of regenerated shoots was also assayed.

Morphological and histological characterizations of the two observed phenotypes were carried out to examine the differences at the cellular and tissue levels.

MATERIALS AND METHODS

Plant material and culture conditions

Selected *P. radiata* seeds were provided by the Forestal Mininco, S.A. nursery. The seeds were surface sterilized by soaking them in 2% (w/v) aqueous $NaHClO_4$ for 10 minutes and in 50% (v/v) aqueous H_2O_2 for 5 minutes, followed by three rinses in sterile distilled water. The seeds were then stratified at 4°C for 24 hours and sterilized again in 50% (v/v) H_2O_2 for 5 min. After rinsing, the embryos were obtained by dissecting the seeds.

The embryos were cultured in an inverted position (2) with the cotyledons immersed in a bud induction medium (20) (LP2.5 supplemented with 3% w/v commercial sugar, 0.8% Gibco agar and 2.5 mg/l BAP). The pH was adjusted to 5.8 before autoclaving for 20 minutes at 121°C. After 3 weeks, explants were passed to elongation steps for approximately four months (2). In this stage, the explants were successively transferred every 4 weeks to glass jars (7 x 10 cm) containing 20 ml of hormone-free $LP_{1/2}$ medium. Cultures were maintained at a 16-hour photoperiod with a light intensity of 60 $\mu Em^{-2}s^{-1}$ at 22 +/- 6°C. Isolated shoots with an average height of 1.2 cm were placed on LP medium containing 0.2% activated charcoal (Merck 2186) for one additional transfer before rooting. Root initiation was induced on a water-agar medium containing 2.0 mg/l indolebutyric acid (IBA) and 0.5 mg/l napthaleneacetic acid (NAA) for 5 days. The induced shoots were then transferred onto a low sugar $LP_{1/2}$ (containing 10 g/l commercial sugar) to evaluate rooting.

Temperature assay

The effect of temperature on shoot induction and the elongation processes was studied. Sixty embryos were pre-cultured as described above for one week. The embryos were then individually cultured in 8.0 x 2.5 cm glass jars containing $LP_{2.5}$ medium and used in the following assays.

Shoot induction: 12 groups of five embryos each were transferred to an illuminated (60 $\mu Em^{-2}s^{-1}$) chamber with a controlled continuous temperature gradient at one of the following temperatures: 13.5°C; 16.3°C; 19.7°C; 22.9°C, 24.1°C; 26.1°C; 28.5°C; 30.8°C; 31.3°C; 31.9°C; 32.8°C; 34.1°C. The explants were shoot induced for three weeks, transferred to $LP_{1/2}$ and then cultured for an additional month in the temperature gradient controlled chamber. After 7 weeks of exposure to regulated temperatures, half of the meristematic tissue of each embryo was transferred to the regular growth chamber (22 +/- 6°C). Four weeks later (week 11) the shoot buds per explant at each temperature treatment were counted.

Shoot elongation: the remaining meristematic clumps were maintained in the temperature gradient controlled chamber, now adjusted to 13.5°C; 17.7°C; 21.8°C; 24.1°C; 26.1°C; 28.5°C; 30.8°C; 31.3°C; 31.9°C; 32.8°C; 34.1°C for another four weeks to identify the best shoot elongation temperature. Since the temperatures set at this stage were not the same as those of the induction step, the following changes were made: the embryos induced at 16.3°C were elongated at 17.7°C; those induced at 22.9°C were elongated at 21.8°C; and those induced at 19.7°C were equally distributed between these two temperatures. All other embryos had the same induction and elongation temperatures. At this point, one-third of the shoots were transferred to the regular growth chamber. The remaining shoots (2/3) were maintained in the temperature-controlled chamber and evaluated after 4 weeks of culture (15 weeks total) to determine the quantity of waxy shoots and the total number of shoots produced at each temperature. This assay was repeated twice. As a control, 35 embryos were cultured for 15 weeks in the regular growth chamber.

Cytokinin effect on shoot induction

Cytokinin concentrations tested on adventitious bud induction were selected from previous reports (2,10,15). Twenty-five embryos were cultured in one of the following treatments: Thidiazuron 0.025 mg/l ($LP_{T0.025}$); Benzyladenine 1 mg/l ($LP_{1.0}$), 2.5 mg/l ($LP_{2.5}$), or 5.0 mg/l ($LP_{5.0}$). After the induction period (3 weeks), the explants were cultured in hormone-free $LP_{1/2}$ for six months. The number of buds per embryo and the percentage of waxy shoots were evaluated. Statistical analysis was done applying the Mann-Whitney test ($P< 0.05$), and the Z test corrected for continuity.

Effect of additional Manganese on the elongation step

Fifteen 7-week-old induced embryos were cultured in LP medium containing 10 mg/l $MnSO_4$ (12, 21) and tested against an identical control group cultured in a normal LP (0.76 mg/l $MnSO_4$) (20). Shoot elongation steps were performed as described above for four months. At the end of this period, the following parameters were evaluated: a) number of buds per embryo, b) total number of shoots produced per treatment, c) percentage of waxy shoots in each treatment, and d) average height of the shoots produced in each treatment.

Induction of axillary shoots

From 33 to 116 isolated shoots with an average height of 1.7 cm were cultured for 2 weeks in the following multiplication media: $LP_{1/2}$: control, intact shoots, $LP_{2.5}$: intact shoots, $LP_{2.5}H$: half-pruned back needles, and $LP_{2.5}A$: apically-pruned shoots. The shoots were then transferred to the elongation steps ($LP_{1/2}$ for four months), after which each treatment was evaluated for its ability to produce axillary shoots as follows: multiplication factor (n° of final shoots / n° of initial shoots). Statistical analysis of the discrete data was carried out by the Mann-Whitney test ($P< 0.05$) to compare means.

Morphological and histological analysis of wet and waxy Pinus radiata shoots

Morphological analysis: Wet and waxy *in vitro* cultured *Pinus radiata* shoots were characterized with a Nikon SMZ-10 stereoscopic microscope and photographed. A scanning electron microscope (SEM) was used to examine the epicuticular needle surface of wet and waxy shoots according to Fowke (11). Needles were fixed in 50% v/v aqueous glutaraldehyde, dehydrated in a graded series of absolute acetone with 20% increments, freeze-dried and coated with gold/palladium using a splutter coating unit. Samples were then examined under a JEOL JSM-25-S-II scanning electron microscope for epicuticular wax development.

Histological analysis: Samples were prepared for optical microscopy according to the O'Brien and McCully modified method

(19). Needles were fixed in FAA solution (formaldehyde 5%, acetic acid 5%, ethanol 63% and water 27%), gradually dehydrated in a graded series of absolute ethanol using 20% increments, vacuum infiltrated, and gradually embedded in Paraplast. Samples were sectioned in a rotating microtome and stained with safranine (0.5% w/v) and fast green (0.5% w/v). Sections were observed and photographed using a Nikon HSX-DX optical microscope.

RESULTS

Temperature assay

The best temperature for the induction and elongation of *Pinus radiata* shoots was determined by evaluating the quantity and quality of single shoots generated per embryo. The number of shoots produced at each temperature is shown in Figure 1. The best shoot bud induction (after 11 weeks of culture) occurred at 26.1°C, obtaining 134 shoots per embryo. This temperature level also yielded the highest percentage of waxy shoots (34%, not shown). These tendencies continued after 6 months of culture. Lower temperatures caused a significant reduction in the response. Conversely, higher temperatures inhibited shoot development (absence of bars above 30.8°C in Figure 1). Shoot elongation was evaluated by assessing the quantity and quality of single shoots with an average height of 1 cm after four months of culture. The best shoot elongation temperature (evaluated after 15 weeks of culture) was obtained at 21.8°C. Elongation was also acceptable at 24.1°C; 26.1°C and 28.5°C (Fig 1). The control treatment carried out in the growth chamber yielded 31 shoots per embryo with 28% waxy shoots.

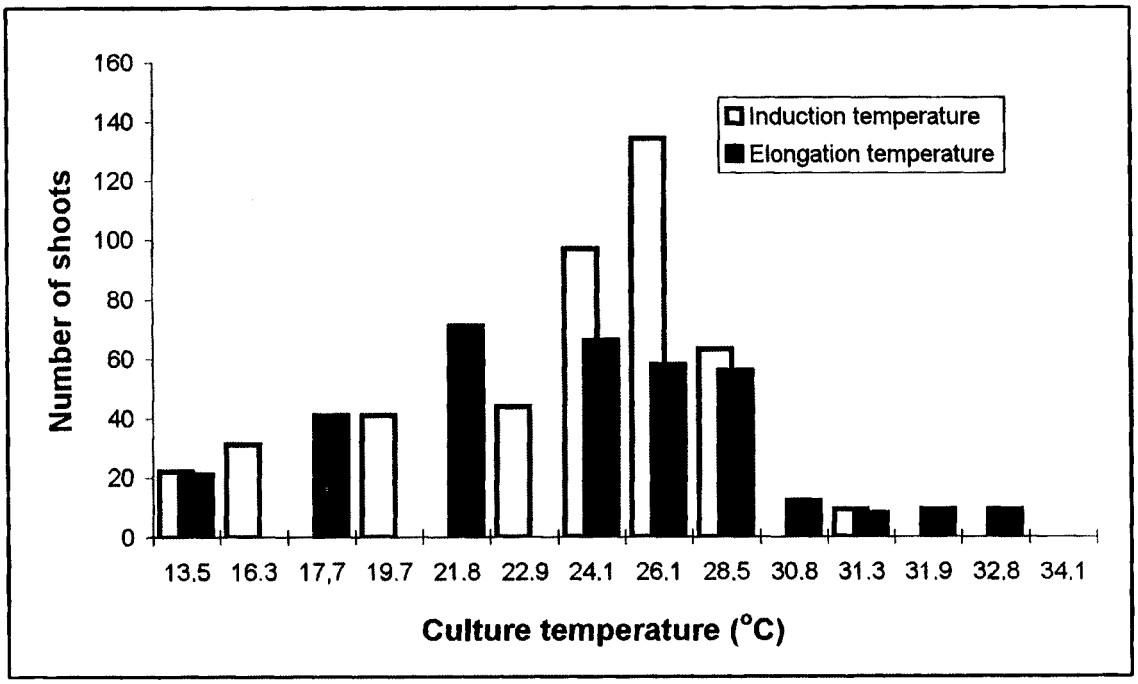

Figure 1: Effect of temperature on the induction and elongation of *P. radiata* shoots*.

Induction temperature: number of shoot buds per embryo at the end of the induction treatment (11 weeks).

Elongation temperature: number of shoots (1cm height) per embryo at the end of the elongation treatment (15 weeks).

* Absence of bars above 30.8 °C means no shoot development.

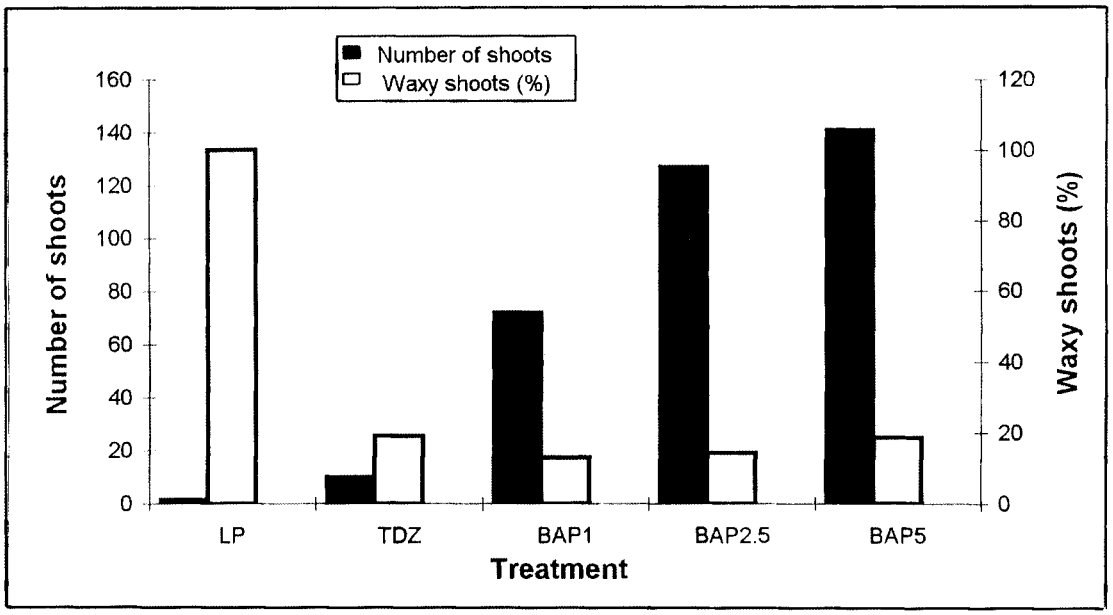

Figure 2: The number of shoots produced per embryo and the percentage of waxy phenotypes obtained from the *in vitro* culture of *P. radiata* with different treatments of BAP or TDZ.

25 embryos were cultured in each treatment and evaluated 6 months later.

Evaluation of cytokinin concentration on bud induction

BAP induced a higher average number of buds per explant than TDZ. Overall, 5 mg/l BAP proved to be the best concentration in terms of number of shoot buds per explant (140.8) (Fig 2) and total number of shoots (1,267, not shown). Although data on the difference in the number of shoot buds produced per explant between 2.5 mg/l and 5 mg/l BAP were not statistically significant, the latter generated better quality shoots (Fig 2). Regeneration of shoots per embryo with 0.025 mg/L TDZ and other previously-tested concentrations (0.001 mg/l and 0.05 mg/l) was 10 times less productive than with 5 mg/l BAP. However, shoots induced with TDZ were larger than those induced with BAP.

Effect increased $MnSO_4$ in LP medium

We observed that greenhouse-acclimatized *in vitro Pinus radiata* plants were deficient in Mn. A comparative foliar analysis of the Mn content of *in vitro* cultured shoots and *ex vitro* plants grown in the greenhouse showed a significant decrease of this microelement in the *in vitro* cultured shoots (20 ppm *in vitro*, 197 ppm *ex vitro*).

Since the Mn concentration in LP medium is 13 times lower than in other media used in *Pinus radiata in vitro* cultures (21), we tested the addition of $MnSO_4$. LP medium was supplemented with 10 mg/l of Mn (as $MnSO_4$) to compare the effect of manganese on the growth and elongation of shoots in relation to LP basal medium (0.76 mg/l $MnSO_4$). After 10 weeks, an increase of 53.8% in the number of shoots per embryo was obtained in the 10 mg/l $MnSO_4$ treatment. The percentage of waxy shoots at this concentration was also greater (80%) (Table 1).

Induction of axillary shoots

Pinus radiata shoots were induced for two weeks on LP medium supplemented with 2.5 mg/l BAP, with or without the pruning of lateral needles and apex, in order to assess large scale propagation by axillary bud multiplication of elongated shoots. A good response was obtained in

TABLE 1

Effect of an increase in the concentration of Manganese in the number and quality *of P. radiata* shoots generated *in vitro.*

Treatments	Total number of shoots produced	Number of shoots produced per embryo	Shoot Quality
$LP_{1/2}$ + 0.76 mg/l $MnSO_4$	782*	52	Size: 1 cm waxy shoots: 14.4%
$LP_{1/2}$ + 10 mg/l $MnSO_4$	1037**	80	Size: 1 cm waxy shoots: 26%

*15 embryos were cultured for 4 months before their evaluation. **13 embryos were cultured for 4 months before their evaluation.

all BAP-treated shoots. An increase of 53% in the number of axillary shoots occurred when they were induced on $LP_{2.5}$, compared to the control $LP_{1/2}$ (Fig 3). $LP_{2.5}$ treatment led to the production of an average of 4 axillary buds per shoot, compared to 1.87 with $LP_{1/2}$ (Fig 3). Removal of the apical meristem ($LP_{2.5A}$) increased the effect of $LP_{2.5}$ (intact needles) by 23%.

Morphological and histological analysis of in vitro wet and waxy Pinus radiata shoots

The *in vitro* culture of *Pinus radiata* embryos produced wet and waxy phenotypes (Figs 4A-B). However, the predominant wet phenotype (70%) was not suitable for greenhouse acclimatization (62%

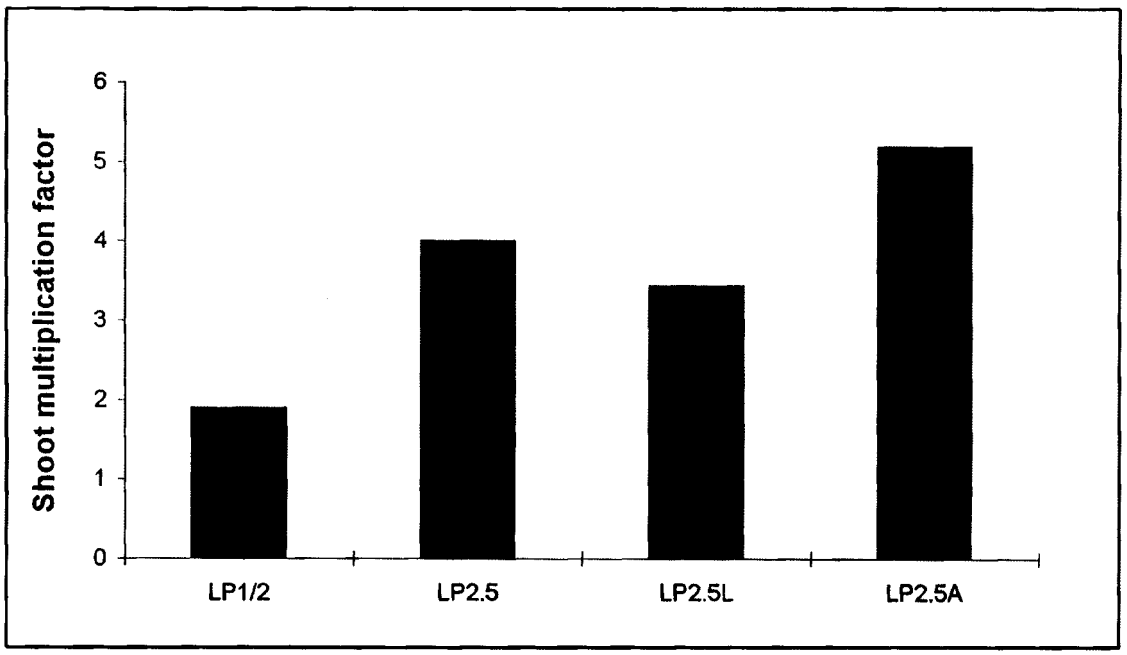

Figure 3: Effect of cutting on shoots and the addition of BAP in LP medium on the induction of lateral buds in *P. radiata* shoots grown *in vitro.*

The shoot multiplication factor was calculated as follows: Number of final shoots produced/ Number of initial shoots.

mortality) due to its high susceptibility to infection and dehydration. Conversely, the waxy phenotype showed a 67% *in vitro* rooting rate and 2% mortality during greenhouse acclimatization.

Histological sections showed similar tissue organization in both phenotypes. Epidermis structure, resin channels, and vascular conducts all showed normal appearance (Fig 4 C-D). Needle surfaces of

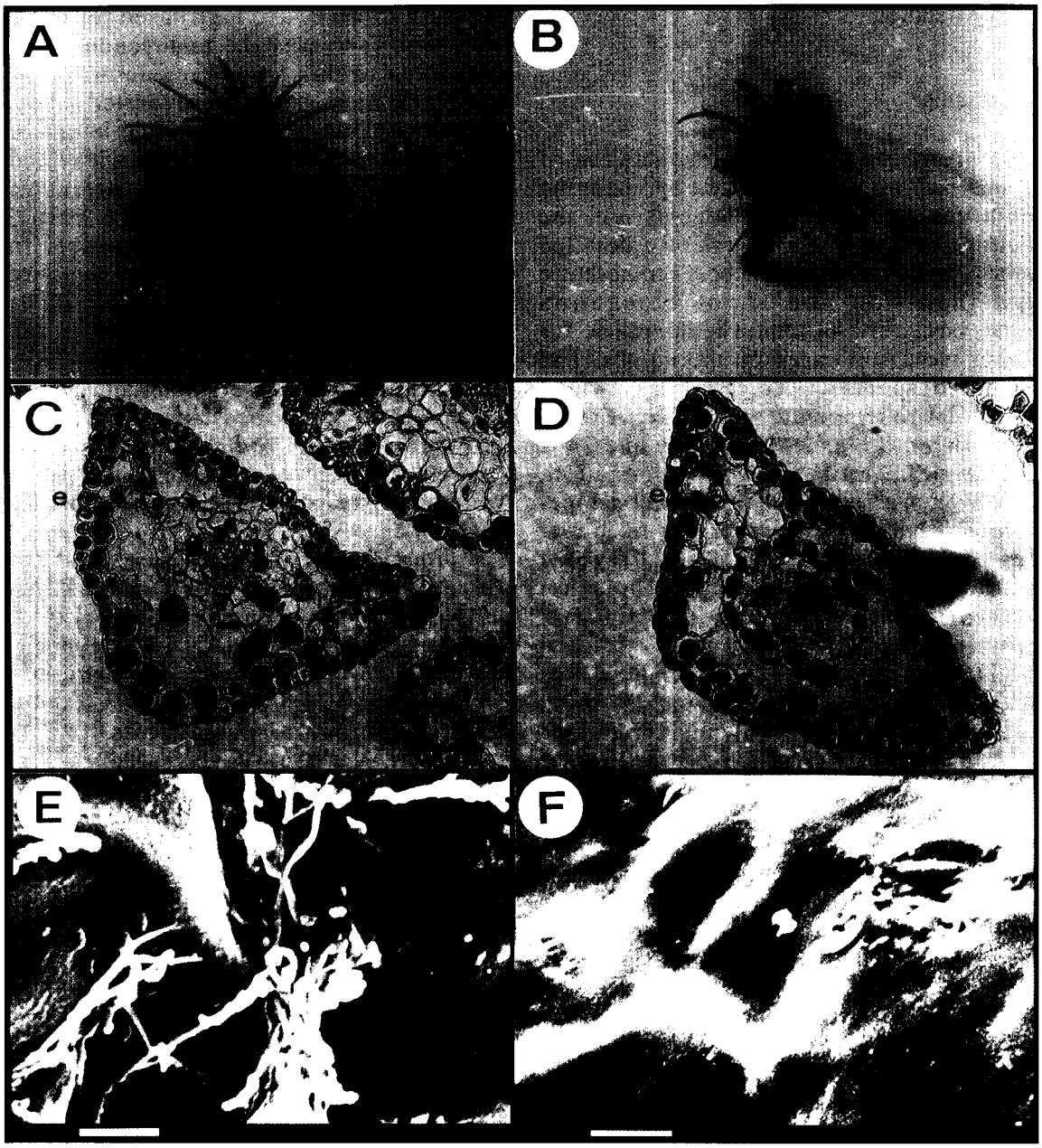

Figure 4: Morphological and histological characteristic of waxy and wet phenotypes. **A-B**. Appearance of waxy and wet phenotypes respectively. **C-D**. Histological cuts of *P. radiata* needles of waxy and wet phenotypes respectively grown *in vitro*. e: epidermis, rc: resinous channels, vb: vascular bundle. **E-F**: Scanning electron microscopy of the epidermal surface of *P. radiata* needles of waxy and wet phenotypes grown *in vitro*. **E**: Detail of globular and tubular waxes that are present in aged needles of waxy phenotype (20,000x). **F**: Needle epidermis of the wet phenotype with a marked reduction in globular waxes and an absence of tubular waxes (20,000x).

wet and waxy shoots are shown in Figures 4E-F. Tubular epicuticular wax deposits were abundant on the epidermis of waxy needles (Fig 4E), but were absent in wet needles (Fig 4F). Globular epicuticular wax was less abundant on wet needles than on waxy needles.

DISCUSSION

The protocol for *in vitro* regeneration of *Pinus radiata* was optimized by changing both environmental components and the culture medium. By means of a detailed study, we determined the best temperature for induction and shoot elongation processes. The optimum temperature for shoot induction based on the number and quality of shoots was 26.1°C (Fig 1), which is similar to temperatures used by Aitken-Christie *et al* (1) (28°C daytime and 24°C nighttime). Optimum shoot elongation, however, was achieved at a lower temperature (21.8°C), with favorable results at temperatures up to 28.5°C. Previous information given by other authors has shown that the elongation is optimal at temperatures of 24°C and 20°C (day and night, respectively) (1,2). Temperatures above 28.5°C considerably affect the shoot elongation process, producing clear signs of dehydration, necrosis and browning of the lower needles.

The stimulating effect of cytokinins on the induction of shoots on *P. radiata* embryos, as described by other authors (5, 22), was also tested in our work. A concentration of 2.5 mg/l of BAP was sufficient to obtain a high number of good quality shoots per embryo. Previous experiments conducted in our laboratory using concentrations of 0.001 mg/l to 0.05 mg/l TDZ did not induce more than 15 shoots per embryo (not shown). However, even when TDZ did not show a significant effect on the number of shoots (Fig 2), those induced were significantly larger and more vigorous than the BAP-induced shoots. Our results differ from other reports (14), which have demonstrated that TDZ stimulates shoot proliferation in various legumes (10) and woody species (14). Through shoot induction with BAP, a high percentage of wet shoots were obtained in all the treatments. This phenotype is not desirable due to its high mortality (62% in our case) during acclimatization in the greenhouse (18). It is possible that the high cytokinin concentration (BAP 5 mg/l) used for shoot induction increases the endogenous concentration of this growth regulator, influencing the number of hyperhydrated shoots (8). We did notice however, that in one of the analyzed families, induction with 1 and 2.5 mg/l BAP also yielded a high percentage of wet shoots, which suggests a partial genetic component for this response. In this sense, the evaluation of the response obtained with different families of *Pinus radiata* showed a high variability in the percentage of wet and waxy shoots produced (not shown). Bergmann and Stomp (4) previously described a genotypic effect on the rooting of *P. radiata*. We, however, have not found genetic differences between wet and waxy phenotypes in preliminary RAPDS analysis (23).

Adding 10 mg/l of manganese ($MnS0_4$) to LP medium showed a significant stimulating effect on the number and quality of generated shoots (Table 1). The concentration of this microelement in the original LP medium (0.76 mg/l) apparently did not cover the *P. radiata* requirements. Important lignin synthesis enzymes, such as peroxidase, require manganese as a cofactor, which could justify adding it in higher concentrations to *in vitro* cultures of woody species (12, 21).

While evaluating the induction of axillary shoots over a 6-month period, we observed that BAP alone in the culture medium increased the number of new shoots per explant by 53% over that of the control group. An additional 23% increase in new shoots was achieved by removing the shoot apex. It is likely that a decrease in the endogenous level of auxins, due to the loss of the apical meristem in conjunction with an increase of the cytokinins level in the culture medium, triggered axillary bud development in *in vitro* cultured shoots. Das *et al* (7) also determined that the regeneration of *Vigna mungo* shoots was observed only when explants were derived from the

axillary shoots of plants germinated in a medium containing cytokinin. A similar response was observed by pruning needles in adult *Douglas fir* shoots cultured *in vitro* (13). Traditionally, *in vitro* culture of forest species only stimulates the induction of axillary shoots in a cytokinin-supplemented medium (16). We found that there is also a synergistic effect on the stimulation of buds if the apical area of the cultured shoot is simultaneously removed (however, no statistical difference was found between pruned and unpruned cytokinin-treated shoots.

Since there was a high incidence of wet type shoots during the *in vitro* culture of *P. radiata* embryos, we characterized this phenotype through morphological, histological, and scanning electron microscopy analysis, using waxy shoots as controls (Fig 4). Histological studies comparing wet and waxy shoots did not yield observable differences in epidermal, mesophyll and vascular cells (Figs 4°C-D), contrary to that described in other species (6). Nevertheless, scanning electron microscopy revealed differences in the amounts and type of wax deposits of epidermal cells (figs 4E-F). Changes in the wax composition of *in vitro* regenerated shoots have been described as a response to different relative humidity conditions (3). The lack of wax on the needle surface could account for the hyperhydric appearance of the wet phenotype. We do not know the factors that inhibit the deposition of epicuticular wax in the wet shoots of *P. radiata*, although temperatures above and below 26.1°C and agar concentrations of less than 0.8% seem to favor hyperhydration in cultures. It is possible to partially revert the presence of wet shoots by adding hydroxyquinoleine (a compound with anti-vitrifying properties) to the culture medium, decreasing the forms of reduced nitrogen (15), increasing the agar concentration (24), or reducing the exposure of embryos to high cytokinin concentrations. Environmental and culture medium factors affecting hyperhydricity of shoots have been shown in micropropagation assays with different *P. radiata* clones (2, 9).

In summary, we have optimized conditions for multiplying *P. radiata* embryos via *in vitro* organogenesis. According to our results, even when genetic components are involved, the number and quality of shoots can be improved by an adequate management of culture conditions and environmental factors.

ACKNOWLEDGEMENTS

We are grateful to Professor Aldo Norero for providing culture facilities in a temperature gradient-controlled chamber. We also thank Berta Puebla and Pamela Flores for their collaboration in the culture of *Pinus radiata* embryos. This work was supported by Forestal Mininco S. A.

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