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BRIEF COMMUNICATION

In vitro organogenesis and plant regeneration from unpollinated ovary cultures of *Azadirachta indica*

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Abstract

A novel method of organogenesis in neem (*Azadirachta indica* A. Juss.) from unfertilized ovaries is described. The Murashige and Skoog's (MS) medium with 9 % sucrose, 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 5 μM 6-benzylaminopurine (BAP) was the best for callus induction from unfertilized ovaries. However, further proliferation of callus occurred better on MS medium supplemented with 0.5 μM 2,4-D either alone or in combination with 4.5 μM kinetin. Maximum shoot regeneration (78 %) was observed when calli, induced from ovaries of 4 mm size flower buds and proliferating on MS + 0.5 μM 2,4-D, were subcultured to MS medium containing 5 μM BAP. Histological analysis revealed that 4 mm sized flower bud corresponds to a 2-nucleate stage of embryo sac. The shoots were then multiplied by forced axillary branching on MS medium supplemented with 1.0 μM BAP and 250 mg dm^{-3} casein hydrolysate. The shoots could be rooted on ¼ strength MS medium supplemented with 0.5 μM indole-3-butyric acid (IBA) at a frequency of 79 %. Cytological analysis by root tip squash preparations revealed that all the plantlets were diploids. These plants were subsequently hardened and established in soil with transplantation rate of 81.8 %.

Additional key words: adventitious shoot proliferation, auxins, callus culture, casein hydrolysate, cytokinins, embryo sac, histology, ovary culture.

Neem (*Azadirachta indica* A. Juss.), an evergreen tropical tree belonging to the family *Meliaceae*, has many important medicinal, agrochemical and economic uses. Due to highly heterozygous nature, long reproductive cycle and poor seed yield, improvement of neem by conventional methods is very limited. In this respect, tissue culture can play an important role. *In vitro* regeneration of neem from various vegetative tissues was published (Chaturvedi *et al*. 2004a, Rout 2005). Recently, some studies have described shoot regeneration from anthers/microspores (Chaturvedi *et al.* 2003a) and endosperm tissues (Chaturvedi *et al.* 2003b) of adult tree origin.

Even though the main objective of the present study was to obtain haploid plants from the unfertilized ovary, regeneration of diploid plants indicated the potential use of this juvenile unfertilized ovary explant for large scale micropropagation. In contrast, the leaves, nodal and internodal segments from mature trees show recalcitrance due to accumulation of secondary metabolites.

Unfertilized ovaries, obtained from closed flower buds of an adult 54-year-old neem tree, were used as explants. Ovaries were excised from flower buds of four sizes (2, 3, 4 and 5 mm) and the corresponding developmental stage of ovary was determined by paraffin sections. The flower buds were surface sterilized with 0.1 % (m/v) solution $HgCl_2$ for 7 min, followed by three washings in sterile-distilled-water. The ovaries were dissected out with the aid of a stereo-microscope (*Nikon SMZ-645*, Tokyo, Japan). Four ovaries were cultured in

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Abbreviations: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; IBA - indole-3-butyric acid; Kin - kinetin (N^6-furfuryladenine).
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pre-sterilized, disposable Petri plates (55 × 15 mm) containing 10 cm^3 of Murashige and Skoog (1962; MS) medium with or without growth regulators for callus induction. The Petri plates were sealed with parafilm.

For induction and multiplication of callus from unfertilized ovaries, MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 0.5, 1.0 μM) either alone or in combination with kinetin (Kin; 4.5 and 10 μM) or 6-benzylaminopurine (BAP; 5 μM) was used. All the media were tested with 3 or 9 % (m/v) sucrose. The degree of callusing was scored in terms of the diameter of the callus.

Calli proliferated on ovaries from flower buds of various sizes (2, 3, 4 and 5 mm) were subcultured on MS medium supplemented with BAP (5 or 7.5 μM) for shoot regeneration. The small shoots were detached from the callus and transferred to 0.5 μM BAP for elongation. These shoots were multiplied via axillary shoot proliferation on MS medium supplemented with 1 μM BAP and 250 - 2000 mg dm^{-3} casein hydrolysate (*HiMedia Laboratories*, Mumbai, India) following the procedure of Chaturvedi *et al*. (2004b). For rooting, terminal portions of elongated shoots, measuring about 4 cm in height with 3 - 4 nodes, were excised and cultured on MS with full, half or quarter strength of the major salts and 0.5 μM indole-3-butyric acid (IBA).

Cultures for shoot regeneration, multiplication and rooting were grown in 150 × 25 mm glass tubes (*Borosil*, Mumbai, India), each containing 20 cm^3 of medium. Unless mentioned otherwise, all media contained 3 % (m/v) sucrose and were solidified with 0.8 % (m/v) agar (*Qualigens*, Mumbai, India). The pH of the medium was adjusted to 5.8 before autoclaving at 104 kPa and 121 °C for 15 min. All the cultures were maintained at temperature of 25 ± 2 °C, relative humidity 50 - 60 %, 16-h photoperiod and irradiance of 50 μmol m^{-2} s^{-1}. 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.

For hardening, the rooted plants were washed to remove the agar, transferred to *Soilrite* (*Chowgule Industries*, Delhi, India) in hycotrays (*Sigma*, St. Louis, USA) and placed in a glasshouse under high humidity. After 4 weeks, the plants were transferred to soil in polythene bags and sprayed with a mixture (1:1) of 0.1 % urea and fungicide *Bavistin* (*BASS*, Ahemdabad, India). After another 4 weeks, the plants were transferred to pots and shifted to a polyhouse. After 3 months of transplantation, the plants were shifted to a shaded area under natural conditions.

For histological analysis, regenerating calli and unfertilized ovaries (at different developmental stages) were sampled and fixed in FAA (5:5:90 v/v/v formalin : acetic acid : 70 % ethanol) for 48 h and stored in 70 % alcohol. The material was passed through the tertiary-butylalcohol (TBA) series for dehydration, infiltrated with paraffin wax (melting point 60 °C, *E. Merck*, Darmstadt, Germany), embedded in pure paraffin wax and 8 - 10 μm thick sections were cut using *Spencer* rotary microtome (Delhi, India). The sections were mounted on microslides, dewaxed and double stained with 1 % safranin and 1 % astra-blue.

For cytological analysis, healthy root tips were pre-treted with 0.02 % 8-hydroxyquinoline (*BDH,* Mumbai, India) at 4 °C for 4 h, and fixed in a modified Carnoy's fluid containing absolute alcohol:chloroform:glacial acetic acid:methanol (7:3:1:1 v/v/v/v) for 48 h. For squash preparation, the root tips were placed in a mixture of nine drops of 2 % aceto-orcein and one drop of 1 M HCl in a glass slide and heated gently. The slides were observed under a *Nikon* photomicroscope, and the cells showing a good separation of chromosomes were studied.

Unfertilized ovaries from 2, 3, 4 and 5 mm sized flower buds were cultured on various media. Though most of the treatments showed ovary enlargement to various extents, further response was not observed. The percentage of ovaries callused and the degree of callusing varied with the sucrose concentration in the medium and 9 % sucrose was significantly better than 3 % sucrose. Of the various hormonal treatments tested, MS medium with 9 % sucrose containing 1 μM 2,4-D and 5 μM BAP induced callusing in 100 % of the cultures. The callus growth was profuse (larger than 15 × 15 mm) in the ovaries excised from 3 and 4 mm flower buds while only moderate from others. On this medium, ovaries enlarged after 4 weeks (Fig. 1*A*) and were completely covered with the light brown and friable callus by the end of 8th week (Fig. 1*B*).

Irrespective of the stage of ovary at culture, none of the primary cultures exhibited any kind of organogenesis. Moreover, in subcultures on the original media, the calli did not show sustained growth. The presence of 2,4-D alone or in combination with kinetin in the MS medium proved better for further callus multiplication. On MS + 0.5 μM 2,4-D medium, 90 % of the cultures supported sustained and good callus growth within 8 weeks (Fig. 1*C*) as compared to 75 % response on MS + 0.5 μM 2,4-D + 4.5 μM Kin.

To achieve shoot regeneration, calli from above mentioned two sources were subcultured on MS basal medium or the same supplemented with either 5 or 7.5 μM BAP. No differentiation occurred on MS basal medium, but shoot-bud differentiation was induced at both the BAP concentrations (Table 1).

The calli from ovaries of 4 mm flower buds, grown on MS + 0.5 μM 2,4-D, showed maximum regeneration on MS + 5 μM BAP. 78 % of these cultures differentiated an average of 11.9 shoots per culture (Table 1). Compared to this, the calli from ovaries of 3 mm flower buds, multiplied on MS + 0.5 μM 2,4-D + 4.5 μM kinetin, exhibited shoot regeneration in 75 % cultures on MS + 7.5 μM BAP with an average of 3.1 shoots per culture

(Table 1). Thus, the optimum concentration of BAP for mature ovary explants in the shoot regeneration medium is 5.0 μM while that for young ovary explants is 7.5 μM.

On the regeneration media, the calli first turned brown, but after 7 weeks green nodulated structures appeared. After 8 weeks, distinct shoot buds were differentiated from nodules on this medium (Fig. 1*D*). Histological sections through nodulated calli revealed the presence of nests of vascular elements surrounded by compact cambium like cells. The vascularized nests were surrounded by loosely arranged empty cells (Fig. 1*E*). Although the callus could be multiplied on the callus

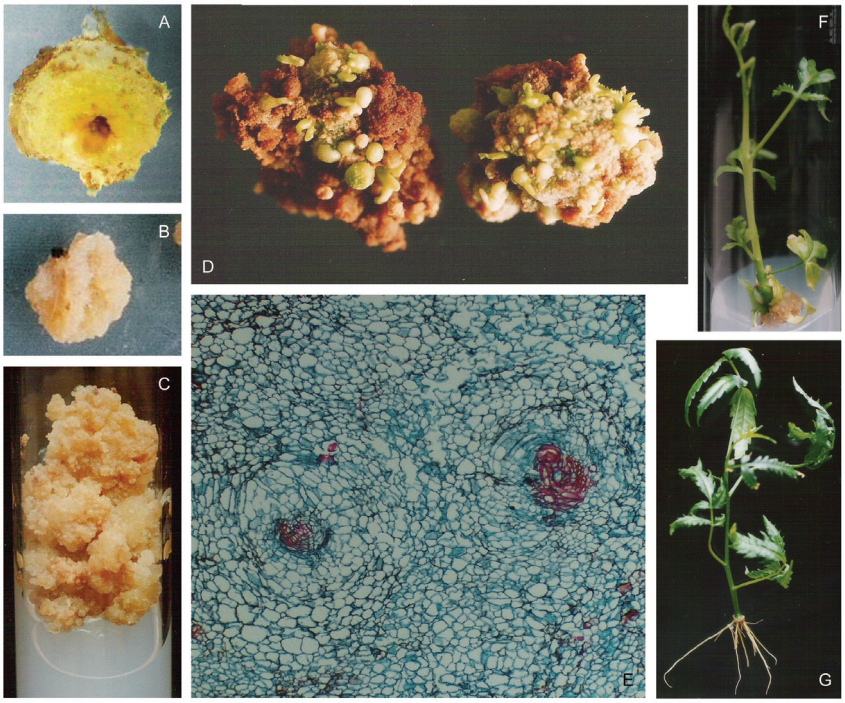

Fig. 1. *A* - the ovary from 4 mm flower bud on MS + 9 % sucrose + 1 μM 2,4-D + 5 μM BAP, showing enlargement after 4 weeks (× 2.2); *B* - the same ovary after 8 weeks, showing the proliferation of light brown and friable callus (× 2.2); *C* - 8-week-old ovary callus after subculture on MS + 0.5 μM 2,4-D, showing good growth (× 2.2); *D* - the callus from MS + 0.5 μM 2,4-D, 8 weeks after transfer to MS + 5 μM BAP, showing differentiation of a large number of shoot buds from nodulated regions (× 2.6); *E* - section of a 7-week-old regenerating callus from MS + 5 μM BAP, showing nests of vascular elements; whereas the internal tissue is compact, the outer tissue comprises loosely arranged empty cells (× 990); *F* - a single node segment from an elongated shoot**,** 3 weeks after transfer to MS + 1 μM BAP + 250 mg dm^{-3} casein hydrolysate. The explant has developed a long solitary shoot with 6 - 7 nodes. The basal end of the explant has callused (× 1.7). *G* - the same shoot 4 weeks after transfer to ¼ MS + 0.5 μM IBA. Healthy and branched roots have developed directly from the basal cut end (× 1.9).

Table 1. Effect of the stage of ovary (excised from various sized flower buds) and the callus multiplication medium (MS with 2,4-D or MS with 2,4-D and Kin) on shoot-bud differentiation in a medium containing MS + BAP (5 or 7.5 μM). Callus was induced from unfertilized ovaries on MS (9 % sucrose) + 1 μM 2,4-D + 5 μM BAP and further proliferated on multiplication medium. Means ± SE; growth period 8 weeks.

Callus multiplication	Flower bud size [mm]	Shoot regeneration [%]		Number of shoots [culture^{-1}]	
		5 μM BAP	7.5 μM BAP	5 μM BAP	7.5 μM BAP
MS	2	0	0	0	0
+ 0.5 μM 2,4-D	3	50.0 ± 1.0	10.0 ± 1.0	11.0 ± 2.0	1.0 ± 0.0
	4	78.0 ± 3.0	12.0 ± 1.0	11.9 ± 2.0	1.0 ± 0.2
	5	76.0 ± 5.0	33.0 ± 2.0	5.6 ± 2.0	1.0 ± 0.0
MS	2	0	0	0	0
+ 0.5 μM 2,4-D	3	25.0 ± 2.0	75.0 ± 4.0	1.0 ± 0.0	3.1 ± 0.2
+ 4.5 μM Kin	4	66.0 ± 2.0	70.0 ± 5.0	2.7 ± 0.5	3.8 ± 0.2
	5	0	0	0	0

maintenance medium for two years, the regeneration potential of the callus declined with age and was completely lost after 6 subcultures of 8 weeks each.

The shoots, regenerated on ovary callus, did not grow beyond 0.5 cm on the regeneration medium. Therefore, these shoots were detached from the callus and transferred individually to MS medium supplemented with only 0.5 µM BAP for elongation. On this medium, 82 % of the cultures attained a height of 3 cm, with each shoot having an average of 4 nodes after 3 weeks. The shoots were multiplied through axillary shoot proliferation and the best multiplication was achieved on MS medium supplemented with 1 µM BAP and 250 mg dm^{-3} casein hydrolysate at a rate of seven fold every 3 weeks. More than 88 % cultures developed shoots that were on average 7.5 cm long after 3 weeks (Fig. 1*F*).

Terminal 4 cm long portions of shoots from 3-week-old cultures on MS + 1 µM BAP + 250 mg dm^{-3} casein hydrolysate were used for rooting. The remaining portion of the shoots were cut into single node segments and utilized for further multiplication. For rooting, ¼ MS medium supplemented with 0.5 µM IBA was found to be the best. This medium supported direct rooting at the base of the shoot without an intervening callus phase. 79 % of the shoots were rooted with 5 - 6 roots per shoot after 4 weeks (Fig. 1*G*) on this medium. The plantlets were successfully established in soil with 81.8 % transplantation survival.

Histological studies revealed that the ovary from 2 mm flower bud contained immature ovule at the integumentary primordia stage (Fig. 2*A*). The megaspore-mother-cell was formed in the ovary from 3 mm size flower bud. The megaspore-mother-cell was relatively small and was filled with the cytoplasm (Fig. 2*B*). Observation throughout the studies revealed that the ovary is at the best stage for callus induction at 2-nucleate-stage of embryo sac, which corresponds to 4 mm size flower bud (Fig. 2*C*). The 4-nucleate embryo sac was present in the ovary from 5 mm size flower bud (Fig. 2*D*).

The uniformity of the plants was confirmed by cytological analysis. Mitotic preparations were made from root-tips of ten plants regenerated from ovary callus cultures. All the ten plants showed diploid chromosome number (2n=2x=24).

The present study, for the first time reported the feasibility of utilizing unfertilized ovary for large scale micropropagation in neem. Plant regeneration *via* ovary culture has also been reported in several plants such as lily (Van Tuyl *et al*. 1991), sweet potato (Ruth *et al*.

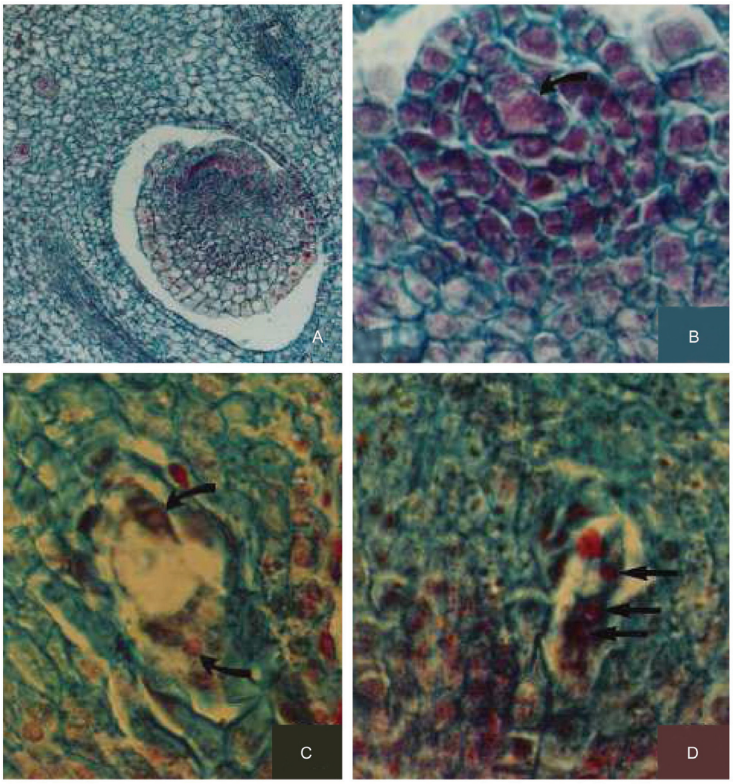

Fig. 2. *A* - a section of an ovary from 2 mm flower bud, showing the presence of immature ovule at the integumentary primordia stage (× 210); *B* - a section of an ovary from 3 mm flower bud, showing megaspore-mother-cell (*arrow*) (× 460); *C* - a section of an ovary from 4 mm flower bud, showing 2-nucleate (*arrow*) embryo sac (× 740); *D* - a section of an ovary from a 5 mm flower bud, showing 4-nucleate embryo sac. Three of the nuclei are clearly seen in the picture (× 820).

1993), onion (Bohanec *et al.* 1995, Luthar and Bohanec 1999), sugar beet (Gurel *et al*. 2000), maize (Tang *et al*. 2006), coconut (Perera *et al*. 2007) and *Psoralea corylifolia* (Chand and Sahrawat 2007). Although the main objective of the above studies, including the present study, was to obtain haploid plants; regeneration of diploid plants revealed the potential use of ovary explants for adventitious shoot proliferation. Depending upon the regenerative pathways, *in vitro* developed plants may be haploid, diploid or mixoploid (San and Gelebert 1986, Yang and Zhou 1990, Chand and Basu 1998, Alan *et al.* 2003). Occurrence of uniformly diploid plants, in the present study, was revealed by cytological analysis. In the present study, besides the stage of ovary at culture, the presence of 2,4-D in combination with BAP in the MS medium promoted callus induction from ovaries; the same either alone or in combination with Kin facilitated massive callus proliferation. Subsequently, these calli from proliferation medium were subcultured on regeneration medium, MS + BAP, to achieve organogenesis. In general, higher percentage of sucrose is known to suppress the divisions in diploid cells and, therefore, facilitates divisions from haploids cells (*see* Bhojwani and Razdan 1996). However, to our surprise, we obtained diploid callus even when the medium contained 9 % sucrose.

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