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Effect of morphological heterogeneity of somatic embryos of *Melia azedarach* on conversion into plants

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Key words: somatic embryo morphology, conversion, histology, scanning electron microscopy.

ABSTRACT: Embryogenic cultures were initiated from immature *Melia azedarach* (Meliaceae) zigotic embryos. Explants were induced on Murashige and Skoog (1962) medium with 4.54 µM thidiazuron or 0.45 µM dichlorophenoxyacetic acid. After 6 weeks of culture on induction medium, somatic embryos were categorized in four morphological classes based on the presence of single or fused embryos and if they remained united or not to the original explant; that were evaluated histologically. The somatic embryos of every category were transferred, in groups or individually, on a 1/4 MS medium. Bipolar embryos, the more typically normal ones, had well defined shoot and root apical meristems and produced single plants; subcultured individually their conversion was 28%, and subcultured in groups the conversion declined to 6.8%. Fused embryos subcultured in groups had only a 2.1% conversion and produced plants with fused stems. None conversion rate in the others classes was associated to poorly developed shoot and root meristematic areas or with their absence. The converted plants were acclimatized and transferred, in a mist, to soil, with an independent of the class 95% survival rate.

Introduction

The paradise tree (*Melia azedarach* L.) is an original species of the South of Asia (Iran, India and South of China) that was introduced in the New World, cultivated and naturalized by tropical America, from Mexico to Argentina (Pennington, 1981). In Misiones province (Argentina) the giant paradise (*Melia azedarach* var. *gigantea*) is cultivated as forest, and it acquires a major development, height and diameter than the common paradise. Paradise is a species with three useful characteristics. Besides offering a very good quality timber, the extracts from several parts of the paradise possess insecticide (Breuer and Schmidt, 1995; Chen *et al*., 1996; Ursi Ventura and Ito, 2000; Andreu *et al*., 2000; Nathan *et al*., 2006), antiviral (Coto and de Torres, 1999; Andrei *et al*., 1986) and fungicide properties (Carpinella *et al*., 2003).

The standard development of the somatic embryos in dicotyledons presents many morphologic characteristics similar to those of the zygotic embryos. Both show the pro-embryonic and embryonic stages of development. This similarity between the somatic and zygotic embryogenesis is surprising because the somatic embryos develop completely without physical and genetic influences of the maternal tissue (Zimmerman, 1993), and not present endosperm.

Although the morphologic similarities between the somatic and zygotic embryogenesis has been demonstrated in several species (Zimmerman, 1993; Pullman *et al*., 2003), it is frequent to find that somatic embryos

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differ from zygotic embryos in physiological vigor (Bornman *et al*., 2003), in their hormonal levels (Hays *et al*., 2001) and that show abnormalities in the development. Numerous authors have shown that embryo morphology directly affected the success of conversion into plants, *in vitro* conditions (Lazzeri *et al*., 1987; Wetzstein and Baker, 1993).

The first report of somatic embryogenesis in *Melia azedarach* was recently documented (Vila *et al*., 2003). However, in this work the development of the somatic embryos showed substantial morphological heterogeneity. The aims of the present study were to characterize the morphological heterogeneity of somatic embryos of *Melia azedarach*, and to study the effect of somatic embryos morphology observed on germination and conversion into plantlets.

Materials and Methods

Collect season, fruit sterilization and tissue culture procedure were done as previously reported (Vila *et al*., 2003). Immature fruits (8-9 weeks after pollination) were collected from paradise trees located in the Campus of the Facultad de Ciencias Agrarias (UNNE), Corrientes, Argentina. Fruits were surface sterilized by a 5 min immersion in 70% ethanol, followed by a 20 min immersion in 3.6% (w/v) sodium hypochlorite with two drops of Tween® and finally, they were washed three times in distilled water. The torpedo and early cotyledonar stage (1-1.5 mm length) of immature zygotic embryos were extracted and cultured on embryogenic induction medium composed of mineral salts, sucrose and vitamins according to Murashige and Skoog (1962) (MS) + 4.54 µM thidiazuron or 0.45 µM dichlorophenoxyacetic acid. The media were solidified with 0.7% agar (Sigma A-1296). Media pH was adjusted to 5.8 with KOH or HCl prior to the addition of agar. The tubes were covered with aluminium foil and autoclaved at 1.46 kg. cm^{-2} 20 min.

The tubes containing the explants were covered with Resinite AF 50® (Casco S.A.C. Company, Buenos Aires) and cultures were incubated in a growth room at 27 ± 2°C, under a 14 h photoperiod (provided by cool-white fluorescent) providing light intensity of 116 µmol $m^{-2}.s^{-1}$ at the tube level.

After 6 weeks of culture on the induction medium (MS + 4.54 µM thidiazuron or 0.45 µM dichlorophenoxyacetic acid), somatic embryos were categorized in morphological classes, with a stereoscopic microscopy and a scanning electron microscopy based on the presence of single or fused embryos and according if they remained united or not to the original explant.

Scanning electron microscopy

The somatic embryos of all categories were fixed 40% formaldehyde-acetic acid-70% ethanol (5:5:90), and then dehydrated with an increasing series of acetone, and dried up to critical point in a Denton Vacuum DCP-1 Critical Point Drying Apparatus. Later they were covered with gold paladium in a sputter Dentum Vacuum Desk II and finally observed under the scanning electron microscopy JEOL 5800 LV.

The classified embryos were used to do the studies on conversion into plants. For this purpose they were subcultivated to the 1/4 MS (with 3% of sucrose) following different conditions detailed in figure 2.

Histological studies

Histological analysis were performed according to Gonzalez and Cristóbal (1997). Samples of classified embryos were fixed with (as above), dehydrated with histological dehydrant ("Deshidratante histológico BIOPUR®"), then embedded in paraffin wax as described by Johansen (1940) and sectioned at 8-10 µm thick serial sections with a rotary microtome. Sections were mounted on glass slides and stained with safranin - Astra blue (Luque *et al*., 1996) and observed under a light microscope.

Results

The somatic embryos of *Melia azedarach*, obtained from immature zygotic embryos after 6 week culture on induction medium, showed a wide variability in their morphology (Fig. 1). Four classes of somatic embryos were appreciated: 1) individual embryos that exhibited a clear bipolarity, with lengthened hypocotyls, separated from the original explant and with serrated cotyledonar leaves (similar to the real leaves of the species) (Fig. 1A-C). These embryos were the most similar morphologically to the zygotic embryos; 2) fused embryos, characterized by shortened hypocotyls and/or by multiple cotyledons (Fig. 1E-G); 3) individual embryos in general, with lengthened hypocotyls, sometimes fused by the hypocotyls, united to the explant (Fig. 1I, J) and 4) individual globular embryos, formed only with auxin in the culture medium, specially dichlorophenoxyacetic acid (Fig. 1K, L).

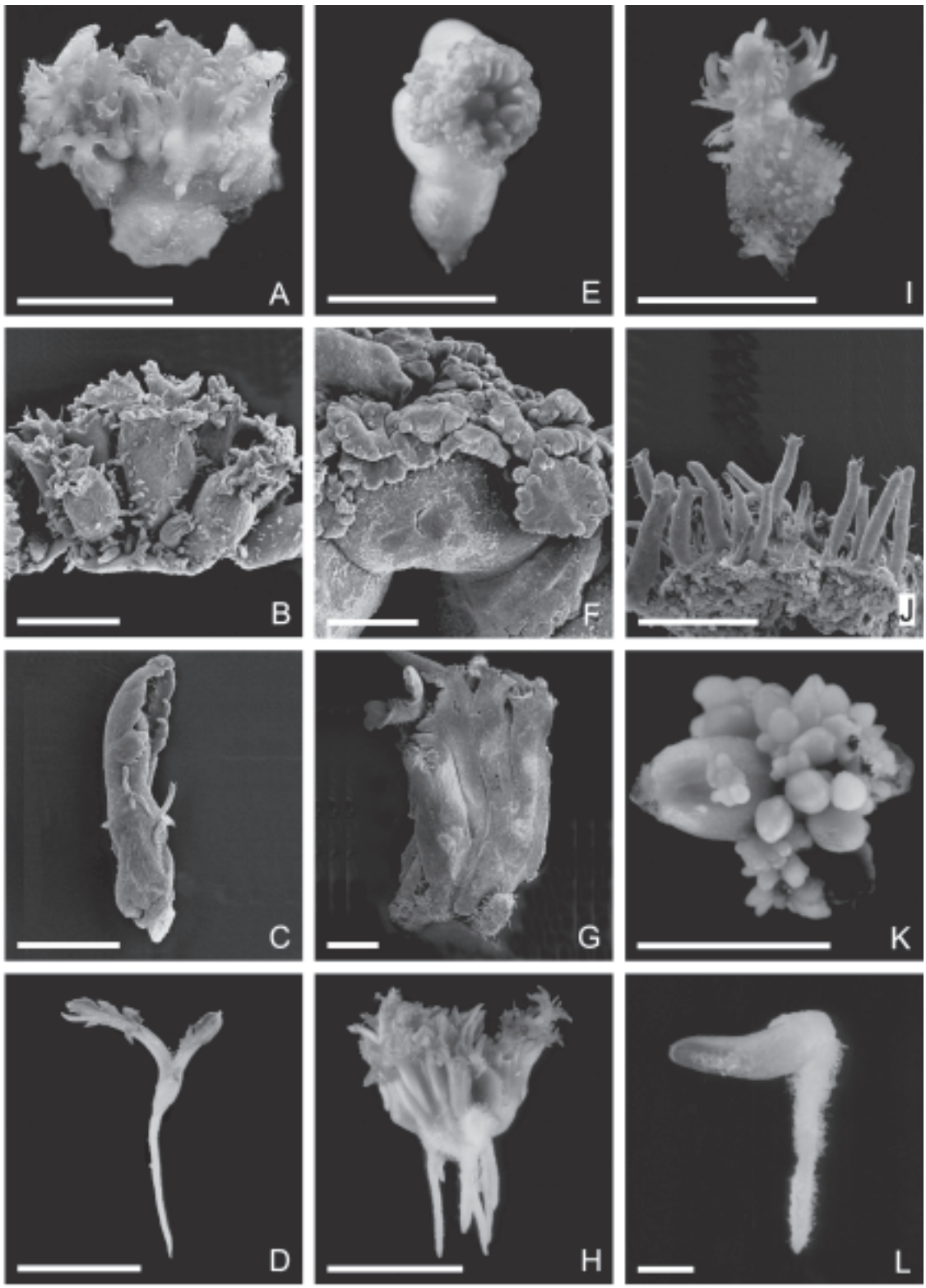
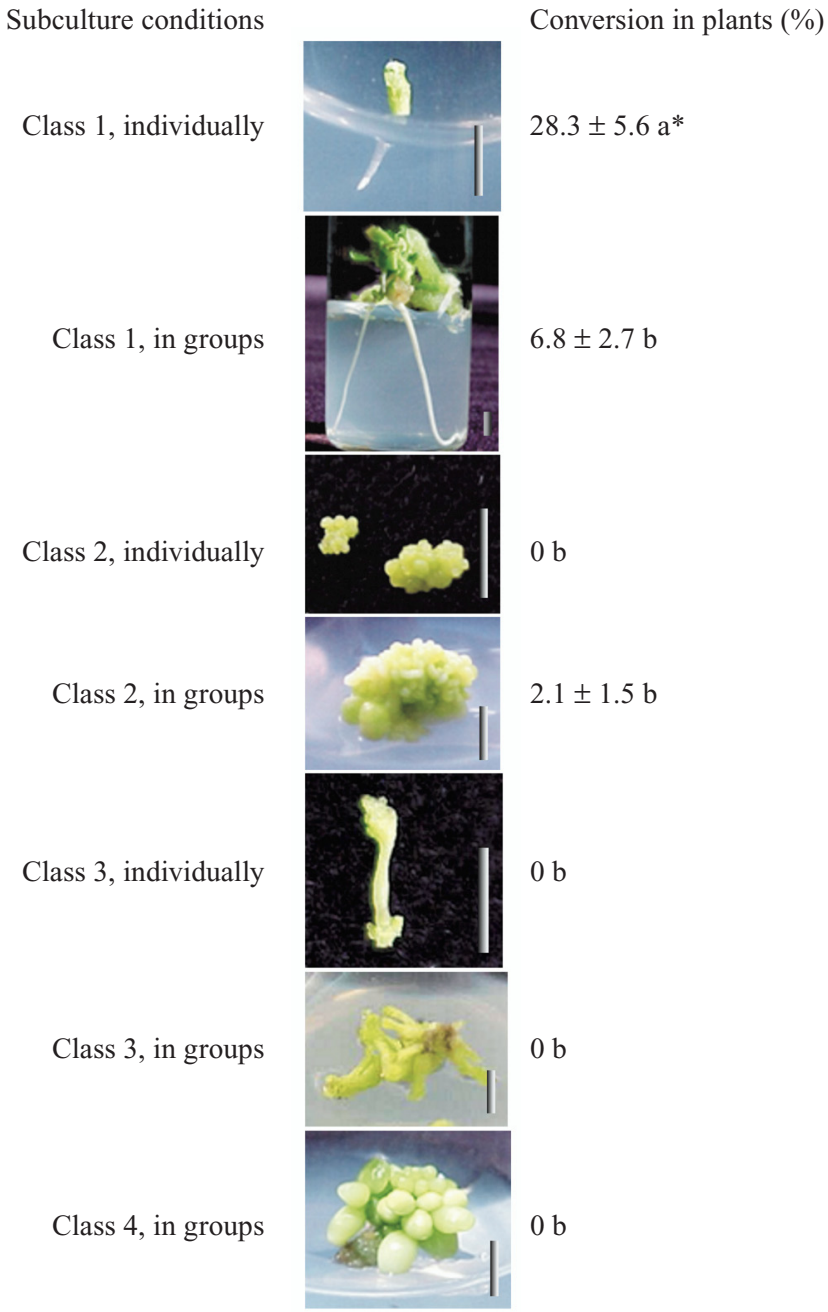


FIGURE 1. Morphology of somatic embryos of *Melia azedarach*. (A) Class 1 of somatic embryos. (B-C) SEM micrograph showing two developmental stages of embryos of the class 1. (D) Class 1 somatic embryo converted into plant. (E) Class 2 of somatic embryos. (F-G) SEM micrograph showing two developmental stages of embryos of the class 2. (H) Class 2 somatic embryos converted into plant. (I) Class 3 of somatic embryos. (J) SEM micrograph showing somatic embryos of the class 3. (K) Class 4 of somatic embryos. (L) Class 4 somatic embryo emitting the radicle. Bars = 5 mm (A, D, E, H, I, K), 1 mm (B, C, F, G, J, L).

When the somatic embryos of *Melia azedarach* of every category were transferred, in groups or individually, to 1/4 MS medium, the determinant effect of the morphology of the embryos on the conversion into plants could be appreciated.

Class 1 somatic embryos offered 28.3% of conversion into plants when they were subcultured individually (Fig. 1D, and Fig. 2), being this rate of conversion statistically superior than that obtained with the remaining classes of embryos and modalities of subculture. When class 1 embryos were subcultured in groups, the percentage of conversion declined to 6.8% (Fig. 2). Class 2 embryos behavior was opposite to that of class 1; when they were transferred in groups a low percentage germinated but remained fused after germination (Fig. 1H), whereas when they were separated there was no



*Different letters indicate significant differences. Duncan's test ($P < 0.05$). Bars = 2 mm

FIGURE 2. Effect of the subculture conditions and somatic embryos categories on the conversion in plants of *Melia azedarach* in 1/4 MS (with 3% of sucrose).

conversion (Fig. 2); if they were separated after emitting the radicle, they might continue growing. In the case of class 3 of somatic embryos, the regeneration of plants was not possible because they turned necrotic after a few days of subculture.

The highest conversion percentages in class 1 embryos was due to the fact that they present clearly bipolar structures without vascular connections with the original explant and showed both meristems (Fig. 3A and B). Meanwhile, in class 2 the majority of the em-

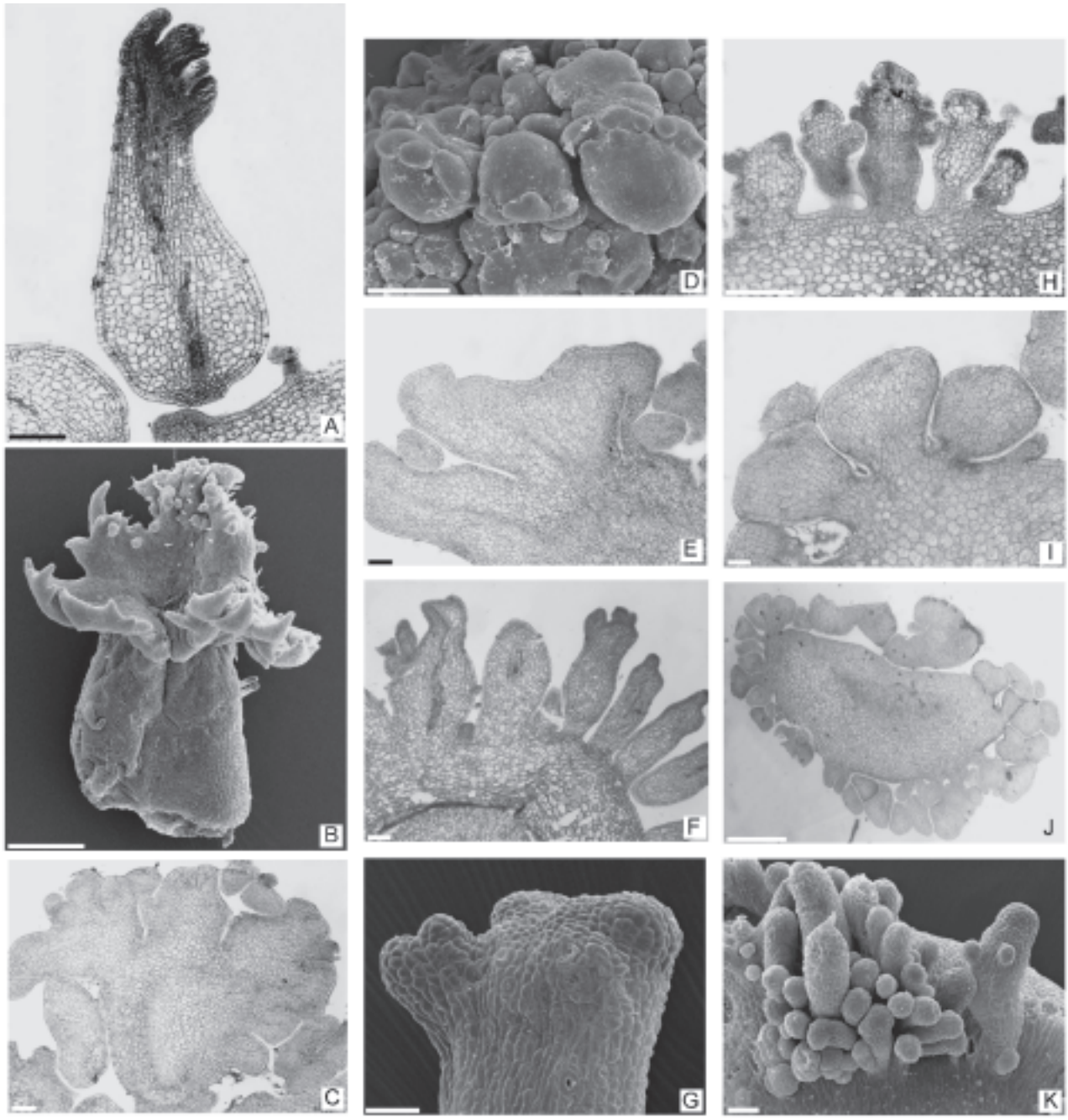


FIGURE 3. Light and SEM micrographs of different somatic embryo morphological classes of *Melia azedarach*. (A) Light micrograph of class 1 somatic embryo showing evident bipolar structure. (B) SEM micrograph showing the class 1 of somatic embryo. (C) Light micrograph of class 2 fused somatic embryos. (D) SEM micrograph of class 2 of somatic embryos showing multiple primordia of foliar structures (arrows). (E) Light micrograph of class 2 embryos showing connection with the explant from procambial tissue. (F) Light micrograph of class 3 somatic embryos without root apex. (G) SEM micrograph of class 3 somatic embryo showing shoot apex poorly developed and stomates. (H) Light micrograph of class 3 somatic embryos showing shoot apex deals (arrows). (I-J) Light micrographs of class 4 of somatic embryos without shoot apex. (K) somatic embryos of class 4. Bars = 50 μm (G, K), 100 μm (A, C, E, F, I), 500 μm (B, D, H, J).

bryos lacked both shoot and root apical meristems (Fig. 3C). The apical zone appeared expanded with an excrescence similar to primordial leaf, that grew laterally (Fig. 3D). In a longitudinal section, some of them, presented a connection with the explant from procambial tissue (Fig. 3E). This characteristic would impede their separation and subsequently the conversion into plants.

Class 3 embryos, in spite of appear as bipolar structures, lacked of root apical meristem (Fig. 3F). In the shoot apex the meristem was poorly developed (Fig 3G), presenting leaf primordia that in few days showed necrosis (Fig. 3H). They possessed procambial tissue in the central region not connected to the explant (Fig. 3F). A prominent characteristic in these structures was the abundance of stomas in the whole surface.

Class 4 globular embryos differed from the previous type in the absence of vascular connection with the explant (Fig. 3I). In general they lacked of shoot meristem and were very numerous (Fig. 3J, K). Although, near 6% of class 4 embryos emitted the radicle, they never developed the shoot apical meristem (Fig. 1L) (data not shown).

The converted plants were acclimatized and transferred to soil in a mist, with a 95% survival rate irrespective of the class. Transference was made in groups for class 2, but previously these somatic embryos had to be carefully separated with a scalpel (after radicle emission) for survival to occur.

Discussion

The study of the morphology of the somatic embryos assumes importance because it has a direct relationship with the conversion into plants in several species, including woody plants. The effect of the morphology of the embryos on the germination (Wetzstein and Baker, 1993; Rodriguez and Wetzstein, 1994; Barry-Etienne *et al*., 2002) or the manner of normalizing the development of the somatic embryos with the objective of improving the production of plants (Niedz *et al*., 2002; Pullman *et al*., 2003) have been studied.

In the present study, it is interesting to note that the percentage of somatic embryos conversion into plants was superior than the percentages obtained in previous works (Vila *et al*., 2003; Vila *et al*., 2007).

Some of the abnormalities showed in somatic embryos of *Melia azedarach* were also observed in somatic embryos of the related species *Azadirachta indica* obtained from zygotic embryos (Chaturvedi *et al*., 2004). These results agree with those obtained in many species of plants in which the somatic embryos showed different morphologic and physiological abnormalities (Tisserat *et al*., 1979).

The highest conversion percentages in class 1 embryos was related to their structures with a clear bipolarity, without vascular connections with the original explant and showing apical shoot and root meristems, result coincident to that described in a previous report (Vila *et al*., 2003).

The behavior of class 4 embryos that emitted the radicle but never developed the shoot apical meristem, was also observed in somatic embryos of *Azadirachta indica* when they were submitted to several treatments of conversion (Chaturvedi *et al*., 2004). In several species it was observed that dichlorophenoxyacetic acid produced somatic embryos with a higher incidence of abnormalities than others plant growth regulators (Rodriguez and Wetzstein, 1998; Lazzeri *et al*., 1987).

In the future, it would be of interest to determine how to stimulate embryos of all categories to develop a morphology comparable to category 1, similar to some studies done in *Melia azedarach* (Vila *et al*., 2007) and in the model species *Picea abies* (Egertsdotter and Von Arnold, 1997).

The experience described in the present study should facilitate any tissue culture applications that require *Melia azedarach* plant regeneration via somatic embryogenesis, such as synthetic seeds production or preservation of germplasm.

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References

Andrei GM, Lampuri JS, Coto CE, De Torres RA (1986). 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.

Barry-Etienne D, Bertrand B, Schlönvoigt A, Etienne H (2002). The morphological variability within a population of coffee somatic embryos produced in a bioreactor affects the regeneration and the development of plants in the nursery. *Plant Cell, Tissue and Organ Culture* **68**: 153-162.

Bornman CH, Dickens OSP, Van Der Merwe CF, Coetzee J, Botha A-M (2003). Somatic embryos of *Picea abies* behave like isolated zygotic embryos *in vitro* but with greatly reduced physiological vigour. *South African Journal of Botany* **69**: 1-10.

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.

Carpinella MC, Giorda LM, Ferrayoli CG, Palacios SM (2003). Antifungal effects of different organic extracts from *Melia azedarach* L. on phytopathogenic fungi and their isolated active components. *Journal of Agricultural and Food Chemistry* **51**: 2506-2511.

Chaturvedi R, Razdan MK, Bhojwani SS (2004). *In vitro* morphogenesis in zygotic embryo cultures of neem (*Azadirachta indica* A. Juss.). *Plant Cell Reports* **22**: 801-809.

Chen C, Chang S, Cheng L, Hou R (1996). Deterrent effect of the chinaberry extract on oviposition of the diamondback moth, *Plutella xylostella* (L.) (Lep., *Yponomeutidae*). *Journal of Applied Entomology* **120**: 165-169.

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

Egertsdotter U, Von Arnold S (1997) Development of somatic embryos in Norway spruce. *Journal of Experimental Botany* **49**: 155-162.

Gonzalez AM, Cristóbal CL (1997). Anatomía y ontogenia de semillas de *Helicteres Lhatzkyana (Sterculiaceae)*. *Bonplandia* **9**: 287-294.

Hays DB, Mandel RM Pharis RP (2001). Hormones in zygotic and microspore embryos of *Brassica napus*. *Plant Growth Regulation* **35**: 47-58.

Johansen DA (1940). Plant microtechnique. New York: McGraw Hill Book Co.

Lazzeri PA, Hildebrand DF, Collins GB (1987). Soybean somatic embryogenesis: effects of hormones and culture manipulations. *Plant Cell, Tissue and Organ Culture* **10**: 197-208.

Luque R, Sousa HC, Kraus JE (1996). Métodos de coloração de Roeser (1972) e Kropp (1972) visando a subtituição do azul do astra por azul de alcião 8GS ou 8GX. *Acta Botanica Brasilica* **10**: 199-212.

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

Nathan SS, Savitha G, George DK, Narmadha A, Suganya L, Chung PG (2006) Efficacy of *Melia azedarach* L. extract on the malarial vector *Anopheles stephensi* Liston (Diptera: Culicidae). *Bioresource Technology* **97**: 1316-1323.

Niedz RP, Hyndman SE, Wynn ET, Bausher MG (2002). Normalizing sweet orange [*C. sinensis* (L.) Osbeck] somatic embryogenesis with semi-permeable membranes. *In Vitro Cellular and Developmental Biology - Plant* **38**: 552-557.

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

Pullman GS, Johnson S, Peter G, Cairney J, Xu N (2003). Improving loblolly pine somatic embryo maturation: comparison of somatic and zygotic embryo morphology, germination, and gene expression. *Plant Cell Reports* **21**: 747-758.

Rodriguez APM, Wetzstein HY (1994). The effect of auxin type and concentration on pecan (*Carya illinoinensis*) somatic embryo morphology and subsequent conversion into plants. *Plant Cell Reports* **13**: 607-611.

Rodriguez APM, Wetzstein HY (1998). A morphological and histological comparison of the initiation and development of pecan (*Carya illinoinensis*) somatic embryogenic cultures induced with naphthaleneacetic acid or 2,4-dichlorophenoxyacetic acid. *Protoplasma* **204**: 71-83.

Tisserat B, Esan EB, Murashige T (1979). Somatic embryogenesis in angiosperms. *Horticultural Reviews* **1**: 1-78.

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.

Vila S, Gonzalez A, Rey H, Mroginski L (2003). Somatic embryogenesis and plant regeneration from immature zygotic embryos of *Melia azedarach (*Meliaceae*)*. *In Vitro Cellular and Developmental Biology - Plant* **39**: 283-289.

Vila SK, Rey HY, Mroginski LA (2007). Factors affecting somatic embryogenesis induction and conversion in Paradise tree (*Melia azedarach* L.). *Journal of Plant Growth Regulation* **26**: 268-277.

Wetzstein HY, Baker CM (1993) The relationship between somatic embryo morphology and conversion in peanut (*Arachis hypogaea* L.). *Plant Science* **92**: 81- 89.

Zimmerman JL (1993). Somatic embryogenesis: a model for early development in higher plants. *The Plant Cell* **5**: 1411- 1423.