Somatic Embryogenesis and Gene Expression
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Somatic Embryogenesis in Neem (*Azadirachta indica* A. Juss.): Current Status and Biotechnological Perspectives

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Abstract

Neem (*Azadirachta indica* A. Juss.) is a remarkable multipurpose, evergreen tree of the mahogany family, Meliaceae. The history of commercial use of Neem is shrouded in the mystery and tradition of Vedic period of India. From the last two decades, the tree has become the focus of attention due to its medicinal, agrochemical and economic uses. From its roots to its spreading crown, Neem contains a plethora of highly valuable biologically active compounds. Neem tree's virtues are, to a large extent, attributable to its chemical constituents, which have been claimed to possess several biological activities, such as immune-stimulation, blood purification, anti- inflammation, anti-tumor, insect repulsion, bactericidal activity, and growth disrupting properties. Despite profound economic and medicinal value of Neem tree, very little scientific work is done on the improvement of this species. Genetic improvement of the plant by traditional breeding methods is hampered by prolonged juvenile period, enormous heterozygosity due to cross pollination and sexual recombination, recalcitrance and poor seed yields. It is almost impossible to keep pace with the increasing demand of the plant only through conventional methods of vegetative propagation such as, layering, cutting and grafting, which are used to maintain the genetic integrity of the selected trees. For improvement of Neem trees by somatic cell genetics, a highly efficient regeneration system is a pre-requisite. Among various *in vitro* methods, somatic embryogenesis is the method of choice, because of certain advantages over shoot regeneration like single cell origin of somatic embryos, a very high proliferation efficiency and potential of development of synthetic seeds. The present chapter reports the state-of-the-art of Neem somatic embryogenesis, stressing the milestones, the most recent

advances and main point of interest. With this chapter, authors aim to offer an experience background to researchers who intend to study Neem somatic embryogenesis.

Keywords: Neem (*Azadirachta indica* A. Juss.), Micropropagation, Somatic embryogenesis.

INTRODUCTION

Azadirachta indica A. Juss. (common names: Neem, Margosa and Indian lilac), a member of the family Meliaceae, is a majestic, evergreen, tropical forest tree, with a broad crown and a height of approximately 25m. It is a most valuable but least exploited multipurpose tree. It can grow on poor soils and wastelands and is famous for its drought resistance (Radwanski, 1977). Neem is believed to be a native to the Indian subcontinent and Myanmar, but due to introduction by the Indian migrants it has become widespread in its geographic distribution, particularly in Africa, Latin America, Fiji and other tropical island countries. Natural propagation of Neem occurs by seeds, and the reproductive phase normally begins after 5 years (Schmutterer, 1995). The timings of flowering and fruiting vary from place to place (Kundu, 1999). In India, Neem generally flowers from January through May, and fruits are available from June to August.

The flowers, which are white with characteristic sweet aroma, are protandrous and cross-pollinated (Nair and Kanta, 1961). Each bisexual flower has 10 stamens, situated at the base of the hypogynous disc, which is annular and nectariferous. The gynoecium is tricarpellary and syncarpous. The ovary is superior and trilocular at the base and unilocular above. Each carpel bears 2 ovules on parietal placentation (Nair and Kanta, 1961), but only single ovule per ovary develops into a seed. Sometimes 2 and, rarely, 3 seeds per fruit have been observed (Schmutterer, 1995) (Figure 1A). The fruits are ovoid drupes, borne in drooping panicles (Figure 1B). The young fruits are green, and turn yellow when ripe. The epicarp is thin and the endocarp is hard and bony. The pulpy mesocarp is eaten by animals and humans. The seeds are recalcitrant and lose their viability after 2-3 weeks, which can be extended to 6-8 weeks by cold storage (Aiyadurai, 1959). The embryology of Neem has been described by Garudama (1957) and Nair and Kanta (1961). They have also reported the occurrence of twin embryos (Figure 1C).

Mangenot and Mangenot (1958) observed consistently 30 chromosomes in the root tip cells of Neem while three Indian cytologists reported the diploid chromosome number of this plant as 28 (Deshmukh, 1959). To generate sufficient information and to throw more light on actual chromosome number of *A. indica.* Chaturvedi et al. (2004) made mitotic and meiotic preparations from root tips of seedlings and young flower buds of adult trees, respectively. The studies revealed that the diploid number of chromosomes in Neem as $2n = 2x = 24$ (Figure 1D) and haploid number is $n = x = 12$ (Figure 1E).

1. ECONOMIC IMPORTANCE

Neem tree is a renewable source of various useful products. Besides being a popular avenue tree, with a large crown, the wood of Neem has been used as timber for house building, furniture and other domestic and agricultural tools. The wood of

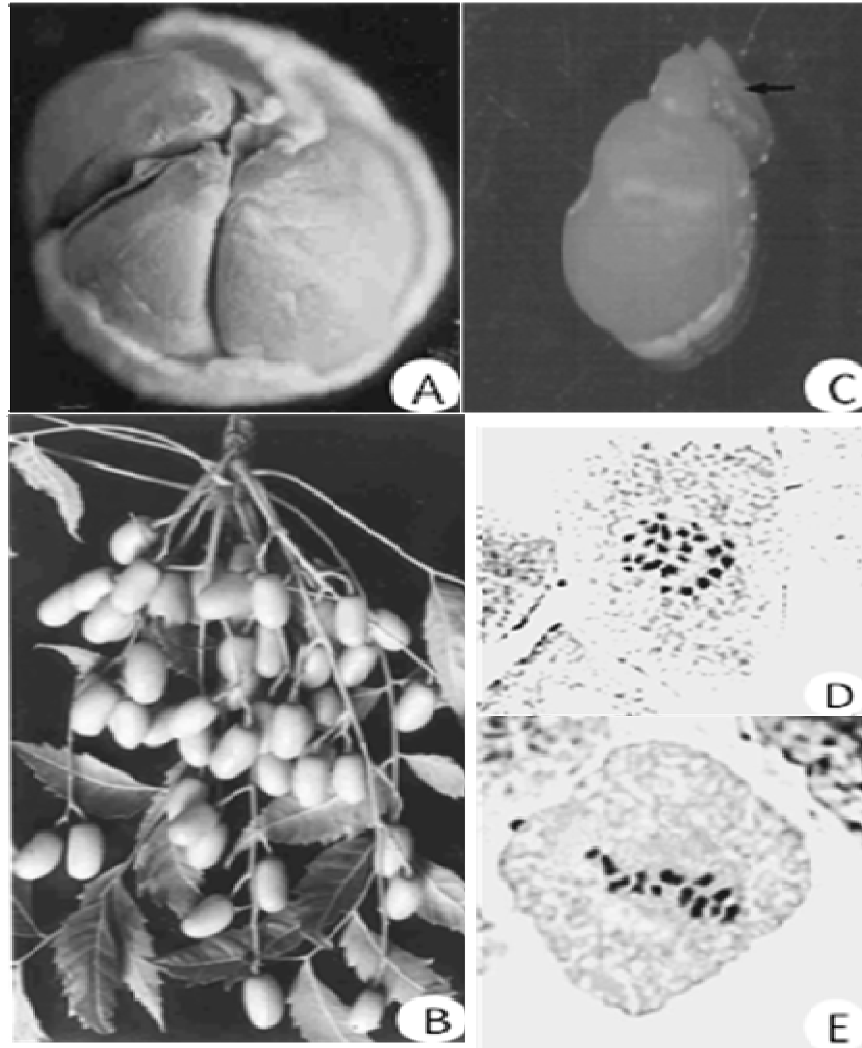

Figure 1: Slide showing a mature fruit, twig of Neem tree, root chromosome number. A) A mature fruit, with the endocarp ruptured to show the occurrence of three seeds; one of the seeds is much larger than the other two (×5). B) A twig of Neem tree bearing green (unripe) olive-like fruits in drooping panicles. Photograph taken in June (×0.6). C) Twin embryos excised from a seed; one of the embryos is considerably smaller (arrow marked) than the other (×12). D) Root tip cell of a seedling, showing diploid chromosome number 2n=2x=24 (×1460). E) Shoot tip cell of a field grown anther-derived plant, showing haploid number of chromosomes (2n=x=12) (×2100).

Neem resembles teak wood in its strength, and is more resistant to shock, fungi, and insect attack. It is immune to termites and is durable even outdoors (Thengane et al., 1995). Various parts of the Neem tree, particularly leaves, bark and fruits have been traditionally used in India in Ayurvedic medicines, and the seed-oil has been used as an antimalarial, antihelminthic, vermifuge, antiseptic, antimicrobial, and wound healing agent, and also to cure various skin disorders.

Insect repellent property of Neem was first reported in India in 1928 but little scientific work was done until late fifties (Blake, 1987). The observation that locusts did not destroy Neem tree (Pradhan et al., 1962) attracted the attention of national and international scientists to investigate the amazing properties of this wonder tree. Over 2,400 plants are known to contain insecticidal and insect repellent constituents but only Neem holds out the promise of providing highly effective, non-toxic and environment friendly means of controlling or eliminating insect pests which cause losses in agricultural production (Govindachari et al., 1992). Today, over 60 Neem- based products are under commercial production or near commercialization. Neem products are finding use in pest control, toiletries, cosmetics, pharmaceuticals, and animal and plant nutrition.

The maximum industrial utilization of Neem is for its seed-oil, which contains several active compounds. Azadirachtin is the most prominent constituent of Neem kernels**.** It has diverse pesticidal activities, such as insect repellent, antifeedant, oviposition deterrent, reduced hatchability of eggs and increased emergence of malformed adults (Thoh et al., 2011). Neem oil is also reported to have antifertility properties. The oilcake, left after extracting oil from the seeds, is a richer source of nutrients than farmyard manure and is, therefore, recommended as natural fertilizer.

With the growth of Neem-based industry due to the changing preference for natural fertilizer as pesticide the demand for Neem fruits is expected to increase sharply. India has about 14 million Neem trees with an average yield of about 50 kg of fruits and 350 kg of leaves per mature tree. However, only about 1/3 of the fruits are collected due to operational problems and quality considerations. Of the fruit yield, only about 10% is attributed to seed kernels (after removing the seed coat), and desired biologically active compounds comprise only 10 grams per kilogram of kernel weight.

More than just the availability, the quality of seeds in terms of azadirachtin content and absence of aflatoxin will become a major concern for the Neem-based industry. Neem tree shows wide genetic variation in terms of tree size, morphology, fruit size and fruit production (Srivasuki et al., 1993). The azadirachtin content of seeds also varies considerably within the natural population because of ecological and, possibly, genetic reasons (Ermel, 1995; Wewetzer, 1998). Studies on genetic diversity using AFLP markers have revealed high polymorphism in the assays, indicating a broad genetic base (Singh et al., 1999).

In view of the importance of azadirachtin content in determining the quality of mature seeds, it is desirable to select high yielding trees and produce adequate planting material of plus trees by clonal propagation. Traditionally, Neem is propagated by seeds,

which yields a heterogeneous population. Moreover, the seeds of Neem are of recalcitrant type and start losing viability after 2 weeks. Keeping quality of the seeds is poor due to high moisture (30-35%) and oil (40-45%) content. On the other hand, vegetative propagation of Neem by root suckers, shoot cuttings, air layering and grafting is difficult and not very successful (Dogra and Thapliyal, 1996). In this respect tissue culture can play an important role.

2. TISSUE CULTURE TECHNIQUES

The totipotency of plant cells has already been predicted in 1902 by Haberlandt and the first true plant tissue culture on agar was established. Since then plant tissue culture techniques have greatly evolved. The technique has developed around the concept that a cell has the capacity and ability to develop into a whole organism. The principles involved in plant tissue culture are very simple and primarily an attempt, whereby an explants can be to some extent freed from inter-organ, inter- tissue and inter-cellular interactions and subjected to direct experimental control. Brief overviews of three tissue culture techniques, involved in rapid multiplication, are presented below.

2.1. Axillary Bud Proliferation

Shoot proliferation from axillary buds is desirable where generation of true-to- type or clonal plants is of interest, like in case of propagation of plus trees of Neem. The best explant for this purpose is the nodal segments. Micro propagation by induction of shoots from pre-existing meristems is a most popular approach to clonal propagation of plants because the cells of the shoots-apex are uniformly diploid and least susceptible to genotypic changes under culture conditions. Thus, it guarantees that the characteristics of source plant are conserved (Rao and Venkateswara, 1985). It offers many advantages over the conventional methods of vegetative propagation: 1) The rate of multiplication is extremely rapid and can continue round the year, independent of the season. Thus, over a million plants can be produced in a year starting from a small piece of tissue. 2) The enhanced rate of multiplication can considerably reduce the period between the selections of plus trees and raising enough planting material for field trials. Consequently, many workers have attempted clonal propagation of Neem using a variety of explants, like nodal segments (Arora et al., 2010), apical buds, axillary buds (Quraishi et al., 2004).

2.2. Adventitious Shoot Proliferation

Adventitious shoot proliferation in cell and tissue cultures, in response to hormonal manipulation of the culture medium, require *de novo* differentiation of meristematic region, randomly, all over the tissue other than the pre-existing meristem. Thus, it may cause a change in ploidy due to chimera formation, hence, less desirable in contrast to

clonal propagation by axillary shoot proliferation. It is a multistep process and a series of intracellular events, collectively called induction that occurs before the appearance of morphologically recognizable organs. Various explants like leaf, cotyledon, embryo and root have been tried with different media combinations by the scientists to obtain adventitious shoot proliferation in various species. Available reports on adventitious shoot proliferation in Neem pertain to regeneration from leaf discs (Singh and Chaturvedi, 2009, Arora et al., 2010), leaf tip (Salvi et al., 2001; Abubacker and Alagumanian, 1999), roots (Salvi et al., 2001) and immature and mature zygotic embryos (Chaturvedi et al., 2004).

3. SOMATIC EMBRYOGENESIS

In tissue cultures, plant regeneration via somatic embryogenesis may offer many advantages over organogenesis, such as single cell origin, possibility to automate large scale production of embryos in bioreactors and their field planting as synthetic seeds. Moreover, the bipolar nature of embryos allows their direct development into plantlets without the need of a rooting stage as required for plant regeneration via organogenesis (Bhojwani and Razdan, 1996). It can also be used for the production of metabolites in species where embryos are the reservoir of important biochemical compounds. Furthermore, epidermal single cell origins of embryos favor the use of this process for plant transformation. This prompted many scientists to achieve regeneration via somatic embryogenesis using various explants.

Despite noteworthy success in various important Indian trees, somatic embryogenesis in Neem is still in its infancy. The optimum requirements at various stages of somatic embryogenesis like, induction, proliferation, maturation, germination and establishment in the soil, are not yet determined. Development of high frequency embryogenic cultures, synchronization of embryogenesis and conversion of embryos into complete plantlets, remain the challenge for Neem tissue culturists. In this chapter, somatic embryogenesis in Neem will be discussed in details by highlighting achievements, incurring problems and its future significance.

3.1. Initiation and Establishment of Somatic Embryogenesis in Neem

The ability to produce morphologically and developmentally normal embryos, and whole plants from undifferentiated somatic cells in culture, through the process of somatic embryogenesis, resides uniquely within the plant kingdom. After the initial description of somatic embryo induction from carrot callus cells Steward et al. (1958) this unique developmental potential has been recognized both as an important pathway for the regeneration of plants from cell culture systems and as a potential model for studying early regulatory and morphogenetic events in plant embryogenesis. Consequently, last 10-20 years have witnessed a rapid increase in the number of species that can now be regenerated from cell culture into whole plants through somatic

embryogenesis. For many recalcitrant species, like cereals, cotton and pines, the embryonal tissues are the most suitable explants to establish somatic embryogenesis because of their juvenile nature (Akhtar et al., 2000). Since the development of somatic embryos closely resembles that of zygotic embryos, both morphologically and temporally, immature zygotic embryos could be utilized to raise rare hybrids, which are normally lost due to post zygotic sexual incompatibility (Bhojwani and Razdan, 1996).To obtain somatic embryogenesis in Neem, various explants have been employed and the most common being the juvenile material, such as excised cotyledons, hypocotyls or immature embryos at various stages of development (Table 1).

Table 1: Summary of somatic embryogenesis in *Azadirachta indica* A. Juss.

Sl. No.	Explant	A/J	Medium		% Embryo Germinated	Monopolar/ Bipolar Germination	References
			Induction	Maturation/Germination			
1	Cotyledons	J	B5 + 4.4 μM BAP	1. B5 semisolid medium devoid of growth regulators. 2. 2. B5 liquid medium devoid of growth regulators	?	Monopolar (R)	Muralidharan and Mascarenhas, 1989
2	Cotyledons	J	MS (with 5% sucrose) + 4.4 μM BAP + 2.9 μM IAA + 1000 mgl^{-1} CH	1. MS (with 5% sucrose) + 8.8 μM BAP + 2.9 μM IAA 2. ½ MS (with 5% sucrose)	60-70%	Monopolar (Sh)	Shrikhande et al., 1993
3	Cotyledons	J	MS + 8 μM NAA	MS + 0.9 μM BAP + 0.03 μM GA_3	11.7%	Monopolar (Sh)	Islam et al., 1993
4	Cotyledons	J	1. MS (with 5% sucrose) + 4.4 μM BAP + 2.7 μM NAA + 1000 mgl^{-1} CH	1. MS (with 5% sucrose) + 8.8 μM BAP + 2.7 μM IAA 2. ½ MS (with 1% sucrose)	?	?	Su et al., 1997
	Hypocotyl	J	2. MS (with 5% sucrose) + 4.4 μM BAP+ 2.7 μM IAA + 1000 mgl^{-1} CH				

			3. MS (with 10 % sucrose) + 0.9 µM Zeatin +1000 mgl^{-1} CH				
5	Cotyledons	J	1. MS + 10 µM TDZ 2. MS + 20 µM TDZ	MS basal	60-70%	?	Murthy and Saxena, 1998
6	Cotyledons Hypocotyl Epicotyl Cotyledona-ry node Leaf	J		MS basal	?	?	Gairi and Rashid, 2004
7	Leaf	J	MS + 2.3-4.5 µM TDZ + 0.5 µM 2,4-D	MS + BAP + GA_3	10-15%	Bipolar	Akula et al., 2003
	Nodes	J	MS basal	MS + BAP + GA_3			
	Roots	J	MS basal	MS + BAP + GA_3			
8	Immature zygotic embroys	A	1. MS + 5 µM BAP 2. MS + 5 µM BAP + 1 µM 2,4-D	MS basal	3%	Monopolar (R)	Chaturvedi et al., 2004b
9	Immature zygotic embryos	A	1. MS + 1.11 µM BAP + 4.52 µM 2,4-D 2. MS + 1.11 µM BAP + 0.45 µM 2,4-D	½ MS (with 2% sucrose) + 0.94 µM ABA	64.2%	Bipolar	Rout, 2005
10	Leaflets	A	MS + 6.9 µM kinetin + 8.6 µM IAA	1. MS + 6.9 µM kinetin + 8.6 µM IAA2. ½ MS	82%	Bipolar	Shekhawat et al., 2009

A = Explant from adult plant; J = Explant from juvenile plants; Sh = Shoot; R = Root; ? = Not mentioned in report

4.2. Embryogenic Tissues

The first attempt to induce somatic embryogenesis in Neem was made by Muralidharan and Mascarenhas (1989**)** where embryo-like structures from cotyledon was obtained. On B5 (Gamborg et al., 1968**)** medium containing 4.4 μ M 6- benzylaminopurine (BAP), the explants formed nodular structures, which appeared bipolar. These structures did not show germination if left on the same medium. Upon transfer to hormone-free B5 semi-solid medium, the nodular structures elongated and leafy appendages developed. However, on hormone-free B5 liquid medium, a tap root with leaf like structures developed. No studies were conducted to confirm the embryonic nature of the bipolar structures. Shrikhande et al. (1993) used immature cotyledons to test the effect of different plant growth regulators and carbohydrates on embryo induction. The best somatic embryo induction was obtained on MS (Murashige and Skoog, 1962) medium containing 5% sucrose, 4.4 μ M BAP, 2.9 μ M indole-3-acetic acid (IAA) and 1000 mgl-l Casein Hydrolysate (CH). Although these authors have furnished histological data in support of embryogenesis, the structures labelled as somatic embryos appear more like shoots, with their vascular traces running into the callus. None of the structures resembles a bipolar embryo with closed radicular and plumular poles. Moreover, on germination medium the so called "embryos" developed only a shoot, which had to be transferred to hormone-free ½ MS medium for rooting. Most surprisingly, the authors have described mature embryos with suspensor. Mature embryos generally lack a suspensor and at no stage can the suspensor be seen in macrophotographs as depicted in this paper. Islam et al. (1997) reported very high incidence of embryogenesis in the cultures of cotyledons on MS medium supplemented with 8 μ M α-naphthalene acetic acid (NAA). However, the germination frequency of the embryos, on MS medium containing 0.9 μ M BAP and 0.03 μ M gibberellic acid (GA3), was very poor (maximum 11.7%) and germination was monopolar. The embryos formed only shoots. The first conclusive evidence for induction of somatic embryogenesis in Neem was demonstrated by Su et al. (1997). They were unable to confirm the results of Shrikhande et al. (1993). Under the culture conditions Shrikhande et al. (1993**)** got somatic embryos, Su et al. (1997) observed only shoot-bud formation. The calli initiated from cotyledons or hypocotyls on MS medium supplemented with 4.4 μ M BAP, 2.7 μ M NAA, 1000 mgl-1 CH and 5% sucrose, differentiated greenish globular detachable structures only when transferred to liquid medium where NAA was replaced by 2.7 μ M IAA. In further subcultures on the same medium, the globular structures differentiated shoots and roots. However, if transferred to MS (5% sucrose) supplemented with only 1000 mgl-1 CH the globular structures produced embryos on the spherical head. Maximum embryo differentiation (73%) occurred on MS (10% sucrose) + 0.9 μ M Zeatin. In this case, germination of embryos occurred on ½ MS medium (major inorganic salts reduced to half strength) containing 1% Sucrose. Murthy and Saxena (1998) observed somatic embryo formation in the cultures of mature seeds directly as well as via callusing.

The cotyledonary callus differentiated nodular structures in suspension cultures on MS medium with or without Thiadiazuron (TDZ; 1 μ M). When planted on hormone-free, semi-solid medium, these structures differentiated embryos, which germinated (60-70%) on the same medium. Gairi and Rashid (2004) reported direct differentiation of somatic embryos from different regions of hypocotyl, epicotyl, cotyledonary-node, cotyledons and leaves of intact seedlings of Neem on MS medium containing 1 μ M TDZ. Individual embryos on transfer to hormone-free medium regenerated readily into plantlets.

The developmental stage of embryo at the time of culture is an important determinant of their morphogenic response (DeMarch et al., 1993). To identify the most responsive stage of embryos for morphogenesis, Chaturvedi et al. (2004) cultured immature embryos of Neem at different stages of development- globular, heart-shape, torpedo-shape and early dicotyledonous stage (early dicotyledonous embryos were 2.5 times smaller than fully developed dicotyledonous embryos) (Figure 2A) on MS medium supplemented with a range of growth regulators. The morphogenic response varied considerably with the stage of embryo at culture. Globular and heart-shape embryos generally turned brown without showing any notable change. The older embryos germinated, formed calli or differentiated into three types of organized structures: shoots, somatic embryos and neomorphs (abnormal structures with varied morphology) (Figure 2B). Often the same explant differentiated more than one kind of regenerants. Maximum somatic embryogenesis and shoot-bud differentiation occurred directly from the explants on MS + 5 μ M BAP medium, and the most responsive embryo stage was found to be early dicotyledonous, followed by torpedo stage. The former showed differentiation of shoots and somatic embryos at higher frequency (57%) (Figure 2C). In the combined presence of 5 μ M BAP and 1 μ M 2,4-D in MS medium, early dicotyledonous embryos showed a fairly high degree (50%) of somatic embryogenesis and neomorph formation from the callused explants (Figure 2D). However, regeneration involving a callus phase runs the risk of introducing variability due to genetic instability of the callus cells. Therefore, only directly differentiated regenerants were utilized by them for micropropagation. Irrespective of the treatment and the stage of zygotic embryo, somatic embryos exhibited considerable morphological abnormalities, such as pluricotyledony (Figure 3A, i), fusion of cotyledons (Figure 3A, ii) and absence of cotyledons (Figure 3A, iii). The occurrence of normal dicotyledonous embryos was extremely rare. The scanning electron microscope (SEM) images of somatic embryo were recorded to study the ontogenic development of somatic embryos (Figure 4A) and their morphogenesis from globular (Figure 4B), early heart (Figure 4C) to the torpedo stage (Figure 4D). On MS basal medium, only 3% of the embryos developed a long tap root within 4 weeks (Figure 3B) but plumular shoots were not observed.

The histological examination of somatic embryos showed a poorly differentiated radicular end, elongated hypocotyl with provascular strands and cotyledons but a distinct plumule was never seen at the junction of the hypocotyl and the cotyledon. Although somatic embryos never germinated to form complete plantlets on any of the

treatments tested, secondary embryogenesis occurred on MS basal medium supplemented with BAP + IAA, or GA_3 + IAA. On MS + 1 μ M BAP + 0.5 μ M IAA, 100% somatic embryos exhibited secondary embryogenesis with a mean number of 14 embryos per primary embryo. In this medium, secondary embryogenesis was preceded by callusing of the primary somatic embryos (Figure 3C).

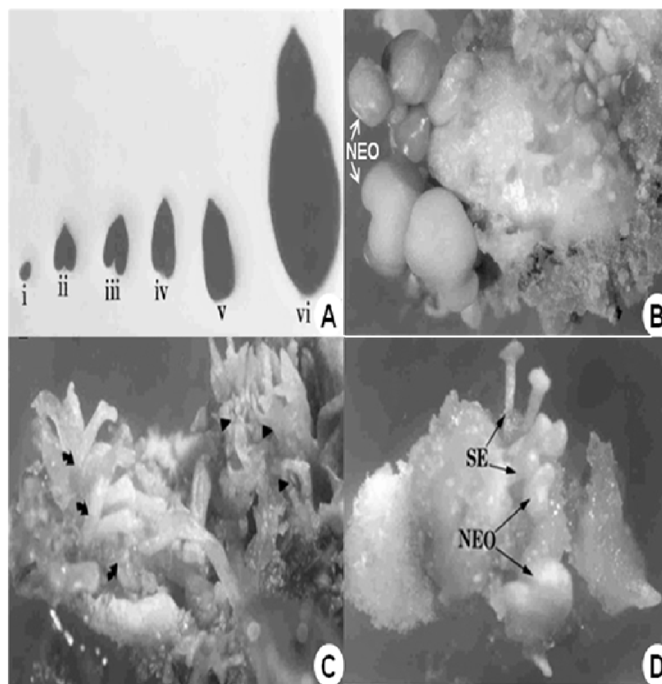

Figure 2: Somatic embryogenesis induction in different growth regulators. A) Different stages of zygotic embryos **i** Globular, **ii** early heart shape, **iii** late heartshape, **iv** torpedo shape, **v** early dicotyledonous, **vi** fully developed dicotyledonous embryo (×7.6). B) 4-week-old culture of a torpedo shape embryo on MS + BAP (5μ M), showing differentiation of neomorphs (NEO) and somatic embryos (SE) at different sites on the same explant (× 8).C) Four-week-old culture of an early dicotyledonous embryo on MS + 5 M BAP, showing differentiation of somatic embryos (arrows) on one side and shoots (arrowheads) on the other side of the explant (×7.5). D) Four-week-old culture of an early dicotyledonous stage embryo on MS + 5M BAP + 1 M 2,4-D showing differentiation of somatic embryos (SE) and neomorphs (NEO) from the callused explant (×8).

However, on 5.0 μ M GA3 + 2.5 μ M IAA, secondary embryos differentiated directly from the primary somatic embryos (Figure 3D, E). Secondary somatic embryos also exhibited morphological abnormalities like primary somatic embryos and failed to germinate on any of the treatments tested. In this context, Merkle et al. (1990) remarked that secondary embryogenesis generally occurs when primary somatic embryos fail to mature normally. Similarly, according to Williams and Maheshwaran (1986), the loss of integrated control results in secondary embryogenesis as budding from the epidermal cells of mature and germinating somatic embryos.

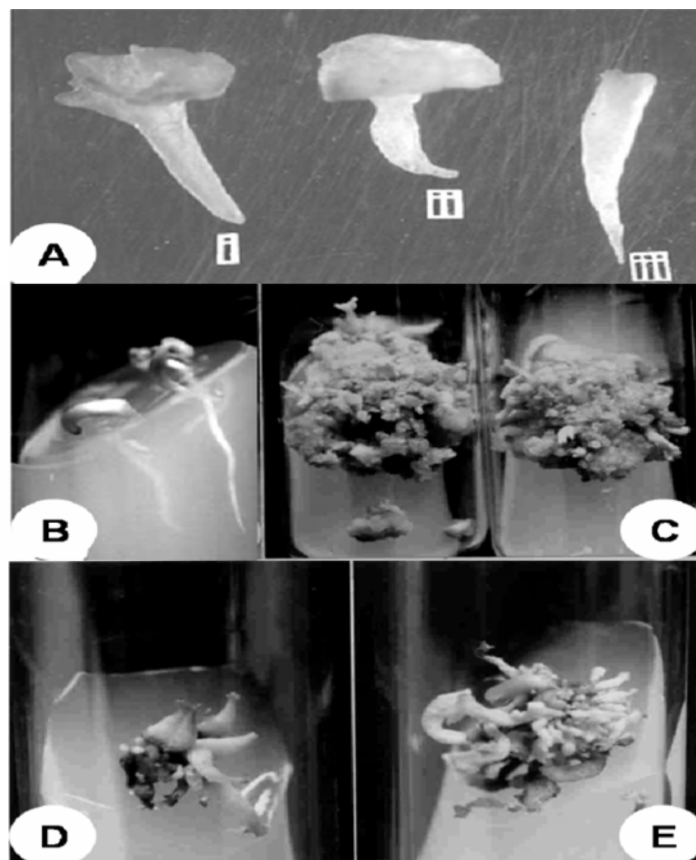

Figure 3: Regeneration and secondardy somatic embryogenesis. A) Somatic embryos excised from cultures showing: **i.** pluricotyledony, **ii.** fusion of cotyledons, and **iii.** absence of cotyledons (×10). B) Somatic embryos cultured on MS basal medium for 4 weeks; very long tap root has developed in both the embryos but the plumular end has not formed a shoot (×1.9). C) Eight-week-old somatic embryos culture on MS + 1 M BAP + 0.5 M IAA, showing callusing of the explant and differentiation of secondary embryos from the callus (×1.3). D, E) Cultures of somatic embryos (5- and 8-week-old) on MS + 5.0 M GA_3 + 2.5 M IAA showing secondary embryogenesis directly without any callus formation (×2.3).

Rout (2005) also reported somatic embryogenesis from immature zygotic embryos that were cultured 40 days after anthesis. Embryogenic callus was proliferated on MS medium supplemented with 1.11 μ M BAP and 4.52 μ M 2,4-D. When these calli were transferred to same medium containing reduced auxin (0.45μ M 2,4-D), numerous embryos proliferated from the surface of callus. Maturation and germination of somatic embryos occurred on half strength MS salts and vitamins supplemented with ABA and 2% sucrose; the maximum percentage (64.2%) of germination was observed with 0.94 μ M ABA within 2 weeks of culture.

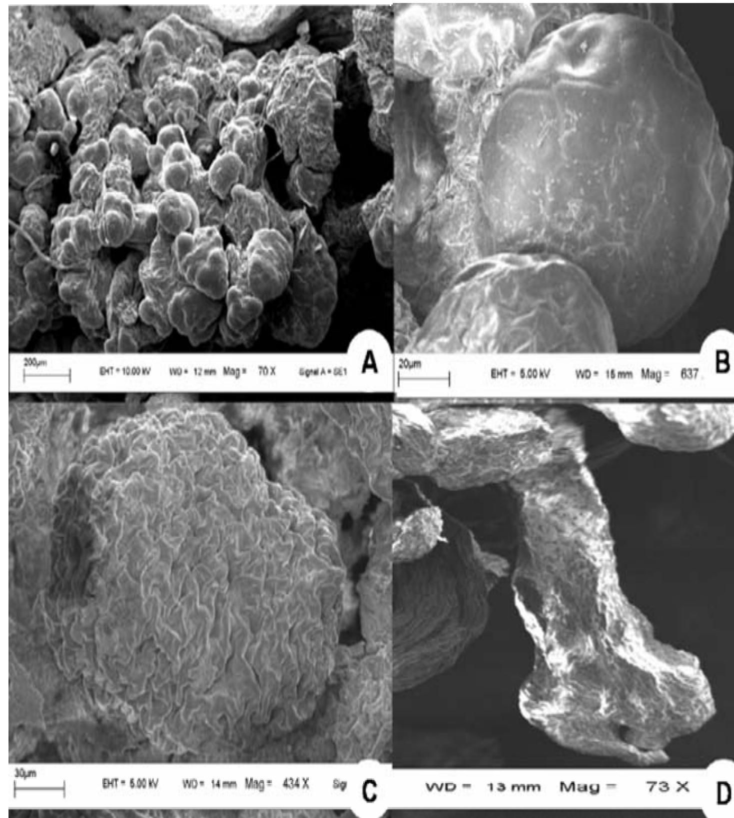

Figure 4: Scanning Electron Microscopy. A) Scanning Electron Micrograph of developing somatic embryos on early-dicotyledonary stage of zygotic embryo. B-D) Scanning Electron Micrographs, showing various developmental stages of somatic embryogenesis, B: globular, C: early heart stage, D: torpedo stage.

4.3. Axenic Cultures

Akula et al. (2003) cultured leaf, single nodal and root explants (1.5-2.5 mm), excised from 1-year-old *in vitro* cultures of seven clones, obtained from different countries China, Zambia, Indonesia, India, Kenya, Mauritius and Thailand, to study somatic embryogenesis. They reported induction of somatic embryogenesis in four out of seven selected clones of Neem. Direct induction of somatic embryogenesis was achieved both from nodal and root segments within eight weeks of culture on MS medium without growth regulators. When these embryos were left on the induction medium, approximately 15% of the somatic embryos developed into whole plantlets after passing through a series of developmental stages. Indirect somatic embryo- genesis via callusing obtained from leaf explants grown on MS medium supplemented with a combination of 2.3-4.5 µ M TDZ and 0.5 µ M 2,4- dichlorophenoxyacetic acid (2,4-D). However, somatic embryos derived from leaf explants showed low conversion rates (< 5%).

4.4. Adult Tissues

Shekhawat et al. (2009) utilized juvenile leaflets (3.5-7.5 cm long) from 6- year-old field grown plants to induce somatic embryogenesis in Neem. Various factors, like auxins, cytokinins, sucrose, inorganic and organic salts, were examined to establish induction and maturation of embryos. Calli induced on MS medium supplemented with 6.9 μ M kinetin and 8.6 μ M IAA were embryogenic which subsequently gave rise to somatic embryos. Maturation of embryos was accomplished on the same medium, after three subcultures. On half strength MS basal medium 82% of the embryos germinated. More than 80% plantlets survived acclimatization.

5. PROBLEMS ASSOCIATED WITH NEEM EMBRYOGENESIS

Though Neem somatic embryogenesis has shown spectacular development in the recent years, the wide spread use of somatic embryos is still not customary due to certain limitations such as (1) asynchronous development of embryos (2) morphological abnormalities (3) poor maturation and (4) low conversion of embryos into plantlets, which have jeopardized the whole tissue culture efforts.

In most of the Neem publications that have described regeneration via somatic embryogenesis, the structures described as embryos do not appear and/or behave like normal mature embryo i.e. with the root-shoot axes and the presence of a hypocotyl and two cotyledons. On germination medium, these embryos showed monopolar germination by giving rise to either shoot (Shrikhande et al., 1993; Islam et al., 1997; Su et al., 1997) or roots (Chaturvedi et al., 2004) but never both. The histological examination revealed the poorly developed vascular strands at the radicular and plumular axes, which resulted in the loss of connection between the two poles all along the length of somatic embryos and, thus, hampering the bipolar germination.

On several treatments, Chaturvedi et al. (2004) observed, green, shining bodies with smooth surface and solid interior differentiated from the zygotic embryo explants (Figure 5A). Whereas some of the neomorphs were spherical with no visible appendages, others appeared like heart shape embryos with an apical notch or foliar protuberance at the tip or arising from inside (Figure 5B-C). In histological sections, the neomorphs appeared to be of epidermal origin and often showed provascular strands (Figure 5D). Occasionally, the neomorphs appeared like a curled cotyledon and developed a shoot from inside (Figure 5E-F). These structures appeared to be similar to the so called "embryos" showing only monopolar shoot development in other reports (Muralidharan and Mascarenhas, 1989; Shrikhande et al., 1993; Islam et al., 1997; Su et al., 1997; Sharma et al., 1999). It was possible by Chaturvedi et al. (2004) to regenerate full plants from most of the neomorphs *via* shoot-bud differentiation on BAP containing medium. Sharma et al. (1999) made similar observations in shoot-tip cultures of Neem. The shoot tips formed globular bodies on MS + 1.11μM BAP + 1.43μM IAA + 135.73 μM Adenine sulphate. In subsequent subcultures on the same medium the globular bodies

differentiated numerous shoot-buds. Some embryo-like structures were also observed but germinable embryos were never formed. Additionally, it is a general observation that embryogenic cultures are never spontaneously synchronized. The heterogeneity in the developmental steps (globular, heart-shaped, torpedo and cotyledonary stages) and eventual morphological heterogeneity is frequently observed at the end of embryo development. Chaturvedi et al. (2004) observed morphological abnormalities, such as pluricotyled-ony fusion of cotyledons and absence of cotyledons in immature zygotic embryo cultures of Neem. Typically, developmental abnormalities such as fasciations and fusion of two or more somatic embryos can occur when cell division in meristematic areas occurs prior to differentiation of the shoot-apex and cotyledons. If cell divisions continue to be stimulated somewhat later when the cotyledonary primordial are initiated, polycotyledonary and fused cotyledons may also result (Ammirato, 1987).

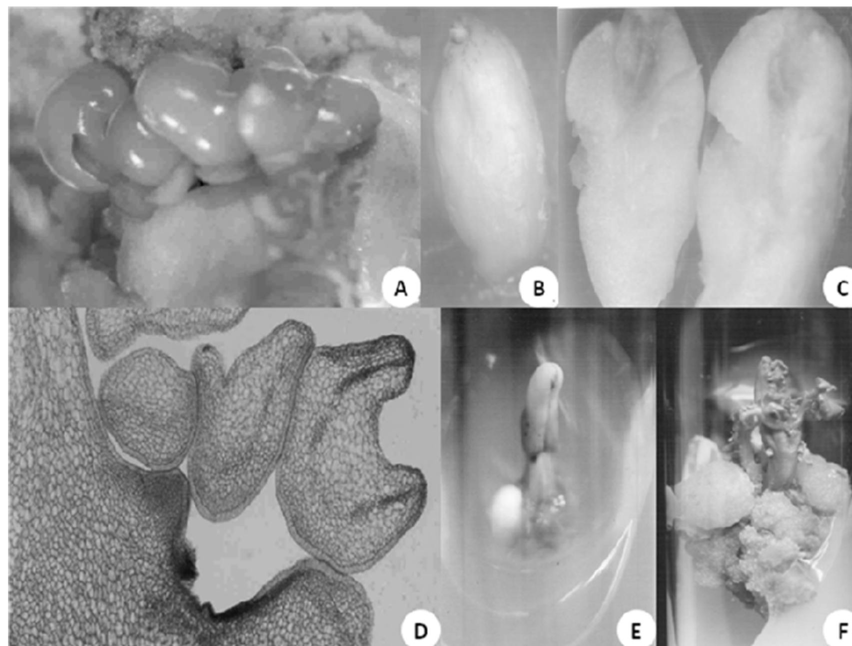

Figure 5: Histological study of somatic embryos. A) 4-week-old culture of a torpedo shape embryo on MS + 2,4-D (5μM), showing direct differentiation of green, shining neomorphs with smooth surface and solid interior. Some of these structures also show cotyledon-like flaps. The portion of the explant in contact with the medium has proliferated into a brownish green calli (× 8). B) An excised neomorph from 4-week-old culture of torpedo shape embryo on MS + 2,4-D (5μM), showing a foliar like protuberance at the tip (× 20). C) Longitudinal halves of B), showing solid central tissue and dark green region towards the apex (× 11). D) Section of a torpedo shape embryo cultured on MS + 2,4-D (5μM), showing epidermal origin of neomorphs of various shapes. These structures are loosely attached to the explant and show provascular strands, a well differentiated epidermis and compactly arranged internal cells (× 115). E) 4-week-old cultures of torpedo shape embryo on MS + BAP (5μM), showing a neomorph like a curled cotyledon (× 1.9). F) Same as E), 3 weeks after transfer to fresh medium, neomorph has elongated and a shoot is emerging from inside. Note the presence of brown callus at the base of the explant (× 2).

Although somatic embryogenesis in Neem has been obtained by many workers, further germination and plant recovery from somatic embryos is not very successful and posing a problem for it's commercial utilization. The maximum percentage of successful conversion of embryos into plantlets is 82% reported by Shekhawat et al. (2009**).** Apart from this other obstacles to normal development of somatic embryos into complete plantlets include precocious germination and secondary embryogenesis (Chaturvedi et al.,2004). For subsequent germination of somatic embryos into complete plantlets, the control of developmental changes is indispensable.

6. EXPLOITATION OF SOMATIC EMBRYOGENIC POTENTIAL FOR IMPROVEMENT OF NEEM

Undoubtedly, somatic embryogenesis is a stable, long term, and highly promising method for rapid and truly clonal propagation of plants, it is also viewed as a valuable tool for genetic transformation, cell line selection and germplasm preservation.

6.1. Genetic Improvement

Neem is a heterogeneous plant showing great amplitude of variation. The wide distribution of Neem and its growth under a variety of climatic conditions sufficiently demonstrate the diversity present within the species. The traditional methods of breeding, including hybridization, can be of very limited use owing to its long reproductive cycles, which include long juvenile periods, and by the complex reproductive characteristics, including self-incompatibility and high degree of heterozygosity. In this context, genetic transformation offers an attractive alternative to breeding because it provides the potential to transfer specific traits into selected genotypes without affecting their desirable genetic background. Recently, these possibilities have become reality, mainly because of advances in *in vitro* culture. Among various *in vitro* methods, somatic embryogenesis is a preferred method for genetic transformation as the embryos are bipolar structures with closed vascular system, possessing both a shoot and a root meristem and always assumed to have a single cell origin.

Genetic transformation in Neem was first attempted by Naina et al. (1989), who used *Agrobacterium tumefaciens* to transform aseptic seedlings. Seedlings were inoculated using *A. tumefaciens* strain K12 × 562 E and K12 × 167, which contained plasmid pCGN 562 and pCGN 167, respectively. Crown galls were formed in 60% seedlings with in 4-5 weeks of culture. The developed shoots synthesized octopine and were kanamycin resistant, suggesting their transformed nature. Later, Allan et al. (2002) established hairy root cultures from aseptic seedlings, stem and leaf explants of Neem and reported the production of azadirachtin, nimbin, salanin and other insect antifeedant compounds from them. Recently, Satdive et al. (2007) observed an enhanced yield of azadirachtin in hairy root cultures raised from aseptic seedlings, in the presence of biotic elicitors.

In majority of above cases seedlings were used for introduction of foreign genes, which is less attractive than transforming somatic embryos because passage through a sexual stage resulted in a drastic reshuffling of the genome and the performance of the transgenic seedlings would be unknown. Moreover, the plant transformation techniques do not transform all cells. Consequently, the plants regenerated from transformed tissues via organogenesis are often chimeras (Krastanova et al., 1995) where the group of cells contributes towards organ development. Whereas, somatic embryos arise from single cells (Toonen and de Vries, 1996) and, for this reason, regeneration via somatic embryogenesis reduces the formation of chimeras. Nevertheless, somatic embryogenesis seems to produce fewer rates of somaclonal variations compared with organogenesis (Heinze and Schmidt, 1995).

6.2. Germplasm Conservation

The storage of seeds in dry and cold conditions offers a convenient method for long-term storage of the germplasm. However, the seeds of Neem cannot be stored at low temperatures or have limited tolerance to desiccation. Somatic embryogenesis provides a potential solution for these problems, for example by maintaining long- term embryogenic calli. However, maintenance of cell cultures by subculture is time consuming and expensive, and involves the risk of loss of valuable materials through contaminations or human or technical errors. Additionally, long-term cell cultures include the risk of genetic changes by somaclonal variation and a decrease in plant regeneration capacity (Fitch and Moore, 1993**).** The frequent initiation of new embryogenic cell cultures can reduce the adverse effects on genetic fidelity and regeneration potential. However, initiation of fresh cultures of Neem is possible from juvenile tissues only during short periods of the year which favours best plant growth.

Cryopreservation (storage at -196°C in liquid nitrogen) of embryogenic cell cultures, or somatic embryos is an efficient alternative to cell culture maintenance (Kartha, 1987). It minimizes the necessity of the establishment and maintenance of the embryogenic cell cultures, reducing manipulations, somaclonal variation, contamination and risks of loss. Moreover, cryopreservation is highly lucrative in comparison to other *in vitro* conservation strategies. As somatic embryogenesis is still difficult to achieve in material beyond the juvenile stage, cryoconservation preclude genotypes from ageing during the whole selection stage of field-tested, clonally propagated seed progenies.

6.3. Cell Selection

Cell selection consists of regeneration of plants from cell population combined with the selection of desirable genetic characteristics. In order to avoid the production of genetic chimeras, it is necessary to use a plant regeneration system able to regenerate plants from single selected cells. Somatic embryogenesis allows regeneration of whole plants from single cells (Toonen and de Vries, 1996), making possible its use in cell selection programs.

Conclusions

During the past decades, concerted efforts have been made to understand the phenomenon of somatic embryogenesis and success has been achieved to some extent. But, the full potential of somatic embryogenesis as a mean of propagating Neem tree is limited by the fact that in maximum studies it was induced from embryonic and juvenile explants, which is not a preferred explant for true-to-type cloning of phenotypically superior trees. Therefore, further research need to be aimed at screening of totipotent cells from mature donor explants of elite Neem trees. Moreover, maturation of somatic embryos has always been a problem in Neem species which have to be overcome to make it a viable method of propagation. More basic research is needed to understand factors affecting induction and regeneration of somatic embryos. The fundamental question of growth, differentiation and development of somatic embryos and different factors responsible for various embryonic growth patterns in Neem need to be answered by continuous investigation in this area. Another future concern is whether the plant originating from somatic embryogenesis genetically identical to the mother plant originally selected, i.e. not affected by any somaclonal variation problems. Furthermore, serious efforts are needed to unravel the molecular and biochemical changes taking place during Neem embryogenesis and to the best of our knowledge, no efforts have been addressed in this regard in Neem. This is probably one of the most promising lines of research for the coming years.

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