

Influence of genotype and explant source on indirect organogenesis by *in vitro* culture of leaves of *Melia azedarach* L.

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ABSTRACT: *In vitro* regeneration of shoots from leaf explants of the Paradise tree (*Melia azedarach* L.) was studied. Three different portions (proximal portion, distal portion and rachis of the leaflets) of three developmental stages (folded, young still expanding and completely expanded) of leaves of 10 – 15 years old plants of seven genotypes were cultured on Murashige and Skoog (1962) medium (MS) supplemented with 1mg.l⁻¹ benzylaminopurine (BAP) + 0.1mg.l⁻¹ kinetin (KIN) + 3 mg.l⁻¹ adenine sulphate (ADS).

The rachis of the leaflets of the completely expanded leaves was found to be the most responsive tissue, in most of the genotypes employed. Shoot regeneration occurred in leaf explants of all the genotypes tested. The best genotype for shoot regeneration was clone 4. Rooting was induced on MS medium supplemented with 2.5 mg.l⁻¹ 3-indolebutyric acid, IBA, (4 days) followed by subculture on MS lacking growth regulators (26 days). Complete plants were transferred to soil.

Abbreviations: ADS: adenine sulphate; BAP: 6-benzylaminopurine; IBA: 3-indolebutyric acid; KIN: kinetin; MS: Murashige & Skoog (1962) medium.

Introduction

The Paradise tree (*Melia azedarach* L.) is native of Southern Asia (Cozzo, 1944), and was introduced and naturalized through tropical America where it has a relatively fast rate of growth (Pennington, 1981). It is used as a decorative tree. The extracts made from its leaves, stems, flowers, seeds and fruits have insecticide properties (Breuer and Schmidt, 1995; Chen *et al.*, 1996;

Andreu *et al.*, 2000; Ursi Ventura and Ito, 2000). The extract of leaves also inhibited the replication in cell cultures of several pathogenic human and animal viruses (Coto and De Torres, 1999) and prevents Tacaribe virus encephalitis in mice (Andrei *et al.*, 1986).

The giant paradise (*Melia azedarach* var. *gigantea*) is a valuable cultivated forest species in the Misiones Province (Argentina). It is easily propagated by seeds, but the populations obtained often show great undesirable genetic variation. This drawback has been solved by using clonal propagation methods, such as *in vitro* axillary bud and nodal explant culture (Domecq, 1988; Ahmad *et al.*, 1990; Thakur *et al.*, 1998; Shahzad and Siddiqui, 2001).

Although plant regeneration through indirect organogenesis from leaves of shoots proliferating *in vitro*

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was accomplished for *Melia azedarach* (Vila *et al.*, 2003), there is no report on plant regeneration from different leaf parts obtained from field grown plants. The aim of present work is to study the influence of genotype and explant type on shoot regeneration of Paradise tree.

Materials and methods

Plant Material

The plant material was kindly provided by Danzer Forestación (Posadas, Misiones Argentina). Leaf explants of *Melia azedarach* L. obtained from plants from seven different genotypes, identified as 4, 14, 24, J1, J2, Lp and R, were used. All the genotypes were obtained by cloning mother plants (10 - 15 years old) and the resulting plants were grown in the garden of the Facultad de Ciencias Agrarias (UNNE), Argentina.

Explant culture and regeneration

Three developmental stages of leaves were chosen: folded (0.5 – 1 cm in length, 1 – 2% of final size) (Fig.

1A), young still expanding (2 - 4 cm in length, 3 – 7% of final size) (Fig. 1B) and completely expanded (10 cm in length, 24% of final size) (Fig. 1C). An approximately 0.2 cm² portion of the proximal and distal end of leaflets as well as rachis portions (Fig. 1C) of each developmental stage were employed as explants. Leaf explants were surface sterilized by immersion in 70% ethanol (10 sec) followed by NaOCl (0.25% active Cl) with two drops of TRITON-X- 100®, during 5 min and finally rinsed three time with sterile distilled water.

Basal medium (MS) was prepared according to Murashige and Skoog (1962). The explants were placed in 11 ml glass tubes with 3 ml of MS + 1 mg.l⁻¹ BAP + 0.1 mg.l⁻¹ KIN + 3 mg.l⁻¹ ADS (Vila *et al.*, 2003) supplemented with agar 0.75% (Sigma A- 1296). The pH of the media was adjusted to 5.8 with, either KOH, or HCl prior to the addition of agar. Tubes were covered with aluminium foil and autoclaved at 1.46 Kg/cm² for 20 min.

The explants were cultured with the adaxial side in contact with the medium. The tubes containing the explants were covered with Resinite AF 50® (Casco S.A.C Company Bs. As.) and incubated in a growth room at 27± 2°C in darkness.

TABLE 1.

Effect of the initial developmental stage of the leaves and portion of the leaflets of seven genotypes of *Melia azedarach* on the shoot regeneration after 5 weeks in MS + 1mg.l⁻¹ BAP + 0.1mg.l⁻¹ KIN + 3 mg.l⁻¹ ADS.

Developmental stage	portions of the leaflets	% of explants with shoots						
		genotypes						
		4	14	24	J1	J2	Lp	R
Folded leaf	proximal	3.3 (±3.3) ^{a1}	0 ^a	3.3 (±3.3) ^a	0 ^a	0 ^a	0 ^a	0 ^a
	distal	6.7 (±3.3) ^a	3.3 (±3.3) ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
	rachis	33.3 (±8.8) ^b	0 ^a	3.3 (±3.3) ^a	0 ^a	3.3 (±3.3) ^a	0 ^a	0 ^a
Young and expanding leaf	proximal	13.3 (±8.8) ^{ab}	0 ^a	16.7 (±3.3) ^b	0 ^a	0 ^a	0 ^a	0 ^a
	distal	16.7 (±3.3) ^{ab}	0 ^a	23.3 (±3.3) ^b	0 ^a	3.3 (±3.3) ^a	0 ^a	0 ^a
	rachis	30 (±11.5) ^b	0 ^a	6.7 (±3.3) ^a	6.7 (±3.3) ^b	3.3 (±3.3) ^a	3.3 (±3.3) ^a	0 ^a
Expanded leaf	proximal	20 (±5.8) ^{ab}	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
	distal	3.3 (±3.3) ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
	rachis	30 (±5.8) ^b	0 ^a	0 ^a	26.7 (±3.3) ^c	13.3 (±6.7) ^b	16.7 (±6.7) ^b	10 (±5.8) ^b

(±) = ± Standard error

¹Different letters, within columns, indicate significant differences at α 0.05, using Duncan's multiple comparison test

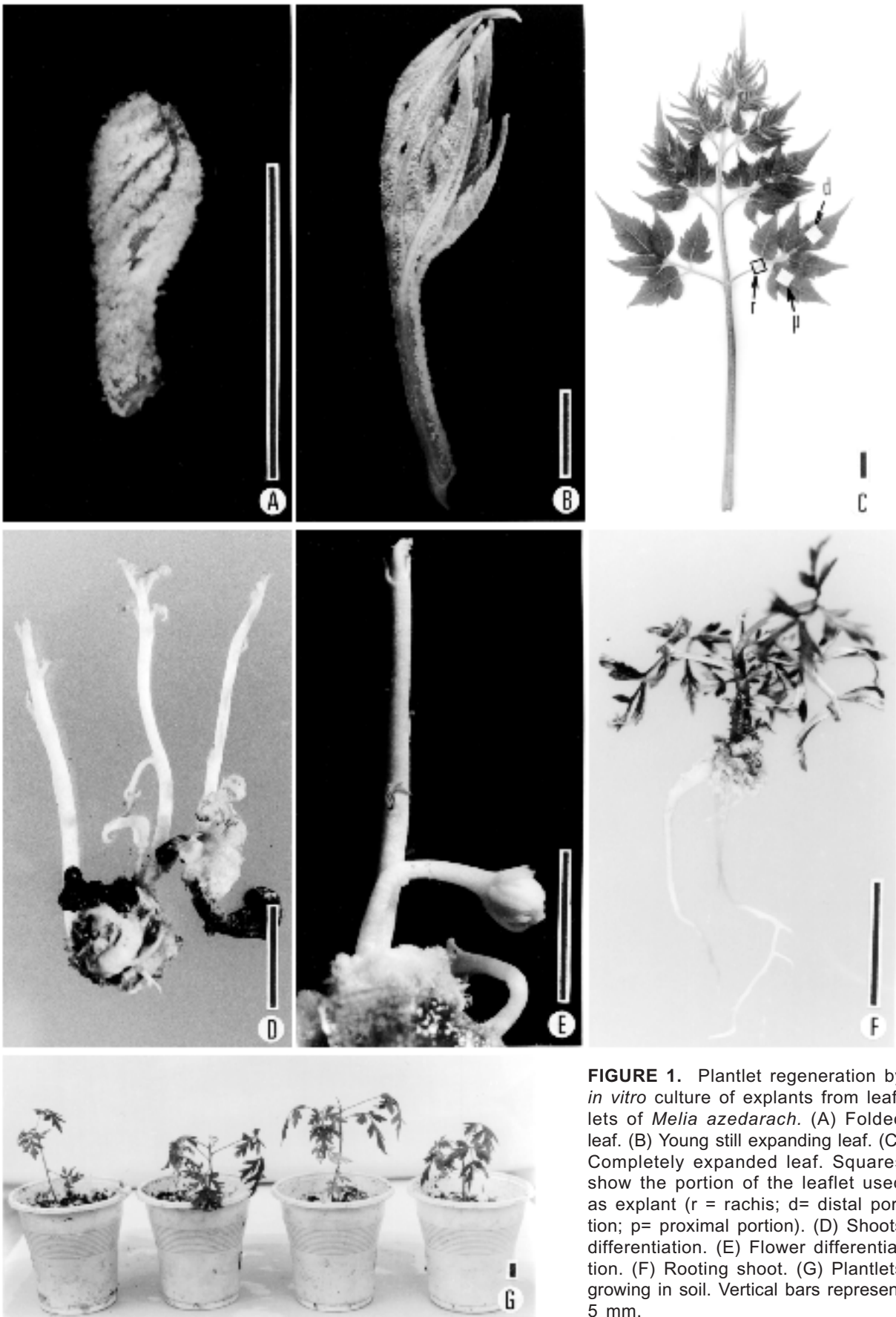


FIGURE 1. Plantlet regeneration by *in vitro* culture of explants from leaflets of *Melia azedarach*. (A) Folded leaf. (B) Young still expanding leaf. (C) Completely expanded leaf. Squares show the portion of the leaflet used as explant (r = rachis; d= distal portion; p= proximal portion). (D) Shoots differentiation. (E) Flower differentiation. (F) Rooting shoot. (G) Plantlets growing in soil. Vertical bars represent 5 mm.

Data analysis

Data analyses was based on the percent of explants that formed shoots (only shoots with more than 5 mm in length were considered) and the number of shoots per explant.

Within each experiment each treatment combination was carried out three times with ten explants per treatment. All data were subjected to analysis of variance (ANOVA) and comparisons of means were made by the Duncan test at $P < 0.05$.

Rooting and transfer to pots

Root differentiation on the shoots was induced by subculture of the regenerated shoots on MS supplemented with 2.5 mg.l^{-1} IBA for 4 days and subsequently in MS without this auxin for 26 days. The tubes containing the shoots were incubated in a growth room at $27 \pm 2^\circ\text{C}$ with a 14 h photoperiod provided by cool-white fluorescent light at $116 \mu\text{m}^2 \cdot \text{s}^{-1}$. Rooted plantlets were transplanted into plastic pots with ster-

ile soil, acclimatized for a week in the growth chamber and then moved to the greenhouse.

Results

After a few days in culture, necrotic areas were evident on some leaf explants, probably due to either sodium hypochlorite or ethanol contact with the leaf during the process of disinfection. However, after 30 - 35 days of culture well developed shoots were found on the leaf explants. Most of the shoots developed on the midrib or on the rachis of the leaflets, after the formation of a sparse callus. Occasionally, shoot regeneration was observed from the leaf mesophyll.

Developmental stage of the leaf

Developmental stage of leaf tissue was an important factor influencing regeneration. Regeneration from Paradise tree leaves enhanced with increasing leaf age. Explants from fully expanded leaves (10 cm in length)

TABLE 2.

Effect of the initial developmental stage of the leaves and portion of the leaflets of seven genotypes of *Melia azedarach* on the number of shoots regenerated per explants after 5 weeks in MS + 1 mg.l^{-1} BAP + 0.1 mg.l^{-1} KIN + 3 mg.l^{-1} ADS.

Developmental stage	Portions of the leaflets	Number of shoots/explant						
		genotypes						
		4	14	24	J1	J2	Lp	R
Folded leaf	proximal	0.7 (± 0.7) ^{a1}	0 ^a	0.3 (± 0.3) ^{ab}	0 ^a	0 ^a	0 ^a	0 ^a
	distal	0.7 (± 0.3) ^a	0.7 (± 0.7) ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
	rachis	2.5 (± 0.5) ^{ab}	0 ^a	0.3 (± 0.3) ^{ab}	0 ^a	0.3 (± 0.3) ^a	0 ^a	0 ^a
Young still expanding leaf	proximal	0.7 (± 0.3) ^a	0 ^a	1.3 (± 0.2) ^c	0 ^a	0 ^a	0 ^a	0 ^a
	distal	2.2 (± 0.9) ^{ab}	0 ^a	1.6 (± 0.5) ^c	0 ^a	1 (± 1) ^a	0 ^a	0 ^a
	rachis	3.6 (± 0.7) ^{ab}	0 ^a	1 (± 0.6) ^{bc}	0.7 (± 0.3) ^b	0.3 (± 0.3) ^a	0.3 (± 0.3) ^a	0 ^a
Expanded leaf	proximal	3.7 (± 1) ^{ab}	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
	distal	2.3 (± 2.3) ^{ab}	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
	rachis	5.9 (± 1.9) ^b	0 ^a	0 ^a	1.4 (± 0.3) ^c	1.3 (± 0.7) ^a	1.1 (± 0.1) ^b	1.7 (± 0.9) ^b

(\pm) = \pm Standard error

¹Different letters, within columns, indicate significant differences at $\alpha 0.05$, using Duncan's multiple comparison test

have permitted the highest values of shoot regeneration, in most of the clones (4, J1, J2, Lp and R), whereas explants from folded and young still expanding leaves (0.5–1 and 2–4 cm in length) produced only a few shoots or none at all. The youngest leaf was less responsive. However, this behaviour depended of the genotype, because in clones 14 and 24 the only responsible explants where the smallest leaves (Table 1).

Portion of the leaf

In all genotypes, the leaf explant permitted shoot differentiation (Fig. 1D). However, the best results were obtained when rachis of the leaflets was employed. When rachis of the leaflets of the clones 4, J1, Lp and R from expanded leaves were cultured, the percent of explants with shoots and the number of shoots per explants were significantly superior than those in the proximal and distal portion (Tables 1 and 2). Whereas in clones 14 (folded leaf) and 24 (young still expanding leaf) the distal portion was more responsive, but the shoots were formed on the midrib.

Effect of the genotype

When comparing the regeneration capacity of seven *Melia* genotypes, significant differences were observed ($P < 0.0001$). Clon 4 regenerated the greatest percent of explant with shoots and number of shoots per explants, when explants employed were rachis of all developmental stage and proximal portion of expanded leaf ($P < 0.05$, using Duncan's multiple comparison test – this statistical analysis is not showed in Tables 1 and 2).

Although the clone 4 was more responsive, most of the tested genotypes showed similar behaviour. In general, the rachis of the expanded leaf was the best explant, except for clones 14 and 24 (Tables 1 and 2).

It is interesting to note that in several of the tested genotypes (4, R, J1, J2 and 24), flowering was observed (Fig. 1E). These flowers arose from calli.

Rooting

Shoots regenerated from leaves differentiated roots (Fig. 1F). Rooting percent was variable, ranging from 0

TABLE 3.

Effect of the initial developmental stage and portion of the leaflet on the rooting percent of regenerated shoots when subcultures for 4 days in induction medium containing MS + 2.5 mg.l⁻¹ IBA and then in MS for 26 days.

Developmental stage	portions of the leaflets	Rooting %						
		genotypes						
		4	14	24	J1	J2	Lp	R
Folded leaf	proximal	22.2 (±22.2)	—	16.7 (±16.7)	—	—	—	—
	distal	16.7 (±16.7)	0	—	—	—	—	—
	rachis	41.3 (±20.8)	—	25 (±14.4)	—	16.7 (±16.7)	—	—
Young and expanding leaf	proximal	14.3 (±14.3)	—	27.8 (±14.7)	—	—	—	—
	distal	22.2 (±22.2)	—	22.2 (±11.1)	—	0	—	—
	rachis	56.6 (±3.6)	—	16.7 (±16.7)	0	22.2 (±22.2)	22.2 (±22.2)	—
Expanded leaf	proximal	63.9 (±21.7)	—	—	—	—	—	—
	distal	28.9 (±21.1)	—	—	—	—	—	—
	rachis	24.2 (±24.2)	—	—	50.2 (±4.9)	30.6 (±19.4)	63.9 (±21.7)	75 (±14.4)

(±) = ± Standard error

to 75% among the shoots regenerated (Table 3). Rooting ability does not reflect inherent differences which existed in the original leaf explant. The shoots arose from rachis as well as those arose from distal and proximal portion were similar in rooting ability. In some way, shoots originated in leaf of different developmental stage had similar behaviour for root differentiation. Among the seven clones studied, differences were observed for their rooting capacity. Although in most of them a considerable percentage of shoots rooted well (clones 4, J1, Lp and R), this percentage was variable within of the genotypes (for example: clone 4 varied between 14.3 and 63.9). There were some clones which rooting percent was lower (clones 24 and J2), while in clone 14, rooting did not occur (Table 3). The rooted plantlets were further transferred to plastic pots with sterile soil. A 85% survival rate was obtained (Fig. 1G).

Discussion

In general, the capacity to vegetatively propagate trees is associated with juvenility (Bonga, 1982). However, our results proved that in Paradise tree the more mature leaf (10 cm long) have not lost the capacity for organogenesis and were more responsive than younger leaves.

The results indicate that the best portion of the leaf was rachis of the leaflets. This results are in agreement with those obtained by Vila *et al.* (2003), indicating that regeneration in the Paradise tree is enhanced in rachis explants obtained from shoots growing *in vitro*. The portion of the leaflets appeared to be an important factor. An explanation for this behaviour would be that the rachis and midrib region of the leaflets of Paradise tree possess some factor that favours shoot organogenesis. The requirement for the presence of vascular tissue in the leaf explant to obtain regeneration is in agreement with the results obtained in several genera including some woody plant species, such as *Ulmus* (Bolyard *et al.*, 1991) and *Pyrus* (Chevreau *et al.*, 1989) where most regenerated shoots originated from both, the vein regions, and petiole. In *Morus alba* the shoots formed in the transitional zone between the midrib and petiole (Oka and Ohyama, 1981). In *Annona squamosa* the petiole and midrib regions contain stimulants that favour shoots proliferation (Nair *et al.*, 1984). Similar results

have been reported for *Populus* hybrid leaf culture. In this species it was found that shoot induction of lower leaf segment with petiole was easier than those of the middle and top segments (Chen *et al.*, 1980).

Genotypic variability in shoot formation was obtained not only in the percentage of explants that formed shoots but also in the number of shoots per explant. Genotypic differences in regeneration potential have been reported in other woody plant species (Korban *et al.*, 1992; Kunze, 1994). A loud feature, in this work, was the capacity of the leaves explants for regenerate flowers in several of the genotypes studied. This phenomena was reported in Paradise tree from youngest tissues (Thakur *et al.*, 1998; Sato and Esquibel, 1995; Handro and Floh, 2001). The addition of cytokinin to the media, specially BA, promoted the flowering *in vitro* in same plants (Simmonds, 1982; Van der Krieken *et al.*, 1986; Roberts *et al.*, 1993; Thakur *et al.*, 1998). However, specific experimentation will be necessary to determine if *in vitro* flowering in *M. azedarach* could be induced by cytokinins. This phenomena assumes significance because *in vitro* techniques provide several ways in which flowering can be examined, most of which can not be carried out *in vivo* (Van Staden and Dickens, 1991).

The results in rooting percent are not in agreement with the highest percent of rooting reported when shoots of the same clones employed in this study were obtained by *in vitro* culture of apical meristems (Vila *et al.*, 2002). Probably, shoots originated from leaves are less vigorous than those originated from meristems. Similar results, in rooting ability, were observed in *Azadirachta indica*, when the explant were leaves (Eeswara *et al.*, 1998).

In conclusion, these studies describes a protocol which permitted plant regeneration of 7 genotypes of *Melia azedarach* through leaf explants cultured *in vitro* under controlled environmental conditions. The results show that the rachis of the leaflets of the expanded leaf is indispensable for shoot regeneration in the most of the clones of Paradise tree.

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