Journal of Chemical, Biological and Physical Sciences

An International Peer Review E-3 Journal of Sciences

*Available online at*www.jcbsc.org

Section E: Plant Biotechnology

CODEN (USA): JCBPAT Research Article

Somatic Embryogenesis and Genetic Transformation Protocols Effective on Neem Trees

Beatriz Zetina-Martínez[1], Sugey Guillen-Vergara[1], Alejandro G. Nila-Méndez[1], Francisco J. Gabino-Roman[1], Elizabeta Hernández-Domínguez[1*].

[1,*]Instituto Tecnológico Superior de Acayucan, Carretera Costera del Golfo Km 16.4, Col. Agrícola Michapan. CP. 96100, Acayucan, Veracruz.

Abstract. To optimize an *in vitro* protocol for neem propagation via somatic embryogenesis, effects of various concentrations of dicamba, kinetin, TDZ and 2, 4-D (0, 0.5, 1.0 and 3.0mg/L) in combination with IAA or BA (0, 0.5, 1.0 and 3.0 mg/L) were studied. In fact, almost all the treatments showed the development callus masses with no evidence of proembriogenic masses. When hypocotyls and cotyledons were cultivated on MSB5 semisolid medium supplemented with 0.5 mg/L 2,4-D, 39% of the cultures displayed somatic embryogenesis. On the other hand, a genetic transformation protocol was developed to transform *Azadirachta indica* A. Juss via *Agrobacterium*. Four-week-old hypocotyls and cotyledons were used as explants in this transformation protocol. More than 35% of cotyledon and 65% of hypocotyl explants produced shoots on MSB5 medium supplemented with 1.0 mg/L 6-benzylaminopurine (BA). These explants were co-cultured with *Agrobacterium tumefaciens*, strain C58C1, harbouring the pCAMBIA 1303 plasmid, carrying the hygromycin resistance gene (*htp II*) as a selectable marker and β-glucuronidase (GUS) as a reporter gene. Factors affecting the transformation efficiency were evaluated; such as age and type of explants, *Agrobacterium* concentration, infection and co-cultivation time period, the presence of growth regulators, and the effect of cefotaxime on regeneration medium. Our results showed a 21% transformation efficiency when a 10-minute co-cultivation period was employed. Putative transformed explants were immediately transferred to a regeneration medium in order to generate shoots.

Keywords: *Azadirachta indica* transformation • somatic embryogenesis • organogenesis

INTRODUCTION

Azadirachta indica A. Juss, a member of the Meliaceae family, is indigenous to southern Asia. This species is popularly called the neem tree and is distributed in tropical and subtropical regions of the world. The neem tree is a multipurpose timber tree from which high value products are extracted for use as insecticides, fertilizers and multipurpose medicines[1]. This tree contains more than 100 bioactive compounds and it is rich in proteins. The most important bioactive compound that the neem tree produces is azadirachtin (repellent); other compounds are gedunin (antimalarial), nimbin (anti-inflammatory, antipyretic), nimbidin (antibacterial), nimbidol (antimalarial, antipyretic), salannun (repellent) and sodium nimbinate (spermicide)[1]. Azadirachtin is a limonoid (tetranotriterpenoid) that occurs in all parts of the neem tree, but is concentrated in the seed kernel [2]. Therefore, the commercial potential of this tree has renewed worldwide research interest in it. Hence, the demand for elite clonal planting stocks is increasing [3]. Neem is traditionally propagated by seeds, which require immediate planting after harvesting as they are recalcitrant.

This fact limits the distance distribution of seeds and their storage for year-round availability of propagules[4]. Seed propagation also has the disadvantage of heterogeneity as a result of cross-pollination. Therefore, the application of a reliable clonal propagation system would greatly aid in the multiplication of such an elite type. Tissue culture could not only overcome these limitations, but could also accelerate the production of clonal material to be transferred and grown in the field. Several reports exist for researchers that have attempted clonal propagation of neem trees[2, 4]. Clonal multiplication of superior elite individuals via somatic embryogenesis may be an alternative propagation method. Somatic embryogenesis (SE) offers an effective system for plant regeneration[3], and may assist in the application of recombinant DNA technology. SE is a process in which plant cells are induced to form somatic embryos through the manipulation of culture medium components and plant growth regulators (PGRs), without the involvement of gamete fusion.

On the other hand, recombinant DNA technology is a powerful tool for introducing foreign genes into long-lived perennial tree species[5]. Therefore, the standardization of *in vitro* regeneration by means of somatic embryogenesis would help to maintain and enhance the multiplication of elite clones of interest for a higher productivity. At present, genetic transformation methods use *Agrobacterium*-mediated gene transfer, a method that has advantages over direct DNA delivery, like the preferential integration of foreign DNA into transcriptionally vigorous chromosomal regions[6]. Several hardwood plant species like *Azadirachta indica*[7] and *Tectona grandis*[8] have been modified by this method, resulting in the insertion of some foreign genes into their genome.

This research aims to standardize two efficient protocols in *Azadirachta indica*; one for *in vitro* regeneration via somatic embryogenesis, and the other for genetic transformation by *A. tumefaciens* in neem trees.

METHODS

Preparation of plant material. *A. indica* seeds were obtained from ~10 year-old trees, selected in the region of Oluta, Veracruz, Mexico (17°56′00″N 94°54′00″W). The seeds were disinfected by 20-min immersion in a sodium hypochlorite solution [NaOCl 10% (v/v)], which contained 50 L/L polyoxyethylene sorbitan monolaurate (Tween 20®). Immediately afterwards the seeds were immersed in 70% (v/v) ethanol for 5 min; after that, three rinses of 5 min each with sterile distilled water were performed. Embryos were isolated aseptically from the seeds, and cultured on semisolid half-strength MSB5 medium [half-strength of MS salts[9], supplemented with B5 vitamin[10], 30g/L sucrose, and 0.7% (w/v) agar]. The pH was adjusted to 5.75

prior to autoclaving at 121°C for 20 min. Cultures were kept at 25°C under a 16 h photoperiod provided by cool-white fluorescent lamps (140 μM/m^2s). After 30 days, seedlings were obtained. *In vitro plantlet regeneration.* The cotyledons and hypocotyls of 30 day-old seedlings, were dissected in segments of about 5-10 mm in length. The explants were cultured on semisolid MSB5 medium supplemented with 0, 1.0, 2.0, or 4.0 mg/L N6–benzylaminopurine (BA) for 30 days for shoot induction. *Induction of somatic embryos*. These explants of cotyledons and hypocotyls were cultured onto MSB5; supplemented with 0, 0.5, 1.0 or 3.0 mg/L dicamba; 0, 0.5, 1.0 or 3.0 mg/L kinetin (KIN) combined with 0, 0.5, 1.0 or 3.0 mg/L indole-3-acetic acid (AIA), 0, 0.5, 1.0 or 3.0 mg/L thidiazuron (TDZ), 0, 0.5, 1.0 or 3.0 mg/L AIA combined with BA and 0, 0.5, 1.0 or 3.0 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) to induce somatic embryos. After somatic embryos were obtained, these were cultivated on MSB5 medium supplement with 1mg/L BA and Indole-3-butyric acid (IBA). *Plant tolerance to cefotaxime*. *A. indica* hypocotyls and cotyledons were plated on semisolid MSB5 medium supplemented with different concentrations of filter-sterilized cefotaxime (0, 100, 250 and 500 mg/L). After 30 days of culture, the results obtained were analyzed. Once the optimal concentration of cefotaxime was determined, it was used to remove *Agrobacterium*, and select for transformants. *Agrobacterium strain*. The *Agrobacterium* strain used was C58C1, a derivative of EHA101. This strain carried the pCAMBIA 1303 plasmid (http://www.cambia.org; Genbank access, AF234299). It was maintained on LB medium supplemented with 50 mg/L kanamycin and 100 mg/L rifampicin. *Transformation procedure*. Transformation experiments were performed in a growth chamber at 28°C with a 16 h photoperiod (140 μM/m^2s). Hypocotyls and cotyledons were excised from 30-40 day-old seedlings of *A. indica*, rinsed in liquid MSB5, dipped in 10 mL of an overnight grown culture of *A. tumefaciens* at different times (5, 10, and 15 min), dried on filter paper to remove excess culture medium, and transferred to co-cultivation medium (semisolid MSB5 medium supplemented with 1.0 mg/L BA). Cultures were kept in darkness. After 3 days, explants were washed with sterile co-cultivation medium at 25°C for 2 h at 50 rpm, dried explants were transferred to co-cultivation medium supplemented with 500 mg/L cefotaxime (selection medium). *GUS histochemical assay*. Histochemical localization of *uidA* expression was performed on transformed tissue. Control and putative shoots were subjected to β-glucuronidase (GUS) histochemical staining[11]. Approximately 15 hypocotyls and cotyledons per experiment were used for the histological assay. After staining, explants were cleared in 70% (v/v) ethanol for 24 h prior to counting the number of GUS foci.

RESULTS

*Regenerated plants from cultured shoots and somatic embryogenesis of A. indica***.** After *A. indica* seeds were germinated, explants were excised from 30-day-old seedlings, and cultured on semisolid MSB5 medium supplemented with BA, at different concentrations to evaluate for shoot induction **(Fig. 1 A & B)**. When a concentration of 1.0 mg/L of BA was used, a high responsive yield was obtained since 37% of the explants displayed shoot formation. Those explants produced from 2 to 4 shoots. On the other hand, another batch of neem seedlings were dissected and the explants were cultured on MS medium in the presence of several types of growth regulators (kinetin, dicamba, AIA, BAP and thidiazuron) at different concentrations (0, 0.5, 1.0 and 3 mg/L) in order to evaluate the formation of somatic embryos. All explants showed the development of callus masses. Although these calli were of the compact type and showed different colors (from light green to brown), they did not show evidence of proembriogenic masses. However; 39% of explants cultured on MS medium supplemented with 0.5 mg/L 2,4-D showed the formation of callus masses after 30 days of culture; after subculturing, new cell masses were observed. These were yellowish and friable suggesting

proembryogenic callus formation **(Fig. 1C)**. A more detailed view of the formed calli showed embryos at all developmental stages **(Fig. 1 D & E)**. We quantified the percentage of each embryogenic structure. These were 57% globular, 9% torpedo, 15% heart-shaped, and 12% early and 6% late cotyledonary structures. Our observations confirm the results found by other researchers, where a growth regulator like 2, 4-D helps to induce embryogenic callus in a number of plants species including woody perennials[4, 5]. Plantlet conversion ability from these embryogenic callus masses was obtained as it is shown on **Fig. 1F**. *Agrobacterium mediated transformation of A. indica***.** *Effect of antibiotics*. To evaluate the effect of cefotaxime on *A. indica* explants previously obtained (see above), sensitivity assays were performed. Our results showed that doses up to 500 mg/L of cefotaxime did not affect the morphogenic capacity of explants; similar results were reported for *Eucalyptus camaldulensis*[12]. After *Agrobacterium* infection, explants were placed on selection medium with the appropriate antibiotic concentration as described above (500 mg/L cefotaxime). *Infection time*. Another factor that was evaluated in this study was the infection time with *Agrobacterium*. We observed that *Agrobacterium* infection for 10 minutes produced the highest percentage of explants that exhibited GUS activity **(Figure 2A-D, and Table 1)**. Under this infection time, resulting transformed explants were able to generate shoots on selection medium **(Fig. 2 C and D)**.

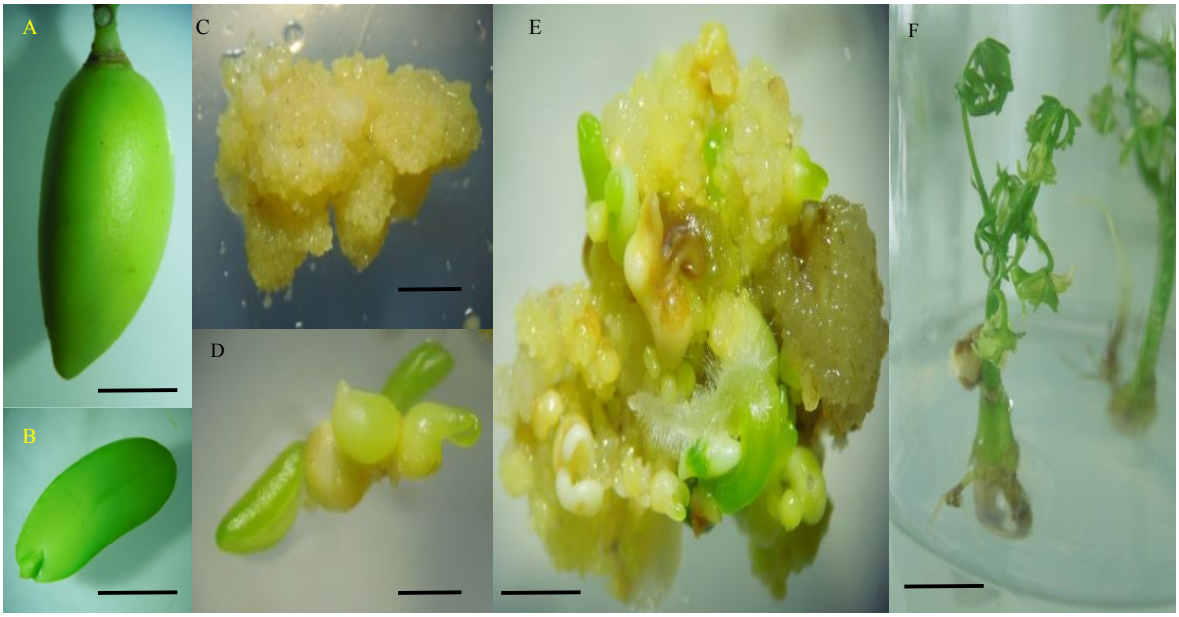

Fig.1: Induction of somatic embryogenesis in *A. indica*. A) Immature seeds, B) Immature zygotic embryo, C) Preglobular and globular somatic embryos, D) Heart shaped and torpedo somatic embryos, E) A more detailed view shows that heart, torpedo and cotyledonary embryos can develop in the same cluster, F) Germinated embryo. Scale bars A) 15 mm, B) 8 mm, C)-E) 1mm, F) 30 mm.

Conversely, no GUS activity was detected on explants infected with *Agrobacterium* for 5 min, suggesting that a shorter time period than 5 min does not ensure integration and working of transferred DNA, while a time period longer than 10 min can also be lethal due to overgrowth of *Agrobacterium*, which may behave as a parasite in explants and decrease nutrient supply[8, 12]. Our results show that the percentage of explants that was positive for this assay diminished with longer infection times, as well as the shoot formation rate **(Table 1)**.

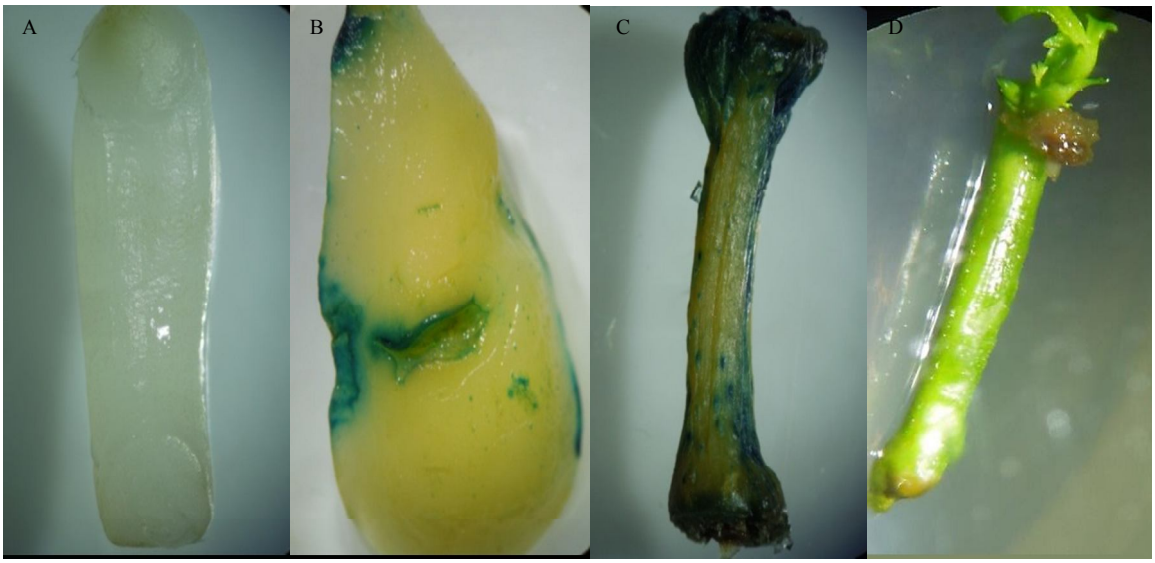

Fig. 2: Regeneration and genetic transformation of *A. indica*. A) Histochemical staining of GUS activity of non-transgenic hypocotyls, B and C) Histochemical staining of GUS activity in transgenic cotyledons and hypocotyls, D) Transgenic plant that was able to generate shoots on selection medium.

Table 1: Evaluation of *A. indic*a explants infected with *Agrobacterium* at different incubation times.

	5 min	10 min	15 min
Number of explants infected	30	30	30
Explant survival (%)	42.3±8.0ab	56.3±4.0ab	31.1±6.9a
Shoot formation rate	11.0±2.6a	25.3±1.5b	29.3±4.0b
GUS positive (%)	0.0±0.0a	30±2.0b	15.3±1.5c
Transformation efficiency (%)	0.0±0.0a	20.33±2.0b	9.3±2.3c

Values with the same letter within lines are not significantly different according to Tukey´s range test at the 5% level.

This study demonstrates the transfer and expression of elements present in the pCAMBIA 1303 vector to *A. indica* tissue via *Agrobacterium*, similar to what has been reported in other studies[7]. In order to exhibit these results, experimental conditions were optimized for the detection of GUS activity in neem (see Methods and **Fig. 2)**.

CONCLUSIONS

We developed a protocol for the induction of indirect somatic embryogenesis in neem by means of the subculture of hypocotyls and cotyledons in MSB5 medium supplemented with 0.5 mg/L 2, 4 D. A successful *A. tumefaciens*-mediated transformation as well as a regeneration protocol of neem are reported in this work. The transformation protocol required hypocotyl and cotyledon explants from 30–day *in vitro* seedlings. A 10-min incubation period with the *Agrobacterium* C58C1 strain, harboring the pCAMBIA 1303 plasmid, proved to be the best condition for tissue transformation and shoot generation. Selection and regeneration of transformed tissue was conducted on selection medium, which included 1 mg/L BAP.

ACKNOWLEDGEMENTS

This work was supported by CONACYT, grants 96183 and 106172. We would like to thank PhD. Gladys Iliana Cassab Lopez-IBT for kindly providing the pCAMBIA 1303 plasmid and the C58C1 *Agrobacterium* strain.

REFERENCES

1. C. Akura, A. Akura, R. Drew. Somatic embryogenesis in clonal neem, *Azdirachta indica*. A. Juss and analysis for *in vitro* azadirachtin production. *In Vitro Cell Dev. Biol. Plant* (2003), 39 (3):304-310.
2. B. Murthy, P. Saxena. Somatic embryogenesis and plant regeneration of neem (*Azadirachta indica* A. Juss.). *Plant Cell Rep.* (1998), 17 (6-7): 469-475.
3. V. Gautham, K. Nanda, S. Gupta. Development of shoots and roots in anther-derived callus of *Azadirachta indica* A. Juss. –a medicinal tree. *Plant Cell Tiss. Org. Cult*. (1993), 34 (1): 13–18.
4. R. Chaturvedi, M. Razdan, S. Bhojwani. *In vitro* morphogenesis in zygotic embryo cultures of neem (*Azadirachta indica* A. Juss.). *Plant Cell Rep*. (2004), 22 (11): 801-809.
5. P. Pijut, R. Beasley, S. Lawson, K. Palla, M. Stevens, Y. Wang. *In vitro* propagation of tropical hardwood tree species – a review 2001-2011. *Propag. Ornam. Plants* (2012), 12 (1): 25-51.
6. C. Ho, S. Chang, J. Tsay, C. Tsai, V. Chiang, Z. Chen. *Agrobacterium tumefaciens*-mediated transformation of *Eucalyptus camaldulensis* and production of transgenic plants. *Plant Cell Rep*. (1989), 17 (9): 675-680.
7. N. Naina, P. Gupta, A. Mascarenhas. Genetic transformation and regeneration of transgenic neem (*Azadirachta indica*) plants using *Agrobacterium tumefaciens*. *Curr. Sci.* (1989), 58 (4): 184–187.
8. S. Widiyanto, A. Sukmawan, A. Haro. Transient expression of β-glucuronidase reporter gene in *Agrobacteriu*m-Inoculated shoots of various teak clones. *Afr. J. Biotechnol*. (2009), 8: 2143-2150.
9. T. Murashige, F. Skoog. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* (1962), 15 (3): 473-497.
10. O. Gamborg, R. Miller, K. Ojima. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res*. (1968), 50 (1): 151-158.
11. R. Jefferson, T. Kavanagh, M. Bevan. GUS fusions: β glucoronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* (1987), 6 (13): 3901-3907.
12. R. Quisen, Y. de Oliveira, M. Pileggi, F. Cuquel, M. Quoirin. Selective agent and *A. tumefaciens* overgrowth-control antibiotics in *Eucalyptus camaldulensis* cotiledonary culture. *Braz. Arch. Biol. Techn*. (2009), 52 (6): 1485-1492.

Corresponding Author: **Elizabeta Hernández-Domínguez,**
Instituto Tecnológico Superior de Acayucan, Carretera Costera del Golfo Km 16.4, Col. Agrícola Michapan. CP. 96100, Acayucan, Veracruz.
e-mail: elihed@gmail.com