

An efficient protocol for the production of triploid plants from endosperm callus of neem, *Azadirachta indica* A. Juss.

Rakhi Chaturvedi^{1,2*}, Maharaj K. Razdan^{1,3}, Sant S. Bhojwani^{1,4}

¹ Department of Botany, University of Delhi, Delhi-110 007, India

² Department of Plant Molecular Biology, University of Delhi South Campus, Benito Juarez Road, Dhaula Kuan, New Delhi-110 021, India

³ Department of Botany, Ramjas College, University of Delhi, University Enclave, Delhi-110 007, India

⁴ Director, Dayalbagh Educational Institute (DEI) (Deemed University), Dayalbagh, Agra-282 005, India

Received July 8, 2002 · Accepted December 6, 2002

Summary

Triploid plants of neem were obtained by immature endosperm culture. Immature seeds, at the early dicotyledonous stage of embryo development, is the best explant to raise endosperm callus on MS + NAA (5 µmol/L) + BAP (2 µmol/L) + CH (500 mg L⁻¹). Maximum shoot bud differentiation from the endosperm callus occurred on MS + 5 µmol/L BAP. Shoots were multiplied by forced axillary branching and rooted *in vitro*. The plants were established in soil. Over 66% of the plants were triploid with chromosome number 2n = 3x = 36. A characteristic feature of the shoots of endosperm origin is the presence of a large number of multi-cellular glands.

Key words: *Azadirachta indica* – endosperm culture – medicinal plant – neem – triploids – woody plant

Abbreviations: BAP = 6-Benzylamino Purine. – CH = Casein Hydrolysate. – CM = Coconut Milk. – 2,4-D = 2,4-Dichlorophenoxyacetic Acid. – FAA = Formalin-Acetic-Alcohol. – IAA = Indole-3-Acetic Acid. – IBA = Indole-3-Butyric Acid. – Kn = Kinetin. – NAA = α-Naphthaleneacetic Acid. – TBA = Tertiary-Butyl-Alcohol. – TDZ = Thidiazuron. – YE = Yeast Extract

Introduction

In recent years, neem (*Azadirachta indica* A. Juss.) has attracted world-wide attention and «has been identified as one of the most promising of all the plants» (National Research Council of USA 1992, Schmutterer 1995, Kundu 1999). It is regarded as an economic tree of India because of the growing use of its products in agriculture, medicine, cosmetics and animal health care (Thengane et al. 1995). Despite the pro-

found economic value of neem tree, very little scientific work has been done on the improvement of this species. Plant tissue culture provides the best means to elicit the cellular totipotency of plant cells and, therefore, it forms the backbone of the modern approach to crop improvement. Even the triploid cells of endosperm are totipotent and provide a direct and easy approach to regenerate triploid plants difficult to raise *in vivo*.

Triploid plants are usually seed-sterile. However, there are many examples where seedlessness caused by triploidy is of no serious concern or, at times, even advantageous. Natural

* E-mail corresponding author: rakhi_chaturvedi@yahoo.co.uk

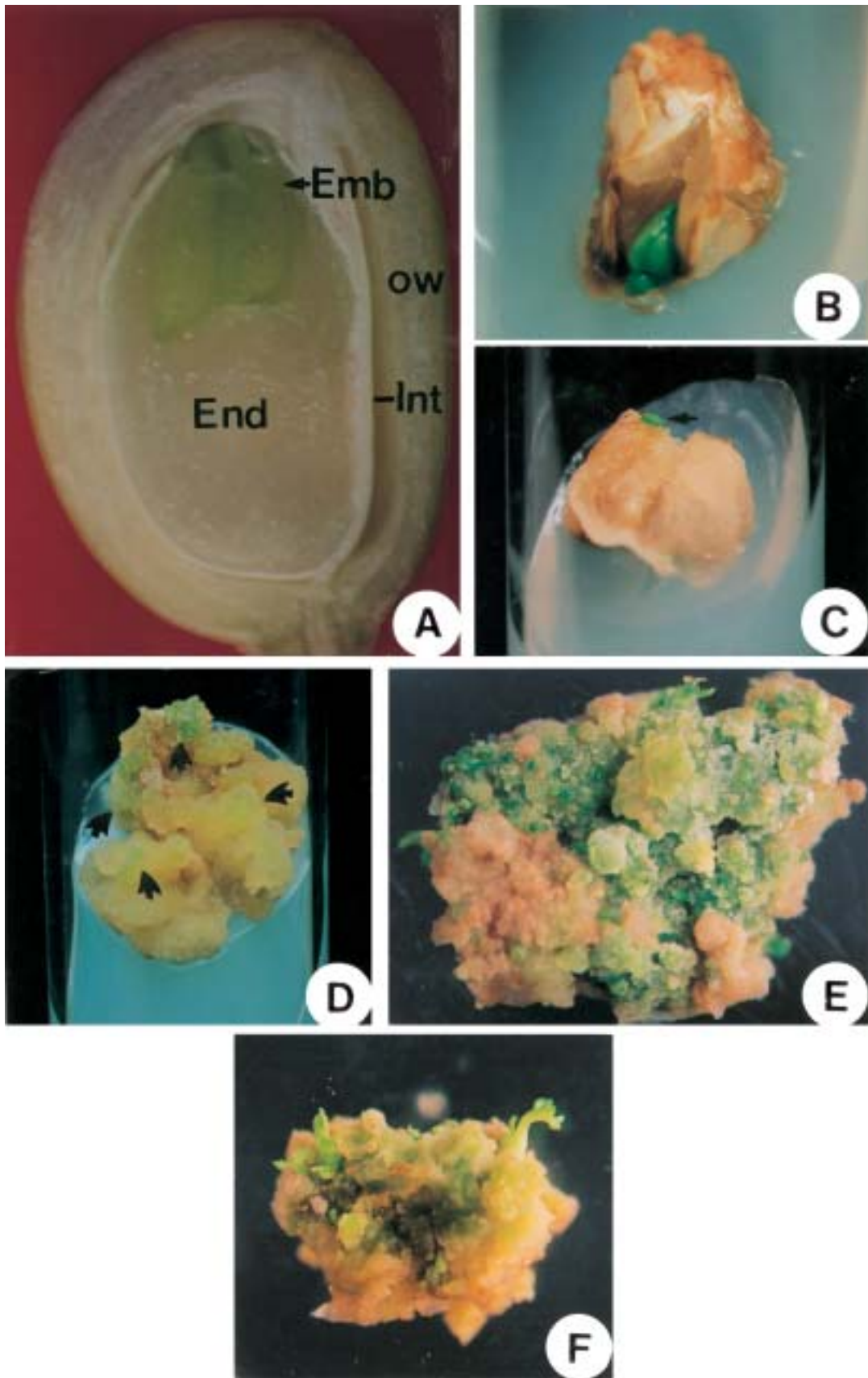


Figure 1.

triploids of tomato produced larger and tastier fruits than their diploid counterparts (Kagan-Zur et al. 1990). Recently, The Central Tuber Crops Research Institute, Thiruvananthapuram, has produced the first triploid of cassava, which is ideal in terms of several desirable characteristics (IPGRI Newsletter for Asia, The Pacific and Oceania, May–August 2001). In neem, azadirachtin is obtained from the kernels and, therefore, triploids may not be good for the elites selected for this tetraterpenoid. However, the elites of neem selected for other secondary metabolite production such as nimbin, nimbinin, nimbidin, nimbindiol and tannins, produced in the bark, and quercetin, nimbosterol and gedunin obtained from leaves (Thengane et al. 1995) triploidy may offer advantages.

Traditionally, triploids are produced by crossing induced superior tetraploids and diploids. This approach is not only tedious and lengthy (especially for tree species) but in many cases it may not be possible due to high sterility of autotetraploids (Esen and Soost 1973, Gupta 1982). In contrast, regeneration of plants from endosperm, a naturally occurring triploid tissue, offers a direct, single step approach to triploid production (Bhojwani and Razdan 1996). The selected triploids, expected to be sexually sterile, can be bulked up by micropropagation. There is no report of *in vitro* production of triploids of neem. This study, therefore, highlights the feasibility of triploid production from the endosperm tissues of neem, a tree species.

Material and Methods

Plant material and initiation of aseptic cultures

Immature fruits, at the early dicotyledonous stage of the embryo, were collected from a 50-year-old tree growing in the botanical garden of the University of Delhi. The fruits were thoroughly washed in 1% savlon (Johnson and Johnson, USA) solution for 5 min, followed by rinsing with sterile distilled water (SDW). All further manipulations were carried out under aseptic conditions in a laminar air-flow cabinet. After rinsing in 90% ethanol for 30 sec and two changes in SDW, the fruits were surface sterilised with 0.1% HgCl₂ solution for 8 min. After washing the fruits thrice with SDW, the fruits were dissected to excise the whole seeds or endosperm with or without embryo. The excised seeds (with enclosed endosperm and embryo) or endosperm with or without embryo were cultured on MS (Murashige and Skoog 1962) basal medium containing 3% sucrose and gelled with 0.8% agar. The pH of the medium was adjusted to 5.8 before autoclaving at 1.5 kg cm⁻² and 121°C for 15 min.

The MS medium, variously supplemented with benzylaminopurine (BAP), kinetin (Kn), thidiazuron (TDZ), 2,4-dichlorophenoxyacetic acid (2,4-D), α -naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA) and casein hydrolysate (CH), was used for induction and multiplication of callus and regeneration of plants from endosperm callus. At least twenty-four cultures were raised for each treatment and all the experiments were repeated three times. The degree of callusing was expressed in terms of volume of callus and is denoted by number of plus signs. Regeneration in the subcultures of callus is expressed as per cent response. The small shoots were detached from the callus and transferred to lower concentration of BAP (0.5 μ mol/L) for elongation.

Multiplication and rooting of shoots

On callus induction medium, the seeds burst open, releasing the entire green embryo and callusing endosperm (see Fig. 1B). However it is not known if some of the callus had proliferated from integument also. The shoots, regenerated from callus, were multiplied on MS medium supplemented with BAP (1 μ mol/L) + CH (250 mg L⁻¹). For rooting, the individual shoots, measuring about 4 cm in height with 3–4 nodes were taken and cultured on MS with full, half (½) or quarter (¼) strengths of the major salts and IBA (0.5 μ mol/L).

Cultures were grown in 150 × 25 mm glass culture tubes (Borosil, India) each containing 20 mL of medium. All the cultures were maintained at 25 °C ± 2 °C, 50–60% relative humidity and diffuse light (1000–2000 lux) with 16 h photoperiod.

Twenty four cultures were raised for each treatment and all the experiments were repeated at least three times. Observations were recorded at weekly intervals and standard error of the mean was calculated which is indicated by ± sign.

Transplantation

The rooted plants were washed to remove the agar, transferred to soilrite in hycotrays and placed in a glasshouse under high humidity and covered with cling film. After 10 days the cling film was removed and 3 weeks later the plants were transferred to soil in polythene bags and sprayed with a mixture of 0.1% Urea and Bavistin (1 : 1). 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.

Cytology

Roots tips (ca 1 cm), from seedlings and very young axillary/apical buds from 5-month-old endosperm-derived plants were collected at around 10.30 AM and used to study chromosomes. Excised root tips

Figure 1. A. Longitudinal half of a neem fruit at the early dicotyledonous stage of the embryo. At this stage it has a massive endosperm. (Emb, Embryo; End, Endosperm; OW, Ovary Wall; Int, integument) (×13). **B.** 2-week-old culture of an immature seed on MS + NAA (5 μ mol/L) + BAP (2 μ mol/L) + CH (500 mg L⁻¹). The seed has burst, releasing the green embryo and callusing endosperm (×3.6). **C.** Same, after 3 weeks; a white fluffy endosperm callus has emerged from the burst seed. The embryo can be seen at one end (arrow marked) (×2.7). **D.** 8-week-old subculture of endosperm callus on MS + NAA (5 μ mol/L) + BAP (2 μ mol/L) + CH (500 mg L⁻¹), showing callus proliferation. Note the appearance of green loci (arrowhead marked) (×2.1). **E.** 5-week-old subcultures of endosperm callus on MS + BAP (5 μ mol/L), showing differentiation of numerous shoots (×3.5). **F.** Same, after 6 weeks, distinct shoots have developed. Some of the smaller shoots are also present (×2.6).

Table 1. Effect of some growth regulators on endosperm callusing in seed cultures. Control: MS; Growth period: 4 weeks.

Treatment ($\mu\text{mol/L}$)	% cultures showing endosperm callusing	Degree of Callusing*
Control	0	0
2,4-D (5)	45.0 \pm 1.0	+
2,4-D (5) + BAP (2)	11.5 \pm 0.5	+
2,4-D (5) + BAP (2) + CH (500mgL ⁻¹)	45.0 \pm 2.0	+
NAA (5) + BAP (2) + CH (500mgL ⁻¹)	53.0 \pm 1.0	++
IAA (5) + BAP (2) + CH (500mgL ⁻¹)	46.0 \pm 1.0	++

\pm = SE.

* The degree of callusing is directly related to the number of + sign: + = (< 10 mm \times 5 mm); ++ = (10 mm \times 5 mm – 10 mm \times 15 mm).

Table 2. Effect of cytokinin/s alone or in combination on shoot bud differentiation from endosperm callus. Control: MS; Growth period: 6 weeks.

Treatment ($\mu\text{mol/L}$)	% cultures showing shoot-bud differentiation	No. of shoot-buds per culture
Control	0	0
BAP 5.0	95 \pm 2.0	14.5 \pm 4.0
7.5	91 \pm 2.0	9.2 \pm 4.0
10.0	83 \pm 3.0	4.8 \pm 1.0
15.0	80 \pm 2.0	4.0 \pm 1.0
Kn 5.0	50 \pm 2.0	3.6 \pm 1.0
10.0	77 \pm 3.0	5.0 \pm 1.5
TDZ 1.0	0	0
2.0	0	0
BAP 5.0 + IAA 1.0	91 \pm 3.0	8.2 \pm 1.0

\pm = SE.

were washed under running tap water to remove the soil. After pre-treating the root tips and buds with 0.02% 8-Hydroxyquinoline (BDH, India), at 4 °C, for 4 h, the materials were fixed in a modified Carnoy's fluid, containing absolute alcohol, chloroform, glacial acetic acid and methanol (7:3:1:1) for 48 h.

The fixed material was placed in a mixture of nine drops of 2% aceto-orcein and one drop of 1N HCl in a watchglass and heated gently. After cooling, the individual root tips or young axillary/apical buds were placed in a drop of fresh aceto-orcein on a glass slide, and a cover slip was placed over it. The slide was warmed gently and the material was squashed. The squash preparations were observed under Nikon Opti-Phot photomicroscope and the cells showing good separation of chromosomes were photographed.

Meiotic preparations were made from young flower buds collected from three different adult trees at around 11.30 AM. After fixing the buds in Carnoy's fluid containing absolute alcohol, chloroform and glacial acetic acid (6:3:1), the anthers were directly squashed in 2% aceto-orcein and the slides were observed for chromosome count.

Histological Studies

For histological studies, wax sections were cut. The materials were 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 TBA series for dehydration, infiltrated with paraffin wax (melting point 60 °C, E. Merck, Germany) and, finally, embedded in pure paraffin wax. The paraffin blocks were mounted on wooden stubs, and 8–10 μm thick sections were cut using a Spencer Rotary Microtome (USA) attached with a steel knife. The sections were mounted on microslides, dewaxed and double stained with safranin (1%) and astrablue (1%).

Results

Neem bears non-endospermous seeds. Therefore, only immature endosperm could be cultured. The seed stage corresponding to early dicotyledonous stage was found convenient to excise the endosperm. In older seeds, the endocarp becomes hard rendering dissections difficult. In the cultures of excised endosperm, with or without the embryo, none of the tested media induced callusing. Therefore, the whole seeds at the early dicotyledonous stage of the embryo were cultured. At this stage the fruit wall and seed coat are fairly thin and endosperm is massive (Fig. 1 A), and the seeds could be dissected out easily.

Callus induction

The excised seeds were cultured on MS and MS medium variously supplemented with 2,4-D, BAP, NAA, IAA and CH (Table 1). On basal medium, all the explants turned brown and died without any sign of growth. However, all hormonal treatments induced endosperm callusing. MS + 2,4-D (5 $\mu\text{mol/L}$) showed good callus proliferation (45%) but best callusing (53%) occurred on MS + NAA (5 $\mu\text{mol/L}$) + BAP (2 $\mu\text{mol/L}$) + CH (500mgL⁻¹).

Initially, the explant turned brown, but after two weeks the responding cultures exhibited bursting of the seed, releasing the green embryo and callusing endosperm (Fig. 1 B). After another week the seed had wide opened and a white fluffy callus had emerged (Fig. 1 C). In primary cultures the callus

Table 3. Rooting response of shoots of endosperm origin on MS at full, 1/2 and 1/4 strength of major salts supplemented with 0.5 $\mu\text{mol/L}$ IBA. Growth Period: 4 Weeks.

Treatment ($\mu\text{mol/L}$)	% Shoots rooted	No. of roots per shoot	Length of the longest root	No. of laterals
MS	50 \pm 2.0	1.5 \pm 0.5	4.0 \pm 1.0	1.0 \pm 0.2
1/2 MS	100	6.0 \pm 0.5	4.7 \pm 0.7	2.4 \pm 0.3
1/4 MS	0	0	0	0

\pm = SE.

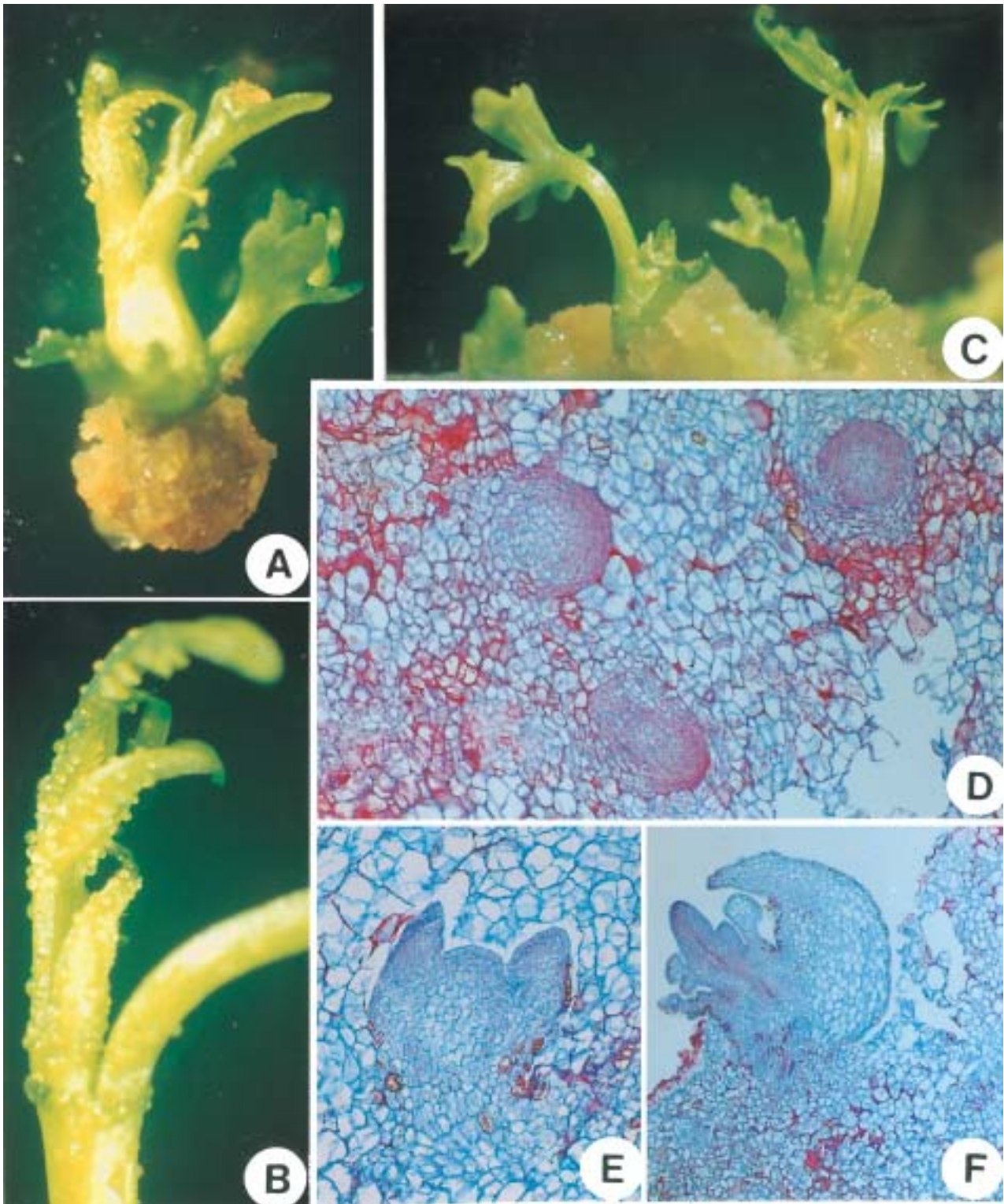


Figure 2. **A.** 3-week-old subculture of endosperm callus on MS + BAP ($0.5\mu\text{mol/L}$), showing two healthy shoots. One of the shoots showing sessile and stalked glands on the surface ($\times 14$). **B.** Same as **A**, enlarged view of shoot tip region showing numerous glands on the surface of the leaf ($\times 18$). **C.** Two shoots have developed from the endosperm callus 3-week after subculture on MS + BAP. Some glands can also be seen on these shoots ($\times 20$). **D.** Section of a 2-week-old regenerating callus from MS + BAP ($5\mu\text{mol/L}$), showing endogenous meristematic pockets ($\times 210$). **E.** Section of a 3-week-old callus from MS + BAP ($5\mu\text{mol/L}$) showing young endogenous shoot bud ($\times 100$). **F.** Same as **E**, showing a distinct shoot, differentiated from the surface of the callus ($\times 60$).

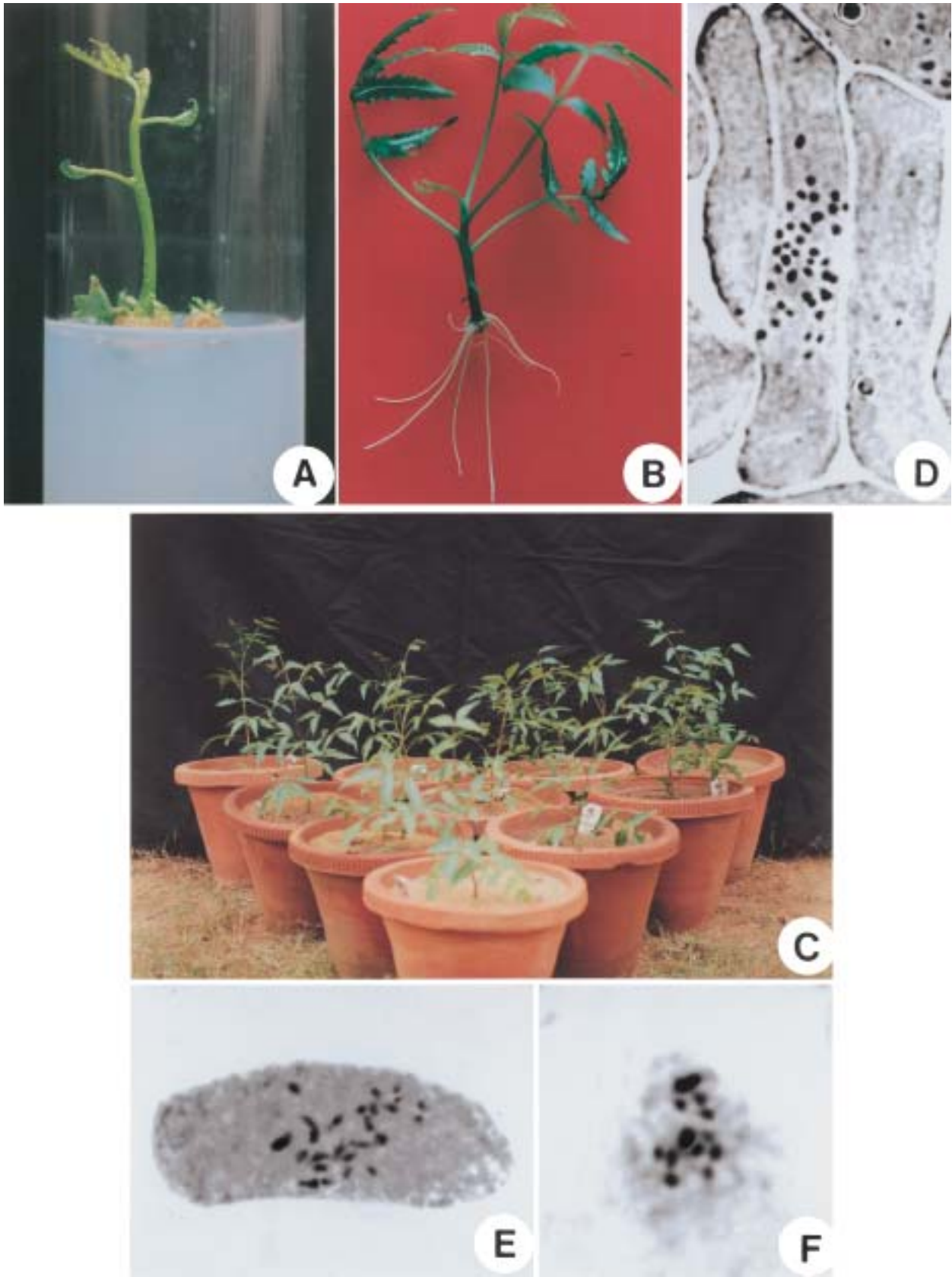


Figure 3.

did not grow much. However, if the de-embryonated callus was transferred to fresh MS + NAA + BAP + CH medium, >87 % cultures developed fast growing, brown and compact callus. In 5-week-old cultures, the calli had developed several green loci, which remained disorganised even after 8 weeks (Fig. 1D).

Shoot Regeneration

To achieve plant regeneration, the calli from MS + NAA (5 $\mu\text{mol/L}$) + BAP (2 $\mu\text{mol/L}$) + CH (500 mg L^{-1}) were sub-cultured on MS alone or MS supplemented with cytokinin (BAP, Kinetin, TDZ). BAP was also tested in combination with IAA.

There was no organogenesis on basal medium. TDZ supported only good callus proliferation. All other treatments induced shoot bud differentiation (Table 2). MS + 5 $\mu\text{mol/L}$ BAP was the best medium for shoot regeneration. On this medium, 95 % calli differentiated more than 14 shoots per culture after 6 weeks.

On the regeneration media, the calli proliferated and turned green, compact and nodulated after two weeks. After 3 weeks, distinct shoot buds appeared all over the green callus. The number of shoot buds increased until 5 weeks (Fig. 1E). However, the shoots regenerating from callus did not grow beyond 1 cm on the regeneration medium even after 6 weeks (Fig. 1F).

A characteristic feature of the shoots from endosperm callus was the preponderance of multi-cellular glands on the surface of the shoots. These glands, occasionally stalked, were found in large numbers on younger leaves close to the shoot apex (Fig. 2A–C). Such structures have never before been observed in studies of shoot regeneration from the anther, ovary and zygotic embryo cultures, or in any publication on tissue culture of neem.

Histological Studies

Serial sections of the regenerating callus revealed that the shoot buds differentiated from the surface of the callus as well as endogenously. Two week old callus showed many meristematic pockets inside the callus (Fig. 2D), which developed into shoot buds after 3 weeks (Fig. 2E). Figure 2F shows that the shoot buds have developed from the peripheral tissues of the callus.

Shoot elongation, multiplication, rooting and transplantation

As the shoots regenerating from endosperm callus did not grow well while attached to the callus, these shoots were detached from the callus and transferred to the elongation medium, with a reduced level of BAP (0.5 $\mu\text{mol/L}$). On the elongation medium, >80 % of shoots elongated and attained a height of 4 cm, with 3–4 nodes, after 5 weeks (Fig. 3A). The elongated shoots multiplied through axillary shoot proliferation on MS medium supplemented with BAP (1 $\mu\text{mol/L}$) + CH (250 mg L^{-1}) at a rate of 7–8 fold every 8 weeks.

To induce rooting, 4 cm long shoots were transferred to MS supplemented with full, half ($\frac{1}{2}$) or quarter ($\frac{1}{4}$) strengths of major salts and 0.5 $\mu\text{mol/L}$ IBA (Table 3). Of these, $\frac{1}{2}$ MS + IBA (0.5 $\mu\text{mol/L}$) was the best for rooting. Root initiation occurred within 3 weeks, and after 4 weeks 100 % shoots had formed an average of 6 roots (Fig. 3B). Transplantation survival of micro-propagated plants was more than 90 % (Fig. 3C).

Cytology

Ploidy determined from 20 plants (from independent seeds), regenerated from endosperm callus, showed that 66 % plants had triploid number of chromosomes ($2n = 3x = 36$; Fig. 3D) and the other 34 % were diploids ($2n = 2x = 24$).

To generate sufficient information and also to throw more light on actual chromosome number of *Azadirachta indica* A. Juss., mitotic and meiotic preparations were made from the root tips of seedlings and the young flower buds of adult trees, respectively (Chaturvedi et al., under communication). These studies showed that the diploid number of Chromosome in neem is $2n = 2x = 24$ (Fig. 3E) and haploid number is $n = x = 12$ (Fig. 3F).

Discussion

Endosperm is a unique tissue in its origin, development and ploidy level. It is a product of double fertilisation, but unlike the embryo it is triploid and develops into a formless tissue (Bhojwani and Bhatnagar 1999). It is, therefore, an interesting tissue for morphogenesis. The earliest attempt to grow endosperm tissue in cultures was made by Lampe and Mills (1933). However, the first tissue cultures of immature maize

Figure 3. **A.** A shoot regenerated from endosperm, 5 weeks after transfer to MS + BAP (0.5 $\mu\text{mol/L}$). The shoot has elongated ($\times 2.2$). **B.** A shoot from **A**, rooted on $\frac{1}{2}$ MS + IBA (0.5 $\mu\text{mol/L}$). ($\times 2.0$). **C.** Hardened micro-propagated plants, 8 months after transfer to soil ($\times 0.1$). **D.** A shoot tip cell of an endosperm-derived plant, showing triploid number of chromosomes ($2n = 3x = 36$) ($\times 2300$). **E.** Root tip cell of a seedling, showing $2n = 2x = 24$ ($\times 1460$). **F.** Meiotic metaphase-I in pollen-mother-cell showing $n = x = 12$ ($\times 4200$).

endosperm were raised in 1949 by LaRue. Since then, immature and mature endosperm of several species has been shown to form continuously growing calli (Bhojwani and Razdan 1996). Totipotency of endosperm cells was first demonstrated by Johri and Bhojwani (1965). To date, differentiation of shoots/embryos/plantlets from endosperm tissue has been reported for at least 15 species belonging to 9 families (Bhojwani and Bhatnagar 1999). In many of these reports, the regenerants were shown to be triploid. Production of triploids from endosperm cultures of neem is reported in the present study for the first time.

Generally, it has been found that mature endosperm requires the initial association of embryo to form callus, but immature endosperm proliferates independent of the embryo. However, in neem the association of the embryo proved essential to induce callusing of immature endosperm; the best explant was immature seeds. Similar observation for mulberry was reported by Thomas et al. (2000).

Endosperm callusing generally requires an auxin, a cytokinin and a rich source of organic nitrogen, such as YE or CH (Nakajima 1962, Rangaswamy and Rao 1963, Bhojwani and Johri 1971, Srivastava 1971, Wang and Chang 1978, Gmitter et al. 1990). Thomas et al. (2000) observed maximum callusing of mulberry endosperm on MS + BAP + NAA + CM or YE. Neem endosperm showed good callus proliferation on MS + 2,4-D (45%), but best callusing (53%) occurred on MS + NAA + BAP + CH. The endosperm callus differentiated shoots, which could be rooted, and full triploid plants have been established in soil. Interestingly, a unique feature of these shoots, not seen on shoots regenerated from other explants, was the presence of numerous multi-cellular glands, particularly on young leaves close to the shoot apex. Some of the glands had developed a small stalk. Dayanandan et al. (1993) have reported the occurrence of multi-cellular glandular trichomes at the shoot apex, on young developing petioles, abaxial surface of the leaflets, and stems, and referred to them as "external resin secretory glands". The protocol described in this paper on the endosperm culture of neem was found to be very effective. 66% of the plants were triploid suggesting their endosperm origin. These triploid plants have been successfully established in soil.

Acknowledgements. Rakhi Chaturvedi thanks Professor S. P. Bhatnagar for critical analysis of the manuscript and Council for Scientific and Industrial Research (CSIR), India, for financial assistance.

References

- Bhojwani SS, Bhatnagar SP (1999) The Embryology of Angiosperms. Vikas Publishing House Pvt. Ltd., New Delhi, 359 pp
- Bhojwani SS, Johri BM (1971) Morphogenetic studies on cultured mature endosperm of *Croton bonplandianum*. *New Phytol* 70: 761–766
- Bhojwani SS, Razdan MK (1996) Plant Tissue Culture: Theory and Practice. Elsevier, Amsterdam, 502 pp
- Chaturvedi R, Razdan MK, Bhojwani SS Chromosome number in Neem (*Azadirachta indica* A. Juss.). (under communication)
- Dayanandan P, Stephen A, Muruganandan B (1993) Location of neem triterpenoids and other secretory structures. In: Proc World Neem Conference, Bangalore, pp 141–149
- Esen A, Soost RK (1973) Seed development in *Citrus* with special reference to 2x, 4x crosses. *Amer J Bot* 60: 448–462
- Gmitter FG Jr, Ling XB, Deng XX (1990) Induction of triploid *Citrus* plants from endosperm calli *in vitro*. *Theor Appl Genet* 80: 785–790
- Gupta PP (1982) Genesis of microspore-derived triploid petunias. *Theor Appl Genet* 61: 327–331
- Johri BM, Bhojwani SS (1965) Growth response of mature endosperm in cultures. *Nature* 298: 1345–1347
- Kagan-Zur V, Mills D, Mizrahi Y (1990) Callus formation from tomato endosperm. *Acta Hort* 280: 139–142
- Kundu SK (1999) The mating system and genetic significance of polycarpy in the neem tree (*Azadirachta indica*). *Theor Appl Genet* 99: 1216–1220
- Lampe L, Mills CO (1933) Growth and development of isolated endosperm and embryo of maize. *Abs Papers Bot Soc, Boston*
- La Rue CD (1949) Culture of the endosperm of maize. *Amer J Bot* 36: 798
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol Plant* 15: 473–497
- Nakajima T (1962) Physiological studies of seed development, especially embryonic growth and endosperm development. *Bull Univ Osaka Perfect Ser B* 13: 13–48
- Rangaswamy NS, Rao PS (1963) Experimental studies on *Santalum album* L. Establishment of tissue culture of endosperm. *Phytomorphology* 13: 450–454
- Schmutterer H (1995) The Neem Tree: Sources of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes. VCH, Weinheim, 696 pp
- Srivastava PS (1971) *In vitro* induction of triploid roots and shoots from mature endosperm of *Jatropha panduraefolia*. *Z Pflanzenphysiol* 66: 93–96
- Thengane S, Joshi M, Mascarenhas AF (1995) Somatic embryogenesis in neem (*Azadirachta indica*). In: Jain SM, Gupta P, Newton R (eds) Somatic Embryogenesis in Woody Plants. Vol II Important Selected Plants. Kluwer Academic Publishers, Dordrecht, pp 357–374
- Thomas TD, Bhatnagar AK, Bhojwani SS (2000) Production of triploid plants of mulberry (*Morus alba* L.) by endosperm culture. *Plant Cell Rep* 19: 395–399
- Wang T, Chang C (1978) Triploid *Citrus* plantlets from endosperm culture. In: Proc Symp Plant Tissue Culture. Science Press, Peking, pp 463–467