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R. Thakur · P. S. Rao · V. A. Bapat

In vitro plant regeneration in *Melia azedarach* L.

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Abstract Nodal explants of 3- 6-week-old seedlings cultured on Murashige and Skoog (MS) medium supplemented with 6-benzyladenine (BA) (17.75 μM) produced multiple shoots. Shoots were isolated and induced to root on ½-strength MS medium supplemented with indole-3-butyric acid (4.92 μM). In-vitro-rooted shoots resumed growth after a short period of acclimatization and resulted in plantlets which were successfully established in soil. In vitro flowering was observed in some of the nodal explants in the above medium, and also in cotyledonary leaves and internodal explants on MS medium supplemented with a combination of indole-3-acetic acid (IAA) (0.06 μM) + BA (4.44 μM) and IAA (0.06 μM) + kinetin (4.65 μM).

Key words *Melia azedarach* · Plant regeneration · Multiple shoots · In vitro flowering

Abbreviations *BA* 6-Benzyladenine · *2,4-D* 2,4-Dichlorophenoxyacetic acid · *GA* Gibberellic acid · *IAA* Indole-3-acetic acid · *IBA* Indole-3-butyric acid · *KN* Kinetin · *MS* Murashige and Skoog medium · *NAA* α-Naphthaleneacetic acid · *RH* Relative humidity

Introduction

Melia azedarach L. is related to *Azadirachta indica*, and has therapeutic and insecticidal properties (Ascher et al. 1995; Schmidt et al. 1997). The tree yields valuable timber resistant to termites (Yaga 1978), fodder, green manure, as well as oil from seeds (Ascher et al. 1995). It is a native of north-western India, and naturalized in many subtropical countries. The tree bears seeds only during the summer and its natural rate of multiplication is limited. In view of its economic uses, attempts have been made to micropropagate this plant by in vitro culture of axillary buds (Domecq 1988). This communication reports investigations with *M. azedarach* with a view to developing a protocol for its micropropagation.

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R. Thakur
Department of Life Sciences, University of Mumbai, Mumbai-400098, India

P. S. Rao (✉) · (V. A. Bapat)
Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai-400085, India
e-mail: biotech@magnum.barct1.ernet.in
Fax: +91-22-5560750

Materials and methods

Explant

Fruits of *M. azedarach* L. (Meliaceae) were obtained from several trees at Mahatma Phule Agricultural University, Ahemadnagar, India. Seeds were first washed in running tap water for 2 min, and then disinfected with 3% (vol/vol) Dettol for 5 min. The disinfected seeds were washed again with tap water and sterilized in 70% alcohol for 5 min, 1% (wt/vol) $HgCl_2$ for 7 min followed by rinsing twice with sterile distilled water. The surface-sterilized seeds were aseptically germinated on moist cotton and on Murashige and Skoog (1962) (MS) medium solidified with 0.8% (wt/vol) agar (Fig. 1A). From 3-week-old seedlings, shoot tips and nodes were isolated and cultured on various nutrient media.

Media preparation and culture conditions

Media were prepared using the MS salt composition, sucrose (3%) and bacteriological-grade agar (0.8%). Growth regulators were incorporated into the media and the pH was adjusted to 5.8. The media were then sterilized in an autoclave at 15 lb pressure and 121°C for 20 min. The growth room conditions maintained for in vitro culture were 25±2°C, 50–60% relative humidity (RH), a 16-h photoperiod with fluorescent light (1000 μmol · m^{-2} · s^{-1} photointensity), followed by an 8-h dark period. Unless otherwise stated, all growth regulators added to MS medium are in micromolar concentrations.

Initiation and proliferation of shoot tip and nodal explants

Shoot tip and nodal explants were cultured on MS medium alone or supplemented with one of the following growth regulators: 6-ben-

zyladenine (BA; 4.44, 8.88, 17.75); kinetin (KN; 4.65, 9.29). Response, viability, intensity of shoot development, and quality of shoots (as measured by the ease of dividing shoot cultures, shoot elongation and manifestation of abnormalities such as fasciation, vitrification or etiolation) was recorded weekly for 7 weeks.

Optimum rooting procedure

Shoots (≥15 mm with four or more leaves) were isolated and transferred to: (1) medium with MS and MS supplemented with one of the following combinations of growth regulators: indole-3-butyric acid (IBA; 4.92) + charcoal (0.1%), α-naphthaleneacetic acid (NAA; 5.37) + charcoal (0.1%), indole-3-acetic acid (IAA; 0.06) + charcoal (0.1%); (2) ½-strength MS alone and on ½-strength MS supplemented with one of the following combinations of growth regulators: IBA (0.49, 2.46, 4.92, 9.84, 19.69, 24.61, 49.21), NAA (5.37), IAA (0.06), IBA (4.92) + charcoal (0.1%), NAA (5.37) + charcoal (0.1%), IAA (0.06) + charcoal (0.1%), IAA (0.06) + IBA (4.92), IAA (0.06) + NAA (5.37), IBA (4.92) + gibberellic acid (GA; 2.89), IBA (4.92), GA (2.89) + thiamine (29.6 μM); and (3) medium with ¼-strength MS supplemented with IBA (4.92) + GA (2.89), IBA (24.16) + GA (8.66). The number of rooted shoots and quality of roots was recorded after 3 weeks.

Acclimatization and field trials

Rooted explants with four or more leaves and three to four roots were washed to remove the adhering agar and medium, and then transferred to tumblers (2.5-in-diameter-wide mouth; 3 in height, 12 2-mm holes at the base) in the greenhouse (temperature 28 ± 5 °C). All plants were bottom irrigated by placing the tumblers in trays filled with ½-in-deep water. Four experiments were conducted for acclimatization, varying light intensity, RH and substrata [garden soil/soil substitute (vermiculite)].

(1) In vitro plants were transferred to garden soil and exposed to natural light and 60 ± 7% RH in the greenhouse.
(2) In vitro plants were transferred to vermiculite (soil substitute) and exposed to natural light and 60±7% RH in the greenhouse.
(3) In vitro plants were transferred to garden soil and exposed to natural light and 82 ± 3% RH by covering them with transparent polythene bags (12.5×21 cm^2). The polythene bags were punched with four holes (5 mm diameter) to allow gaseous exchange. After 1 week, the plants were exposed to 60 ± 7% RH (polythene cover was removed).
(4) In vitro plants were transferred to vermiculite, the other conditions were as in (3).

Fifteen plants were taken in each experiment and their viability, leaf number and height were recorded weekly for up to 3 weeks. After 3 weeks, all the surviving plants were transferred to polythene bags with garden soil and taken out to the nursery. Their growth was compared to that of zygotic seedlings after 7 weeks and again after 13 weeks.

Callus establishment

Segments of leaves, internodes, roots and cotyledonary leaves lacking any meristematic buds were placed on media with MS alone and MS supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 4.52), 2,4-D (4.52) + BA (4.44), 2,4-D (4.52) + KN (4.65), 2,4-D (4.52) + BA (8.88), 2,4-D (4.52) + KN (9.29), IAA (0.05) + BA (4.44), IAA (0.05) + KN (4.65), NAA (5.37) + BA (4.44), NAA (5.37) + KN (4.65). Response, intensity of callus development, and callus quality (friability and colour) were recorded weekly for 8 weeks. Fresh and dry weights were measured on a weekly basis for 8 weeks using callus from six culture tubes each week. Callus was placed on preweighed aluminium foil and weighed immediately to obtain the fresh weight. It was then kept in an oven at 70 °C for 17 h and weighed again to obtain the dry weight. The mean values for percentage increase in fresh and dry weight were calculated.

Results

Seedling germination

Seeds started germinating within 1 week, and seedlings up to 8-cm-long, suitable for culture, developed within a period of 3–6 weeks (Fig. 1A). Seed germination was better using MS medium supplemented with 3% sucrose than on moistened cotton. The seedlings were healthier and retained their cotyledonary leaves for up to 6 weeks in contrast to those on moistened cotton, which senesced within 1 week of germination.

Initiation and proliferation of nodal explants

On MS supplemented with BA (4.44, 8.88, 17.75 μM), multiple shoots (5–25 mm) developed within 4–7 weeks in 74% of cultures (Table 1). Considerable shoot elongation was observed in the presence of BA (17.75 μM). At lower concentrations of BA, shoot length was reduced, and profuse leaf development occurred (Fig. 1B). KN was ineffective in inducing shoot proliferation. Generally, nodal explants were more responsive than shoot tip explants. On media supplemented with various BA concentrations, 80% nodal explants and 24% of shoot tip explants developed ≥15 shoots (Fig. 1C). Flowering was observed in some of the cultures (Fig. 1C). Flowers were immature and lacked carpels and stamens.

Optimum rooting procedure

Among the various auxins investigated, IBA (4.92 μM) was most effective in initiating roots on shoots (Fig. 1F, G, Table 2). Long and branched roots devoid of root hairs, developed on this medium. Higher concentrations of IBA led to the formation of profuse stout roots. IAA and NAA were less effective in inducing root development. Addition of charcoal (0.1%) in both MS and ½-strength MS medium with IAA (0.06 μM), IBA (4.92 μM), or NAA (5.37 μM) did not support root development. As an adsorbant, charcoal may have decreased the effective growth regulator concentration in the media. Since rooting was obtained on media without charcoal, experiments with charcoal and different growth regulator concentrations were not conducted.

Fig. 1 **A** Germinated seedlings on MS medium (1 cm ≅ 1.1 cm). **B** Profuse leaves on reduced shoots developed from shoot tip and nodal explants on MS+BA (4.44 μM) (1 cm ≅ 1.3 cm). **C** Multiple shoots and a flower developed from shoot tip and nodal explants on MS+BA (17.75 μM) (1 cm ≅ 0.57 cm). **D** Flower induced from cotyledonary leaf explant on MS+IAA (0.06 μM)+KN (4.65 μM) (1 cm ≅ 0.57 cm). **E** Callus tissue developed on MS+2,4-D (4.52 μM)+BA (4.44 μM) (1 cm ≅ 0.92 cm). **F, G** Excised shoots rooted on ½-strength MS medium with IBA (4.92 μM) (**F** 1 cm ≅ 0.78 cm; **G** 1 cm ≅ 1.25 cm). **H** Hardened plants in polythene bags (1 cm ≅ 11.5 cm)

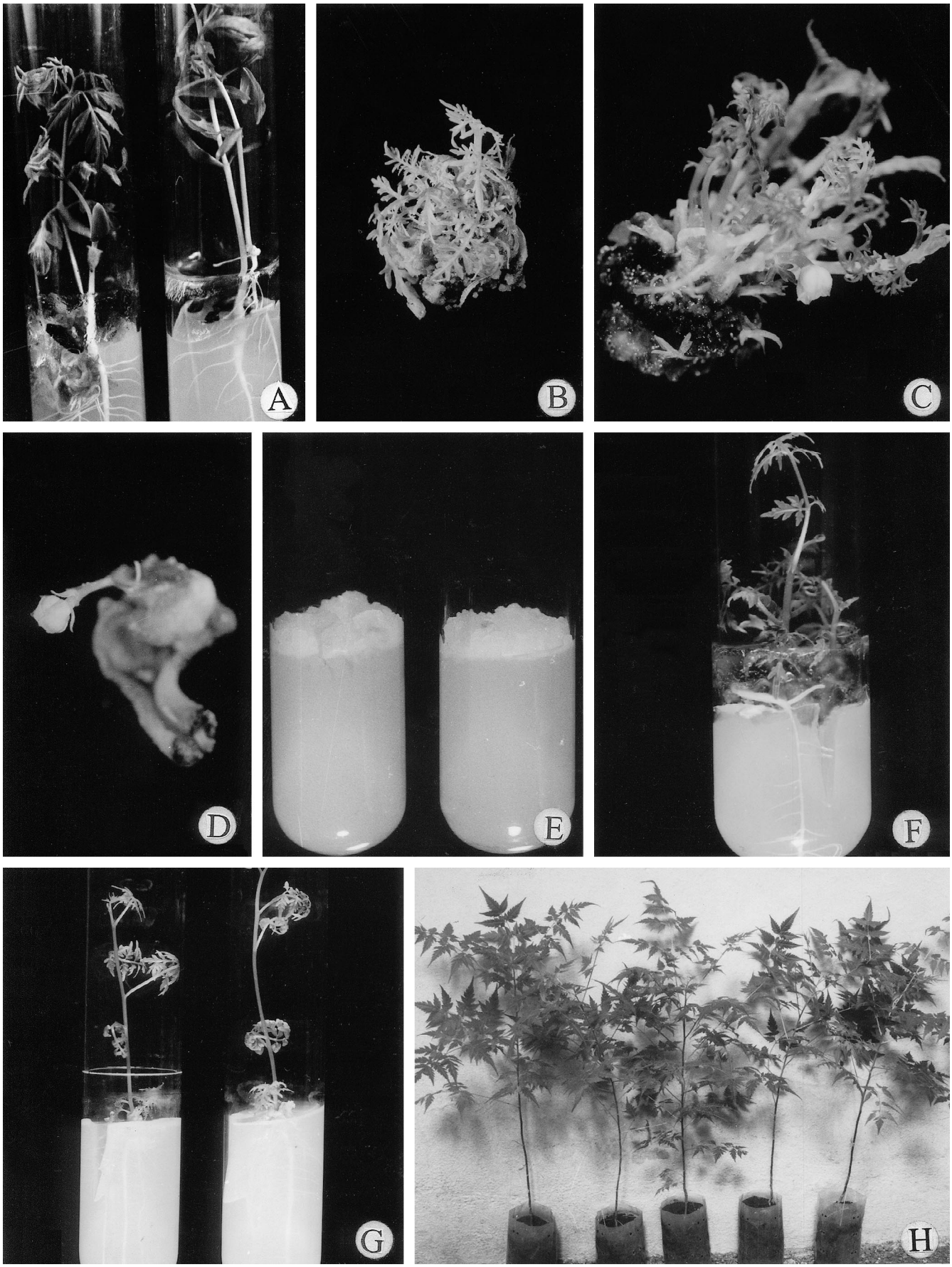
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Table 1 Multiplication of shoots in explants of *Melia azedarach* in various media after 7 weeks

Treatment (μM)	Explant (number of replicates)	Mean number of shoots generated per explant±SD	Number of explants showing more than 15 shoots	Number of flowering explants
MS (control)	Shoot apices (12)	0	0	0
	Nodes (12)	0	0	0
MS+BA (4.44)	Shoot apices (24)	6.35±4.02	3 (12%)	0
	Nodes (39)	14.94±8.26	29 (74%)	8 (20%)
MS+BA (8.89)	Shoot apices (7)	10.36±5.58	4 (57%)	0
	Nodes (7)	15.21±7.31	6 (86%)	1 (14%)
MS+BA (17.75)	Shoot apices (6)	9.17±4.49	2 (33%)	0
	Nodes (14)	23.75±7.49	13 (93%)	2 (14%)
MS+KN (4.65 or 9.29)	Shoot apices (14)	0	0	0
	Nodes (26)	0	0	0

Table 2 Root induction in shoots of *M. azedarach* in various media after 3 weeks

Treatment (μM)	Total number of replicates	Number of cultures showing rooting	Rooting (%)
MS (control)	24	0	0
½MS (control)	12	0	0
½MS+IBA (0.49)	12	0	0
½MS+IBA (2.46)	12	10	(83)
½MS+IBA (4.92)	24	20	(83)
½MS+IBA (9.84)	12	10	(83)
½MS+IBA (19.69)	12	7	(58)
½MS+IBA (24.61)	12	6	(50)
½MS+IBA (49.21)	12	5	(42)
½MS+IAA (0.06)	24	17	(71)
½MS+NAA (5.37)	24	21	(88)
½MS+IAA (0.06)+IBA (4.92)	24	17	(71)
½MS+IAA (0.06)+NAA (5.37)	24	13	(54)
½MS+IBA (4.92)+GA (2.89)	12	5	(42)
½MS+IBA (4.92)+GA (2.89)+ thiamine (10 mg/l)	12	5	(42)
¼MS+IBA (4.92)+GA (2.89)	12	11	(92)
¼MS+IBA (24.6)+GA(8.66 μM)	12	4	(33)

Acclimatization

The survival rate was 100% in both the experiments where the RH was high. However, plant growth was better in vermiculite (Table 3). The light weight and porous texture of the soil substitute prevents root damage, allows better root growth and hence development of a healthier plant. Plants directly exposed to low-humidity conditions could not withstand the shock and died following desiccation. In-vitro-cultivated plants lack the necessary anatomical features to withstand variations in the natural environment. Since the growth of plants was not affected by sudden exposure to natural light in the greenhouse, experiments at lower light intensities were not conducted. All the plants transferred to polythene bags with garden soil established well (Fig. 1H). The growth of the in-vitro-cultured plants in the fields was comparable to that of the zygotic plants (Table 4). Leaves were not counted because an exact leaf number could not be determined due to fall of older leaves.

Initiation and proliferation of callus

An actively growing and friable callus was induced from internodal segments on a basal medium fortified with 2,4-D (4.52 μM) in combination either with BA (4.44 μM) or KN (4.65 μM) (Fig. 1E). The same media was used to study callus growth (Fig. 2). The growth rate was maximum during the 6th and 7th week and declined afterwards. Flowering was observed in cotyledonary leaf and internodal explants in response to IAA (0.06 μM) supplemented with BA (4.44 μM) and KN (4.65 μM) (Fig. 1D).

Discussion

The present study indicates that nodes with axillary buds may be convenient, accessible and efficient explants for micropropagation in *Melia*. Several advantages of this system include the ease of obtaining explant material, a strong tendency for profuse multiple-bud induction, the relative ease with which the shoots could be induced to root and the high percentage of ex vitro plant survival after acclimatization.

We observed in the present study that a transient period of increasing daily temperature variation, decreasing humidity and increasing light was not essential for the survival of the tissue-cultured *M. azedarach* plantlets. The period of exposure to high humidity was sufficient. Maintenance of high humidity is necessary for some time after transplantation, for the continued turgidity of the tissue-cultured leaves, until the freshly acclimatized leaves develop (Donnely and Vidaver 1984). The leaves from in-vitro-developing plants are less able to control stomatal transpiration than normal leaves especially at RH less than 90%. Therefore, their exposure to higher light intensities, while maintaining a high RH stimulates faster development of acclimatized leaves (Sutter et al. 1988). Furthermore, irrigation of the ex vitro plantlets

Table 3 Effect of acclimatization (3 weeks) on the survival of *M. azedarach* plants transferred in vitro to in vivo

Treatment	Total number of surviving plants out of 15	Survival on the 21st day (%)	Mean increase in height±SD (cm)	Mean increase in leaf number ±SD
(1) 60±7% RH and garden soil	0	0	0	0
(2) 60±7% RH and vermiculite	1	6.7	4.7±0.00	4±0.00
(3) 82±3% RH and garden soil	15	100	6.11±2.58	4.07±1.61
(4) 82±3% RH and vermiculite	15	100	7.31±2.15	5.07±1.53

Table 4 Field performance comparison of 13 ex vitro and zygotic plants of *M. azedarach*

	Mean height±SD (cm)	
	7 weeks	13 weeks
Ex vitro plants	21.2±5.1	39.0±11.3
Zygotic plants	20.1±6.5	36.2±14.1

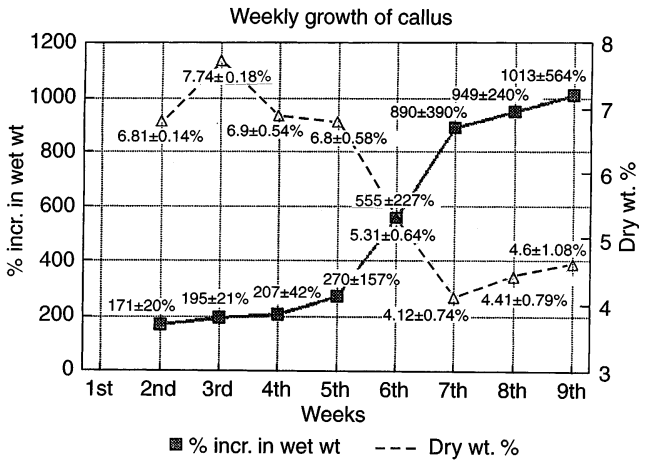

Fig. 2 Weekly growth of callus on MS medium supplemented with 2,4-D (4.52 μM) and BA (4.44 μM)

with ½ MS during the early days after transfer was not essential.

An interesting feature of the present study was the potential of seedling explants to embark upon flowering in vitro in response to BA and combinations of IAA along with BA and KN. The phenomena assumes significance considering the fact that the explants were obtained from seedlings and in nature there is a maturation period spanning a few years before a plant bears flowers. There have been reports of BA promotion of flowering in some plants, especially in various species *Lemna* (Fujioka et al. 1986) and bamboo (Nadgauda et al. 1990), and the relevance of in vitro flowering has been discussed by Staden and Dickens (1991).

During the last two decades, considerable importance has been focused on neem (*A. indica*) worldwide due to its several commercial uses. Recently, neem tissue cultures have been established and plants have been regenerated (Thengane et al. 1995). *M. azedarach* is taxonomically related to neem, and a protocol for its production by micropropagation, as described here, represents a promising approach for cost-effective commercial application.

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