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# Factors Affecting Somatic Embryogenesis Induction and Conversion in "Paradise Tree" (*Melia azedarach* L.)

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**Abstract** Factors affecting somatic embryogenesis induction and conversion in paradise tree (*Melia azedarach*) were evaluated. Somatic embryogenesis was influenced by plant growth regulators, explant stage, carbohydrate source and concentration, gelling agents, light, and induction times. MS medium with 4.54 μM thidiazuron (TDZ) was optimal for the induction of embryogenic tissue. Zygotic embryos that were 1-1.5 mm long (torpedo and early cotyledonal stage) had a greater embryogenic response than smaller or larger embryos and better conversion of somatic embryos into plants. In general, embryos that formed in medium containing 1% or 5% carbohydrate were hyperhydrics or fused, respectively, whereas those that formed in medium with a carbohydrate concentration of 3% had better morphology. Raffinose at 3% yielded satisfactory somatic embryo induction with good morphology and the best values of conversion into plants. Induction and conversion of somatic embryos were superior on medium solidified with agar A-1296. The explants maintained under 160 μmol $m^{-2}$ $s^{-1}$ or 1 week in darkness and later 160 μmol $m^{-2}$ $s^{-1}$ produced a significantly higher embryogenic index. Only 4 days of treatment on induction medium, with either raffinose or sucrose at 3% as a carbohydrate source, were required to induce somatic embryogenesis, but longer exposure, until 18 days, increased the yield and improved the morphology of somatic embryos.

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**Keywords** Paradise tree · Somatic embryogenesis · Explants stage · Carbohydrate source

## Introduction

Paradise tree, *Melia azedarach* L., a member of the Meliaceae family, is native to southern Asia. It was introduced into the New World and spread throughout tropical America, from Mexico to Argentina (Pennington 1981). Paradise tree is a multipurpose timber tree from which products are extracted to make products such as insecticides and fungicides (Breuer and Schmidt 1995; Andreu and others 2000; Ursi Ventura and Ito 2000; Carpinella and others 2003). The extract of leaves has an antiviral factor that inhibits the replication of several viruses (Andrei and others 1986).

Although zygotic embryos present great variability, they can be used as a source of explants for somatic embryogenesis because this regeneration system is widely considered very efficient for conservation, cryoconservation, transformation, and transgenic plant recovery. When a breeding program is in place, embryogenesis can be carried out from genetically superior control-pollinated seed. Somatic embryogenesis is frequently regarded as the best system for propagation and can produce plants that perform as seed-derived plants (Etienne and Berthouly 2002; Stasolla and Yeung 2003), mostly because both root and shoot meristems are present simultaneously.

In a preliminary report (Vila and others 2003), we demonstrated that paradise tree could be regenerated via somatic embryogenesis by culture of immature zygotic embryos. In that report, however, a major limitation of the paradise tree embryogenic system was the malformed production of somatic embryos, which were often fused and had a relatively low rate of somatic embryo conversion.

In other woody plant species some factors have been shown to improve somatic embryo development. These include the effect of carbohydrate addition on the induction medium (Blanc and others 1999) and the role of the carbohydrate metabolism (Lipavská and Konrádová 2004); the addition of abscisic acid and maltose to the developmental medium (Reidiboym-Talleux and others 1999); the effect of the gelling agents, ammonium nitrate, and light on the development of somatic embryos (Tremblay and Tremblay 1991); and the addition of abscisic acid and silver nitrate to the induction medium (Pullman and others 2003).

In this study the objective was to improve the present paradise tree embryogenic system by studying the effect of several factors such as plant growth regulators, explant stage, carbohydrate source and concentration, gelling agents, light, and induction times on somatic embryo induction and conversion. In the experiments described below we have attempted to find the optimum combination of selected factors affecting paradise tree somatic embryogenesis that will substantially minimize the limitations mentioned.

## Materials and Methods

The season for collection, fruit sterilization, tissue culture procedure, and incubation conditions were as reported previously (Vila and others 2003).

### Effect of Plant Growth Regulators

For somatic embryo induction, explants (immature zygotic embryos) were placed on MS medium (Murashige and Skoog 1962) supplemented with different plant growth regulators (PGRs): thidiazuron (TDZ): 0.0004, 0.004, 0.04, 0.45, 4.54, 13.62, 27.24, and 40.86 μM; zeatin (ZEA): 0.04, 0.46, and 4.56 μM; 6-($\gamma$,$\gamma$-dimethylallylamino)-purine (2iP): 0.04, 0.49, and 4.92 μM; kinetin (KIN): 0.04, 0.46, and 4.65 μM; 6-benzyladenine (BA): 0.44, 4.44, and 44.4 μM; 2,4-dichlorophenoxyacetic acid (2,4-D): 0.04, 0.45, 4.52, 13.56, 27.12, and 40.68 μM; picloram (PIC): 4.14, 20.7, and 41.4 μM; $\alpha$-naphthaleneacetic acid (NAA): 0.05, 0.54, and 5.37 μM; indole-3-butyric acid (IBA): 0.04, 0.49, and 4.90 μM; indole-3-acetic acid (IAA): 0.05, 0.57, and 5.71 μM; and four combinations (2,4-D 0.04 μM with BA and TDZ 0.04 μM; PIC 20.7 μM with TDZ 4.54 μM and BA 4.44 μM).

When embryogenic tissue appeared, sections containing a group of 5-10 embryos (10-15 mg fresh weight) were cultured on one-quarter-strength mineral salts and vitamins of MS (¼ MS) medium (Vila and others 2003).The tubes were covered with Resinite AF 50® (Casco S.A.C., Buenos Aires) and were incubated in the same physical conditions as above.

### Effect of Developmental Stage of the Explants

The fruits were collected beginning after 9 weeks of fertilization, at different developmental stages, from the immature zygotic embryos in the globular stage to the cotyledonal stage, when the endocarp of the fruits turned hard. The fruits were collected at 3–4-day intervals during the maturation period of the embryos (2 weeks). Four developmental stages of embryos were cultured on embryo induction medium: stage **a**, late globular stage (0.3–0.5 mm long); stage **b**, heart stage (0.5–1 mm long); stage **c**, torpedo and early cotyledonal stage (1-1.5 mm long); and stage **d**, cotyledonal stage (1.5-2.5 mm long). Somatic embryos obtained were transferred in groups of 5-10 onto conversion medium (¼ MS).

### Effect of the Carbohydrate Source

The explants (zygotic embryos 1-1.5 mm long) were cultured on embryo induction medium (MS with 4.54 μM TDZ, without sucrose) supplemented with sucrose, fructose, galactose, maltose, glucose, raffinose, mannose, and lactose, all at 1%, 3%, and 5%. When embryogenic tissue appeared, groups of 5-10 somatic embryos (10-15 mg fresh weight) were transferred onto conversion medium (¼ MS) with the same carbohydrate source and concentration.

### Effect of Gelling Agents

Different gelling agents were tested for embryo induction and conversion of *Melia azedarach*. The induction medium (MS with 4.54 μM TDZ and raffinose or sucrose at 3%) was solidified with one of the following: agar A-1296 (0.7%), agar A-9799 (0.5%), agar A-7921 (0.7%), agar A-8678 (0.6%), agarose A-0169 (0.89%), agargel A-3301 (0.4%), or phytagel P-8169 (0.18%) (all from Sigma, St. Louis, MO). Liquid medium was evaluated. It was maintained on a gyrotary shaker at 80 rpm. When embryogenic tissue appeared, portions (10-15 mg each) were transferred onto the conversion medium (¼ MS) solidified with the same gelling agents. Somatic embryos formed in liquid medium were transferred to liquid conversion medium and were maintained on a gyrotary shaker at 80 rpm. In each case, 3% sucrose or 3% raffinose was added.

### Effect of Light Intensities

The embryogenic explants (zygotic embryos 1-1.5 mm long) were incubated on embryo induction medium (MS with 4.54 μM TDZ) in darkness or under a 14-h photoperiod

(provided by cool-white fluorescent Philips TLD 36W/840) providing light intensities of 5, 160, or 215 μmol $m^{-2}$ $s^{-1}$ at the tube level. The frequency of somatic embryos was recorded after a total of 6 weeks of culture.

### Effect of Induction Times

Immature zygotic embryos (torpedo and early cotyledonal stage), 1 mm long, were cultured on induction medium (MS with 4.54 μM TDZ, containing either 3% of sucrose or 3% of raffinose) for 1, 4, 7, 14, 18, or 30 days and later were transferred to ¼ MS supplemented with 3% of sucrose or raffinose.

### 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 repeated independently 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 Duncan comparison test at the 5% level of probability.

The embryogenic (EI), fused embryogenic (FEI), and individual embryogenic (IEI) indexes were defined as follows:

EI = (% explants with somatic embryos × number of somatic embryos/explant) ÷ 100

FEI = (% explants with somatic embryos × number of fused somatic emb/explant) ÷ 100

IEI = (% explants with somatic embryos × number of individual somatic emb/explant) ÷ 100

### Plant Acclimatization

The plantlets 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 at 25°C and 80% relative humidity for 20 days and finally transferred to plastic pots with soil and moved to the greenhouse.

## Results and Discussion

### Effect of Plant Growth Regulators

After a few days in culture, paradise tree zygotic embryos began to swell. At 5-15 days later in culture, with all the tested PGRs and depending on the doses, the first globular embryos were differentiated on the surface of the swollen embryos. In general, in those explants in which embryogenic tissue was differentiated, the formation of callus did not occur and the embryos appeared directly on the explants (Figure 1A). When the explants formed callus, somatic embryos never differentiated from them (Figure 1B). These calluses originated in the media containing the higher doses of 2,4-D and PIC (data not shown). The embryos obtained with each one of the tested PGRs showed great variability in morphology. For example, somatic embryos that originated in BA-containing medium were very similar to the zygotic embryos in their cotyledonal shape (Figure 1C), unlike those formed in media with 2,4-D (Figure 1A) or TDZ (Figure 1D), which showed no morphologic similarity with the zygotic embryos. Many embryos exhibited morphologic abnormalities with fused cotyledonary leaves (Figure 1E).

Although with all the tested PGRs the formation of embryogenic tissue was possible, depending on the doses, with lower concentrations of 2iP, NAA, IBA, and IAA and the highest concentrations of KIN, BA, 2,4-D, and PIC and without PGR (control), no induction of somatic embryos occurred (data not shown). On the other hand, the differentiation of somatic embryos was induced at all the concentrations of TDZ (0.0004-40.86), and the highest EI value (14.6) was obtained with a concentration of 4.54 μM. The requirement of TDZ for somatic embryogenesis in paradise tree was previously reported (Vila and others 2003). With 0.45 μM TDZ, 0.44 μM BA, 0.45 μM 2,4-D, and 0.54 μM NNA, high EI values were also obtained (range = 11.6-12.8); nevertheless, conversion rates declined with respect to those that originated with 4.54 μM TDZ. Similar results were obtained in *Azadirachta indica* when these PGRs were used to induce somatic embryogenesis from mature seeds (Murthy and Saxena 1998), but with a difference: buds formed when BA was added to the medium. The addition of ZEA, KIN, PIC, IBA, and IAA to the media, in all the tested concentrations, decreased the differentiation of somatic embryos (between 0 and 3.6, data not shown). However, some of these PGRs were excellent inducers of somatic embryogenesis in several woody plants (Biahoua and Bonneau 1999). In *Azadirachta indica* IAA was needed in the medium to differentiate somatic embryos from calluses originated from hypocotyls or immature cotyledons (Su and others 1997) and to induce secondary embryogenesis from immature zygotic embryos (Chaturvedi and others 2004). Although it is more common to find studies in which the combination of auxins (especially 2,4-D) and cytokinins allows a major production of somatic embryos, our results showed that when auxins or cytokinins were added to the medium alone, the yield of somatic embryos was superior or similar to that of when some of them were combined (Table 1). These results agree with others obtained from

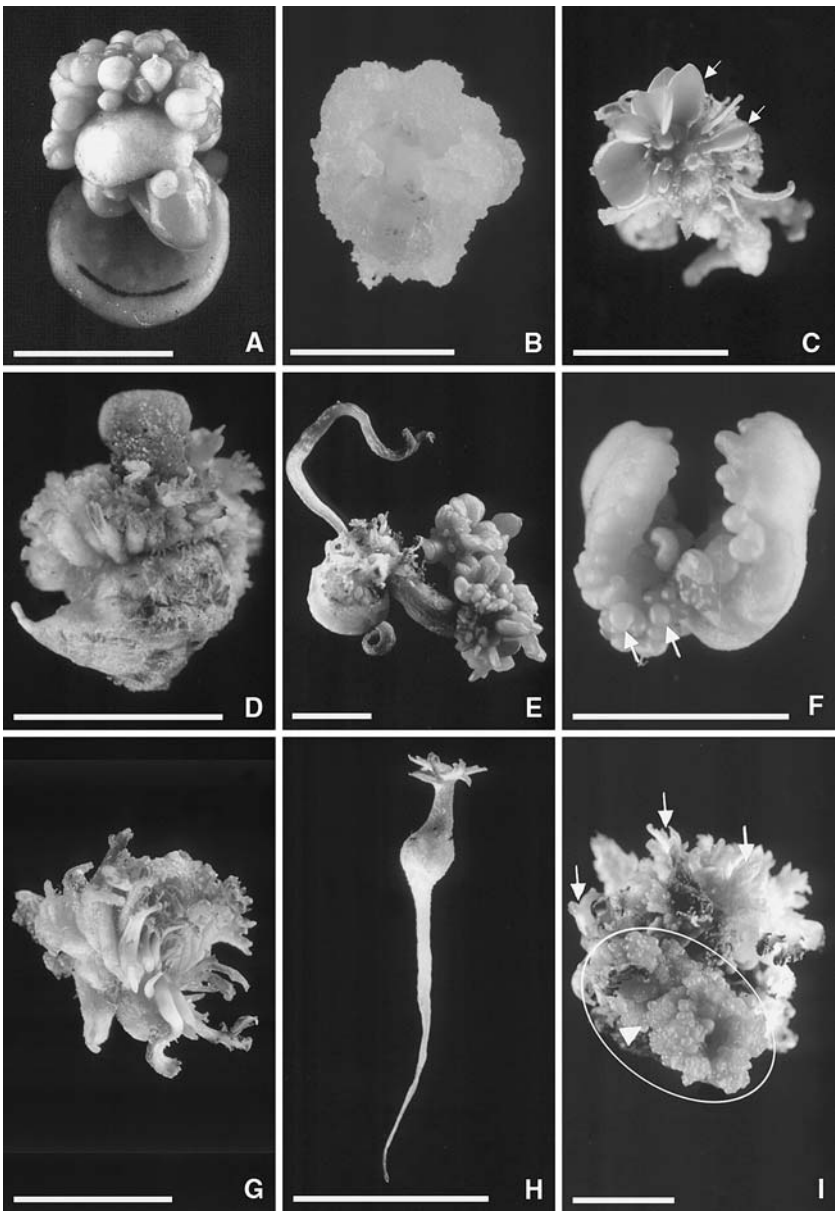


**Fig. 1** Morphogenic responses obtained from immature zygotic embryos influenced by several factors. **A** Somatic embryos obtained in MS medium supplemented with 0.45 μM 2,4-D. **B** Callus originated on MS + 27.12 μM 2,4-D. **C** Somatic embryos in cotyledonal stage (arrows) obtained on MS + 0.44 μM BA. **D** Somatic embryos induced on MS + 4.54 μM TDZ. **E** Abnormal somatic embryos originated on MS + 4.14 μM PIC. **F** Somatic embryos (arrows) obtained from immature zygotic embryos less than 1 mm in length (in late globular stage). **G** Somatic embryos obtained from immature somatic embryos 1 mm long (in torpedo and early cotyledonal stage). **H** Conversion of individual somatic embryo into plant, obtained from zygotic embryos 1 mm long. **I** Individual somatic embryos (arrows) and fused (circle and arrowhead showing globular embryo) formed from an explant 1.5 mm long. Scale bars = 4 mm

other woody plants such as *Prunus incisa* (Cheong and Pooler 2004), *Acacia sinuata* (Vengadesan and others 2002), *Myrtus communis* (Parra and Amo-Marco 1998), and *Eucalyptus globulus* (Pinto and others 2002). When embryogenic tissue sections containing groups of 5-10 somatic embryos were transferred to ¼ MS, their conversion rate was very low (<10%) (Table 1). It is probable that these low conversion values could be due to the great variability in the morphology of the regenerated somatic embryos, because many of them possessed abnormal structures.

## Developmental Stages of the Explants

In this experiment, after 20-30 days of culture, all the developmental stages of the zygotic embryos of paradise tree cultured *in vitro* differentiated somatic embryos. Nevertheless, the morphogenic responses varied considerably with the developmental stage of the zygotic embryos at the inception of the culture. Zygotic embryos in stage **a** (globular stage) differentiated only direct fused somatic embryos (Figure 1F), whereas explants in the more advanced stages regenerated individual somatic embryos, which were easily separated from the explant and included lengthened hypocotyls (Figure 1G). These embryos were the most appropriate for the regeneration of complete plants (Figure 1H). However, between 10% and 30% of stages **b**, **c**, and **d** differentiated both morphogenic responses in the same explant, in general, fused embryos at the base of the cotyledons, hypocotyl, and radicle, and individual embryos in the cotyledons of the zygotic

**Table 1** Effect of 11 induction media on EI and on percentage conversion of somatic embryos of *Melia azedarach* in ¼ MS after 4 weeks in culture

| PGR (in μM) | EI (mean ± SE) | Conversion (%) |
|---|---|---|
| None (control) | 0[a] | 0 |
| TDZ 0.0004 | 4.8 (± 1)[abc] | 8.6 (± 8.6) |
| TDZ 0.004 | 6.7 (± 3.2)[abcd] | 0 |
| TDZ 0.04 | 10.8 (± 4.6)[bcd] | 5.6 (± 2.7) |
| TDZ 0.45 | 12.1 (± 3.9)[cd] | 3.9 (± 2) |
| TDZ 4.54 | 14.6 (± 4.4)[d] | 4.4 (± 1.2) |
| TDZ 13.62 | 6.1[abc] | 5.9 (± 4.3) |
| TDZ 27.12 | 9.3 (± 1)[bcd] | 6.6 (± 6.6) |
| TDZ 40.86 | 3 (± 1)[ab] | 0 |
| 2.4-D 0. 04+TDZ 0. 04 | 10.7 (± 1)[bcd] | 3.4 (± 1.9) |
| PIC 20.7 + TDZ 4.54 | 2.9 (± 0.8)[ab] | 0 |

Different letters within columns indicate significant differences. Duncan's test ($P \leq 0.05$)

embryos (Figure 1I). In several woody species the suitable selection of the developmental stage of the zygotic embryos at the start of the culture is critical for the induction of somatic embryogenesis (Arya and others 2000; Tonon and others 2001; Mauri and Manzanera 2003; Igasaki and others 2003). Different morphogenic responses from different developmental stages of immature zygotic embryos were obtained in *Azadirachta indica* (Chaturvedi and others 2004). Nevertheless, these somatic embryos never germinated. The EI of the fused (FEI) and individual (IEI) somatic embryos are provided in Figure 2. The morphogenic response of stage **d** explants was inferior to that of **b** or **c** explants. Nevertheless, stage **c** (1-1.5 mm long, torpedo and early cotyledonal stage) was the most responsive for induction and development of somatic embryos because it produced the highest value of IEI (Figure 2A) and, consequently, the highest percentage of conversion into plantlets (Figure 2B). On the other hand, stage **a** of zygotic embryos gave the highest value of FEI but they never germinated (Figure 2B). These results agree with those obtained with other woody plants, where the very early stages of the zygotic embryos used as explants were inappropriate for the induction of somatic embryogenesis (Canhoto and others 1999; Kim and others 1999; Arya and others 2000; Mauri and Manzanera 2003).

## Effect of Carbohydrate Source

When eight carbohydrate sources were tested for their effect on embryogenesis, no somatic embryos developed on medium containing mannose. This suggests that the cells of the explant were incapable of metabolizing this sugar.

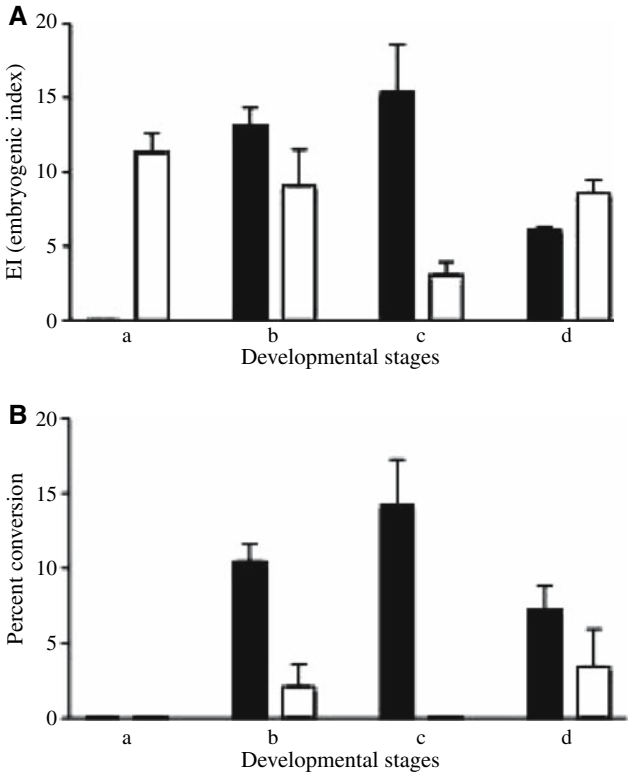


**Fig. 2** Effect of developmental stage of the zygotic embryos cultured *in vitro* on the induction of somatic embryos in MS + 4.54 μM TDZ (**A**) and on percentage conversion in ¼ MS (**B**). Individual embryos (■), fused embryos (□)

With all of the remaining carbohydrates, the explant formed somatic embryos regardless of the carbohydrate concentration in the medium (Table 2), although qualitative differences in the embryos were apparent. Embryos that were induced on medium with 1% carbohydrate were single, hyperhydric, and bigger than those induced on medium containing higher concentrations of carbohydrates (Figure 3A). Explants maintained on medium with 3% carbohydrate developed single or fused somatic embryos but no hyperhydric embryos (Figure 3B). Except for raffinose, with 5% carbohydrate on induction medium, the somatic embryos in general were fused (Figure 3C). These results were reflected in the embryogenic index values of individual (IEI) and fused embryos (FEI) which appear in Table 2. Qualitative variations in somatic embryos obtained with different concentrations of carbohydrates in the medium have been observed in *Prunus incisa* (Cheong and Pooler 2004).

The sum of the IEI and FEI showed that in general 3% was the most suitable concentration for the production of embryos independent of the carbohydrate source added to the medium (Table 2). These results are in agreement with those obtained in other woody plant species (Norgaard 1997; Reidiboym-Talleux and others 1999; Vengadesan

and others 2002). Although glucose and maltose offered the highest values of EI, the embryos were hyperhydric or fused and this feature was translated into a low percentage of conversion of embryos into plantlets (Table 2). In spite of that, glucose has not been mentioned in studies of woody species as a carbohydrate that improves the somatic embryogenesis when compared to sucrose; however, it behaved similarly to sucrose in *Ulmus glabra* (Corredoira and others 2003) and in *Prunus incisa* (Cheong and Pooler 2004). Maltose was used in several species, producing favorable results on embryogenesis. In alfalfa, for example, it stimulated the production of embryos and improved their morphology at low concentrations and was superior to sucrose, at equal osmolarity (Strickland and others 1987). In some woody plant species such as *Hevea brasiliensis*, only maltose gave a significantly higher somatic embryo yield than sucrose among the four sugars tested (Blanc and others 1999), and in *Prunus avium* the quality of the somatic embryos improved (Reidiboym-Talleux and others 1999), whereas in *Abies nordmanniana* (Norgaard 1997) it had a beneficial effect on maturation and regeneration of plants. Fructose, galactose, and lactose showed a low response in the induction of somatic embryos of paradise tree. Nevertheless, some of them were used successfully in the induction of embryos in several cultivars of *Citrus* (Tomaz and others 2001).

Although raffinose showed an intermediate EI in the induction of somatic embryos, similar to sucrose, this sugar produced embryos with better morphology (Figure 3D) when compared with those obtained in media with sucrose (Figure 3E). In addition, these embryos showed a major ability to turn into complete plantlets (Figure 3F, Table 2). In several species of *Picea*, the raffinose family oligosaccharides (RFOs) are substantially responsible for desiccation tolerance acquisition and/or increasing the conversion of the somatic embryos (Bomal and others 2002; Pond and others 2002; Lipavská and Konrádová 2004). In *Picea abies* the RFO are present in mature zygotic embryos (Gosslová and others 2001), as well as in somatic embryos submitted to desiccation or cold treatment (Konrádová and others 2003). This fact and the results obtained by Downie and Bewley (2000) in *P. glauca* suggest that the RFOs, which are rapidly degraded, are used as a source of energy during the initial process of germination (Lipavská and Konrádová 2004). The simplest method of adding raffinose into the maturation media proved to be unsuccessful for somatic embryos of *P. abies* and did not result in the accumulation of raffinose in the somatic embryos (Konrádová and others 2003). In contrast, in paradise tree the addition of raffinose to the medium produced beneficial effects on somatic embryo conversion. Nevertheless, more study is necessary to determine if the positive effect of this carbohydrate is via a direct pathway or by some product of its metabolism in the somatic embryos.

## Effect of Physical Conditions of the Culture Medium

In general, the physical state of the media had little effect on the induction of somatic embryogenesis of paradise tree. With all the gelling agents tested and in liquid medium, it was possible to obtain somatic embryos (Table 3). However, when agar A-1296 was used, high EI values were obtained with sucrose and raffinose as carbohydrate sources. The EI values also were high with agar A-8678 and agarose in media containing raffinose (Table 3), whereas zygotic embryos cultured on liquid medium showed the lowest EI values.

These results agree with those obtained in *Picea mariana* where agar led to a better production of embryos than other gelling agents, and liquid media proved inappropriate for somatic embryogenesis induction (Tremblay and Tremblay 1991). However, in some other woody plants liquid medium presented advantages over the semisolid medium in the process of embryogenesis (Etienne and Berthouly 2003; Mauri and Manzanera 2003).

In the early 1980s, the information that existed at the time suggested that, in general, the physical conditions of the culture medium had a small effect on somatic embryogenesis (Ammirato 1983). Nevertheless, investigations in several species such as *Picea mariana*, *P. rubens* (Tremblay and Tremblay 1991), *Cucumis sativus* (Ladyman and Girard 1992), and *Pinus strobus* (Klimaszewska and others 2000) have demonstrated that the physical conditions or some inhibitor present in the gelling agents can have a marked effect on somatic embryogenesis.

Nearly 10% of the somatic embryos converted into plantlets in the media with either sucrose or raffinose when they were maintained with agar A-1296. Some embryos also germinated in media gelled with agarose with both carbohydrate sources. With agar A-8678 and with phytagel, conversion was obtained only in media with raffinose as a carbohydrate source. In the remaining tested media there were no conversion rates (Table 3).

## Effect of Light Conditions

Although in paradise tree somatic embryogenesis was induced under all the light intensities studied, the explants maintained under 160 μmol $m^{-2}$ $s^{-1}$ or 1 week in darkness and later under 160 μmol $m^{-2}$ $s^{-1}$ produced a significantly higher EI (Table 4). These results agree with those obtained in other woody plants where it has been demonstrated that the induction or maturation of the somatic

**Table 2** Effect of three concentrations of eight carbohydrate sources on the induction of somatic embryos of *Melia azedarach* in MS + 4.54 μM TDZ and on the conversion to plants in ¼ MS

| Carbohydrate source | (%) | IE (± SE) | | | Conversion (± SE) |
|---|---|---|---|---|---|
| | | IEI | FEI | IEI + FEI | |
| Sucrose | 1 | 6.8 ± 0.8 | 0 | 6.8 ± 0.8$^{bcde}$ | 0$^{a}$ |
| | 3 | 1.6 ± 0.2 | 8.6 ± 1.5 | 10.1 ± 1.3$^{def}$ | 0.3 ± 0.3$^{a}$ |
| | 5 | 0.2 ± 0.2 | 4.3 ± 0.7 | 4.6 ± 0.8$^{abcd}$ | 0.8 ± 0.8$^{a}$ |
| Glucose | 1 | 16.5 ± 3.2 | 0 | 16.5 ± 3.3$^{gh}$ | 0$^{a}$ |
| | 3 | 0 | 23.0 ± 3.4 | 23.0 ± 3.4$^{i}$ | 0.3 ± 0.3$^{a}$ |
| | 5 | 0 | 9.2 ± 1.0 | 9.2 ± 1.0$^{cdef}$ | 0$^{a}$ |
| Raffinose | 1 | 0.9 ± 0.7 | 0 | 0.9 ± 0.7$^{ab}$ | 0$^{a}$ |
| | 3 | 12.3 ± 1.1 | 0.8 ± 0.8 | 13.1 ± 0.4$^{fgh}$ | 8.7 ± 2.9$^{c}$ |
| | 5 | 10.8 ± 2.2 | 2.4 ± 1.3 | 13.2 ± 3.3$^{fgh}$ | 7.2 ± 3.7$^{bc}$ |
| Fructose | 1 | 8.4 ± 1.6 | 0.3 ± 0.2 | 8.7 ± 1.5$^{cdef}$ | 1.7 ± 1.7$^{a}$ |
| | 3 | 4.4 ± 2.0 | 7.2 ± 1.1 | 11.6 ± 1.8$^{efg}$ | 0.8 ± 0.8$^{a}$ |
| | 5 | 0.3 ± 0.3 | 3.7 ± 1.3 | 4.0 ± 1.1$^{abcd}$ | 0$^{a}$ |
| Galactose | 1 | 10.7 ± 1.4 | 0.2 ± 0.2 | 10.9 ± 1.3$^{efg}$ | 0$^{a}$ |
| | 3 | 6.3 ± 3.3 | 2.8 ± 1.8 | 9.1 ± 3.0$^{cdef}$ | 0$^{a}$ |
| | 5 | 1.5 ± 0.4 | 0.7 ± 0.2 | 2.2 ± 0.2$^{ab}$ | 0.4 ± 0.4$^{a}$ |
| Maltose | 1 | 8.5 ± 2.4 | 0.5 ± 0.5 | 9.0 ± 2.9$^{cdef}$ | 3.3 ± 3.3$^{ab}$ |
| | 3 | 2.9 ± 0.5 | 16.0 ± 3.0 | 19.0 ± 3.3$^{hi}$ | 0.02 ± 0.02$^{a}$ |
| | 5 | 3.0 ± 1.0 | 11.9 ± 3.9 | 15.0 ± 3.0$^{fgh}$ | 5.3 ± 3.7$^{abc}$ |
| Mannose | 1 | 0 | 0 | 0$^{a}$ | 0$^{a}$ |
| | 3 | 0 | 0 | 0$^{a}$ | 0$^{a}$ |
| | 5 | 0 | 0 | 0$^{a}$ | 0$^{a}$ |
| Lactose | 1 | 3.6 ± 0.4 | 0 | 3.6 ± 0.4$^{abc}$ | 0$^{a}$ |
| | 3 | 5.2 ± 0.7 | 1.0 ± 1.0 | 6.3 ± 1.2$^{bcde}$ | 7.5 ± 3.8$^{bc}$ |
| | 5 | 2.8 ± 0.6 | 0.5 ± 0.5 | 3.3 ± 1.0$^{abc}$ | 0$^{a}$ |

Different letters within columns indicate significant differences. Duncan's test ($P \leq 0.05$)

embryos can be influenced (Tremblay and Tremblay 1991; Cheong and Pooler 2004) by different light intensities. When the somatic embryos were induced in darkness, after 4 weeks of culture only fused embryos survived and the individual embryos became brown and died (Figure 2G). In this study the highest percentage of conversion was obtained under 160 and 215 μmol $m^{-2}$ $s^{-1}$ (Table 4).

## Induction Time

For induction of somatic embryogenesis in paradise tree, the minimal time that explants must be maintained on the induction media was 4-7 days when sucrose was used as a carbohydrate source. After this period the EI remained stable or decreased slightly but not significantly at the time (Figure 4). When raffinose was added to the medium instead of sucrose, the minimal induction time to obtain an EI similar to sucrose was 18 days (Figure 4). The somatic embryos obtained from explants kept for 2 or 4 days on induction medium produced a morphology different from those that were kept for a longer time; they possessed bigger cotyledons and were slightly succulent with reduced hypocotyls (Figure 3H).

For several embryogenic systems an optimal period of induction has already been defined. A period of 3-7 days was the ideal time for induction of embryos of *Arachis hypogaea* from leaf explants (Baker and Wetzstein 1998), whereas in *Medicago sativa* the largest number of somatic embryos was observed with 10 days of incubation (Denchev and others 1991), and in *Feijoa sellowiana* more than 14 days of exposure of the explants to an auxin was necessary for a positive response (Cruz and others 1990).

## Plant Regeneration

Well-developed paradise tree plantlets (1.5-2.5 cm high) with more than two fully expanded true leaves and with a healthy root system measuring more than 1.5 cm long survived 90% of the time when they were transferred to potting soil in a greenhouse. The somatic embryo-derived plantlets appeared morphologically similar to seed-derived plants.

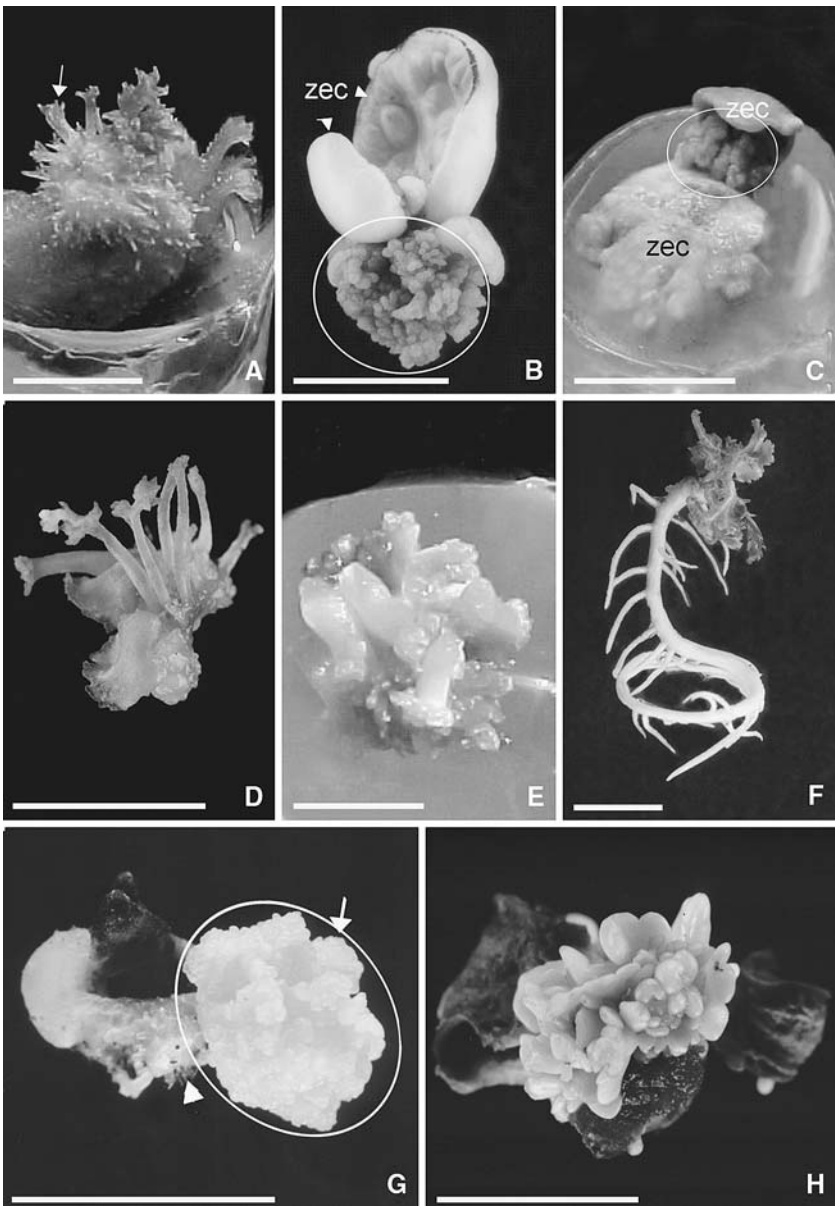


**Fig. 3** Somatic embryos of paradise tree obtained from immature zygotic embryos influenced by several factors. **A** Individual somatic embryo hyperhydrics (arrow) obtained on induction medium with 1% glucose as a carbohydrate source. **B** Fused somatic embryos (circle) obtained on induction medium with 3% glucose as a carbohydrate source. **C** Fused somatic embryos, of corny appearance (circle), obtained on induction medium with 5% glucose as a carbohydrate source. **D** Individual somatic embryos obtained on induction medium with 3% raffinose. **E** Somatic embryos obtained on induction medium with 3% sucrose as a carbohydrate source. **F** Plant obtained in ¼ MS with 3% raffinose. **G** Fused somatic embryos (circle and arrow) and dead individuals (arrowhead) induced and maintained under complete darkness. **H** Somatic embryos obtained from zygotic embryos kept by 4 days on induction medium and then transferred to ¼ MS with 3% sucrose. Scale bars = 5 mm. Zec = zygotic embryo cotyledons

**Table 3** Effect of seven gelling agents and liquid medium on the induction of somatic embryos of *Melia azedarach* in MS + 4.54 μM TDZ and on the conversion to plants on ¼ MS (with 3% sucrose or raffinose)

| Gelling agents | EI (± SE) | | Conversion (% ± SE) | |
|---|---|---|---|---|
| | Sucrose | Raffinose | Sucrose | Raffinose |
| Agar A-1296 (0.7%) | 10.8 ± 1.9 | 10.4 ± 1.5 | 9.8 ± 5.3 | 9.3 ± 4.8 |
| Agar A- 9799 (0.5%) | 7.8 ± 1.8 | 5.8 ± 1.6 | 0 | 0 |
| Agar A- 7921 (0.7%) | 7.7 ± 2.3 | 4.6 ± 2.5 | 0 | 0 |
| Agar A- 8678 (0.6%) | 7.9 ± 2.9 | 11.5 ± 3.2 | 0 | 5.1 ± 3.8 |
| Agarose (0.89%) | 6.2 ± 0.9 | 13.3 ± 3.9 | 1.5 ± 0.8 | 8.7 ± 1.3 |
| Agargel (0.4%) | 9.0 ± 1.8 | 3.6 ± 0.6 | 0 | 0 |
| Phytagel (0.18%) | 5.0 ± 2.0 | 6.5 ± 2.7 | 0 | 3.5 ± 3.5 |
| Liquid | 4.5 ± 0.7 | 3.1 ± 0.8 | 0 | 0 |

**Table 4** Effect of light intensities on the induction of somatic embryos of *Melia azedarach* on MS + 4.54 μM TDZ and on the conversion to plants on ¼ MS (with 3% sucrose)

| Light intensities (μmol $m^{-2}$ $s^{-1}$) | EI (± SE) | Conversion (± SE) |
|---|---|---|
| 0 | 10.4 ± 1.6[a] | 3.9 ± 2.1 |
| 0 for 7 days then at 160 | 17.3 ± 2.0[b] | 2.1 ± 0.5 |
| 5 | 5.6 ± 1.6[a] | 0.2 ± 0.2 |
| 160 | 21.0 ± 3.0[b] | 5.5 ± 2.7 |
| 215 | 8.6 ± 1.6[a] | 7.7 ± 3.3 |

Different letters within columns indicate significant differences: Duncan's test ($P \leq 0.05$)

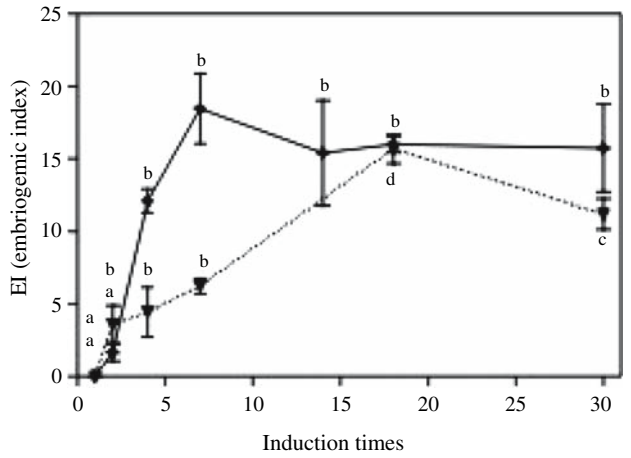


**Fig. 4** Effect of the induction times on MS + 4.54 μM TDZ on the induction of somatic embryos in media with 3% sucrose ( — ) or 3% raffinose (- - -). *Different letters in the lines indicate significant differences: Duncan's test ($P \leq 0.05$)

In conclusion, based on the results presented here we can recommend the most effective combination of individual factors studied as follows:

1. Induction of somatic embryos by *in vitro* culture of immature zygotic embryos in stage **c** (1-1.5 mm long, torpedo and early cotyledonal stage) on MS medium + 4.54 μM TDZ with either sucrose or raffinose at 3%, in medium solidified with 0.7% of agar A-1296, during 18 days of induction and kept under light conditions (160 μmol $m^{-2}$ $s^{-1}$).
2. Conversion of somatic embryos into plants on ¼ MS with 3% raffinose or sucrose, maintained under the same physical conditions of the medium and incubation mentioned above, and subsequently, transfer of the plantlets to plastic pots.

The work presented here shows that it is possible to modify somatic embryo induction and development through manipulation of culture medium factors and incubation conditions. However, more studies are needed with the objective of reducing the morphological variability of the somatic embryos of *Melia azedarach*.

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