In Vitro Adventitious Shoot Formation on Cotyledons of *Pinus pinea*

María Victoria González¹, Manuel Rey^{2,3}, Raffaella Tavazza, Stefano La Malfa, Luigi Cuzzo, and Giorgio Ancora

ENEA—C.R. Casaccia, Biotechnology and Agriculture Division, Section of Biochemistry, Biophysics and Molecular Biology, C.P. 2400, 00100 Rome A.D., Italy

Additional index words. conifer, micropropagation, tissue culture, plant regeneration, Italian stone pine

Abstract. Plant regeneration was obtained from adventitious buds induced in isolated cotyledons of Italian stone pine (*Pinus pinea* L.). The best results for bud induction were obtained by using half-strength LePoivre medium with 4.5 μ M 6-benzyladenine for 30 days. Shoot elongation was achieved in the same medium without growth regulators but with the addition of 0.5% activated charcoal. The induction medium was the best also for shoot multiplication, but it was necessary to include subcultures on elongation medium. The slow elongation rate of adventitious shoots remains the greatest obstacle to multiplication. Root formation (15%) after 5 months was observed when shoots were cultured on elongation medium for long periods.

Pinus pinea L. (Italian stone pine) is an economically important species in the Mediterranean region because of its ecological and landscape significance, and its value in reforestation. *Pinus pinea* is also highly regarded in Spain and Italy for its edible seeds. The species has been the subject of a few reports dealing with in vitro propagation (Diamantoglou et al., 1990; García-Ferriz et al., 1994). Capuana and Giannini (1995) published a protocol for in vitro plantlet regeneration from cotyledons. The present research reports improvements to previous protocols for *P. pinea* by using modified LePoivre (LP) medium, as employed in tissue culture of other species of *Pinus* (Aitken-Christie et al., 1988; Bergmann and Stomp, 1992; Rey et al., 1996).

Materials and Methods

Seeds of *P. pinea* were collected from open-pollinated trees growing at the ENEA research center in Casaccia (Rome) and stored at 4 °C. After removal of the testa, the remain-

ing tissue was surface sterilized by immersion in 2% sodium hypochlorite for 15 min, kept overnight under tap water, and then soaked for 15 min in 6% H₂O₂. Afterwards they were rinsed three times with sterile distilled water and maintained for 48 h at 2–4 °C. Finally, they were soaked again in 6% H₂O₂ for 15 min followed by two rinses with sterile distilled water before extracting the embryos aseptically.

For bud induction, cotyledons were excised from embryos and placed horizontally on half-strength LP medium (1/2 LP), as modified by Aitken-Christie et al. (1988), containing 4.5 μ M BA (6-benzyladenine) and 3% sucrose. After 15, 25, or 30 d, cotyledons were transferred to 1/2 LP without growth regulators supplemented with 0.5% activated charcoal (Sigma, St. Louis). Explants were transferred monthly to the same fresh medium. The numbers of surviving and bud-forming explants were recorded 60 d after culture initiation, and the numbers of shoots per explant 90 d after culture initiation.

For shoot elongation, five media were used by altering the concentrations of basal medium (1/2 or 1/4 LP, without growth regulators), activated charcoal (0.1%, 0.5%, or 1%), and sucrose (2% or 3%). Elongation experiments were performed using isolated shoots at least 0.5 cm in length. The number of shoots elongating at least 0.5 cm over their initial length was recorded after 30 d.

Fig. 1. Adventitious shoot formation on excised cotyledons of *Pinus pinea*. Explants were cultured for 30 d on half-strength modified LePoivre medium containing 4.5 μ M BA and then transferred to the same medium without BA containing 0.5% activated charcoal. (A) Bud induction after culture for 60 d. (B) Shoot formation after culture for 90 d. Bars = 0.3 cm.

Received for publication 18 Aug. 1997. Accepted for publication 20 Jan. 1998. We wish to thank Dr. M.A. Pombal (Dept. Biología Funcional y Ciencias de la Salud, Universidad de Vigo, Spain) for his excellent photographic assistance. This work was partially supported by grants from the FOREST program of the European Union (No. MA2B-CT91-0040 "Upgrading of Southern Pines"), and from the Italian Ministry of Agriculture (MIRAAF). M. Rey gratefully acknowledges the support of a postdoctoral fellowship from the FLAIR program of the European Union. S. La Malfa acknowledges partial support by a short-term fellowship from the Italian National Research Council (CNR). The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

¹Current address: Dept. Biología Vegetal, Escuela Politécnica Superior, Universidad de Santiago de Compostela, Campus de Lugo, E-27002 Lugo, Spain.

²Current address: Lab. Fisiología y Biotecnología Vegetal, Fac. Biología y Ciencias del Mar, Universidad de Vigo, E-36200 Vigo, Spain. E-mail: mrey@uvigo.es.

³To whom reprint requests should be addressed.

Table 1. Effect of duration of exposure to 4.5 μ M BA on *Pinus pinea* cotyledon survival and morphogenesis in vitro.

Exposure to cytokinin (days)	Period of culture (days)	
	60	90
	Surviving cotyledons ² (%)	No. shoots per surviving cotyledon
15	33 b ³	2.4 b
25	42 b	4.4 ab
30	85 a	6.1 a

²All surviving cotyledons formed buds.³Mean separation within columns by Duncan's multiple range test, $P < 0.01$.Table 2. Percentage of elongation of *Pinus pinea* shoots grown for 30 d on modified LP medium supplemented with varying levels of basal salts, activated charcoal, and sucrose.

Salt dilution	Sucrose concn (%)	Activated charcoal concn (%)	No. shoots	Elongated shoots (%)
1/4	3	0.5	83	39 a ²
1/2	3	0.5	143	44 a
	3	0.1	78	28 b
	3	1.0	82	24 bc
	2	0.5	91	17 c

²Mean separation by Duncan's multiple range test, $P < 0.01$. Fig. 2. Shoot multiplication of *Pinus pinea* after 30 d on 1/2 LP medium with 4.5 μ M BA. Bar = 1 cm.

Shoot multiplication was accomplished using two protocols. In the first, isolated 0.5-cm shoots were cultured vertically on 1/2 LP with 4.5 or 25 μ M BA and 3% sucrose, and transferred every 40 d to the same fresh medium. In the second, shoots were cultured successively on these media for 40 d, followed by culture on 1/2 LP without growth regulators and with 0.5% activated charcoal (elongation medium) for 40 d.

All media were adjusted to pH 5.8 before autoclaving and were solidified with 0.8% Difco Bacto-agar. Culture media were autoclaved at 120 °C and 98 kPa for 15 min. Culture vessels were plastic jars (70 × 35 mm) containing 7 mL of medium. Cultures were incubated in a growth chamber at 26 ± 2 °C day/20 ± 2 °C night temperature with a 16-h photoperiod (cool-white light fluorescent tubes, 60 μ mol·m⁻²·s⁻¹).

All experiments were repeated at least three times with a minimum of 30 explants per treatment and repetition. Every experiment consisted of at least six replicates (jars) with five explants each per treatment, cotyledons from different embryos in the case of induction, or shoots for elongation and multiplica-

tion. Data were analyzed statistically using one-way analysis of variance (ANOVA) and the differences contrasted using Duncan's multiple range test ($P < 0.01$). Percentage data were converted by arcsin transformation prior to ANOVA.

Results and Discussion

Bud induction occurred in *P. pinea* cotyledons without intermediate callus formation (Fig. 1A). Exposure to 4.5 μ M BA for 30 d (Table 1) was optimum for explant survival (85%) after culture for 60 d. All surviving explants formed buds in all treatments tested. The average number of buds per cotyledon after exposure to BA for 30 d (19.7 ± 2.4) was greater than previously reported (Capuana and Giannini, 1995). Our results indicate the great morphogenic potential of these explants, as well as the effectiveness of the LP medium on *P. pinea*. After culture for 90 d (Fig. 1B), 6.1 shoots per explant were obtained following the 30-d induction treatment (Table 1).

The percentage of elongated shoots differed among the five media used (Table 2). Our observations on adventitious shoot elon-

gation agree with those of Capuana and Giannini (1995). The greatest percentage of elongating shoots was obtained with 1/2 or 1/4 LP without growth regulators plus 0.5% activated charcoal and 3% sucrose. The medium chosen for subsequent experiments as the elongation medium was 1/2 LP.

Multiplication of shoots of *P. pinea* (Fig. 2) was similarly low after one subculture on 1/2 LP with 4.5 μ M (79% with 1.6 new shoots per explant) or 25 μ M BA (76% with 1.9). In subsequent subcultures, both proliferation and shoot length declined. Using the second protocol, including subcultures on elongation medium, the rate of proliferation increased to 94% on 1/2 LP with 4.5 μ M BA, although the number of shoots per explant did not increase. This proliferation rate was maintained for three subcultures. Proliferation might be improved by using only those shoots at least 1 cm in length for multiplication, maintaining the shorter ones in elongation medium. This view is supported by Capuana and Giannini (1995), who used for multiplication only shoots that were 1–1.5 cm in length after elongating for 11–12 weeks.

Root formation was observed when shoots were cultured on elongation medium for long periods. The first rooted shoots were observed after 2–3 months of culture. After 5 months, 10 of 67 shoots (15%) had rooted.

In conclusion, cotyledons of *P. pinea* produced high numbers of adventitious buds on modified LP medium. This medium, in combination with longer periods of shoot multiplication, provides an improved protocol for plant regeneration and micropropagation of this species. The slow elongation rate of adventitious shoots remains the greatest obstacle to efficient shoot multiplication.

Literature Cited

- Aitken-Christie, J., A.P. Singh, and H. Davies. 1988. Multiplication of meristematic tissue: A new tissue culture system for radiata pine, p. 413–432. In: J.W. Hanover and D.E. Keathley (eds.). Genetic manipulation of woody plants. Plenum Press, New York.
- Bergmann, B.A. and A.-M. Stomp. 1992. Influence of taxonomic relatedness and medium composition on meristematic nodule and adventitious shoot formation in nine species. Can. J. For. Res. 22:750–755.
- Capuana, M. and R. Giannini. 1995. In vitro plantlet regeneration from embryonic explants of *Pinus pinea* L. In Vitro Cell. Dev. Biol. - Plant 31:202–206.
- Diamantoglou, S., I. Panagopoulos, A. Munoz-Ferriz, and S. Rhizopoulou. 1990. In vitro studies of embryo growth, callus formation and multiple bud induction of *Pinus pinea* L. J. Plant Physiol. 137:58–63.
- García-Férriz, L., L. Serrano, and J.A. Pardos. 1994. In vitro shoot organogenesis from excised immature cotyledons and microcuttings production in stone pine. Plant Cell Tissue Organ Cult. 36:135–140.
- Rey, M., M.V. González, R.J. Ordás, R. Tavazza, and G. Ancora. 1996. Factors affecting transient gene expression in cultured radiata pine cotyledons following particle bombardment. Physiol. Plant. 96:630–636.