

## PLANT REGENERATION, ORIGIN, AND DEVELOPMENT OF SHOOT BUDS FROM ROOT SEGMENTS OF *MELIA AZEDARACH* L. (*MELIACEAE*) SEEDLINGS

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### SUMMARY

*In vitro* regeneration of plants from root culture of *Melia azedarach* seedlings was obtained. The origin and mode of development of the regenerated shoot buds were studied by means of histological analysis and scanning electron microscopy (SEM). Maximum shoot bud regeneration was achieved when root segments were cultured on Murashige and Skoog (MS) medium at quarter strength with 3% sucrose and 0.44  $\mu\text{M}$  benzyladenine (BA) and kept under light ( $116 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Shoot bud elongation was achieved on MS with 0.44  $\mu\text{M}$  BA, 0.46  $\mu\text{M}$  kinetin (KIN), and 3.26  $\mu\text{M}$  adenine sulphate (AD). Regenerated shoots were rooted on MS with 12.26  $\mu\text{M}$  indole-3-butyric acid (IBA) for 4 d and subsequently in MS lacking plant growth regulators for 26 d. Plants were established in a potting substrate. Histological analysis of roots from intact seedlings (without treatment) demonstrated that during the early life of the roots, *M. azedarach* lacks preformed buds. In contrast, when the roots were excised and cultured *in vitro*, the histology and SEM observations revealed that buds originated from meristematic groups of cells, which had been formed from the pericycle and several layers beneath. These meristematic groups of cells grew towards the periphery of the cortex by crushing the outer layer of cortical cells. Further development led to the differentiation of leaf primordia and a shoot apical meristem.

**Key words:** histology; shoot buds; root culture; scanning electron microscopy.

### INTRODUCTION

The genus *Melia* contains about five poorly defined species in the Old World tropics (Pennington and Styles, 1975). *Melia azedarach* was introduced in the New World and spread throughout tropical America, from Mexico to Argentina (Pennington, 1981). It is important mainly because of the value of its timber. *M. azedarach* is also valued for its insecticide property (Breuer and Schmidt, 1995; Chen et al., 1996; Andreu et al., 2000; Ursi Ventura and Ito, 2000) and for an antiviral factor in its leaves (Andrei et al., 1986; Coto and de Torres, 1999).

Previous studies with tissue culture of *M. azedarach* are limited. However, plantlets have been obtained from nodal explants (Thakur et al., 1998; Shahzad and Siddiqui, 2001), axillary buds (Domecq, 1988; Ahmad et al., 1990), apical meristems (Vila et al., 2002), leaf explants (Vila et al., 2003a), and from somatic embryogenesis using immature zygotic embryos (Vila et al., 2003b). Nevertheless, there is no report on successful *in vitro* plant regeneration from roots of this species. Usually, roots are avoided as explants for *in vitro* plant regeneration because they are generally unresponsive, and consequently, these studies are scarce in comparison with those made from aerial parts of the plants. The regeneration of shoot buds from isolated roots has been mainly observed in species that normally have this capacity in natural conditions (Kerbaui, 1998).

In *M. azedarach*, no pre-existing shoot buds could be found on the roots in natural conditions. However, the damaged roots of adult individuals can differentiate *de novo* shoot buds (Tourn et al., 1999).

This paper reports, for the first time a protocol to regenerate plants through root culture of *M. azedarach*. This work focuses on the origin and mode of development of the regenerated shoot buds by means of histological analysis and scanning electron microscopy (SEM) observations.

### MATERIALS AND METHODS

**Plant material.** Fruits were collected from plants of *M. azedarach* that have grown in the garden of the Agronomy Faculty, Corrientes, Argentina.

After removing the endocarp of the fruit, seeds were surface-sterilized with 70% ethanol (1 min), followed by a solution of 0.8% sodium hypochlorite and finally rinsed with sterile distilled water three times. Seeds were placed in a 350 ml flask with 15 ml of semi-solid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) without plant growth regulators (PGRs). When seedlings were about 1 mo. old, the root systems were surface-sterilized, by a 20 s immersion in 70% ethanol followed by a 5 min immersion in 0.28% sodium hypochlorite, and finally, they were rinsed three times in sterile distilled water. Two centimeter long secondary roots were excised under sterile conditions and immediately transferred into 11 ml glass tubes containing 3 ml of culture medium.

**Induction media.** The nutrient medium employed was quarter-strength MS medium with 3% sucrose (1/4 MS). Basal medium was supplemented with several concentrations of either thidiazuron (TDZ: 0.04, 0.45, 4.54, and 13.62  $\mu\text{M}$ ) or benzyladenine (BA) alone (0.44 or 4.44  $\mu\text{M}$ ), or BA (0.44; 4.44, 8.88, 13.32, and 26.64  $\mu\text{M}$ ) combined with 0.46  $\mu\text{M}$  kinetin (KIN) and 16.29  $\mu\text{M}$  adenine sulfate (AD). These PGR concentrations and combinations were selected because they were near optimal for plant

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regeneration through somatic embryogenesis (Vila et al., 2003b) and organogenesis (Vila et al., 2003a) in cultures of zygotic embryos and leaf explants of *M. azedarach*, respectively. The media were solidified with 0.7% agar (Sigma A-1296, MO, USA). The pH of the media was adjusted to 5.8 prior to the addition of agar. The tubes were covered with aluminum foil and autoclaved at  $1.46 \text{ kg cm}^{-2}$  for 20 min.

The tubes containing the explants were covered with Resinite AF 50<sup>®</sup> (Casco SAC Company, Buenos Aires, Argentina) and incubated in a growth room at  $27 \pm 2^\circ\text{C}$  with a 14 h photoperiod provided by cool white fluorescent lamps ( $116 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) or in darkness.

**Elongation media.** Shoot buds obtained in each initiation medium after 1 mo. were separated from the mother tissue and cultured individually in 11 ml glass tubes containing 3 ml of bud elongation medium consisting of full strength MS with  $0.44 \mu\text{M}$  BA,  $0.46 \mu\text{M}$  KIN, and  $3.26 \mu\text{M}$  AD. The tubes were sealed with Resinite AF 50<sup>®</sup> and incubated in a growth room at  $27 \pm 2^\circ\text{C}$  with a photoperiod of 14 h ( $116 \mu\text{mol m}^{-2} \text{s}^{-1}$ , cool white fluorescent light). Bud elongation was evaluated after 1 mo.

**Rooting and acclimatization.** The production of roots by shoots obtained from elongated buds was examined on MS medium supplemented with  $12.26 \mu\text{M}$  indole-3-butyric acid (IBA) for 4 d and subsequently on MS without growth regulators for 26 d. The physical conditions were the same as those described above for bud induction under light conditions.

Rooted plantlets were transferred into pots containing perlite, sand, and soil [1:1:1 (v/v)] and were kept in a growth chamber. The pots were covered with polyethylene bags for 2 wk, after which plants were acclimatized.

**Statistical analysis.** For culture initiation after 4 wk of *in vitro* growth, data were collected on bud number and percent of explants with shoot buds (frequency). Each root induction experiment consisted of 10 explants and the experiment was repeated at least three times. Measurement was made after 30 d of culture. All data were subjected to analyses of variance (ANOVA) and comparisons of means were made by the Duncan test at  $P < 0.05$ .

**Histology.** For the histological examination, 40 control roots (without treatment, taken directly from the seedlings) and treated roots (excised and cultured *in vitro* in 1/4 MS with  $0.44 \mu\text{M}$  BA), sampled every 3 d, were excised and fixed in FAA (formaline, acetic acid, 70% ethanol; 5:5:90). The tissues were dehydrated with a solution for histological dehydration BIOPUR<sup>®</sup> (S.R.L., Santa Fe, Argentina), and embedded in paraffin blocks as described by Johansen (1940). Serial sections were mounted on glass slides, stained with Safranin Astra-Blue C.I. 50240; (Luque et al., 1996) and observed under a light microscope.

**SEM observations.** Root segments with shoot buds originated from direct process at different developmental stages were fixed in FAA, and later dehydrated through a graded acetone series, then critical point-dried, gold palladium sputter-coated, and observed under a JEOL 5800 LV scanning electron microscope.

## RESULTS

Various concentrations of PGRs were tested on root explants from 1-mo.-old seedlings of *M. azedarach*. In some explants, shoot bud formation was a direct process and in others it involved associated callus formation. After 3 wk in culture, shoot buds developed on root explants (Fig. 1A) but elongated shoots were rarely formed (Fig. 1B).

In the first experiment, the production of shoot buds was greatly affected by the medium and conditions of incubation. Bud differentiation was obtained in light as well as in the dark (Fig. 2). However, the frequency was lower when the explants were cultured in the dark, and the differentiated shoot buds turned brown after the first week of formation and necrotized by the third week. Meanwhile, when explants were cultured in light, shoot bud regeneration was enhanced and was significantly superior to that in the dark.

The optimal response was obtained with  $0.45 \mu\text{M}$  TDZ, where 50.2% of explants produced an average of  $3.2 (\pm 0.33)$  buds when cultured in light. No bud regeneration was observed in the media with BA at concentrations higher than  $4.4 \mu\text{M}$ . Similarly, the

response at the highest TDZ level ( $13.6 \mu\text{M}$ ) was poor when explants were incubated in light and markedly inferior when cultured in darkness (Fig. 2). Since incubation in the light permitted the greatest bud production in the first experiment, this condition was employed when studying the effect of several PGRs on *in vitro* bud regeneration.

The results obtained when root segments of *M. azedarach* were cultured in a medium similar to that used in the previous experiment, but with lower concentrations of BA and TDZ, are presented in Table 1. Although bud regeneration was readily achieved in all media tested (Fig. 1B), the significantly highest bud regeneration frequency (66.8%) was observed on medium containing BA at  $0.44 \mu\text{M}$  and a combination of  $0.44 \mu\text{M}$  BA,  $0.46 \mu\text{M}$  KIN, and  $16.29 \mu\text{M}$  AD.

The highest level of regeneration, in terms of bud number per explant, was obtained in medium containing TDZ at  $4.54 \mu\text{M}$ . However, some of the regenerated buds turned brown after 4 wk in culture. While  $0.44 \mu\text{M}$  BA stimulated bud initiation, higher concentrations of BA depressed bud formation. Although the frequency and number of buds formed were similar to those obtained in the control medium (1/4 MS, 3% sucrose), overall, medium containing  $0.44 \mu\text{M}$  BA was considered to be the most suitable for bud induction (Table 1).

**Elongation of shoot buds.** In preliminary experiments where several bud elongation media were tested, elongation occurred in MS with  $0.44 \mu\text{M}$  BA,  $0.46 \mu\text{M}$  KIN, and  $3.26 \mu\text{M}$  AD after 1 mo. (data not shown); this medium was therefore employed for elongation of buds regenerated on all induction media (Table 2).

Except for shoot buds formed on 1/4 MS supplemented with  $8.88 \mu\text{M}$  BA,  $0.46 \mu\text{M}$  KIN, and  $16.3 \mu\text{M}$  AD, buds from all induction media elongated (Fig. 1C). Bud elongation was optimal when induction medium was 1/4 MS with  $0.44 \mu\text{M}$  BA (Table 2).

**Rooting and acclimatization.** When regenerated shoots were transferred to a rooting medium, higher rooting rates were obtained with shoots taken from the elongation medium and from all those induction media that contained BA at 0.44 and  $4.44 \mu\text{M}$  (alone or combined with  $0.46 \mu\text{M}$  KIN and  $16.29 \mu\text{M}$  AD). When buds originated in medium containing  $0.44 \mu\text{M}$  BA, 100% of the shoots rooted (Table 2). The rooted plantlets (Fig. 1D), when transferred to pots, survived at 80% (Fig. 1E).

**Histological and SEM observations.** While bud regeneration was observed in 1/4 MS without growth regulators, no bud initiation was observed in control roots when these were analysed anatomically (Fig. 3A). Only the normal development of adventitious roots was observed, and these were formed from the unilayered pericycle (Fig. 3A, arrow). The lateral root primordia were formed by periclinal and anticlinal divisions of a group of pericycle cells (Fig. 3B), and these primordia emerged through the cortex and epidermis (Figs 1F and 3C). The root apical meristem and its vascular connection with the central cylinder of the root were observed (Fig. 3B, C).

In the treated roots, simultaneously with the development of lateral roots the formation of meristematic cells inside the central cylinder was observed (Fig. 3D, E). These meristematic cells differed from those that produced lateral roots because they involved several layers of cells beneath the endodermis, not only the unilayered pericycle. These meristematic formations did not present the characteristic organization of a root apical meristem (Fig. 3D,

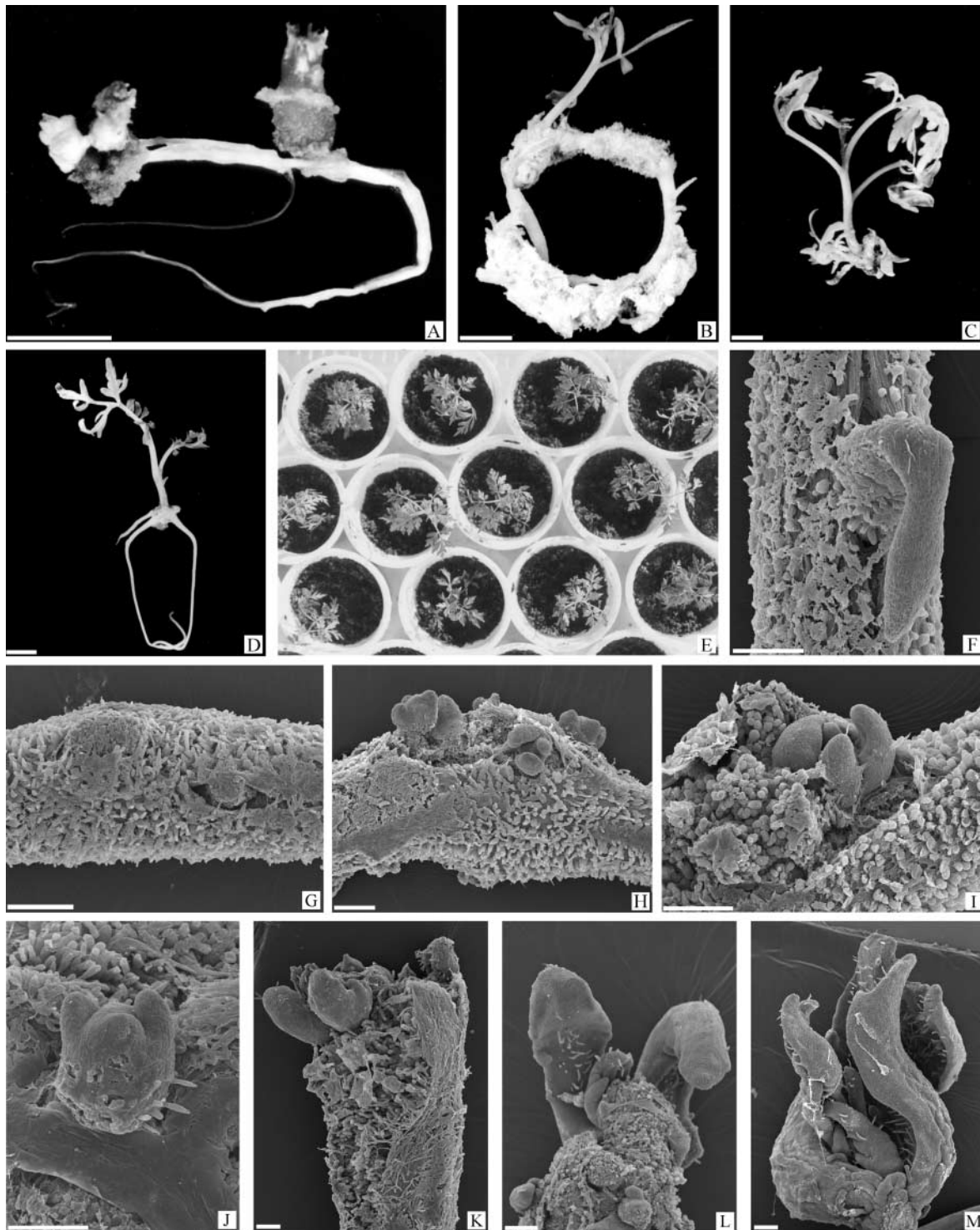


FIG. 1. Plantlet regeneration from cultured root segments of *Melia azedarach*. *A*, Direct bud differentiation. *B*, Callus associated with shoot differentiation. *C*, Shoot obtained in elongation medium. *D*, Rooted shoot. *E*, Regenerated plants in pots during greenhouse acclimation. *F*, SEM micrograph showing lateral root formed from control root. *G*, SEM micrograph showing beginning of meristematic cell groups. *H–M*, SEM micrograph of the sequence of development of shoot buds from root segments from treated roots. Bars = 5 mm (*A–D*) and 200  $\mu$ m (*F–M*).

*E*), underwent divisions and formed spherical structures (Fig. 3*F*, *G*) that progressively expanded through the cortex (Figs 1*G* and 3*H*), and originated buds by a direct process. The development continued with the differentiation of leaf primordia and the shoot

apical meristem, both typical in structure (Fig. 1*H–J*). This resulting bud was connected with the root by parenchymatic tissue. A weak vascular connection with the explant was observed (Fig. 3*I*) in the first stages. Further differentiation led to the formation of

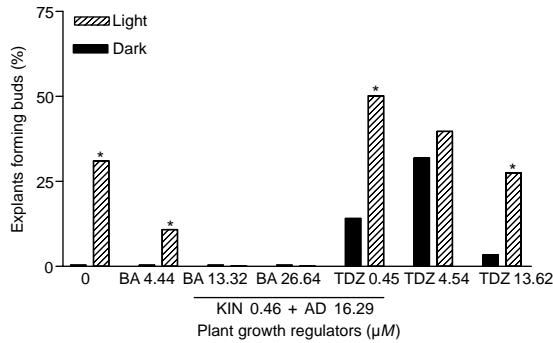


FIG. 2. Effect of growth regulators supplemented to 1/4 MS medium on the percentage of shoot buds per root explant of *M. azedarach* cultured either in darkness (■) or in light (▨). \*Statistically different  $P < 0.05$  by Duncan's multiple comparison test.

TABLE 1

EFFECT OF PGRs ON THE INDUCTION OF SHOOT BUDS FROM ROOT SEGMENTS OF *M. AZEDARACH* AFTER 4 WK CULTURE

PGRs ( $\mu M$ )	Explants forming buds (%) (mean $\pm$ SE)	Number of buds obtained per explant (mean $\pm$ SE)
None	40.3 $\pm$ 5.2 a	2.8 $\pm$ 0.3 a
BA 0.44	66.8 $\pm$ 1.8 b	3.6 $\pm$ 0.9 ab
BA 4.44	45.2 $\pm$ 2.9 a	3.0 $\pm$ 0.3 a
BA 0.44 + KIN 0.46 + AD 16.29	66.8 $\pm$ 3.3 b	3.8 $\pm$ 0.4 ab
BA 4.44 + KIN 0.46 + AD 16.29	40.0 $\pm$ 0.0 a	3.0 $\pm$ 0.0 a
BA 8.88 + KIN 0.46 + AD 16.29	36.7 $\pm$ 6.7 a	3.7 $\pm$ 1.0 ab
TDZ 0.045	53.3 $\pm$ 3.3 ab	3.5 $\pm$ 0.3 ab
TDZ 0.45	40.0 $\pm$ 10.0 a	2.7 $\pm$ 0.2 a
TDZ 4.54	50.0 $\pm$ 5.7 a	5.4 $\pm$ 1.1 b

Different letters within columns indicate a significant difference at  $P < 0.05$  by Duncan's multiple comparison test.

independent buds with their own vascular system and several leaves (Figs 1K–M and 3J, K).

In addition, the differentiation of nodular structures that formed from the outer cortical parenchyma was observed. These structures did not differentiate any buds (Fig. 3L).

DISCUSSION

The use of roots as a source of explants for *in vitro* regeneration is limited to a small number of plant species. Our experiments indicate that root cultures of *M. azedarach* have the potential to form adventitious buds. Even in the absence of PGRs, shoot regeneration was obtained. These results indicated that wounding (by cutting) of roots was sufficient to induce adventitious shoot buds, as occurs in adult trees of *M. azedarach* when the roots are severed by animals or major disturbances such as fire or tree felling (Tourn et al., 1999). Similar results were obtained in *Albizia julibrissin*, where excised roots formed shoot buds on a medium without PGRs (Sankhla et al., 1996). However, in this tree, no adventitious shoot formation occurred when roots from intact seedlings were cultured in medium without PGRs (Sankhla et al., 1994). The inclusion of BA in the medium enhanced bud regeneration in *M. azedarach*. The promoting effect of BA on bud differentiation from root explants has been reported in other woody plant species such as *Citrus mitis* (Sim et al., 1989), *Populus tremula* (Vinocur et al., 1997), and *Citrus aurantifolia* (Bhat et al., 1992). In all these cases, high concentrations of BA were inhibitory for bud induction or bud elongation, which is similar to the responses obtained for *M. azedarach*.

In this study, basal medium supplemented with TDZ was detrimental to the regeneration of shoots, because several buds turned brown and finally died. These results were in contrast with reports on the *in vitro* culture of roots, from other woody species (Sankhla et al., 1996; Vinocur et al., 2000), where TDZ was the best inducer of shoot bud regeneration.

TABLE 2

INFLUENCE OF INDUCTION MEDIA ON SHOOT BUD ELONGATION IN MS WITH 0.44  $\mu M$  BA, 0.46  $\mu M$  KIN, AND 3.26  $\mu M$  AD AND ON FREQUENCY OF ROOTED SHOOTS IN MS WITH 12.3  $\mu M$  IBA CULTURED FOR 4 D, FOLLOWED BY CULTURE IN MS LACKING PGRs

PGRs in the induction media ( $\mu M$ )	Shoots obtained in elongation medium (mean $\pm$ SE)		Percentage of rooted shoots (mean $\pm$ SE)
	%	Height (mm)	
None	66.7 $\pm$ 8.8 ab	15.6 $\pm$ 1.7	84.3 $\pm$ 7.9 ab
BA 0.44	90.0 $\pm$ 5.8 a	26.5 $\pm$ 2.9	100.0 $\pm$ 0.0 a
BA 4.44	80.0 $\pm$ 5.7 ab	20.3 $\pm$ 4.1	96.3 $\pm$ 3.7 a
BA 0.44 + KIN 0.46 + AD 16.3	52.2 $\pm$ 24.8 abc	6.2 $\pm$ 0.4	100.0 $\pm$ 0.0 a
BA 4.44 + KIN 0.46 + AD 16.3	42.1 $\pm$ 4.8 abc	6.3 $\pm$ 0.7	100.0 $\pm$ 0.0 a
BA 8.88 + KIN 0.46 + AD 16.3	8.3 $\pm$ 8.3 c	5.0 $\pm$ 0.0	50 $\pm$ 0.0 c
TDZ 0.045	44.5 $\pm$ 17.8 abc	11.6 $\pm$ 2.6	8.3 $\pm$ 8.3 d
TDZ 0.45	69.2 $\pm$ 10.8 ab	8.9 $\pm$ 2.1	59.7 $\pm$ 15.3 bc
TDZ 4.54	38.3 $\pm$ 21.3 bc	8.3 $\pm$ 1.7	83.3 $\pm$ 16.7 ab

Different letters within columns indicate a significant difference at  $P < 0.05$  by Duncan's multiple comparison test.

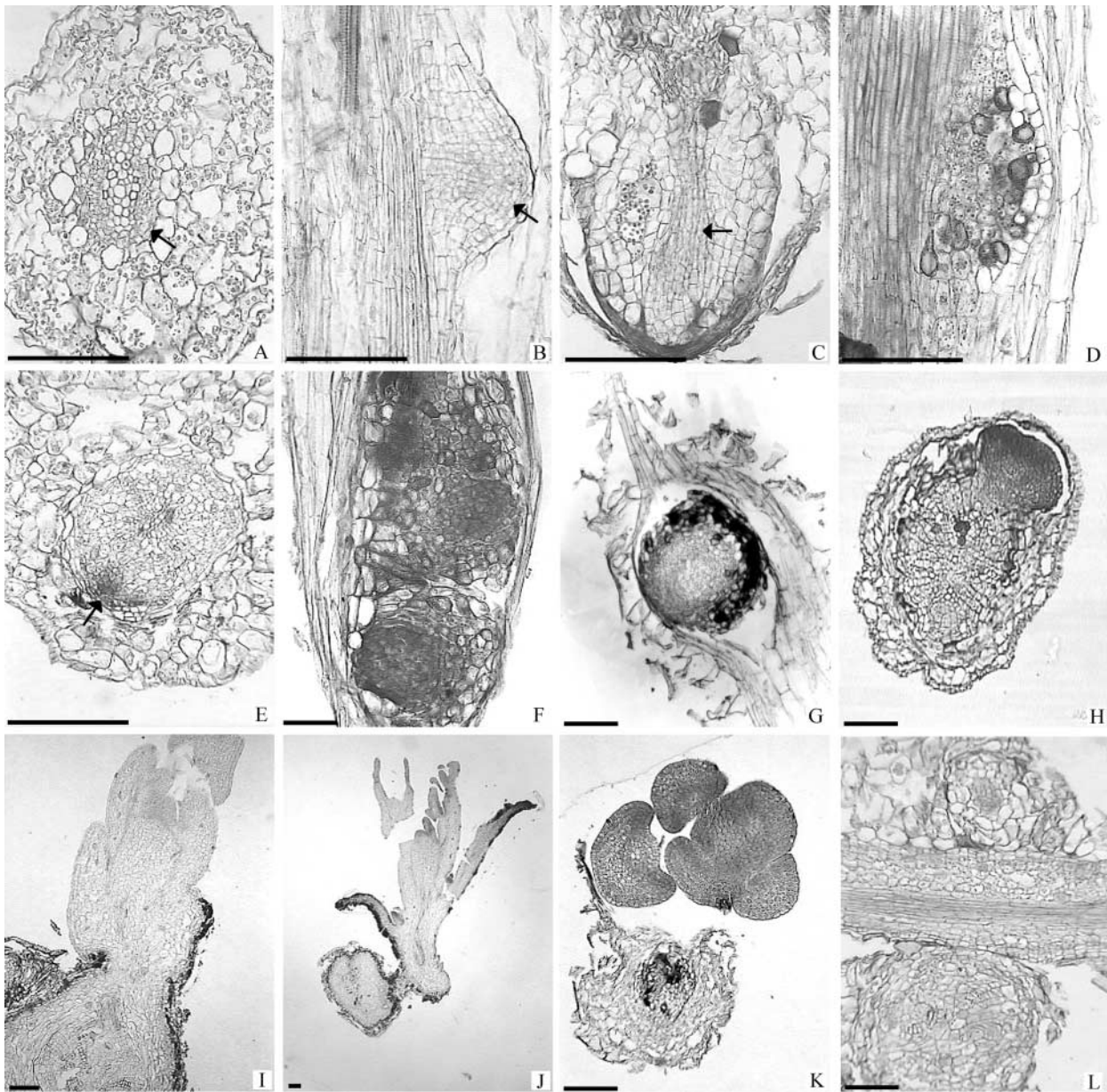


FIG. 3. Histological micrographs of cross-sections of control and treated roots of *M. azedarach*. *A*, Transverse section of control root showing pericycle cells (arrow). *B*, Longitudinal section of control root showing the lateral root meristem (arrow). *C*, Transverse section of control root showing vascular bundle of a secondary root (arrow). *D–G*, Longitudinal (*D*) and transverse (*E*) sections of treated roots showing meristematic cells (arrows) initiated from central cylinder and pericycle cells. *F–H*, Meristematic tissues forming spherical structures. *I*, Longitudinal section of bud connected to explant showing leaf primordia and the shoot apical meristem. *J*, Histological section of independent shoot bud. *K*, transverse section of regenerated bud. *L*, Nodular structures initiated in the cortex. Bars = 100  $\mu\text{m}$ .

Shoot bud regeneration was enhanced by light on all media that promoted shoot bud regeneration.

Histological investigations confirmed that young roots of *M. azedarach* lacked preformed shoot buds, and only the beginning of lateral roots from the pericycle was observed in control plants (Fig. 3*B, C*). In this species, shoot bud formation could be induced by wounding caused by the cut and was enhanced by PGRs in the medium. In contrast to this, the *ex vitro* formation of shoots on roots of *Liquidambar styraciflua* originated from buds that had formed during the early life of the root (Kormanik and Brown, 1967).

Histological examination also showed that buds originated from spherical structures developed from the pericycle and several layers of cells beneath. The origin of buds from the pericycle was also demonstrated in other woody plant species such as *C. aurantifolia* and *P. tremula* (Bhat et al., 1992; Vinocur et al., 2000). In the latter species, all buds originated in close proximity to lateral roots that emerged from the pericycle tissue in the main root, whereas in *A. julibrissin*, buds developed on callus tissue originating from some pericyclic cells (El Maataoui et al., 1998).

Nodular proliferation and subsequent shoot bud formation from root explants was observed in *Acacia albida* (Ahee and Duhoux, 1994) and *Brassica napus* (Sharma et al., 1993). In *M. azedarach*, spherical structures that formed in the internal tissues (cells inside the central cylinder) regenerated buds, whereas nodular proliferation formed in the cortex never differentiated buds. Additional studies are necessary to determine the further development of the nodular structures formed in the cortex.

The protocol described in this publication is novel, simple, and reproducible for shoot bud induction from root explants and further plant regeneration. Our study also revealed the adventitious formation of shoot buds from seedling roots of *M. azedarach*.

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