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ORIGINAL ARTICLE

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In vitro plantlet regeneration of *Schinopsis balansae* (Anacardiaceae)

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Abstract A protocol for the in vitro regeneration of rooted plants from nodal single bud segments of 10-year-old *Schinopsis balansae* trees was developed. Nodal segments were harvested from actively growing shoots of plants grown from seeds and maintained in pots under greenhouse conditions, and from epicormic shoots obtained by forced flushing of branches. Culture of nodal segments on nutrient medium containing the mineral salts and vitamins of Murashige and Skoog medium at 1/4 strength (1/4 MS), supplemented with 100 mg l^{-1} ascorbic acid, 3% sucrose, and 5–15 μM 6-benzyladenine resulted in regeneration of multiple shoots. Rooting of regenerated shoots was observed in 1/4 MS medium with vermiculite as the substrate and supplemented with 7.5 μM indolbutyric acid.

Keywords *Schinopsis balansae* · Micropropagation · Nodal explants · Tissue culture

Introduction

Schinopsis balansae Engl. ("Quebracho"), a member of the Anacardiaceae, is an economically important species of southern South America. It is a large tree with extremely hard wood. In Argentina it is used for posts as well as for the production of tannin (Barkley 1962) and charcoal (Lewis et al. 1997). It is commonly propagated by seed. However, seed viability decreases significantly during storage (Valentini 1960). Attempts to propagate this tree by cuttings have failed .Therefore, in vitro techniques could represent a very useful tool for the propagation of large numbers of desirable trees for research studies and planting out.

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Plant regeneration through either organogenesis or somatic embryogenesis has been accomplished in some members of the Anacardiaceae, such as *Mangifera indica* (Bhaskaran and Prabhudesai 1989; Litz et al. 1984), *Anacardium occidentale* (Bhaskaran and Prabhudesai 1989; Das et al. 1996; Gogte and Nadgauda 2000), *Pistacia atlantica* (Mederos et al. 1997) and *Pistacia vera* (Dolcet-Sanjuan and Claveria 1995; Parfitt and Almehdi 1994; Yang and Lüdders 1993). However, up to now there are no publications on the tissue culture of *Schinopsis balansae*. The present communication describes the procedure developed for the clonal propagation of 10-year-old *S. balansae* trees through the induction of in vitro shoot proliferation from nodal explants and subsequent elongation and rooting of the regenerated shoots.

Materials and methods

Plant material

All plant material was provided by Unitan (Barranqueras, Chaco, Argentina). Nodal segments (0.5 to 1.0-cm-long stem segments bearing a node and a portion of underlying internode) of *Schinopsis balansae* Engl. were used for in vitro culture. Explants were taken from nonlignified branches of two lots of plant material obtained by germination of seeds of one tree. Plants were maintained in pots in a greenhouse for 10 years (lot I). Epicormic shoots were obtained by forced flushing of branches (Fig. 1) of trees (10–12 m height) growing in the field for 10 years (lot II). The branches (1.0–2.5 cm thick) were collected during November–December, cut into 25 to 30-cm segments and laid horizontally in a humid chamber (45 μmol m^{-2} s^{-1}) on a bed of sand and vermiculite (2:1 w/v) and forced to flush epicormic shoots. After 5–7 weeks the flushed shoots (5–10 cm long) were used as the source of explants. Both explant sources were sprayed with a mixture of 0.5‰ Agrimicina and 2‰ Kaptan.

Explants were surface-disinfected by successive soaking for 1 min in 70% ethanol and 30 min in 1.5% NaClO (with 0.1% Triton). Finally, the explants were washed five times with sterile distilled water.

Fig. 1 Forced flushing of a branch segments from a 10-year-old *Schinopsis balansae* tree. *Bar* 1 cm

Culture medium

For establishment, the nodal segments were cultured on quarter-strength Murashige and Skoog (1962) medium with 3% sucrose, 0.6% agar (1/4 MS) lacking growth regulators and supplemented with various antioxidant agents at 100 mg l^{-1} each (L-cysteine, HCl-cysteine, ascorbic acid or citric acid). Activated charcoal (2 g l^{-1}) was also used in some culture media. All the chemicals of the media were provided by Sigma Chemical.

After 4 weeks of culture, the established explants were subcultured to a shoot-multiplication medium consisting of 1/4 MS with 100 mg l^{-1} ascorbic acid, supplemented with 0; 1; 5; 10 or 15 μM of BA (benzyladenine), kinetin, TDZ (thidiazuron) or isopentenyladenine.

The pH of the media was adjusted to 5.8 using a few drops of either 0.1 *N* HCl or 0.1 *N* KOH prior to the addition of agar. Tubes were covered with aluminium foil and autoclaved at 1.46 kg cm^{-2} for 20 min.

Physical culture conditions

Each explant was placed individually in a 40-ml glass tube containing 10 ml of synthetic medium. Tubes were sealed with Resinite AF 50 (Casco, Buenos Aires) and incubated for 10 days in darkness before transfer to a growth room at 27±2°C under a 14/10 h (day/night) daylight cycle with an irradiance of 4.5 μmol m^{-2} s^{-1} at the culture level provided by cool white fluorescent lamps.

For shoot multiplication and rooting of regenerated shoots the cultures were maintained at 27±2°C with lighting (cool white fluorescent lamps) at 116 μmol m^{-2} s^{-1} and a 14 h photoperiod.

Experimental design and statistical analyses

For the establishment phase, ten nodal segments were cultured per treatment. Each experiment was repeated at least 3 times. After 4 weeks of culture, the number of established explants was recorded. At the shoot multiplication phase, each treatment consisted of ten explants. Treatments were arranged randomly on the shelves in the growth room. Each experiment was repeated 3 times. After 4 weeks of culture, the following data were recorded: micropropagation rate (MR), defined as number of regenerated buds/ bud cultured; numbers of explants forming single or multiple shoots. Means are given with the standard error (±SE). Analysis of variance (Tukey's multiple range test) was used for comparing the group means between treatments.

Rooting

Each regenerated shoot (2–3 cm long) was transferred to a 170-cm^3 glass jar containing 60 cm^3 of various substrates (perlite, vermiculite, perlite + vermiculite, or 0.7% agar) with 30 ml of rooting medium consisting of 1/4 MS supplemented with 0; 2.5; 5.0; 7.5; 15.0 or 17.5 μM IBA (3-indolebutyric acid). Results were recorded 60 days after the beginning of experiment. For each treatment ten shoots were used and the experiments were repeated 3 times.

Plantlets with well-developed roots were transferred to pots containing peat, perlite and sand (1:1:1 v/v) and were maintained at high humidity by regular water sprays.

Results and discussion

After 1–2 weeks of culture, most of the buds swelled, turned green and eventually sprouted. Some buds became brown or necrotic and a few buds (less than 10%) were contaminated with bacteria and/or fungi. The browning of the nodal segments was the main cause of losses during establishment of the explants. More than 50% of the nodal segments removed from lot I and all the nodal segments from lot II showed browning when cultured with only 1/4 MS (Fig. 2). Similar results have been obtained with other woody plant species, where explant tissues become brown or secrete black/brown pigments to the culture medium. These pigments, composed mainly of tannins or oxidised polyphenols, inhibit bud growth and usually kill the explants (Compton and Preece 1986; Thorpe et al. 1991). We obtained optimum establishment when nodal segments were cultured on 1/4 MS medium containing antioxidant agents. In most cases, 100% of the explants from lot I and up to 30% of the explants of lot II were established. On the other hand, the use of medium with 2 g/l^{-1} activated charcoal also improved the percentage of explants successfully established (Fig. 2). Antioxidants or activated charcoal have been widely used to control browning in tissue cultures of many plant species (Compton and Preece 1986; Preece and Compton 1991).

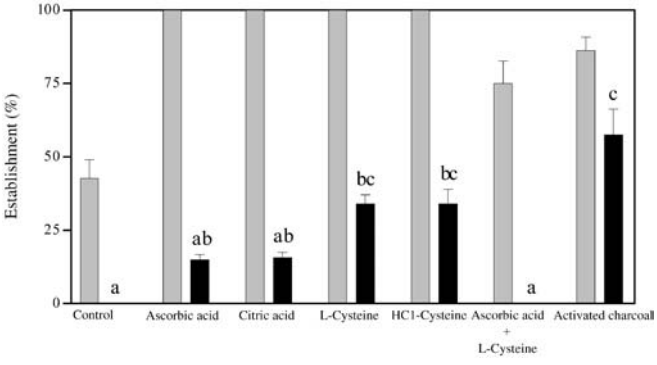

Fig. 2 Influence of antioxidant compounds and activated charcoal on in vitro establishment of buds from 10-year-old *S. balansae* tree. *Clear square* Explant derived from lot I: Plants obtained by germination of seeds. *Black square* Explant derived from lot II: epicormic shoots obtained by forced flushing of branches. Values represent the mean ± SEM of 3 replicates. ANOVA, Tukey's multiple range test. A different letter indicates a significant difference at $P<0.05$

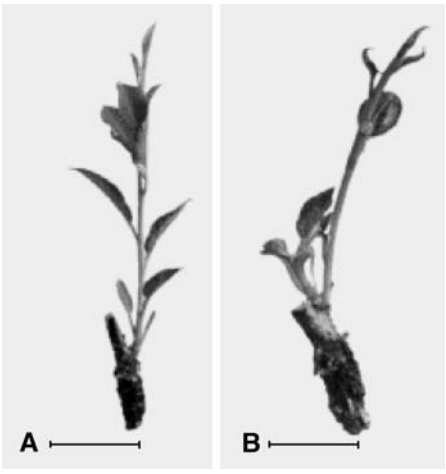

Fig. 3A, B Shoot development from a nodal segments of 10-year-old *S. balansae* trees. **A** Single shoot **B** two shoots. *Bar* 1 cm

Although the explants removed from epicormic shoots obtained by forced flushing of branches, exhibited a relatively low percentage of establishment, this system can be a useful tool for micropropagating mature trees which are growing in the field. This procedure has been used for in vitro plant regeneration of mature trees of various woody plants including *Eucalyptus grandis* (Ikemori 1987) and *Quercus robur* (Evers et al. 1993; Vieitez et al. 1994).

After the first transfer to fresh medium, the presence of one or several shoots originating from axillary buds was detected in various media. Thereafter, new shoots developed quickly and attained a mean length of 3–4 cm within 4 weeks (Fig. 3A, B). The effects of four concentrations of three cytokinins on shoot development from nodal segments are shown in Table 1. Almost 75% of the explants produced shoots when incubated in medium lacking cytokinin, suggesting that exogenous cytokinin is not required for shoot regeneration. In most cases, the addition of a cytokinin decreased the proportion of nodal segments that produced shoots (Table 1). However, exogenous cytokinin, increased the percent of nodal segments producing multiple shoots. The highest number of explants with multiple shoots was obtained on 1/4 MS+10μM BA. MRs were also promoted by inclusion of 5–15 μM BA in the culture medium. An MR of 10 was obtained in medium containing 10 μM BA, whereas an MR of 6 was recorded for the medium lacking cytokinin (Table 2). Several other studies have shown that BA stimulates the initiation of shoot growth from nodal segments of some woody plant species (see reviews by Aitken-Christie and Connett 1992; Einset 1991; Thorpe et al. 1991).

Media containing 1, 5, 10 or 15 μM TDZ were ineffective in inducing bud break and shoot regeneration (data not presented). These results contrast with those of Huetteman and Preece (1993) who found that TDZ stimulated axillary shoot proliferation in nodal-segment explants of many woody species.

In vitro rooting of the regenerated shoots (Fig. 4) was only successful when the rooting substrate was vermiculite. Perlite, alone or with vermiculite, as well as agar were ineffective for inducing root initiation. Similarly, *Ilex paraguariensis* initiated roots when grown in vermiculite in sterile jars (Sansberro et al. 2000). Sánchez et al. (1997) reported that regenerated shoots of chestnut rooted in a peat: perlite: vermiculite (1:1:1) substrate, and Aldrufeu (1987) that regenerated shoots of *Pelargonium* rooted in cellulose, perlite or vermiculite. With reference to the effect of addition of IBA to the 1/4 MS medium, it can be noted that the best rooting response was obtained in shoots cultured with 7.5 μM

Table 1 Influence of various concentrations of three cytokinins on shoot development from nodal segment of*Schinopsis balansae*. Values represent the mean (±SEM) of 3 replicates. ANOVA, Tukey's multiple range test: a different letter indicates a significant difference at$P$<0.01. Only shoots of more than 5 mm in length were scored. *BA* 6-Benzyladenine, *KIN* kinetin,*2iP* isopentenyladenine

Cytokinins	μM	Explants forming shoots (%)		
		One shoot/explant	Two or more shoots/explant	
Control	–	73 (7.2)	0	a
BA	1	50 (6.5)	17 (3.4)	ab
	5	7 (1.3)	58 (11.7)	cd
	10	9 (1.2)	75 (6.1)	d
	15	5 (0.7)	45 (4.7)	bc
2iP	1	38 (3.7)	20 (7.0)	ab
	5	43 (3.9)	10 (3.5)	a
	10	38 (3.8)	14 (1.7)	a
	15	53 (7.3)	20 (2.4)	ab
KIN	1	66 (5.5)	0	a
	5	60 (8.2)	3 (0.6)	a
	10	38 (4.9)	1 (0.3)	a
	15	34 (3.9)	5 (1.4)	a

Table 2 Effect of four concentrations of three cytokinins on the micropropagation rate of a 10-year-old *S. balansae* tree. Values represent the mean (±SEM) of 3 replicates. ANOVA, Tukey's multiple range test: a different letter indicates a significant difference at P<0.05

Cytokinins	μM	Micropropagation rate	
Control	–	6 (0.6)	ab
BA	1	6 (1.2)	ab
	5	9 (0.5)	ab
	10	10 (0.4)	b
	15	9 (0.3)	ab
2iP	1	5 (0.8)	ab
	5	5 (1.7)	ab
	10	7 (1.1)	ab
	15	7 (0.9)	ab
KIN	1	6 (0.7)	ab
	5	6 (1.2)	ab
	10	5 (0.6)	a
	15	5 (0.8)	a

Fig. 4 Root development on an in vitro regenerated shoot of *S. balansae* after 2 months in vermiculite with rooting medium

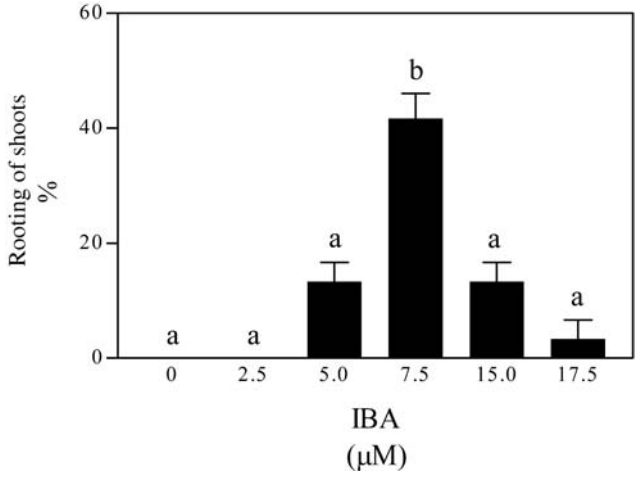

Fig. 5 Effect of indolbutyric acid concentration on in vitro rooting of regenerated shoots of *S.*. Values represent the mean ± SEM of 3 replicates. ANOVA, Tukey's multiple range test. A different letter indicates a significant difference at $P<0.05$

IBA (Fig. 5). In such cases, 40% rooting could be obtained in 60 days. Like many other woody plant species (Moncousin 1991), in our study, the presence of IBA in rooting media is a pre-requisite for root initiation. Roots generally began to appear during the first 30 days after the beginning of the subculture in rooting medium. In most cases, roots emerged directly from the shoots without a visible callus formation. Plantlets were successfully transferred to pots and grown under greenhouse conditions.

In conclusion, the protocol described above permits plant regeneration from adult *Schinopsis balansae* trees by in vitro culture of nodal segments. This system could be used for the clonal propagation of the superior genotypes and preservation of germplasm.

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