Effective Organogenesis, Somatic Embryogenesis and Salt Tolerance Induction *In Vitro* in the Persian Lilac Tree (*Melia azedarach* L.)

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ABSTRACT

An effective tissue culture system to regenerate *Melia azedarach* (Meliaceae), an important multipurpose – including ornamental value – tree, was established. The optimized protocol resulted in plant formation from cotyledon explants via organogenesis and somatic embryogenesis. Embryogenic callus induction occurred on full strength (salts and vitamins) MS medium containing 3 mg/L 1-naphthaleneacetic acid (NAA) and 1 mg/L 6-benzyladenine (BA) with 3% sucrose with or without 5 mg/L giberrellic acid (GA_3). Somatic embryos – induced at 447/g fresh weight of callus – resulted in 100% conversion of somatic embryos to plants, all of which survived in the greenhouse. Organogenic callus was induced on full Murashige and Skoog (MS) medium supplemented with 0.5 mg/L NAA, 1 mg/L BAP, 1 g/L casein hydrolysate (CH) and 5% sucrose. Proliferation and elongation of adventitious shoots – derived from both embryogenic and organogenic callus – was achieved on full MS medium to which 1 mg/L BAP, 200 mg/L CH, 40 mg/L adenine, 80.5 mg/L putrescine and 2% sucrose, or 0.2 mg/l BAP, 0.01 mg/L GA_3, 161.1 mg/L putrescine and 3% sucrose were added. The addition of putrescine to the culture media had a positive effect on shoot proliferation. About 160 shoots/g (fresh weight) callus were produced. Rooted shoots were acclimatized and successfully transferred to soil with 100% survival and whole plants were obtained in ~4 months. Regenerated plants were phenotypically normal. In addition, we developed methods for the *in vitro* selection for salt tolerance. Organogenic callus cultures were screened for salt tolerance using direct selection methods and growth responses were examined. Despite the presence of salt in culture medium resulting in the loss of regeneration potential in organogenic callus, plantlets were obtained from culture media with 42.7 mm and 85.57 mm NaCl.

1. INTRODUCTION

Melia azedarach L. (Meliaceae), "chinaberry", or "persian lilac" is an Asiatic multipurpose tree, of worldwide cultivation, mainly for its ornamental beauty and landscape value. It is also important in timber production. Great interest has been focused on this species because of its insecticidal properties and the occurrence of several limonoid compounds (Itokawa *et al*. 1995, Huang *et al*. 1996, D'Ambrosio and Guerriero 2002, Zhou *et al*. 2004).

1.1. Tissue culture and micropropagation

Raising plants from seeds is a conventional method of propagation, but mass propagation of chinaberry by seed on a commercial scale has limitations (Thakur *et al*. 1998). Conventional vegetative propagation have had limited results (Domecq 1988, Sharry and Abedini 2001). Therefore, micropropagation offers an important alternative of multiplication, as a reliable method for mass production of plants in a shorter time without seasonal constraints. However, the success of micropropagation systems is effectively measured by the percent of plantlets that are successfully transferred from tissue culture vessels to the greenhouse or field conditions and by their reproducibility. Despite the importance of chinaberry, relatively few reports have been published describing the tissue culture of this plant. At the moment, the culture of node segments from adult plants for the production of axillary shoots (Domecq 1988), production of clones from tissue cultures of seedlings (Ahmed *et al*. 1990, Sugatha and Chandra 1997), production of shoots and plants, and calli, from seedling shoot tips and nodes (Thakur *et al*. 1998), shoot

Abbreviations: **2,4-D**, 2,4-diclorophenoxyacetic acid; Ade, adenine; CH, casein hydrolysate; BAP, N^6-benzylaminopurine (= 6-benzyladenine, or BA); GA_3, giberrellic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; KIN, 6-furfuryl aminopurine; MS, Murashige and Skoog's medium; NAA, -naphthaleneacetic acid; NaOCl, sodium hypochloride; PGR, Plant growth regulator; Put, putrescine

organogenesis from leaf explants (Vila *et al*. 2003), cryopreservation process (Bernard *et al* 2002, Scocchi *et al*. 2004), *neo*-formation of flower buds in hypocotyl segments (Sato and Esquibel 1995) and the occurrence of precocious flowering in tissue cultures (Handro and Floh 2001) have been achieved. However, the efficiency of those protocols for regeneration of *M. azedarach* were limited and unreproducible. On the other hand, in the related species *Azidarachta indica* (Indian lilac, neem), there are many studies on tissue and cell culture (Allan *et al*. 1999). Such studies would be of great interest in *M. azedarach* for micropropagation, induction of somaclonal variability and production of secondary compounds *in vitro* (Handro and Floh 2001).

1.2. *In vitro* selection for salt tolerance

Every year more land loses productivity due to the accumulation of salts. Approximately 25% of cultivated land around the world have excess salinity, mainly due to NaCl (Shannon *et al*. 1999). Attempts to enhance tolerance have involved conventional breeding programmes, the use of *in vitro* selection, pooling physiological traits, interspecific hybridization, using halophytes as alternative crops, the use of marker-aided selection, and the use of transgenic plants (Chandler and Thorpe 1986, Flowers 2004). *In vitro* culture constitutes a valid system for the study of the response of the plant to conditions of salinity and for the isolation and the selection of tolerant lines (Vijayan *et al*. 2003). The application of this methodology can be an alternative way for classic genetic improvement, allowing a precocious and quick screening of germplasm. *In vitro* selection on NaCl-containing media seems a promising approach for selecting cell lines which tolerate salt in their nutritional environment. Despite the possibly complex nature of salt tolerance expressed by plant cells, many examples are reported in the literature on the *in vitro* isolation of salt tolerant cell lines with the eventual recovery of regenerates displaying acquired traits of tolerance at the whole plant level (Croughan *et al*. 1981, Winicov 1996). Therefore, studies on *in vitro* screening for obtaining NaCl tolerant organogenic lines might help in elucidating the genetic and physiological aspects of tolerance, and possibly the recovery of the acquired trait in the regenerates. The explant type (callus or cell suspension culture), the type of salt (only NaCl, CaCl or a combination of salts) and their concentration, as well as the applied selection strategy can vary considerably. The selective agent (salt) has been previously applied in many cases to the induction of callus or the initiation of the suspension culture. In some cases the selection system includes several explants of which callus, embryos or plantlets were induced to form directly in saline. The selection strategies could be: 1) step-wise or short-term; 2) in several steps or for long periods, and 3) indirect selection.

Most forest species are particularly sensitive to a low concentration of sodium. Compared with agricultural crops, response of woody species to salt exposure in tissue culture has received less attention. The potential of physiological responses to differentiate between salt sensitive and salt tolerant species or individuals is now being examined (Woodward and Bennett 2005). Research in woody perennials include poplars (Li *et al*. 1984, Watanabe *et al*. 2000), *Prunus cerasifera* (Lucchesini and Vitagliano 1993), pear and quince (Marino and Molendini 2005) and Eucalyptus (Morabito *et al*. 1994, Chen *et al*. 1998). Shoot cultures of salt-tolerant *Eucalyptus microcorys* were able to withstand higher levels of salinity than salt-sensitive shoots, the salt tolerant shoots withstanding up to 150 mM NaCl (Chen *et al*. 1998, Keiper *et al*. 1998). Morabito *et al*. (1994) examined the response of *E. microtheca* clones to salinity in tissue culture and found that survival and physiological responses were mixed, with one clone showing increased survival, while others showed less change in water potential. Of considerable use for the development of salt tolerance in *M. azedarach* would be the availability of a technique that could identify salt tolerance within a species. An examination of the physiological responses shown within a species by individual clones may provide the capacity to do this without the need to conduct extensive glasshouse and field trials (Niknam and McComb 2000). Looking at such a response *in vitro* may also assist in providing information about the salt tolerance mechanisms, leading to further capacity to increase salt tolerance.

Therefore, the aim of the first part of this work was to develop an efficient and reproducible protocol for *in vitro* propagation via somatic embryogenesis and organogenesis of *M. azedarach* in order to obtain vigorous acclimatized plants that might be used for commercial and urban silviculture.

We also report here, in the second part of this chapter, the responses of *M. azedarach*, selected for varying degrees of salt tolerance, after exposure of *in vitro* organogenic callus culture to salt. In this work, we examined the possibility of obtaining chinaberry plants tolerant to NaCl through the *in vitro* selection of callus, and their subsequent regeneration.

2. MATERIALS AND METHODS

2.1. Plant material for callus initiation

Mature fruits of *Melia azedarach* were collected from 20-30 year-old trees with desirable forestry characteristics (fast growing, straight stem, healthy) in Irapuato, Guanajuato, and México. The seeds were excised by removing fruit coats mechanically and they were used as the explant source. Seeds were surface-sterilized in 70% ethanol for 3 min, then in a 20% (v/v) Clorox (6% NaOCl) solution with Tween 20 (4 drops/100 ml) for 30 min, and rinsed three times in sterile distilled water. The seeds were germinated *in vitro* and cotyledons (10/Petri dish) were excised and put directly on induction medium (20 ml) for callogenesis or somatic embryogenesis. Each treatment consisted of 35 replicates and each experiment was repeated three times.

2.2. Culture media and growth conditions

Murashige and Skoog (1962) vitamins, macro and micronutrients (MS) at full or half concentration gelled with 2.5 g/L Gelrite™ or 7.0 g/L Phytoagar (Gibco BRL) was used with sucrose (10-40 g/L) or glucose (60 g/L) as the carbon source. A broad range of PGRs and other additives (casein hydrolysate (CH), putrescine (Put)) were tested. Cotyledon dedifferentiation was induced on two different media: a) *Embryogenic callus induction medium* (ECIM) consisted of full strength (salts and vitamins) MS medium containing 3 mg/L -naphthaleneacetic acid (NAA) and 1 mg/L 6-benzyladenine (BA) with 3% sucrose with or without 5 mg/L GA_3. b) *Organogenic callus induction medium* (OCIM) consists of full strength MS medium with 0.5 mg/L NAA and 1 mg/L BAP supplemented with 1 g/L CH and 5% sucrose.

The pH of all media was adjusted to 5.8-6.0 before autoclaving and were sterilized by autoclaving for 17 min at 121ºC at 15 lbs. Cultures were incubated under a 16 h photoperiod (50 µm.m^2.sec, provided by cool-white day-light Sylvania fluorescent lamps, USA) at 27°C. Plantlets

were grown under continuous cool, white light fluorescent tubes (General Electric) at 25ºC in a plant tissue culture chamber (Model CU-36L, Percival Scientific) before their transplantation to the greenhouse.

2.3. Somatic embryo induction, plant regeneration and acclimatization

Freshly-formed embryogenic callus (20-30 days) from cotyledons was subcultured on *Embryogenesis Subculture and Development Medium* (ESDM). This medium consists of full strength MS salts and vitamins containing 10 mg/L BAP, 6% glucose, 40 mg/L adenine (Ade) and 161.1 mg/L Put. Mature somatic embryos were subcultured to obtain synchronous and normal germination on one of three different media: 1) MS salt-and-vitamin-free hormone medium (MS); 2) *Embryo Germination Medium* (EGM), containing full strength MS salts and vitamins with 0.2 mg/l BA, 0.01 mg/L GA_3, 3% sucrose and 161.1 mg/L Put; 3) *Secondary Embryogenesis Medium* (SEM) that consisted of MS salts and vitamins at full strength, 1 mg/L BAP, 200 mg/L CH, 2% sucrose, 161.1 mg/L Put and 40 mg/L Ade. Plants were subcultured into 500 ml vessels with half strength hormone-free MS medium supplemented with 1% sucrose, to promote their elongation. After approximately 10 weeks, plants were directly acclimatized by putting into pots filled with Peat Most (Sunshine, USA). The plants were covered with nylon and sprayed once with fungicide solution (Benlate, 10 mg/L). Plants were grown in a Plant Tissue Culture Chamber Model CU-36L at 25ºC and continuous light provided by cold-white GE fluorescent lamps. After two weeks, they were transferred to pots with soil under greenhouse conditions, and 1 month later were transplanted to the field.

2.4. Organogenic callus induction and shoots proliferation

The freshly formed organogenic callus from cotyledons was subcultured after 10-15 days on 5 different full strength (salts and vitamins) MS shoot proliferation and elongation media. The first medium (MY) had 1 mg/L BAP, 200 mg/L CH, 40 mg/L Ade, 80.5 mg/L Put and 2% sucrose. The second (MG) was supplemented with BAP 0.2 mg/l, 0.01 mg/L GA_3, 3% sucrose and 161.1 mg/L Put. All other media were supplemented with: 10 mg/L BAP and Ade (IEM); 10 mg/L BAP and 0.1 mg/L KIN (INF) or 2mg/L KIN (M7). Callus fresh weight was measured under sterile conditions on an analytic balance (Sciencetech).

2.5. Rooting and gardening

Elongated shoots – 7-8 cm in length with 4 or 5 compound leaves – from calli were transferred to seven different half strength MS rooting media (MR). They were supplemented with NAA, IAA, or IBA (0.01-1 mg/L). Activated charcoal, fluoroglucinol, Put and Ade were probed. Rooted shoots were subcultured on half strength hormone-free MS medium with 1% sucrose for 15-30 days in order to improve plant growth. After this, plantlets were washed throughly in running tap water to remove traces of gelled agent and other constituents of the culture medium to prevent fungal and/or bacterial contamination during weaning. Then they were transferred to pots containing Peat Most (Sunshine, sphagnum) and were kept under culture chamber conditions covered with nylon for acclimatization. During this process, plants were sprayed once with fungicide (1 g/L Benlate) and then with sterilized water. Once acclimatized the whole plants were transplanted to pots under greenhouse conditions and after 1 month, were transplanted to the field.

2.6. *In vitro* selection of salt tolerance

In vitro selection of salt tolerant plants of *Melia azedarach* L. (Persian lilac) has been accomplished by screening highly morphogenic cotyledon explants cultured on high NaCl media. For this work the selection strategy was a short time-frame by using direct selection methods. Calli were exposed (several times) to different concentrations of salt; callus lines that grew similarly to the control were transferred to regeneration medium. Organogenic calluses were induced from cotyledons in S10 solid medium consisting of MS + 3 mg/l NAA + 1 mg/l BAP + 30 g/l sucrose + 40 mg/l Ade + 8 g/l agar-agar), pH 6. Media were prepared with different concentrations of NaCl (0, 42.7, 85.57, 128.35, or 171.1 mM. Three successive subcultures (3 weeks each) were performed at each NaCl concentration.

Petri dishes with the explants were arranged in a totally randomized design with a complete factorial distribution (5 x 1) for 5 treatments and 10 repetitions giving a total of 50 experimental units. To determine the growth from the exposed cotyledons to the different concentrations of NaCl, the difference in fresh weight between initial and 30, 60 and 90 days was measured. The treatments in which organogenic callus survived were subcultured in Petri dishes with medium for bud proliferation (MS + 10 mg/l BAP + 40 mg/l Ade + 161.1 mg/l Put + 60 g/l glucose + 8 g/l agar-agar), pH 6 with the addition of NaCl (42.7 or 85.57 mM) since too high a concentration resulted in necrosis of the explants. The control treatment was callus cultivated on medium without NaCl. After a period of 30 days the calli were subcultured onto medium for bud elongation (MS + 1.5 mg/l BAP + 30 g/l sucrose + 8 g/l agar-agar), pH 6, without NaCl. The buds were put on rooting medium (MS/2 + 0.01 mg/l NAA + 30 g/l sucrose + 8 g/l agar-agar). Petri dishes with callus were arranged in a totally randomized block design with a factorial distribution (3 x 1) for a total of 3 treatments and 10 repetitions giving a total of 30 experimental units. Student's t-test was applied to determine significant differences between the averages of the different salt concentrations. The cultures were maintained at 25±2°C under a 16/8 h (day/night) photoperiod with light supplied at an intensity of 45 mol $m^{-2}s^{-1}$. Data on survival, aspect, organogenesis and fresh weight were recorded after each week of culture. The electric potential of the different culture media was expressed in mmhos/cm, while the concentration of ion interchangeable sodium was determined analytically (Table 1). Regenerated and control plants were transferred to greenhouse conditions.

Table 1 Electical conductivity of MS medium under different salt concentrations. Culture conditions: 25°C; the normal values of a soil according to the USDA is in the order of 2-4 mmhos. Control: electric conductivity = 3.65 mmhos.

Salt concentration (mM NaCl)	Electric conductivity
42.7	6,37 mmhos
85.57	9.1 mmhos
128.35	11.8 mmhos
171.1	14.53 mmhos
Control	3.65 mmhos

3. RESULTS AND DISCUSION

3.1. Somatic embryogenesis

3.1.1. Induction and propagation of embryogenic calli

Embryogenic calli were induced on ECIM. Tissues started to de-differentiate at 7 culture days. After 20 days in culture, embryogenic calli had formed from cotyledons in 93% of the explants on ECIM medium. Somatic embryos developed over the surface of embryogenic callus and occasionally, directly from cotyledon explants without an intervening callus phase. In the first developmental pathway, embryogenic callus was heterogeneous, light brown to green in color, friable and with a high number of somatic embryos easily separable on its surface (Figs. 1A, 1B). These were carefully removed and subcultured to SEM medium for somatic embryo development. In a second developmental pathway, the cotyledon explants produced tiny tube-like protrusions, or embryoids (Fig. 1C), which, at a later stage were recognizable as bipolar structures that developed into mature somatic embryos when they were sub-cultured onto PGR-free MS medium. The two pathways taken together demonstrate a high efficiency of embryogenic calli formation per initial explant. The second pathway of indirect somatic embryogenesis produced mature somatic embryos at a high frequency – an average of 447 embryos/g of callus fresh weight – and was thus the selected pathway for the rest of the experiments. All the different stages of somatic embryogenesis – globular, heart, torpedo, mature somatic embryos – were easily observed after 15-20 days on SEM (**Fig. 1D**). In general, somatic embryogenesis consists of callus formation on a medium containing auxins and then subculture to hormone-free medium (Reinert 1967), but the presence of an auxin is usually required in the medium in order to maintain the growth of subcultures (Halperin 1995). Moreover, the type and concentration of auxin employed are critical for the induction and formation of somatic embryos. The auxin, 2,4-D has been the most efficient PGR for the induction of somatic embryogenesis (Merkle 1995) even though in some cases, this auxin was not necessary in the development of somatic embryos (Smith and Krikorian 1990). In our work, it is clear that although 2,4-D has a positive effect on callus formation, it has no effect on the induction of morphogenic callus (data not show, preliminary experiments). It is possible that somatic embryogenesis in *Melia* is an example of regeneration from pre-embryogenically determined cells (Evans *et al*. 1981), and does not depend on a 2,4-D requirement (Litz *et al*. 1987). On the other hand, we found that cytokinin concentration is very important for somatic embryo development in this species. Induction of calli occurred with either NAA or BA (i.e. ECIM) and a high concentration of BA (10 mg/L) plus Ade was important for somatic embryo differentiation, i.e. ESM medium. This inductive effect of cytokinins in somatic embryogenesis was also reported by Kavathekar *et al*. (1978), Jha *et al*. (1981) and Desai *et al*. (1986). BAP was the most suitable cytokinin for *Melia* because KIN induced only non-organogenic calli (data not shown, preliminary experiments). This is similar to results obtained by Nirmalakamuri *et al*. (1993) and Drew (1993) in neem (*Azadirachta indica* Juss.) where KIN alone in the media induced only non-morphogenic callus induction.

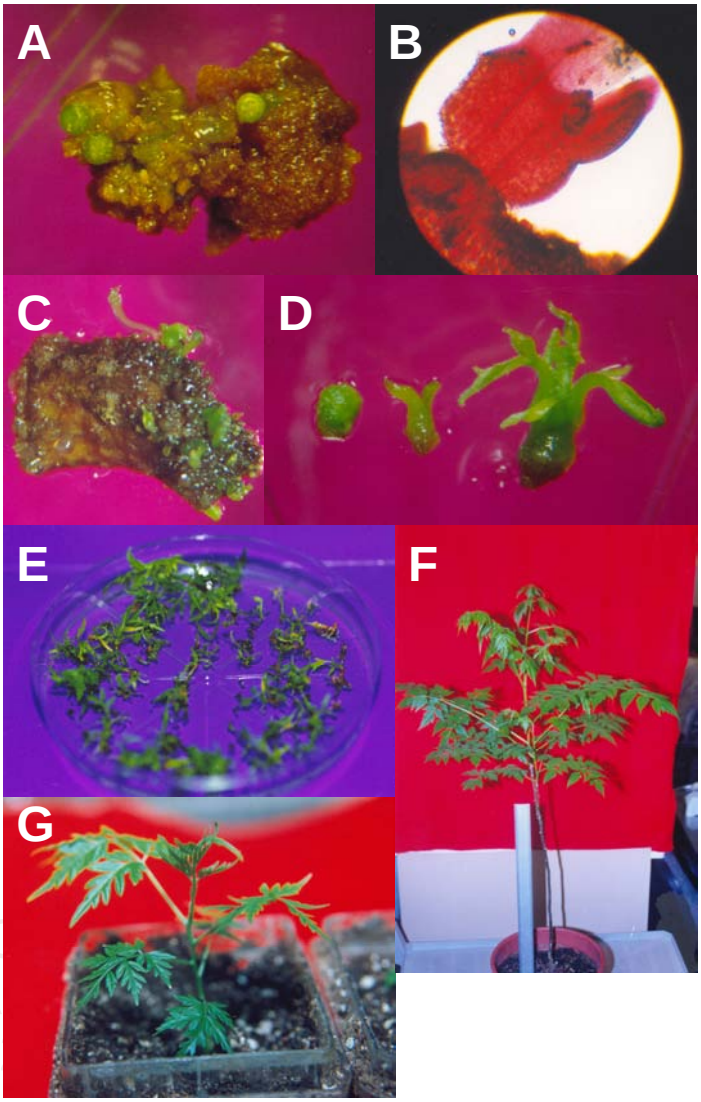

Fig. 1 (A) Heterogeneous embryogenic callus from cotyledons of Melia azedarach L. (B) Somatic embryos of Melia azedarach from embryogenic callus surface. (C) Direct somatic embryogenesis from cotyledon, showing tiny tube-like protrusions. (**D**) Different stages of somatic embryos. (E) Somatic embryo germination on EGM Medium (see text for details). (F) Complete plants from somatic embryos after 1 month in acclimatization. (G) One year old plant from somatic embryos.

3.1.2. Somatic embryos germination and conversion

Somatic embryos were green and had to be separated from callus and subcultured to fresh medium, otherwise necrosis occurred. The best somatic embryogenic stage to subculture somatic embryos was either the torpedo or cotyledon stage. For somatic embryo germination (Fig. 1E), transfer to hormone-free medium or with a low concentration of GA_3 (0.01 mg/l) and BAP (0.2 mg/L) without auxin, were effective (i.e. EGM). Germination occurred on all three different culture media tested (hormone-free MS (full or half strength) with Ade at different concentrations; EGM medium; SEM medium). Somatic embryo conversion to phenotypically normal plants was achieved at 100% after 1 month of culture in the light (Fig. 1F). Secondary somatic embryogenesis was observed on primary somatic embryos or on the stem base of new plantlets in SEM medium. This process could have importance in establishing a genetic transformation system, which permits a high frequency of regeneration (Fernández da Silva and Menédez Yuffa 2003). Our results show a remarkably higher efficiency compared to other reports, which refer mainly to the related tree, "neem". Nirmalakamuri *et al*. (1993) obtained 8-10 plantlets per explant through direct somatic embryogenesis during 4-5 months; Shrikande *et al*. (1993) and Murthy *et al.* (1998) obtained somatic embryogenesis with 60-70% conversion of embryos to plants and 80% survival in field conditions, and Joshi *et al*. (1993) obtained somatic embryos from cotyledons, recovering 20-25 embryos per explant with 2-5% germination on MS/2 medium.

Table 1 Rooting of shoots after 1 month following different treatments.

Culture media + additives (mg/L)	Number of Shoots	Rooting %	% of rooting shoots	Nature of morphogenetic responses
PGR-free	37	4	11	Several roots without principal axis
NAA (0.01)	60	60	100	Long roots. One axis with hairs
IBA (0.01)	41	7	17	Large axis with hairs
IAA (0.01)	27	10	37	Long roots
Floroglucinol (4)	15	0	0	Improve shoot and leaves aspect, but roots are not formed
CA	11	11	100	Very thin roots
Put (161.1)	29	0	0	No roots. No calli
IAA,NAA,IBA (1)	60	53	88	Roots and callus

3.1.3. Hardening-off and acclimatization

When whole plants were transferred to pots, 100% survival occurred in chamber culture, greenhouse and field conditions. Plants were phenotypically normal (Fig. 1G) and roots were vigorous and normal, too. Plants with fungal contamination did not present problems when subcultured to soil. This may be related to the antifungal active principles of this species (Carpinella *et al*. 1999) which inhibited the fungi's growth. The rooting percentage *in vitro* was also shown to show remarkable differences (Table 1), depending on the treatment, and this may also affect, at an *ex vitro* stage, the effectiveness of acclimatization.

3.2. Organogenic callus induction and shoot proliferation

The induction of organogenic calli from chinaberry explants was effective on OCIM medium with 0.5 mg/L NAA and 1 mg/L BAP supplemented with 1 g/L CH, 5% sucrose and 2.5 g/L gelrite. This callus was light brown, with a compact green center with several buds. Generally, nodular adventitious buds became visible within 3 or 4 weeks of culture on OCIM medium. When portions of these calli were subcultured on MG or MY medium, many shoots formed (Fig. 2A). Both MG and MY contained BAP at a low concentration. Accord to Joarder *et al*. (1993), BAP was a suitable cytokinin for the proliferation of shoots in neem (*Azadirachta indica* Juss. Meliaceae). The superiority of BAP to other cytokinins for multiple shoot formation in trees has also been reported by other researchers (Zaman 1991, Thakur *et al*. 1998). At an initial stage, shoots appeared as nodular structures on the surface of calli. At a later stage, shoot buds were recognizable as monopolar structures developing procambial strands, which established a connection with preexisting, callus vascular tissues (Fig. 2B). At the end of 7 weeks most of the adventitious buds developed into phenotypically normal shoots on both MG and MY media: ~160-200 shoots/g of fresh weight of callus were obtained. All shoots could develop into plants. Considering that the average weight of each cotyledonary leaf was 0.88 g at the start of culture, and that the weight increment in one week was 90% (data not shown), the multiplication rate is very high. Shoot multiplication of *M. azedarach* was previously achieved, however the rates of shoot proliferation and their conversion to plants were lower than our protocol. Domeqc (1988) used *M. azedarach* var. *gigantea* shoot tips for micropropagation. The multiplication rate was 6 after 4 subcultures. Prasad *et al.* (1993) induced *in vitro* multiplication of *M. azedarach* from nodal explants. They obtained shoot clusters with one long shoot of 4.6 cm surrounded by numerous tiny shoots. These reports do not indicate the percentage of surviving plants. Thakur *et al*. (1998) obtained multiple shoot development within 7-9 weeks in 74% of cultures; 93% of cotyledon leaves produced ~240 shoots each in 3 months and all of them were converted to whole plants. Although it is considered that callus is a source of genetic variability, the rate of somaclonal variation can be significantly reduced by shortening the culture period (Larkin, 1987). Moreover, in higher plants, regeneration from callus is sometimes a necessary step to manipulate cells and tissues under *in vitro* conditions (Nirmalakamuri *et al.* 1993) and when a great number of plants are necessary in a short period of time.

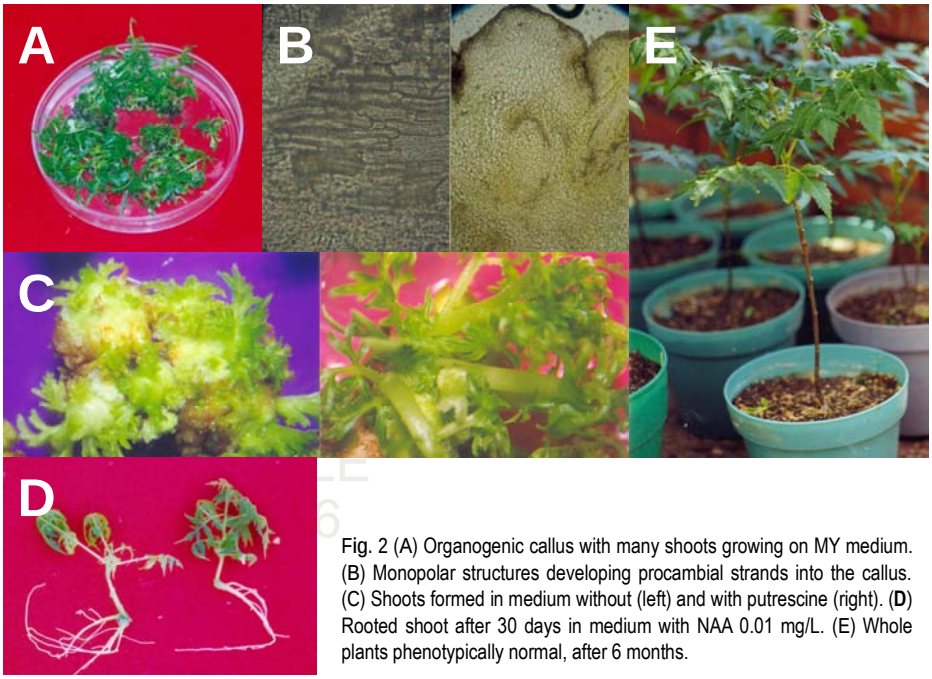

Fig. 2 (A) Organogenic callus with many shoots growing on MY medium. (B) Monopolar structures developing procambial strands into the callus. (C) Shoots formed in medium without (left) and with putrescine (right). (**D**) Rooted shoot after 30 days in medium with NAA 0.01 mg/L. (E) Whole plants phenotypically normal, after 6 months.

The addition of Put to the shoot proliferation medium (see 2.4.) increased the number (data not shown) and quality of shoots (Fig. 2C). When Put was not used, shoots were short, light green, and with abnormal leaves (Fig. 2C). Polyamines are present in all living cells and are required for growth and development in many biological systems (Rajam and Galston 1985, El Hadrami *et al.* 1992); they seem to play a key role in active cellular proliferation (Bagni *et al.* 1982). Polyamines were considered as antioxidants (El Hadrami *et al.* 1993) and there is evidence that they act by controlling the biosynthesis of ethylene (Bradley *et al.* 1984). In addition, there is evidence that polyamines are involved in the regulation of transcription (Moruzzi *et al.* 1975, Jacob *et al*. 1976). Feirer *et al*. (1985) observed that an increase in polyamine level is specific to the pattern of growth rather than to growth rate. *In vitro* shoots of *M. azedarach* do not have browning problems but the plant is dramatically affected by ethylene in *in vitro* culture (Lede *et al.* 1998), and all of these factors may play a role in normal proliferation of *M. azedarach* shoots.

3.3. *In vitro* selection of salt tolerance

Explant necrosis was observed at 171.1 mM and 128.35 mM of ion interchangeable sodium. At 42.7 mM the response of callus was similar to the control. At 85.57 mM, organogenic callus formed hyperhydric buds. *In vitro* selection of salt tolerant plants of *Brassica juncea* L. (Indian mustard) cv. Prakash has been accomplished by screening highly morphogenic cotyledon explants cultures on high NaCl media. Out of a total of 2,620 cotyledons cultured on high salt medium, 3 survived, and showed sustained growth and regenerated shoots (Jain *et al.* 1991). In our work, high NaCl media also resulted in explant necrosis.

An increment in fresh weight indicated that the relative growth rate of callus decreased with an increase in NaCl concentration. The callus on 42.7 mM NaCl gave a higher growth mass and was significantly higher than other treatments. Initially, differences were not observed, but at 30 and 60 days the differences were significant, while at 90 days they were highly significant.

The presence of a high concentration of salt in the culture medium produces a loss in regeneration potential (**Figs. 3a-c**). Work with callus adapted to salinity is characterized by their poor regeneration (Binh *et al*. 1992). The loss of organogenic potential may be due to increased osmotic potential of the saline medium: a higher osmotic potential affects water and nutrient uptake, which may in turn inhibit the metabolic activities necessary for bud initiation and growth (Vijayan *et al*. 2003). Callus that did not lose its regeneration capacity was subcultured twice onto medium with NaCl. Sprouting was not delayed by increasing salinity of the medium in relation with the control (Fig. 3d). In *Morus* sp., Vijayan *et al*. (2003) observed a delay in sprouting by increasing salinity in the medium.

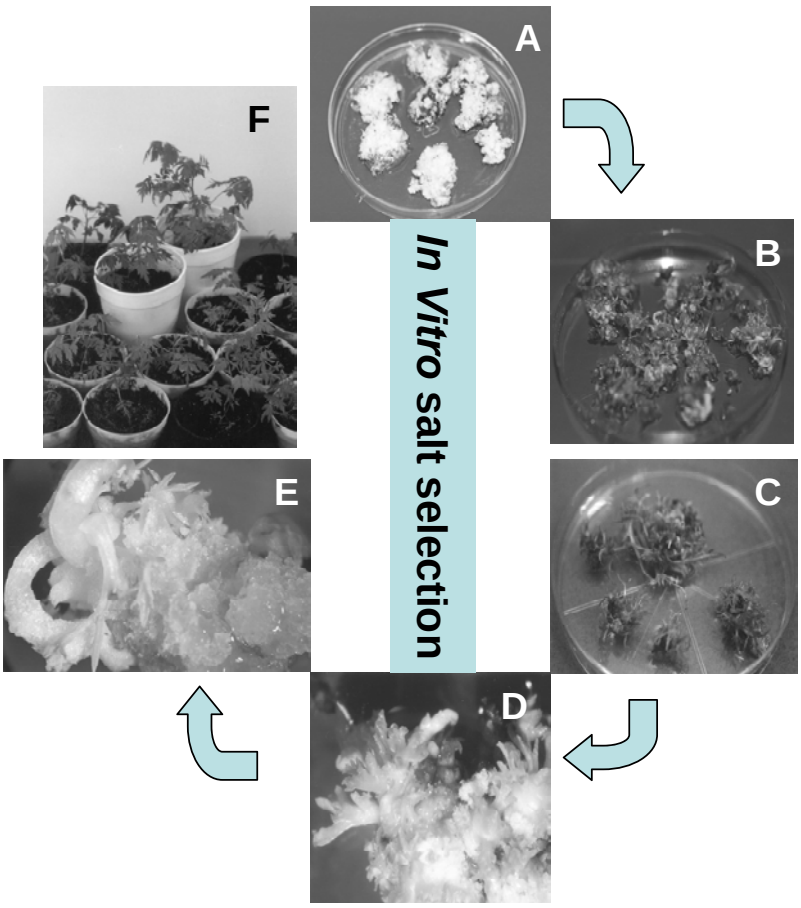

Fig. 3 *In vitro* salt tolerance selection of *Melia azedarach*. Different regeneration potential of callus in culture media with 85.57 mM (A), 42.7 mM (B), and control (C). Sprouting of the control (**D**) and organogenic callus (E) grown on medium with 42.1mM NaCl. (F) Plants obtained from *in vitro* NaCl selection.

Whole plants from NaCl-free medium were grown under greenhouse conditions (Fig. 3e), but their performance on saline soil was not carried out to ascertain if their salinity tolerance that was expressed under *in vitro* conditions could also be expressed in saline soil.

This task will be carried out in the near future.

A possible explanation for the increase in tolerance to salt in organogenic callus observed in our work may be due to the variability already present at the cellular original level of the explants, from which tolerant cellular variants are then selected. Another explanation could be that tolerance to salt is a mutation of nuclear genes, whose resulting cellular mutants lines are then selected in the presence of NaCl.

3.4. Rooting and acclimatization

Rooting of chinaberry and its subsequent establishment outdoors is better achieved with a low concentration of auxins. Of the various auxins tested for root induction, NAA proved to be the most effective. This is in contrast with Thakur *et al*.'s (1998) work, where IBA was the most effective auxin in initiating roots on shoots. In our work, the best roots were obtained with 0.01 mg/L NAA in 1 month (**Fig. 2D**). Prassad *et al.* (1993) obtained good rooting with 1.0 mg/L NAA in 1 week, but in our work, this concentration of auxin produced callus at the base of 80% of shoots. Domeqc (1988) reported *in vivo* rooting with IBA, but only 30% of shoots converted to whole plants. In our experiments, 100% of shoots subcultured into MR with 0.01 mg/L NAA formed normal roots. Whole plants were grown at 27ºC and 50±5% relative humidy in the culture chamber. In agreement with Thakur *et al*. (1998), we observed that high relative humidity is necessary for plantlet survival. However, in our experiments (different to Thakur *et al.* 1998), a maintenance period of high humidity was not necessary for some time after transplantation to the greenhouse. Plants were subsequently transferred to larger pots and gradually acclimated under greenhouse conditions with variable relative humidity (35-60%). Plants were phenotypically normal (Fig. 2E) and had a good growth rate. All plants transferred to soil conditions survived.

4. CONCLUSIONS

The protocol we developed established the potential to produce plantlets from cotyledon explants through indirect organogenesis and somatic embryogenesis. The general procedure and period for plant regeneration of chinaberry developed from both organogenesis and embryogenesis is shown in **Diagrams 1** and 2. From the mature seed culture to plant regeneration, only 5-6 months are needed. This technique produces a high frequency of plantlets in a short period of time and is more efficient than any other protocol reported to date. Somatic embryogenesis with a high percentage conversion of embryos to plants will allow adjustment of the methodology to produce synthetic seeds and its use *in vitro* selection for abiotic stress resistance. In addition, successful differentiation and multiplication of shoots and subsequent rooting of *M. azedarach* shows the

feasibility of this technique for large-scale production of planting stock. It is very important to note the high shoot proliferation rate, conversion to plants and survival under field conditions using our system. Plant regeneration is an important step in the success of any tree improvement program using tissue culture technology. A well-defined, reproducible, and highly efficient plant regeneration scheme is a prerequisite for genetic transformation. These features are fundamental for the application of any tissue culture protocol to forestry industrial projects.

There has been much interest in the development of woody plants tolerant to a biotic stress, primarily salinity. Tissue culture techniques may prove valuable as a means of achieving this goal. This study provides an understanding of the response of persian lilac callus to salinity, which is important for future studies aimed at developing strategies for selecting and characterizing somaclonal variants tolerant to salt stress. The overall conclusion that can be drawn from this work is that *in vitro* screening is clearly effective for identifying salinity-adapted organogenic callus of *Melia*.

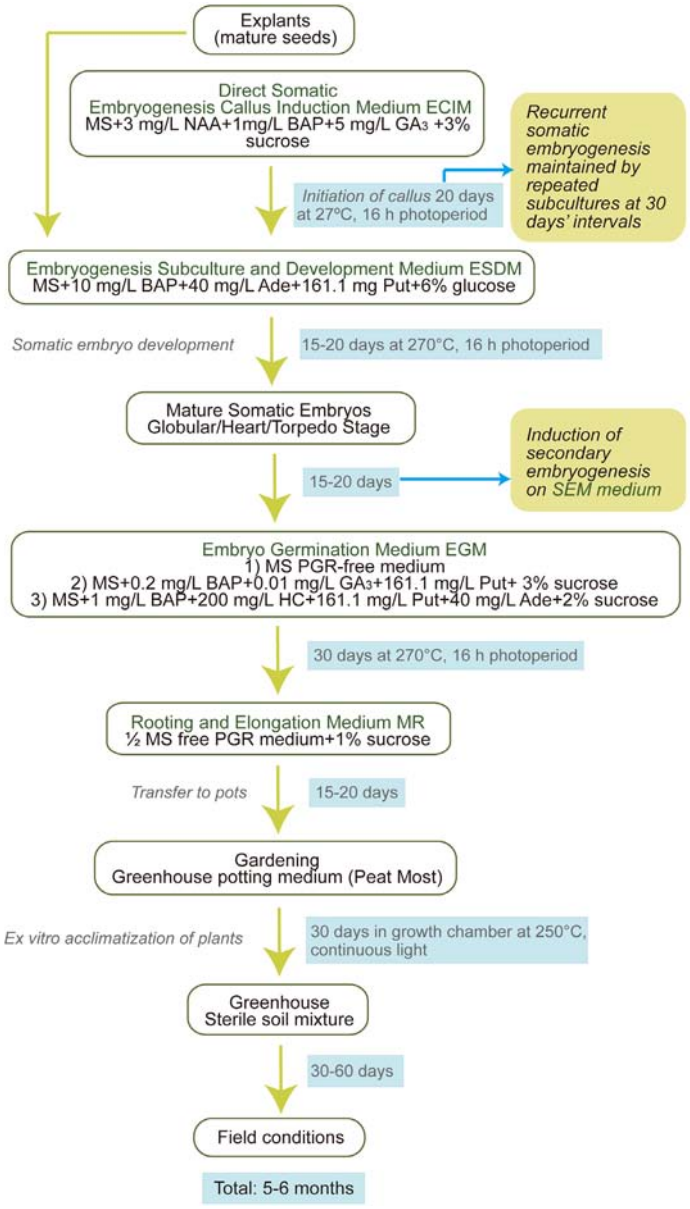

Diagram 1 Diagramatic representation of the optimized somatic embryogenic pathway for *Melia azedarach* L.

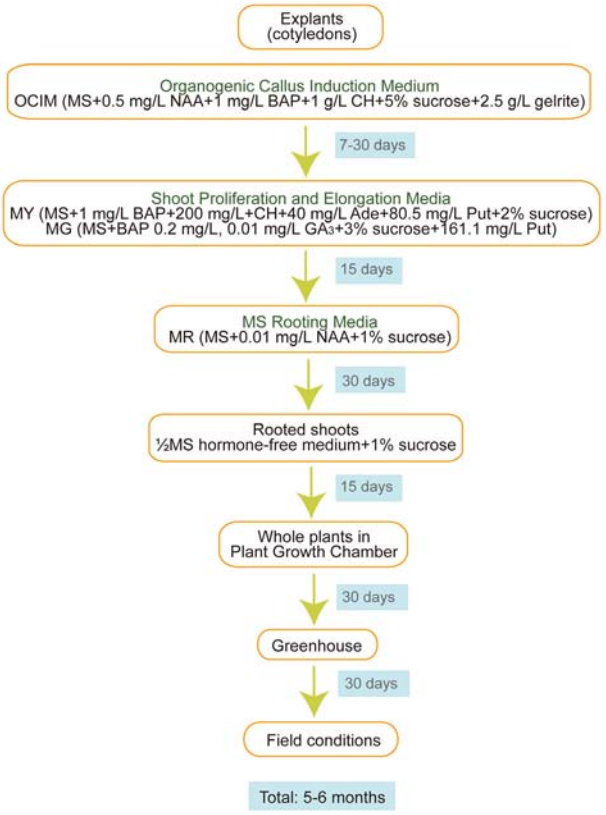

Diagram 2 Diagramatic representation of the optimized organogenetic pathway for *Melia azedarach* L.

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