

An Efficient Protocol for Cyclic Somatic Embryogenesis in Neem (*Azadirachta indica* A. Juss.)

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Abstract— Neem is a highly heterozygous and commercially important perennial plant. Conventionally, it is propagated by seeds which lose viability within two weeks. Strictly cross pollinating nature of the plant causes serious barrier to the genetic improvement by conventional methods. Alternative methods of tree improvement such as somatic hybridization, mutagenesis and genetic transformation require an efficient *in vitro* plant regeneration system. In this regard, somatic embryogenesis particularly secondary somatic embryogenesis may offer an effective system for large scale plant propagation without affecting the clonal fidelity of the regenerants. It can be used for synthetic seed production, which further bolsters conservation of this tree species which is otherwise very difficult.

The present report describes the culture conditions necessary to induce and maintain repetitive somatic embryogenesis, for the first time, in neem. Out of various treatments tested, the somatic embryos were induced directly from immature zygotic embryos of neem on MS + TDZ (0.1 μ M) + ABA (4 μ M), in more than 76 % cultures. Direct secondary somatic embryogenesis occurred from primary somatic embryos on MS + IAA (5 μ M) + GA₃ (5 μ M) in 12.5 % cultures. Embryogenic competence of the explant as well as of the primary embryos was maintained for a long period by repeated subcultures at frequent intervals. A maximum of 10 % of these somatic embryos were converted into plantlets.

Keywords— *Azadirachta indica* A. Juss., Cytokinin, Somatic embryogenesis, zygotic embryo culture.

Abbreviations— ABA - abscisic acid; BAP - 6-benzylaminopurine; CH - casein hydrolysate; 2,4-D - 2,4-dichlorophenoxyacetic acid; GA₃ - gibberellic acid; IAA - indoleacetic acid, IBA - indole-3-butyric acid; Kn - N⁶-furfuryladenine, NAA - α -naphthalene acetic acid.

I. INTRODUCTION

A *ZADIRACHTA Indica* A. Juss., commonly known as neem is an evergreen, multipurpose forest tree of India; it is valuable for its leaves, bark and fruits from which compounds are extracted to make pharmacy products. Today, numerous studies are available describing the insecticidal, antifeedant, growth inhibiting and antifertility activities of azadirachtin against a broad spectrum of insects. In spite of

having such valued properties, improvement of neem by conventional methods is very limited owing to its highly heterozygous nature, long reproductive cycle and poor seed yield. In this regard, somatic embryogenesis particularly secondary somatic embryogenesis may offer an effective system for plant regeneration and improvement.

In cyclic somatic embryogenesis, unlimited numbers of embryos are proliferated in a repetitive manner from single culture of primary embryos. This process is reported to have some advantage over primary somatic embryogenesis such as high multiplication rate, long term repeatability and independency of an explant source. Moreover, by repeated somatic embryogenesis selected embryogenic lines can be maintained for long period, in large quantities until the lines have been tested in field conditions particularly in perennial plants [1]. Somatic embryogenesis also overcomes post fertilization barriers of the embryo; immature embryos of interspecific plants from incompatible crosses may be rescued by culturing them for somatic embryogenesis and simultaneously the plant can be multiplied [2]. It can also be used in species where zygotic embryos contain important secondary metabolites. Furthermore, an epidermal single cell origin of embryos favor the use of this process for plant transformation, by repeated embryogenesis chimeric embryos after few division changes to fully transformed solid embryos [1].

There are number of reports on somatic embryogenesis of neem ([3], [4], [5], [6]). Almost all of the literature on neem somatic embryogenesis is specific to primary somatic embryogenesis and information on secondary somatic embryogenesis is relatively new in neem. Therefore, the main purpose of this study was to improve the present neem embryogenic system by secondary somatic embryogenesis.

II. MATERIALS AND METHODS

A. Plant Material and Initiation of Aseptic Cultures

Immature fruits of neem, collected from 35-year old experimental plant, were thoroughly washed in 1 % (v/v) antiseptic savlon solution for 5 minutes, followed by rinsing with sterile distilled water (SDW). Fruits were then rinsed with 90 % ethanol for 30 seconds before surface sterilizing with 0.1 % mercury chloride solution for 8 minutes. After washing with SDW three times, immature embryos at early dicotyledonous stage were dissected out with the aid of a stereo-microscope. Four embryos were cultured in each 55 × 15 mm pre-sterilized, disposable Petriplates (Tarsons, India)

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containing 10 ml of MS [7] medium with or without growth regulators for embryogenesis. The Petriplates were sealed with parafilm (American National Can Greenwich, N.Y). Unless mentioned otherwise all media contained 3 % sucrose. The pH of the medium was adjusted to 5.8, followed by addition of 0.8 % agar (Himedia, India), before autoclaving at 121 °C and 1.06 Kg/cm² for 20 minutes. All the cultures were incubated at 25 ± 2 °C under 16-h photoperiod provided by cool day light fluorescent tubes. Sixty-four cultures were raised for each treatment and each experiment was repeated at least three times. Observations were recorded at weekly intervals, and standard error of the mean was calculated.

To find the best medium for secondary somatic embryogenesis and embryo maturation, primary embryos were used as the explant, and cultured onto different media combination. The influence of various growth regulators on secondary somatic embryo induction was investigated. Twenty-four cultures were raised for each treatment, and each experiment was repeated at least three times. Observations were recorded at weekly intervals, and standard error of the mean was calculated.

III. RESULTS AND DISCUSSION

In the present study, we have developed an efficient protocol for primary and secondary somatic embryogenesis which can be utilized for synthetic seed production or in genetic transformation. Primary embryos have shown to be an excellent source of secondary embryos, where some cells break away from group control and start initiating new somatic embryos [8]. Secondary somatic embryos usually, proliferate from superficial cells of primary embryos particularly from hypocotyls and cotyledons [2]. Cyclic somatic embryogenesis that is the basis of plant cloning has been reported in many species such as *Dianthus caryophyllus* [9], *Piper nigrum* L. [10], *Morus alba* L. [11], *Coffea sp.* [12], *Phalaenopsis amabilis* [13], *Arachis hypogea* [14], *Medicago sp.* [15], *Myrtus communis* L. [16] and in *Dalbergia sisso* [17]. However, in neem, this is the first report in repetitive somatic embryogenesis.

A. Primary Somatic Embryogenesis

In this study, we have seen the effects of plant growth regulators on somatic embryogenesis. Out of different combinations of growth regulators, the maximum primary somatic embryogenesis occurred on MS + TDZ (0.1 μM) + ABA (4 μM) where more than 76 % cultures responded and embryos were differentiated directly from cotyledonary region of the early dicotyledonary stage of zygotic embryos (Fig. 1A). On another medium, MS + TDZ (0.5 μM) + GA₃ (1 μM), 67.8 % cultures showed embryogenesis with 5.83 ± 0.8 somatic embryos per culture (Fig. 1B). However, in this medium, most of the somatic embryos were proliferated from hypocotyls of germinated immature embryos.

On MS + BAP (10 μM) + IAA (3 μM) + GA₃ (1 μM) + CH (1000 mg/l), 62 % cultures showed somatic embryogenesis with 4.0 ± 0.6 somatic embryos per culture (Fig. 1C), whereas in MS + BAP (5 μM) + ABA (4 μM), from 58.48 ± 0.5 cultures somatic embryogenesis occurred with 4.4 ± 0.5 embryos per culture (Fig. 1D). In this study, we have

seen the significant effects of cytokinins on somatic embryogenesis which is found to be mandatory for the differentiation of somatic embryos (Table I). Many reports have described the positive effect of cytokinin on somatic embryogenesis ([19]- [20]). Reference [1] reported that BAP was the most frequently used cytokinin for the induction of somatic embryos. Thidiazuron, a cytokinin that belongs to the phenylureas, is emerging as successful alternative for high-frequency direct regeneration of somatic embryos. It has been observed that TDZ could substitute auxin and cytokinin required for induction and proliferation of somatic embryos [18].

TABLE I
EFFECT OF DIFFERENT PLANT GROWTH REGULATORS ON PRIMARY SOMATIC EMBRYOGENESIS AFTER 4 WEEKS.

Media	Primary somatic embryogenesis (%)	No. of somatic embryos/culture
MS + TDZ(0.5μM) + GA ₃ (1μM)	67.77 ± 1.0	5.83 ± 0.8
MS + BAP(10 μM) + IAA(3 μM) + GA ₃ (1μM) + CH (1000 mg/l)	62.5 ± 0.5	4.0 ± 0.6
MS + BAP (5 μM)+ ABA (4 μM)	58.48 ± 0.5	4.4 ± 0.5
MS + TDZ (0.1 μM) + ABA (4μM)	76.16* ± 1.0	14.16 ± 1.0

±=SE

*Somatic embryogenesis from cotyledonary region

B. Secondary Somatic Embryogenesis

Three distinct responses were observed when primary somatic embryos were cultured on MS alone or supplemented with different growth regulators (a) secondary somatic embryogenesis (b) germination (c) callusing along with secondary somatic embryogenesis.



Fig. 1 Direct somatic embryogenesis from immature zygotic embryo of neem (*A. indica* A. Juss.): (A) Cluster of somatic embryos originated from cotyledonary region of germinated immature embryo on MS + TDZ (0.1 μM) + ABA (4 μM). (B) Somatic embryo formation from the hypocotyl of germinated immature embryo on MS + TDZ (0.5 μM) + GA₃ (1 μM). (C) Somatic embryo formation from the plumular region of germinated immature embryo on MS + BAP (10 μM) + IAA (3 μM) + GA₃ (1 μM) + CH (1000 mg/l). (D) Somatic embryo formation from the hypocotyl of germinated immature embryo on MS + BAP (5 μM) + ABA (4 μM).

Secondary somatic embryogenesis did not occur when the primary embryos were cultured on medium devoid of growth regulators. On MS basal medium 10 % of the embryos germinated. Contrary to this, reference [21] reported secondary somatic embryogenesis in hormone free medium. The maximum secondary somatic embryogenesis occurred when MS medium was supplemented BAP and IAA. On MS + BAP (1 µM) + IAA (0.5 µM), 100 % cultures responded with 14 ± 2.0 somatic embryo per primary embryos after 8 weeks, which is preceded by callusing (Table II). In contrast to this, reference [23] reported embryo germination in MS medium supplemented with IAA and BAP. The primary embryos formed a brown, friable callus within two weeks, and after 5 weeks secondary somatic embryos were differentiated from the callus. Additional embryos continued to appear up to 8 weeks. On MS + GA₃ (5 µM) + IAA (2.5 µM), 8.3 % cultures showed direct secondary somatic embryogenesis with 7 ± 1.0 somatic embryos per culture. However, when IAA concentration was increased to 5 µM, 12.5 % cultures showed secondary somatic embryogenesis but at this concentration the number of embryos per culture decreased to 3.6 ± 0.5 (Table II). Most of the secondary somatic embryos appeared from hypocotyl region. Similarly, reference [22] reported large number of secondary somatic embryos all around the hypocotyls of the embryo axis.

TABLE II
EFFECT OF DIFFERENT PLANT GROWTH REGULATORS ON SECONDARY SOMATIC EMBRYOGENESIS AFTER 8 WEEKS.

Media	Secondary somatic embryogenesis (%)	No. of somatic embryos/culture
MS + IAA (2.5 µM) + GA ₃ (5µM)	8.3 ± 1.0	7.0 ± 1.0
MS + IAA (5 µM) + GA ₃ (5 µM)	12.5 ± 1.0	3.6 ± 0.5
MS + IAA (7 µM) + GA ₃ (14 µM)	0	0
MS + IAA (0.5 µM) + BAP (1µM)	100*	14.0 ± 0.5

±SE *Somatic embryogenesis was preceded by callusing

IV. CONCLUSION

This report describes the culture conditions necessary to induce and maintain repetitive embryogenesis in neem. The method is useful in cases where large numbers of cloned embryos are needed for propagation or for artificial seed production. Poor germination of embryos is a major limitation of somatic embryogenesis in many tree species ([17], [11]). Therefore, the process of germination needs to be studied in detail for successful plantlet production. Embryogenic competence was maintained for long period by repeated subcultures. MS basal medium supported maximum conversion of embryos into plantlets.

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