BIOLOGIA PLANTARUM 47 (1): 13-19, 2003/4

In vitro plant regeneration of *Melia azedarach* L.: shoot organogenesis from leaf explants

S.K. VILA*, A.M. GONZALEZ, H.Y. REY and L.A. MROGINSKI

Instituto de Botánica del Nordeste, Facultad de Ciencias Agrarias (UNNE), Casilla de Correos 209, Corrientes RA-3400, Argentina

Abstract

In vitro regeneration of *Melia azedarach* L. was studied. Shoots were regenerated from calli initiated from leaflets of *in vitro* growing plants. The best medium for establishment of cultures was Murashige and Skoog (MS) medium with 4.44 μM benzylaminopurine (BAP) + 0.46 μM kinetin (KIN) + 16.29 μM adenine sulphate (ADE). Regenerated shoots were multiplied in MS + 0.44 μM BAP + 0.37 μM KIN + 3.26 μM ADE. Maximal rooting of 89 % was achieved by culture of regenerated shoots in MS + 12.26 μM indole-3-butyric acid for 3 d and subsequently in MS lacking growth regulators for 27 d. Rooted shoots were acclimatized and successfully transferred to soil.

Additional key words: auxin, cytokinin, gibberellic acid, leaf culture, rooting.

Introduction

Melia azedarach L., known in Argentina as "paraíso", is an economically important plant in the *Meliaceae* family. It was originally introduced to Argentina from Southern Asia. Although, conventionally propagated by seeds, micropropagation through axillary bud culture was developed because sexual reproduction can produce genetic variation (Domecq 1988, Ahmad *et al.* 1990, Thakur *et al.* 1998). Although, plant regeneration through either organogenesis or somatic embryogenesis has been accomplished in a range of forest species (for review see Thorpe *et al.* 1991) there is no report on successful *in vitro* plant regeneration through organogenesis or somatic embryogenesis of *M. azedarach*.

The aim of the present work was to describe a general protocol to regenerate plants through organogenesis in callus cultures derived from leaves of *M. azedarach*. The influences of media, explants and genotypes on plant regeneration were studied. Histological analysis was undertaken to ascertain the origin of the regenerated shoots.

Materials and methods

Plants: Danzer Forestación S.A. (Posadas, Misiones) provided all the plants. Unless otherwise stated, leaf explants obtained from *Melia azedarach* L. clone Lp were used. Other clones, 14, 11, H, E, 12, J1, 4, 20 and 3 were used in one experiment. All the clones were obtained by stakes of field grown adult plants (10 - 15 years old).

The source of explants were *in vitro* shoots (Fig. 1*a*) obtained by culture of axillary buds according the protocol described by Domecq (1988) consisting of: *1*) disinfection of isolated axillary buds (1.3 mm length) with 70 % ethanol (3 min), followed by a solution of sodium hypochlorite and 0.1 % TRITON X-100®, for 20 min, and finally rinsing with sterile distilled water;

Received 19 November 2001, *accepted* 27 May 2002.

Abbreviations: ADE - adenine sulphate; BAP - 6-benzylaminopurine; IAA - indoleacetic acid; IBA - indolebutyric acid; GA_3 - gibberellic acid; KIN - kinetin; MS - medium after Murashige and Skoog (1962); NAA - α-naphthaleneacetic acid.

Acknowledgments: The authors are grateful to Mario Slukwa for the plant material. The authors wish to express their gratitude to Aldo Goytia for the photographic plates. CONICET and SGCyT (UNNE) provided financial support for this research. This paper is a part of the thesis of the senior author.

*Corresponding author; fax: (+54) 3783 427131, e-mail: skvila@agr.unne.edu.ar

2) establishment of cultures in MS supplemented with 2.20 μM BAP + 0.49 μM IBA + 0.29 μM GA_3, and *3*) multiplication of the regenerated shoots by culturing them in MS + 2.20 μM BAP + 0.29 μM GA_3. The first three expanded leaves (Fig. 1*b*) were used as source of explants.

Culture initiation: Pieces of leaflets (4 mm^2), containing the midrib were cultured individually into 11 cm^3 glass tubes containing 3 cm^3 of MS supplemented with various concentrations and combinations of BAP, KIN and ADE. Similar media were employed for subculture of calluses. The pH of each medium was adjusted to 5.8 with either KOH or HCl prior the addition of agar. Tubes were covered with aluminum foil and autoclaved at 1.46 kg cm^{-2} for 20 min.

The tubes containing the explant were covered with *Resinite AF 50*® (*Casco S.A.C. Company Bs.*, As, Argentina) and incubated in a growth room at 27 ± 2 °C in darkness or with 14-h photoperiod provided by cool-white fluorescent lamps, with a irradiance of 116 μmol m^{-2} s^{-1}.

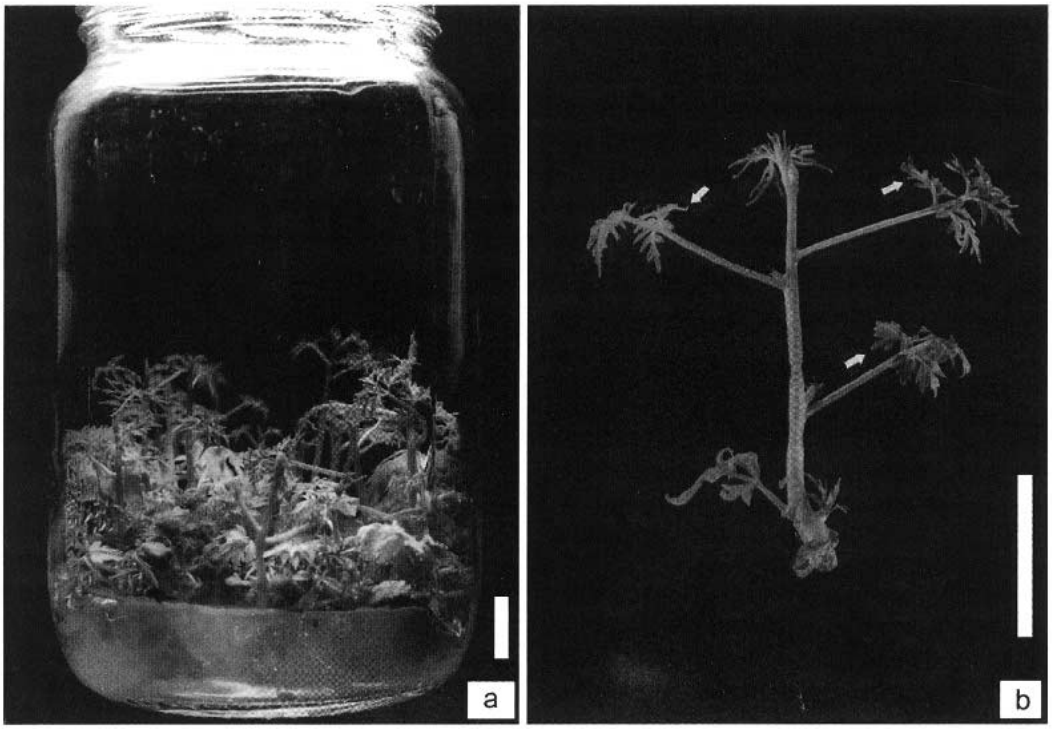

Fig. 1. Source of explants for plant regeneration from leaves of *Melia azedarach* (*bars* represent 1 cm): *a* - *in vitro* shoots obtained by culture of axillary buds, *b* - the leaves used as explant marked by *arrows*.

Multiplication: Shoots obtained in three establishment media, after 1 month, were divided into three nodes explants and were cultured in a 11 cm^3 glass jars containing 3 cm^3 of a shoot multiplication medium consisting of MS + 0.44 μM BAP + 0.36 μM KIN + 3.26 μM ADE (Eeswara *et al.* 1998). The jars were covered with *Resinite AF 50*® and incubated in a growth room at 27 ± 2 °C in either darkness or 14-h photoperiod, with a irradiance of 116 μmol m^{-2} s^{-1}. Shoot multiplication was evaluated after 1 month and the multiplication rate represents the mean number of usable shoots (with three or more nodes) for following induction of rooting.

Rooting and acclimatization: For *in vitro* rooting, individual shoots (length approximately 1.5 - 2 cm, Fig. 2*c*), were transferred on *1*) MS + 4.90 μM IBA + 5.71 μM IAA for 10 d and then subculturing in MS lacking hormones for 20 d, or *2*) MS + 12.26 μM IBA for 3 d and subsequently in MS alone for 27 d, or *3*) MS + 4.90 μM IBA for 30 d. The regenerated shoots were cultured in glass tubes (15 cm^3) containing 3 cm^3 of medium. The physical conditions were the same as described above for multiplication under 14-h photoperiod. The rooted shoots were removed from the culture tubes, washed with tap water and transferred to plastic pots with sand, peat moss and perlite (1:1:1). The plantlets were placed in a mist for 20 d and were finally transferred to soil.

Experimental design and results analysis: For the culture initiation 10 explants were cultured per treatment. Treatments were arranged randomly on the shelves of the growth room. All the experiments were independently repeated three times and the means and the standard error

(SE) were compared. Data were subjected to analysis of variance (*ANOVA*) and comparisons of means were made with the least significant difference test at the 5 % level of probability.

Histological observations: Histology was performed according to Gonzalez and Cristóbal (1997). Samples of cultured explants were fixed in FAA (formaline + acetic acid + 70 % ethanol 5:5:90), dehydrated with a solution for histological dehydratation *BIOPUR®*, followed by paraffin embedding as described by Johansen (1940). The embedded material was then sectioned at 8 - 10 μm thick serial sections. These sections were mounted on glass slides and stained with Safranin (C.I. 50240)-Astra blue (Luque *et al.* 1996) and observed under a light microscope.

Results and discussion

The earliest visible signs of callus growth from leaf explants in several media was noticeable within 7 - 10 d of culture. Calli were soft and white. Most of the calli developed first at the cut end of the explants. However, some of them appeared on the midribs. The frequency of explants which produced calli was about 80 %. After 30 d of incubation, single or multiple shoot differentiation occurred (Fig. 2*a,b*). Their frequency, was greatly affected by the medium and the conditions of incubation. Only the highest concentrations of BAP tested (4.44 μM) alone or in combinations with KIN induced calli with shoots either in darkness or light. On the contrary, when 4.44 μM BAP was combined with ADE, shoot differentiation only occurred in darkness (Table 1). All the combinations of KIN and ADE tested were completely ineffective for shoot production (data not shown). Leaf derived calli showed maximum explants forming shoots when incubated in darkness and the MS medium contained 4.44 μM BAP + 0.46 μM KIN + 16.29 μM ADE (Table 1).

Table 1. Shoot regeneration of *Melia azedarach* from leaf explants after 5 weeks on MS medium with 4.44 μM BAP, and different concentrations of KIN and ADE. Values having the same letter are not significantly different at the $P < 0.05$ (Duncan's multiple comparison test).

Growth regulator [μM]		Explants forming shoots [%]	
KIN	ADE	darkness	light
0	0	13^{abc}	0^{a}
0	5.43	27^{abcd}	0^{a}
0	16.29	13^{abc}	0^{a}
0	32.58	20^{abc}	0^{a}
0.04	0	20^{abc}	0^{a}
0.04	5.43	40^{bcd}	0^{a}
0.04	16.29	13^{abc}	7^{ab}
0.04	32.58	40^{bcd}	7^{ab}
0.46	0	7^{ab}	13^{abc}
0.46	5.43	27^{abcd}	13^{abc}
0.46	16.29	53^{d}	7^{ab}
0.46	32.58	47^{cd}	7^{ab}
4.65	0	33^{abcd}	20^{abc}
4.65	5.43	27^{abcd}	20^{abc}
4.65	16.29	20^{abc}	7^{a}
4.65	32.58	33^{abcd}	20^{abc}

These requirements of exogenous growth regulators are similar to the ones of other woody plants in which shoot regeneration was obtained by the addition in the culture medium of BAP alone (Thorpe *et al.* 1991, Babber *et al.* 2001). Interaction between BAP and light also occurred in other plant species (Baraldi *et al.* 1988, Lercari *et al.* 1986). The use of a mixture of two cytokinins for shoot differentiation has been pointed out (Cheng 1977, Abdullah *et al.* 1989). However, although ADE has been shown to stimulate bud formation (Skoog and Tsui 1948, Dorée *et al.* 1971, Skoog 1971) and it is not frequently used for shoot differentiation (Evans *et al.* 1981), our results show that the addition of ADE to the culture medium, in combination with BAP and KIN, results in a stimulation of the shoot production. These results agree with those of Eeswara *et al.* (1998) for *Azadirachta indica.*

After 1 week of culture, small friable callus presented cells in active divisions. Some cells exhibited dense cytoplasm with small vacuoles, and conspicuous centrally positioned nucleus. They were easily distinguished from the surrounding larger cells (Fig. 3*a*). Some of these cells underwent divisions, and in succeeding days, gave rise to small meristematic groups (Fig. 3*b*). Those groups located mainly in the surface of the callus continued to divide and formed globular structures which were easily detectable because of the cytoplasm densely stained of their cells (Fig. 3*c*). These meristematic structures remained connected with the parenchymatic tissue of the callus (Fig. 3*d*). After a few more days, these meristematic structures gradually developed into bud primordia surrounded by a pair of leaf primordia (Fig. 3*e*). Its vascular connection with the explant was observed (Fig. 3*f*). The progressive differentiation of the shoot apical meristem and leaf primordia led to the regeneration of well-developed shoot and at least two pairs of leaves by the end of the 4th week of culture. These structures remained connected with the callus (Fig. 3*g*). The apical meristem resembles the typical dicotyledonous meristem (Fig. 3*h*), and a transversal section showed its phyllotaxis (Fig. 3*i*). These studies revealed that shoots developed following a pattern of

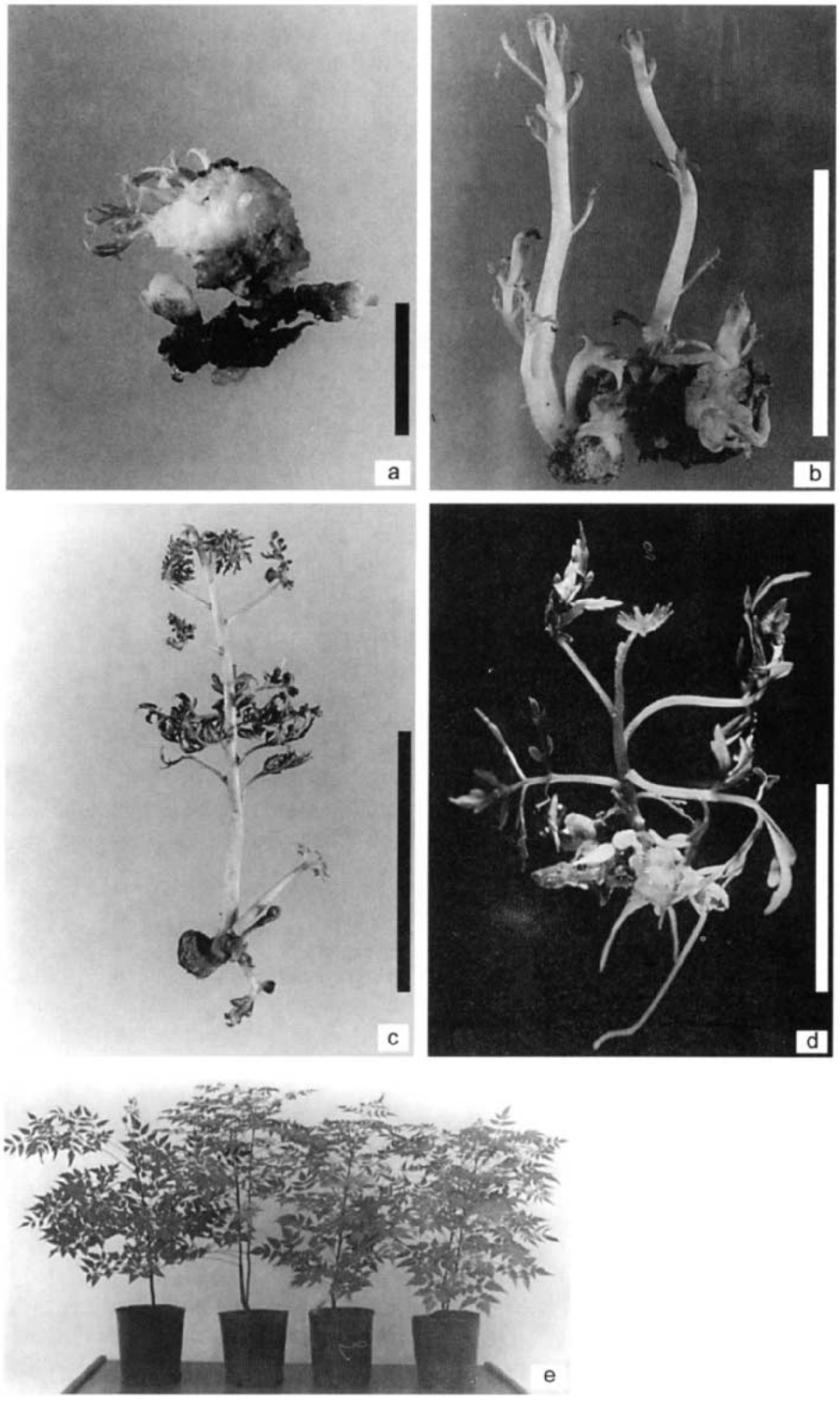

Fig. 2. *a* - callus derived from leaflets of *Melia azedarach*; *b* - callus with multiple shoots obtained on MS + 4.44 μM BAP + 0.46 μM KIN + 16.29 μM ADE; *c* - shoots obtained after multiplication in MS + 0.44 μM BAP + 0.37 μM KIN + 3.26 μM ADE; *d* - rooting of a shoot on MS + 12.26 μM IBA for 3 d and subsequently on MS lacking growth regulator for 27 d; *e* - regenerants growing in the greenhouse. *Bar* = 1 cm.

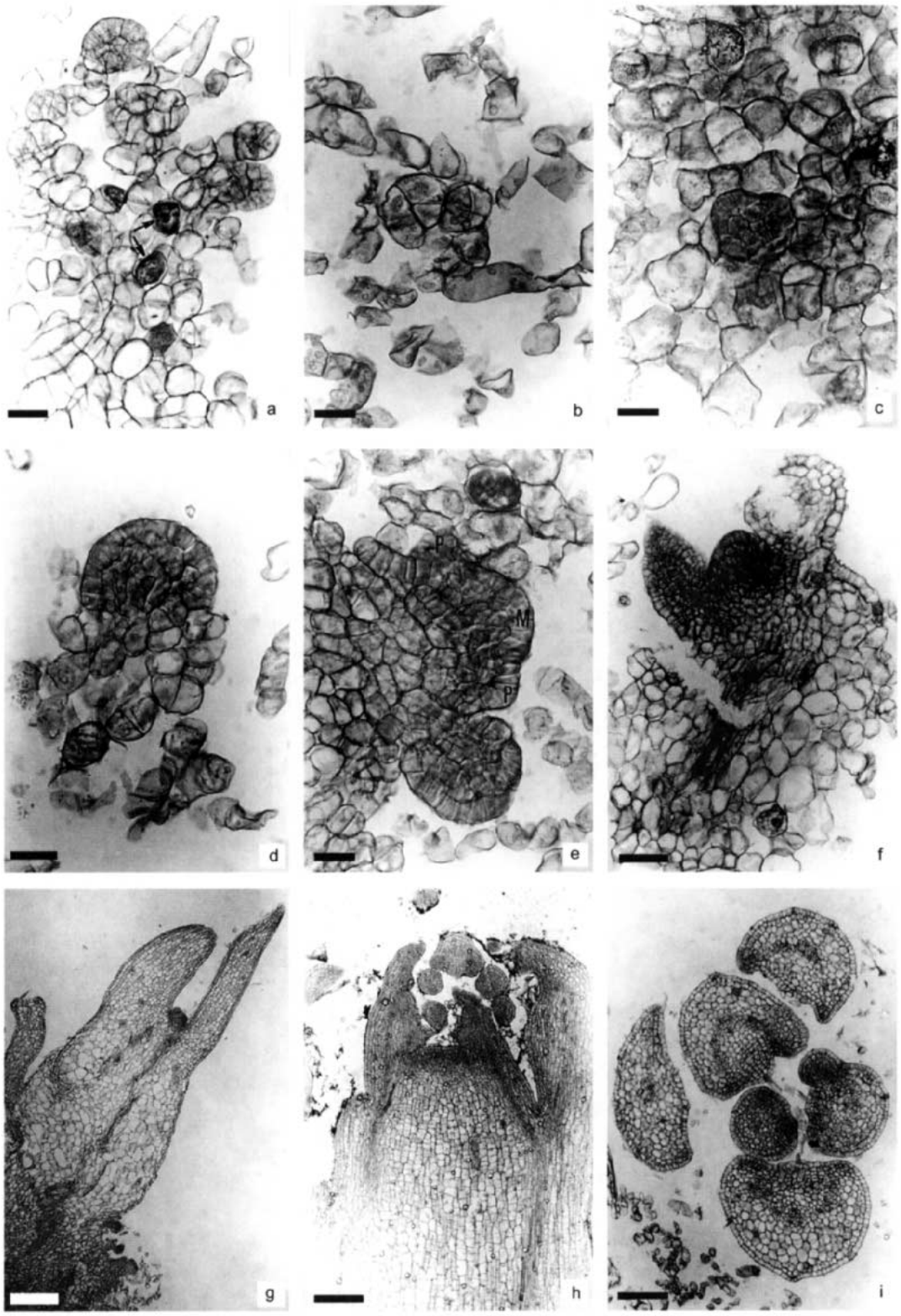

Fig. 3. Organogenesis in leaflets of *Melia azedarach* cultured *in vitro*: *a* - callus, *arrows* showing initial cells (*bar* = 20 μm); *b*, *c*, *d* - small meristematic groups (*bar* = 20 μm); *e* - bud primordium (*bar* = 20 μm, P - leaf primordium, M - meristem); *f*, *g* - bud primordium connection with the explant (*bar* in *f* = 50 μm, in *g* = 100 μm); *h* - typical meristem of naturally grown *M. azedarach* (*bar* = 100 μm); *i* - phyllotaxis (*bar* = 100 μm).

organogenesis very similar to that previously described for other woody species (Thorpe 1980). Although, both explants (rachis and blade of leaflet) permitted shoot differentiation, the best results were obtained when rachis of the leaves were used (data not shown).

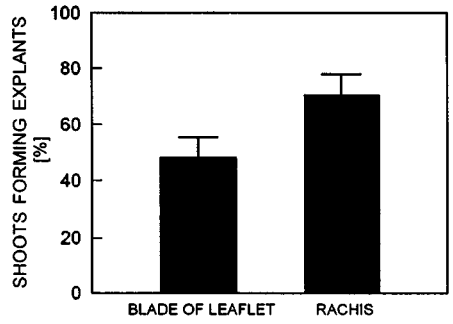

Fig. 4. Shoot regeneration through *in vitro* culture of leaflets of 10 clones of *Melia azedarach*. *Vertical bars* represent SE. Different letters indicate a significant difference at $P < 0.05$.

Because MS + 4.44 μM BAP + 0.46 μM KIN + 16.29 μM ADE produced the greatest overall mean number of shoots in the previous experiments, this medium was employed in the study of the effect of genotype on *in vitro* shoot regeneration. The experiment was performed in darkness. Their ability of shoot production ranged from 15 to 80 % (Fig. 4). Great differences among clones in organogenic capacity *in vitro* has been described repeatedly in tissue cultures of woody species (Bordón *et al.* 2000, Coleman and Ernst 1989).

When the shoots regenerated were subcultured in the multiplication medium multiple shoots were produced. This phenomenon was not significantly affected by neither the medium employed for establishment of cultures nor the conditions of incubation (data not shown). It was observed a strong dominance of the main shoot, which developed faster and attained a length of 1.5 - 2 cm in 1 month, while the rest developed poorly (Fig. 2*c*). As resulted of this the highest multiplication rate is the only 1.5.

When regenerated shoots were induce to differentiate roots, all the treatments used promoted rhizogenesis, ranged from 76 to 89 % of the shoots regenerated either in darkness or in light. The majority of the shoots forming roots had a callus in the base (Fig. 2*d*). These results showed that rooting of shoots derived from tissue culture of *Melia azedarach* is not the critical point for a successfull plant regeneration. Domecq (1988) and Thakur *et al.* (1998) also found that half strength-liquid MS medium supplemented with 2.45 μM IBA permitted more than 50 % of rooting. Ahmad *et al.* (1990) also reported that root initiation could be obtained by using a medium with 4.90 μM IBA. Similar results were reported for *Azadirachta indica* (Eeswara *et al.* 1998) while half-strength MS medium with 2.69 μM naphthaleneacetic acid + 4.90 μM IBA was recommended for successful rooting of *A. excelsa* microshoots (Kool *et al.* 1999).

The acclimatization of the rooted shoots was accomplished and approximately 95 % of the plants were successfully transferred to pots in greenhouse conditions (Fig. 2*e*).

In conclusion this paper reports the first regeneration from leaf explants of *Melia azedarach* via organogenesis.

References

Abdullah, A.A., Yeoman, M.M., Grace, J.: Calabrian pine (*Pinus brutia* Tenore). - In: Bajaj, Y.P.S. (ed.): Biotechnology in Agriculture and Forestry. Vol. 5, Trees II. Pp. 507-525. Springer-Verlag, Berlin 1989.

Ahmad, Z., Zaidi, N., Shah, F.H.: Micropropagation of *Melia azedarach* from mature tissue. - Pakistan J. Bot. **22**: 172-178, 1990.

Babber, S., Mittal, K., Ahlawat, R., Varghese, T.M.: Micropropagation of *Cardiospermum halicacabum*. - Biol. Plant. **44**: 603-606, 2001.

Baraldi, R., Rossi, F., Lercari, B.: *In vitro* shoot development of *Prunus* GF 655-2: interaction between light and benzyladenine. - Physiol. Plant. **74**: 440-443, 1988.

Bordón, Y., Guardiola, J.L., García-Luis, A.: Genotype affects the morphogenetic response *in vitro* of epicotyl segments of *Citrus* rootstocks. - Ann. Bot. **86**: 159-166, 2000.

Cheng, T.Y.: Factors affecting adventitious bud formation of cotyledon culture of Douglas fir. - Plant Sci. Lett. **9**: 179-187, 1977.

Coleman, G.D., Ernst, S.G.: *In vitro* shoot regeneration of *Populus deltoides*: effect of cytokinin and genotype. - Plant Cell Rep. **8**: 459-462, 1989.

Domecq, C.: Cultivo *in vitro* de yemas axilares de paraíso gigante *(Melia azedarach* L. var. *gigantea*). - Phyton **48**: 33- 42, 1988.

Dorée, M., Leguay, J.J., Terrine, C., Sadorge, P., Trapy, F., Guern, J. Adaptation a l'adénine exogène. - In: Colloques Internacionaux C.N.R.S. N° 193. Les Cultures de Tissus de Plantes. Pp. 345-365. Centre National de la Recherche Scientifique, Paris 1971.

Eeswara, J.P., Stuchbury, T., Allan, E.J., Mordue (Luntz), A.J.: A standard procedure for the micropropagation of the neem tree (*Azadirachta indica* A. Juss). - Plant Cell Rep. **17**: 215-219, 1998.

Evans, D.A., Sharp, W.R., Flick, C.E.: Growth and behaviour of cell cultures: embryogenesis and organogenesis. - In: Thorpe, T.A. (ed.): Plant Tissue Culture. Methods and Applications in Agriculture. Pp. 45-113. Academic Press, New York 1981.

Gonzalez, A.M., Cristóbal, C.L.: Anatomía y ontogenia de semillas de *Helicteres Lhotzkyana (Sterculiaceae)*. - Bonplandia **9**: 287-294, 1997.

Johansen, D.A.: Plant Microtechnique. - McGraw Hill Book Co., New York 1940.

Kool, L.T., Keng, C.L., Hoe, C.T.K.: *In vitro* rooting of sentang shoots (*Azadirachta excelsa* L.) and acclimatization of the plantlets. - *In Vitro* cell. dev. Biol. Plant **35**: 396-400, 1999.

Lercari, B., Tognoni, F., Anselmo, G., Chapel, D.: Photocontrol of *in vitro* bud differentiation in *Saintpaulia ionantha* leaves and *Lycopersicon esculentum* cotyledons. - Physiol. Plant. **67**: 340-344, 1986.

Luque, R., Sousa, H.C., Kraus, J.E.: Métodos de coloracão de Roeser (1972) e Kropp (1972) visando a subtituicão do azul do astra por azul de alciao 8GS ou 8GX. - Acta bot. bras. **10**: 199-212, 1996.

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

Skoog, F.: Aspects of growth factors interactions in morphogenesis of tobacco tissue cultures. - In: Colloques Internationaux C.N.R.S. N° 193. Les Cultures de Tissus de Plantes. Pp. 115-135. Centre National de la Recherche Scientifique, Paris 1971.

Skoog, F., Tsui, C.: Chemical control of growth and bud formation in tobacco stem segments and callus cultured *in vitro*. - Amer. J. Bot. **35**: 782-787, 1948.

Thakur, R., Rao, P.S., Bapat, V.A.: *In vitro* plant regeneration in *Melia azedarach* L. - Plant Cell Rep. **18**: 127-131, 1998.

Thorpe, T.A.: Organogenesis *in vitro* structural, physiological and biochemical aspects. - Rev. Cytol. **11A**: 71-111, 1980.

Thorpe, T.A., Harry, I.S., Kumar, P.P.: Application of micropropagation to forestry. - In: Debergh, P.C., Zimmerman, R.H. (ed.): Micropropagation. Pp. 311- 336. Kluwer Acad. Publ., Dordrecht 1991.