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BRIEF COMMUNICATION

Role of phytohormones and nitrogen in somatic embryogenesis induction in cell culture derived from leaflets of *Azadirachta indica*

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Abstract

A protocol for somatic embryogenesis in *Azadirachta indica* A Juss. has been standardized using *in vivo* leaflets. Experiments were carried out to examine the effect of various auxins, cytokinins, sucrose, inorganic and organic salts on subsequent somatic embryo induction and maturation. Embryogenic calli were induced on Murashige and Skoog (MS) medium supplemented with 1.5 mg dm^{-3} kinetin and 1.5 mg dm^{-3} indole-3-acetic acid and subsequently all the stages of somatic embryo development (globular, cordate, torpedo and cotyledonary) were observed. Maturation of these embryos was accomplished with the same growth regulators after three subcultures. The histological study of somatic embryos showed resembles to zygotic embryos. The matured somatic embryos were transferred onto half strength MS-medium devoid of growth regulators for their germination (82 %). Plantlets were acclimatized in the field with a survival rate of 80 - 83.5 %.

Additional key words: indole-3-acetic acid, kinetin, KNO_3, neem.

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Azadirachta indica A. Juss (neem) is recognized as one of the most valuable arid zone tree because of its ability to grow in nutrient deficient land. Neem produces a large array of biologically active compounds (*e.g.* azadirachtin, nimbin, nimbidine, salanin, margosane, meliacin; Satdive *et al.* 2001) which are highly variable in field condition due to plant open pollination nature (Tiwari 1992). Besides this, viability of seeds is low, plants are susceptible to various pathogens, and variation occurs in leaf morphology, tree size, seed size and seed production. Thus, there is a need for alternative method of multiplication of *A. indica*. There are only few earlier reports on *in vitro* plant regeneration in neem (Thengane *et al*. 1995, Su *et al*. 1997, Murthy and Saxena 1998, Shekhawat *et al*. 2002, Akula *et al*. 2003, Srivastava *et al*. 2009). The aim of present research was to establish a viable method for rapid multiplication of *A. indica* through somatic embryogenesis from leaflet culture.

Fresh, juvenile leaflets (3.5 - 7.5 cm long) were picked directly from 6-year-old neem tree planted near the Aravali hills, Rajasthan. The tree with best growth, morphology and highest leaf chlorophyll, sugar and protein contents was selected. The leaflets were washed with 2 % (m/v) *Teepol* for 10 min and rinsed thrice with distilled water, disinfected with 0.1 % (m/v) aqueous $HgCl_2$ for 5 - 7 min and again rinsed four times with sterile distilled water. Thereafter sterilized leaflets cut into small segments (approximate 0.5 cm) were aseptically inoculated on Murashige and Skoog (1962; MS) medium augmented with indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene-acetic acid (NAA) and indole-3-butyric acid (IBA) alone or in combination with benzylaminopurine (BAP) or kinetin (KIN) for embryogenic callus induction. Optimum embryogenic response was obtained on MS + 1.5 mg dm^{-3} kinetin and 1.5 mg dm^{-3} indole-3-acetic acid. The embryogenic calli (200 mg fresh mass) were sub-cultured at 3 - 4 week intervals. The maturation of somatic embryos were achieved on the same media and plant growth regulators, and germination of these somatic

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; BAP - N^6-benzylaminopurine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; KIN - kinetin; MS - Murashige and Skoog medium; NAA - naphthaleneacetic acid.
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embryos were obtained on ½ MS medium without any plant growth regulators. Different concentrations of sucrose (1.0 - 5.0 %), KNO_3 and $(NH_4)_2SO_4$ (50 - 300 mg dm^{-3}) were tested for their ability to stimulate somatic embryo formation and maturation. Ten explants were taken for each treatment. All cultures were maintained at temperature 25 ± 2 °C, relative humidity of 55 ± 5 % and 16-h photoperiod with irradiance of 20.5 µmol m^{-2} s^{-1}. The regenerated plantlets (5 cm in height) were washed in tap water in order to remove agar from the roots, and hardened in pots containing soil and *Vermiculite* (3:1), at temperature 25 ± 2 ºC and relative humidity 60 - 70 %. The acclimatized plants were transferred to earthen pots containing a mixture of soil, manure and sand (1:1:1), and were finally exposed to the natural environment.

Histological study was performed of developed somatic embryos. Samples were fixed in FAA (formalin : acetic acid : 60 % ethanol, 5:5:90), dehydrated in acetone series (25, 50, 70, 90, 100 %) at 30 min intervals. Wax blocks were prepared, and sectioned at 6 µm on a rotary microtome. Section were stained with safrainin and fast green blue and observed under *Olympus* microscope.

All experiments were repeated thrice and 10 replicates per treatment were considered. Somatic embryogenesis induction rates were recorded 4 weeks after culture initiation, and maturation and germination rates 2 weeks after subculture. Statistical analysis was performed by submitting transformed data to one way analysis of variance (*ANOVA*) and the significantly different means were selected by using Student's *t*-test ($P = 0.05$).

The leaflets developed embryogenic calli within three weeks after inoculation on MS medium supplemented with various concentrations of IAA and KIN. The use of cytokinins has generally given conflicting results; although zeatin and 2,4-D enhanced somatic embryogenesis in *Arachis hypogaea,* the addition of other cytokinins (benzylaminopurine or kinetin) decreased the embryogenic response (Eapen and George 1993). Initially callus was green but later it turned brown. After regular sub-culturing at 3 - 4 week intervals on fresh medium containing IAA (1.5 mg dm^{-3}) + KIN (1.5 mg dm^{-3}) + 3 % sucrose, numerous globular structures developed on the surface of the callus (Fig. 1*A*). Media containing higher (>1.5 mg dm^{-3}) and lower (<1.0 mg dm^{-3}) IAA and KIN concentrations produced fewer embryos (Fig. 2). Somatic embryos were 3 - 4 mm long and were characterized by the presence of well-developed cotyledons and an elongated hypocotyl. Besides normal embryos, some abnormal embryos were also formed, but they gave rise to aberrant forms. These structures were compact, nodular and creamy-white to pale-yellow. Such abnormalities are also common in somatic embryogenesis of other dicot species (Rout *et al.* 1991, Garin *et al.* 1997).

Sucrose concentration greatly affects the embryo development and germination (Martin *et al.* 2001, Emons *et al.* 1993). Sucrose at 3 % concentration produced the

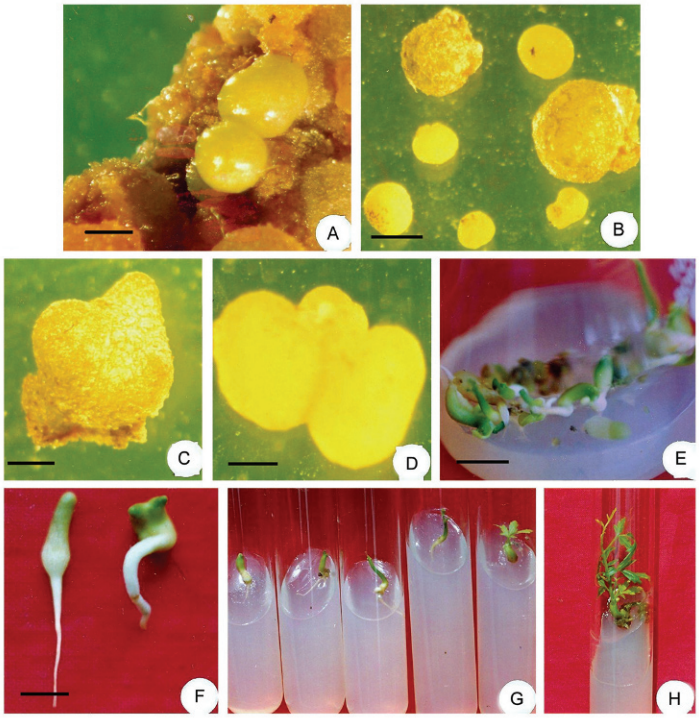

Fig. 1. Different stages of plant regeneration *via* somatic embryogenesis in *Azadirachta indica* A. Juss. *A*. - Embryogenic callus clump showing globular embryos (*bar* = 4.0 mm); *B* - torpedo stage of somatic embryos (*bar* = 2.0 mm); *C* - globular stage (*bar* = 3.5 mm); *D* - cordate stage (*bar* = 4.0 mm); *E* - germinating somatic embryos after 2 - 3 subcultures (*bar* = 6.0 mm); *F* - isolated germinating embryos with elongated roots (*bar* = 6.0 mm); *G,H* - sequential development of plantlets *via* somatic embryo germination.

maximum number of cotyledonary embryos (Table 1). Embryogenesis was inhibited at 4 % or higher concentrations of sucrose in the induction medium, higher concentration of sucrose resulted in browning of the cultures probably due to enhanced production of polyphenols. In contrast to present finding, Cuenca *et al.* (1999) found that higher concentrations of sucrose (up to 8 %) along with abscisic acid improved the percentage of somatic embryogenesis induction in *Quercus robur*. All regular forms of embryos viz., globular (0.2 mm), cordate (0.35 mm), torpedo (0.49 mm) and cotyledonary (0.63 mm) were observed on the same medium (Fig. 1*B-D*).

Table 1. Effect of sucrose concentration on percentage of embryogenic calli and number of somatic embryos of different stages in neem callus cultures (MS medium + 1.5 mg dm^{-3} IAA + 1.5 mg dm^{-3} KIN). At higher concentrations of sucrose browning of calli occurred. Mean ± SE, n = 3. Means followed by the same letters are not significantly different from each other at the 5 % level.

Sucrose [%]	Response [%]	Heart shaped	Torpedo	Cotyledonary
1	28.0	10.0 ± 0.01^{b}	7.2 ± 0.43^{ab}	5.5 ± 0.1^{a}
2	58.2	15.0 ± 0.20^{c}	10.0 ± 0.53^{b}	7.5 ± 0.25^{ab}
3	81.7	30.4 ± 0.44^{e}	20.4 ± 0.48^{d}	10.0 ± 0.53^{b}
4	60.2	10.0 ± 0.53^{b}	6.6 ± 0.24^{a}	4.12 ± 0.27^{a}

Proliferation and maturation of somatic embryos were tested at varying concentrations of nitrate and ammonium-nitrogen (Table 2). The maximum number of somatic embryos was produced on the MS medium containing 50 mg dm^{-3} $(NH_4)_2SO_4$ and 100 mg dm^{-3} KNO_3. Higher concentrations of these chemicals were inhibitory to growth and embryogenesis. The above observation was in agreement with the report of Arora *et al*. (1999).

Histological sections showed the formation of embryogenic tissue and somatic embryos. The embryogenic callus was characterized by small cells with dark stained nuclei. The somatic embryos were observed in the periphery of embryogenic callus at different stage of development. Some proembryos and globular stage embryos showed a suspensor like structure, and no vascular connection between the embryogenic callus. Thus, present investigation supports that ontogeny of *Azadirachta indica* begins when an embryogenic single cell divides to form proembryos. This process resembles zygotic embryogenesis and it is different from other reports on indirect somatic embryogenesis where embryos are formed from proembryogenic masses (*e.g.* Terzi and Loschiavo 1990, Ganesh Kumari *et al.* 2008, Vijaya Kumar *et al.* 2008, Yang *et al.* 2008).

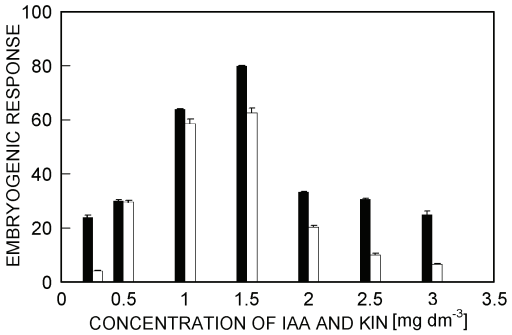

Fig. 2. Effect of IAA and KIN on neem somatic embryogenesis (*full columns* - response percentage, *open columns* - number of somatic embryos) after 21 d of culture.

Conversion of somatic embryos into plantlets is a major problem in tree species (Chengalrayan *et al.* 1994, Akula *et al*. 2003, Mathur *et al*. 2008). The development of complete plantlets was obtained in absence of auxins. Moreover, high endogenous contents of cytokinins may also serve as an enhancer for developmental capabilities of somatic embryos (Wilhelm 2000). In our findings germination of somatic embryos was accomplished on half strength MS medium without growth regulators (Fig. 1*E-H*).

The plantlets (80 - 83.5 %) survived transfer to pots and hardening and final transfer to field conditions. From fresh callus (10.5 g), we produced 3135 somatic embryos, of which 1505 germinated into *in vitro* plantlets and 1255 plantlets were successfully transferred to the soil.

Table 2. Effect of different concentration and combinations of KNO_3 and NH_4SO_4 [mg dm^{-3}] added to MS medium supplemented with IAA(1.5 mg dm^{-3}) and KIN (1.5 mg dm^{-3}) on somatic embryogenesis (response percentage and number of somatic embryos per explant) after 3 - 4 weeks of culture. Means ± SE, n = 3. Means followed by the same letters are not significantly different at 5 % level.

	NH_4SO_4	KNO_3 50	KNO_3 100	KNO_3 200	KNO_3 300
Response [%]	50	86.4 ± 1.1	89.7 ± 0.7	65.7 ± 1.0	54.0 ± 0.2
Number		62.7 ± 1.7^{g}	87.0 ± 0.21^{h}	43.0 ± 0.9^{e}	38.4 ± 0.4^{d}
Response [%]	100	55.0 ± 0.2	65.7 ± 1.0	45.0 ± 0.2	36.6 ± 0.2
Number		58.6 ± 1.8^{g}	46.6 ± 0.5^{ef}	40.4 ± 0.4^{d}	47.2 ± 0.4^{ef}
Response [%]	200	58.2 ± 0.3	44.7 ± 3.4	38.3 ± 0.2	20.2 ± 0.3
Number		48.2 ± 0.4^{f}	43.0 ± 0.9^{e}	49.2 ± 0.2^{f}	44.7 ± 3.4^{e}
Response [%]	300	18.3 ± 0.3	12.6 ± 0.2	8.2 ± 0.4	2.2 ± 0.2
Number		19.2 ± 1.3^{e}	16.3 ± 1.5^{b}	12.4 ± 0.4^{b}	6.2 ± 0.7^{a}

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