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

S.K. Vila*, H.Y. Rey and L.A. Mrogrinski

Instituto de Botánica del Nordeste, Facultad de Ciencias Agrarias (UNNE). C.C. 209. (3400) Corrientes, Argentina.

**Key words:** genotype, leaf explants, organogenesis, paradise tree, plant regeneration.

**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*...
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.**

| Developmental stage          | portions of the leaflets | genotypes |
|------------------------------|--------------------------|-----------|
|                              |                          | 4         | 14        | 24         | J1        | J2        | Lp        | R        |
| Folded leaf                  | proximal                 | 3.3 (±3.3)a | 0ø        | 3.3 (±3.3)a | 0ø        | 0ø        | 0ø        | 0ø        |
|                              | distal                   | 6.7 (±3.3)a | 3.3 (±3.3)a | 0ø        | 0ø        | 0ø        | 0ø        | 0ø        |
|                              | rachis                   | 33.3 (±8.8)b | 0ø        | 3.3 (±3.3)a | 0ø        | 3.3 (±3.3)a | 0ø        | 0ø        |
| Young and expanding leaf      | proximal                 | 13.3 (±8.8)b | 0ø        | 16.7 (±3.3)b | 0ø        | 0ø        | 0ø        | 0ø        |
|                              | distal                   | 16.7 (±3.3)b | 0ø        | 23.3 (±3.3)b | 0ø        | 3.3 (±3.3)a | 0ø        | 0ø        |
|                              | rachis                   | 30 (±11.5)b | 0ø        | 6.7 (±3.3)a | 6.7 (±3.3)a | 3.3 (±3.3)a | 3.3 (±3.3)a | 0ø        |
| Expanded leaf                 | proximal                 | 20 (±5.8)b  | 0ø        | 0ø        | 0ø        | 0ø        | 0ø        | 0ø        |
|                              | distal                   | 3.3 (±3.3)a | 0ø        | 0ø        | 0ø        | 0ø        | 0ø        | 0ø        |
|                              | rachis                   | 30 (±5.8)b  | 0ø        | 0ø        | 26.7 (±3.3)a | 13.3 (±6.7)b | 16.7 (±6.7)b | 10 (±5.8)b |

(±) = ± Standard error

* Different letters, within columns, indicate significant differences at α 0.05, using Duncan’s multiple comparison test.
FIGURE 1. Plantlet regeneration by \textit{in vitro} culture of explants from leaflets of \textit{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 $27\pm 2^\circ C$ with a 14 h photoperiod provided by cool-white fluorescent light at 116 $\mu$m$^{-2}$s$^{-1}$. Rooted plantlets were transplanted into plastic pots with sterile 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)

| Developmental stage | Portions of the leaflets | 4 | 14 | 24 | J1 | J2 | Lp | R |
|---------------------|---------------------------|---|----|----|----|----|----|---|
| Folded leaf         | proximal                  | 0.7 ($\pm 0.7$)$^a$ | 0$^a$ | 0.3 ($\pm 0.3$)$^b$ | 0$^a$ | 0$^a$ | 0$^a$ | 0$^a$ |
|                     | distal                    | 0.7 ($\pm 0.3$)$^a$ | 0.7 ($\pm 0.7$)$^a$ | 0$^a$ | 0$^a$ | 0$^a$ | 0$^a$ | 0$^a$ |
|                     | rachis                    | 2.5 ($\pm 0.5$)$^b$ | 0$^a$ | 0.3 ($\pm 0.3$)$^a$ | 0$^a$ | 0.3 ($\pm 0.3$)$^a$ | 0$^a$ | 0$^a$ |
| Young still expanding leaf | proximal                  | 0.7 ($\pm 0.3$)$^a$ | 0$^a$ | 1.3 ($\pm 0.2$)$^c$ | 0$^a$ | 0$^a$ | 0$^a$ | 0$^a$ |
|                     | distal                    | 2.2 ($\pm 0.9$)$^b$ | 0$^a$ | 1.6 ($\pm 0.5$)$^c$ | 0$^a$ | 1 ($\pm 1$)$^a$ | 0$^a$ | 0$^a$ |
|                     | rachis                    | 3.6 ($\pm 0.7$)$^b$ | 0$^a$ | 1 ($\pm 0.6$)$^b$ | 0.7 ($\pm 0.3$)$^b$ | 0.3 ($\pm 0.3$)$^a$ | 0.3 ($\pm 0.3$)$^a$ | 0$^a$ |
| Expanded leaf       | proximal                  | 3.7 ($\pm 1$)$^b$ | 0$^a$ | 0$^a$ | 0$^a$ | 0$^a$ | 0$^a$ | 0$^a$ |
|                     | distal                    | 2.3 ($\pm 2.3$)$^b$ | 0$^a$ | 0$^a$ | 0$^a$ | 0$^a$ | 0$^a$ | 0$^a$ |
|                     | rachis                    | 5.9 ($\pm 1.9$)$^b$ | 0$^a$ | 0$^a$ | 1.4 ($\pm 0.3$)$^c$ | 1.3 ($\pm 0.7$)$^a$ | 1.1 ($\pm 0.1$)$^a$ | 1.7 ($\pm 0.9$)$^b$ |

($\pm$) = ± Standard error
$^1$Different 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.

| Developmental stage | portions of the leaflets | 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 | proximal                 |           | 14.3 (±14.3) | —        | 27.8 (±14.7) | —        | —        | —        | —       |
| leaf                | 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) |

($\pm$) = ± 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 clon 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 explan 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.

Acknowledgements

The authors wish to thank Aldo Goytía for the photographs plates.
References

Ahmad Z, Zaidi N, Shah FH (1990). Micropropagation of Melia azedarach from mature tissue. Pak J Bot 22: 172-178.

Andrei GM, Lampuri JS, Coto CE, De Torres RA (1996). An antiviral factor from Melia azedarach L. prevents Tacaribe virus encephalitis in mice. Experientia 42: 843-845.

Andreu J, Sans A, Riba M (2000). Antifeedant activity of fruit and seed extract of Melia azedarach and Azadirachta indica on larvae of Sesamia nonagrioides. Phytoparasitica 28: 311-319.

Bolyard MG, Srinivasan CS, Cheng J, Sticklen M (1991). Shoot regeneration from leaf explants of American and Chinense Elm. Hortscience 26: 1554-1555.

Bonga JM (1982). Vegetative propagation in relation to juvenility, maturity and rejuvenation. In: Tissue Culture in Forestry. J.M. Bonga and S.N. Durzan, Eds. Martinus Nijhoff, The Netherlands, pp 387-412.

Breuer M, Schmidt GH (1995). Influence of a short period treatment with Melia azedarach extract on food intake and growth of the larvae of Spodoptera frugiperda (J. E. Smith) (Lep., Noctuidae). Journal of Plant Diseases and Protection 102: 633-654.

Coto CE, De Torres RA (1999). El paraíso (Melia azedarach L.): Fuente de productos bioactivos. Dominiguez 15: 5-15.

Cozzo D (1944). Arboles para parques y jardines. Suelo Argentino, Bs. As., pp.83-84.

Chen CH, Chang S, Cheng L, Hou R (1996). Deterrent effect of the chinaberry extract on oviposition of the diamondback moth, Plutella xylostella (L.) (Lep., Yponomeutidae). J Appl Ent 120: 165-169.

Chevreau E, Skirvin RM, Abu-Qaoud HA, Korban SS, Sullivan JG (1989). A standard procedure for the micropropagation of the neem tree (Azadirachta indica A. Juss). Plant Cell Rep 17: 215-219.

Handro W, Floh EIS (2001). Neo-formation of flower buds and other morphogenetic responses in tissue cultures of Melia azedarach. Plant Cell, Tissue & Organ Cult 64: 73-76.

Korban SS, O'Connor P A, Elobeidy A (1992). Nair S, Gupta PK, Shirgurkar MV, Mascarenhas AF (1984). Kunze I (1994). Influence of the genotype on growth of Norway Spruce (Picea abies L.) in vitro meristem culture. Silvae Genetica 43: 36-41.

Korban SS, O'Connor PA, Elobeidy A (1992). Effects of thidiazuron, naphthaleneacetic acid, dark incubation and genotype on shoot organogenesis from Malus leaves. Journal of Horticultural Science 67: 341-349.

Kunze I (1994). Influence of the genotype on growth of Norway Spruce (Picea abies L.) in vitro meristem culture. Silvae Genetica 43: 36-41.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497.

Nair S, Gupta PK, Shirgurkar MV, Mascarenhas AF (1984). In vitro organogenesis from leaf explants of Annona squamosa Linn. Plant Cell Rep 4: 29-40.

Oka S, Ohyama K (1981). In vitro initiation of adventitious buds and its modification by high concentration of benzyladenine in leaf tissues of mulberry (Morus alba). Can J Bot 59: 68-74.

Pennington DT (1981). Flora Neotropica. Monograph number 28 Meliaceae. The New York Botanical Garden, New York, pp 24-25.

Roberts NJ, Luckman GA, Menary RC (1993). In vitro flowering of Boronia megastigma Nees. and the effect of 6- benzylaminopurine. J Plant Growth Regul 12: 117-122.

Sato A, Esquibel MA (1995). In vitro precocious flowering of Melia azedarach hypocotyl segments. R Bras Fisiol Veg 7: 107-110.

Shahzad A, Siddiqui SA (2001). Micropropagation of Melia azedarach L. Phytomorphology 51: 151-154.

Simmonds J (1982). In vitro flowering on leaf explants of Streptocarpus nobilis. The influence of culture medium components on vegetative and reproductive development. Can J Bot 64: 1461-1468.

Thakur R, Rao PS, Bapat VA (1998). In vitro plant regeneration in Melia azedarach L. Plant Cell Rep 18: 127-131.

Ursi Ventura M, Ito M (2000). Antifeedant activity of Melia azedarach (L.) extracts to Diabrotica speciosa (Genn.) (Coleoptera: Chrysomelidae) beetles. Brazilian Archives of Biology and Technology 2: 215-219.

van der Krieken WM, Barendse GWM, Croes AF (1986). BAP metabolism during in vitro flower bud formation in tobacco. In: Conjugated Plant Hormones Structure. K. Schreiber, H.R. Schütte and G Sembdner, eds., VEB Deutscher Verlag der Wissenschaften, Berlin, pp.153-157.

Van Staden J, Dickens CWS (1991). In vitro induction of flowering and its relevance to micropropagation. In: High- Tech and Micropropagation I Biotechnology in Agriculture and Forestry. Y.P.S. Bajaj, ed., Springer- Verlag, Berlin, New York, Vol 17 pp. 85-115.

Vila S, Scocchi A, Mroginski L (2002). Plant regeneration from shoot apical meristems of Melia azedarach L. (Meliaceae). Acta Physiologiae Plantarum 24: 195-199.

Vila SK, Gonzalez AM, Rey HY, Mroginski LA (2003). In vitro plant regeneration of Melia azedarach L.: Shoot organogenesis from leaf explants. Biologia Plantarum 46: 13-19.
