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Production of haploids of neem (*Azadirachta indica* A. Juss.) by anther culture

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Abstract Androgenic haploids of the neem tree (*Azadirachta indica* A. Juss.) were produced by anther culture at the early- to late-uninucleate stage of pollen. Haploid formation occurred via callusing. The best medium for inducing callusing in the anther cultures was Murashige and Skoog's basal medium (MS) (9% sucrose) supplemented with 1 μ*M* 2,4-D, 1 μ*M* NAA and 5 μ*M* BAP, while anther callus multiplied best on MS medium supplemented with 1 μ*M* 2,4-D and 10 μ*M* Kn. These calli differentiated shoots when transferred to a medium containing BAP; 5 μ*M* BAP was optimum for young calli (75% cultures differentiated shoots), but older calli showed the best regeneration with 7.5 μ*M* BAP. Shoots elongated at a lower concentration of BAP – 0.5 μ*M*. These shoots were multiplied by forced axillary branching and rooted in vitro. The plants were subsequently established in soil. Of the plants that regenerated from anther callus 60% were haploid, 20% were diploid and 20% were aneuploid.

Keywords Androgenesis · *Azadirachta indica* · Haploids · Medicinal plant · Woody plant

Abbreviations *BAP* 6-Benzylaminopurine · *CH* Casein hydrolysate · *2,4-D* 2,4-Dichlorophenoxyacetic acid · *FAA* Formalin acetic alcohol · *IBA* Indole-3-butyric acid · *Kn* Kinetin · *NAA* α-Naphthaleneacetic acid · *TBA* Tertiary-butyl-alcohol

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Introduction

Neem (*Azadirachta indica* A. Juss.), an evergreen tropical forest tree, is a renewable source of various useful products. Almost every part of this tree – seeds, leaves, roots, bark, trunk and branches – has multiple uses. Though many plants produce insecticidal and insect repellent agents, neem holds out the promise of providing highly effective, non-toxic and environment-friendly means of controlling or eliminating insect pests that cause losses in agricultural production (Govindachari et al. 1992). Of the several bioactive ingredients isolated from various parts of the tree, most notable is azadirachtin, which can be extracted from the seed kernels (Thengane et al. 1995). However, improvement of this valuable tree is hampered by its highly heterozygous nature, long reproductive cycle and recalcitrant and poor seed yield. Tissue culture offers the solution to many of these problems. The in vitro production of haploids is extremely valuable in plant breeding and genetics, as with haploids, homozygosity can be achieved in a single step by a doubling of the chromosomes. This is particularly important for a highly heterozygous, long-generation tree species such as neem. Despite considerable efforts, to date there has been no report of the in vitro production of haploids of neem. The study reported here, therefore, highlights the feasibility of haploid production in neem using anthers at the early- to late-uninucleate stage of pollen.

Materials and methods

Plant material and initiation of aseptic cultures

Young inflorescences from a 50-year-old neem tree (*Azadirachta indica* A. Juss.) growing in the botanical garden of the University of Delhi were collected between 7.30 a.m. and 8.30 a.m. Two-millimeter-long flower buds with microspores at the early- to late-uninucleate stage were taken to the laminar air-flow cabinet, and further operations were carried out under aseptic conditions. These buds were surface sterilised with a 0.1% solution of $HgCl_2$ for 7 min, followed by three washings with sterile distilled water. The

buds were dissected out with the aid of a binocular microscope using pre-sterilised Petriplates, forceps and fine needles. Damaged anthers, if any, were discarded. Twenty anthers, from two buds, were cultured in 55×15-mm pre-sterilised, disposable Petriplates (Laxbro, India) containing 10 ml of MS (Murashige and Skoog 1962) medium. The Petriplates were sealed with Parafilm (American National Can, Greenwich, N.Y.). The pH of the medium was adjusted to 5.8 before autoclaving at 1.06 kg cm^{-2} and 121°C for 15 min.

MS medium supplemented with varying concentrations and combinations of BAP, Kn, 2,4-D and/or NAA was used for the induction and multiplication of callus and the regeneration of plants from anther callus. Unless mentioned otherwise, all media contained 3% sucrose and were solidified with 0.8% agar (Qualigens, India). The degree of callusing was scored in terms of the diameter of callus, denoted by the number of plus signs. Regeneration in the subcultures of callus is expressed as percentage response.

The small shoots were detached from the callus and transferred to a lower concentration of BAP (0.5 μ*M*) for elongation.

Multiplication and rooting of shoots

Shoots regenerated from anther callus were multiplied on MS medium supplemented with 1 μM BAP and 250 mg l^{-1} CH. For rooting, terminal 4-cm-long portions of elongated shoots with three to four nodes were excised and cultured on 1/4-strength MS (major salts reduced to 1/4 strength) with 0.5 μ*M* IBA.

Cultures for callus multiplication, regeneration and rooting of shoots were grown in 150×25-mm glass culture tubes (Borosil, India), each containing 20 ml of medium. All cultures were maintained at 25±2ºC and 50–60% relative humidity under a 16/8-h (light/dark) photoperiod with diffuse light (1,000–2,000 lux). Anther cultures were initially kept continuously in the dark, but after 8 weeks the calli that had developed from these cultures were transferred to multiplication medium and maintained in light.

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.

Transplantation

The rooted plants were washed to remove the agar, transferred to Soilrite (Chowgule Industries, India) in hycotrays (Sigma, St. Louis, Mo.) and placed in a glasshouse under high humidity. The hycotrays were initially 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 (BASS, India) and Bavistin (BASS, India) (1:1). After a further 4 weeks, the plants were transferred to pots and shifted to a polyhouse. Three months following transplantation, the plants were shifted to a shaded area under natural conditions.

Cytology

Roots tips (approx. 1 cm) from seedlings raised from seeds and from 5-month-old potted plants regenerated from anther cultures and very young axillary/apical buds from 2-year-old anther-derived plants growing in the ground of Tissue Culture Pilot Plant were collected at around 10.30 a.m. and used to study chromosomes. Excised root tips were washed under running tap water to remove the soil. After the root tips/buds had been pretreated with 0.02% 8-hydroxyquinoline (BDH, India) at 4°C for 4 h, they 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 1 *N* HCl in a watchglass and heated gently. After cooling, an individual root tip or young axillary/apical bud was 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 squashed. The slides were observed under a Nikon Opti-Phot photomicroscope, and the cells showing a good separation of chromosomes were photographed.

Meiotic preparations were made from young flower buds collected from three different adult trees at around 11.30 a.m. After the buds had been fixed 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 observed for chromosome counting.

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 24 h and stored in 70% ethanol. The material was passed through the TBA series for dehydration, infiltrated with paraffin wax (melting point of 60°C; Merck, Germany) and, finally, embedded in pure paraffin wax. The paraffin blocks were mounted on wooden stubs, and 8–to 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 astra-blue (1%).

Results

Callus induction

Anthers at the early- to late-uninucleate stages of microspore development were cultured to raise androgenic haploids of neem. Of the various hormonal treatments tested, MS supplemented with 1 μ*M* 2,4-D, 1 μ*M* NAA and 5 μ*M* BAP induced maximum callusing. However, the percentage of anthers callused and the degree of callusing varied with the sucrose concentration (3%, 9%, 12%): when the medium contained 9% sucrose the percentage of anthers callused and the degree of callusing were distinctly better than at 3% or 12% sucrose (Table 1). With 9% sucrose, 100% of the anthers callused, and callus growth was profuse (Fig. 1A). The callus was brown, friable, soft and wet on this combination. Callusing started after 4 weeks after culture initiation, and after 8 weeks the entire anther was covered with a brown callus (Fig. 1A). Histological studies revealed that in 4-week-old cultures the anther-wall cells had started dividing, while the microspores appeared to be unchanged (Fig. 1B). However, in 8-week-old cultures the entire an-

Table 1 Effect of sucrose concentration on callusing in anther cultures grown on MS + 1 μ*M* 2,4-D + 1 μ*M* NAA + 5 μ*M* BAP. Growth period: 8 weeks

Sucrose concentration (%)	Number of anthers cultured	Percentage of anthers callused[a]	Degree of callusing[b]
3 (control)	300	56.6±0.0	++
9	400	100±0.0	++++
12	200	75.0±0.0	+

[a] ±Standard error

[b] Size of callus is directly related to the number of plus signs: +, smaller than 10×5 mm; ++, between 10×5 mm and 10×15 mm; ++++, larger than 25×15 mm

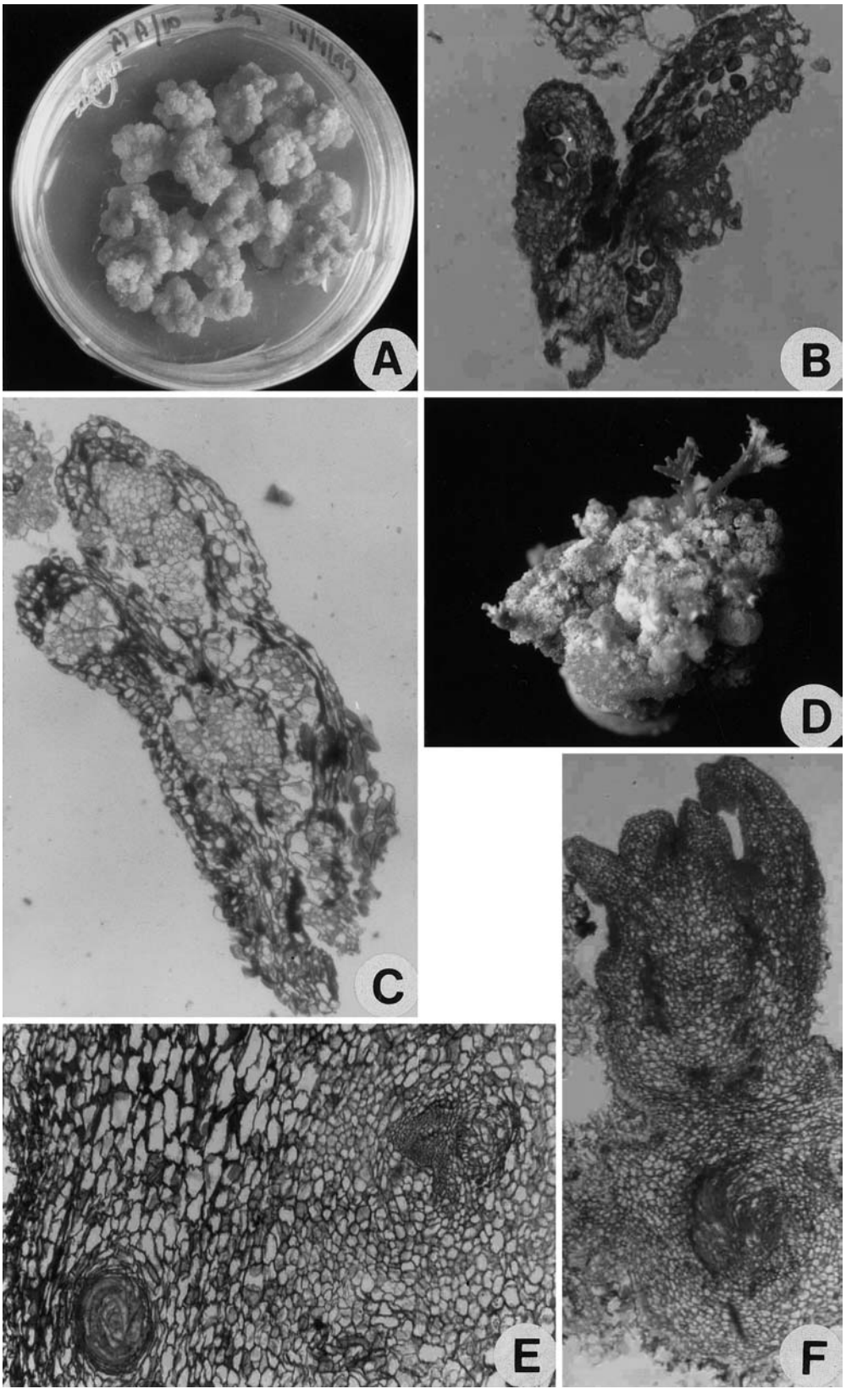

Fig. 1 **A** An 8-week-old anther culture on MS (9% sucrose) supplemented with 1 μ*M* 2,4-D, 1 μ*M* NAA and 5 μ*M* BAP; anthers are completely covered by proliferating brown callus (×1.2). **B** Transverse section of an anther from a 4-week-old anther culture on MS (9% sucrose) containing 1 μ*M* 2,4-D, 1 μ*M* NAA and 5 μ*M* BAP showing the beginning of callusing at the surface of the anther; the microspores appear to be unchanged (×220). **C** Same as **B**, after 8 weeks: the anther locules are filled with callus; microspores are no longer visible (×135). **D** Anther calli 8 weeks after subculture on MS + 5 μ*M* BAP, showing distinct shoots which did not grow beyond 1 cm (×2.3). **E** Section of a 6-week-old regenerating callus from MS + 5 μ*M* BAP showing nests of vascular elements; whereas the internal tissue is compact, the outer tissue comprises loosely arranged empty cells (×950). **F** Shoot buds differentiated from vascularised nodules (×54)

Table 2 Effect of BAP concentration and the age of the source callus cultured on MS + 0.5 μ*M* 2,4-D on shoot differentiation. Growth period: 8 weeks

Age of the callus (weeks)	BAP concentration (μ*M*)			
	5.0		7.5	
	Percentage of cultures showing regeneration[a]	Number of shoots per culture	Percentage of cultures showing regeneration	Number of shoots per culture
13	75.0±0.23	4.5±0.04	70.0±0.23	2.0±0.04
28	45.0±0.81	1.5±0.09	29.0±0.21	0.79±0.04
36	0	0	25.0±0.21	0.25±0.02
68	0	0	16.0±0.81	0.20±0.0

[a] ±=Standard error

Table 3 Effect of BAP concentration and the age of the source callus cultured on MS + 1.0 μ*M* 2,4-D + 10 μ*M* Kn on shoot differentiation. Growth period: 8 weeks

Age of the callus (weeks)	BAP concentration (μ*M*)			
	5.0		7.5	
	Percentage of cultures showing regeneration[a]	Number of shoots per culture	Percentage of cultures showing regeneration	Number of shoots per culture
13	25.0±1.5	0.35±1.0	25.0±0.50	0.5±0.0
28	25.0±0.40	0.37±0.03	25.0±0.81	1.0±0.06
36	62.5±1.0	3.37±0.12	62.5±0.81	2.75±0.04
68	0	0	0	0

[a] ±Standard error

ther locules were filled with callus (Fig. 1C). Regeneration of haploid plants from these calli suggested microspore callusing. However, it was difficult to separate the calli of the two origins. Irrespective of the culture medium the calli remained unorganised in primary cultures.

Callus multiplication

The anther callus (measuring approx. 5×5 mm) was subcultured 8 weeks after culture initiation on the original medium; the callus did not exhibit much growth at this point. However, interaction of 2,4-D with Kn proved better for callus multiplication. On MS medium supplemented with 1 μ*M* 2,4-D and 10 μ*M* Kn, 95% of the cultures showed sustained and massive callus proliferation. Since the origin of the callus often affects the frequency of regeneration, the calli for shoot regeneration were also taken from MS with 0.5 μ*M* 2,4-D. The callus was allowed to multiply on these two media for more than 18 months.

Shoot regeneration

The effect of BAP concentration, age of the callus and callus maintenance medium on regeneration was studied. To induce shoot regeneration, we subcultured anther calli from the above-mentioned two sources on MS medium supplemented with either 5 μ*M* or 7.5 μ*M* BAP. No differentiation occurred on basal medium, but shoot-bud differentiation was induced at both BAP concentrations. Irrespective of the source of the callus, for 13-week-old calli, 5 μ*M* BAP was the most effective concentration for shoot-bud differentiation; at 7.5 μ*M* it was only slightly less effective. At this age, the calli derived from culture on MS medium with 0.5 μ*M* 2,4-D were the most regenerative and those from culture on MS with 1 μ*M* 2,4-D and 10 μ*M* Kn the least (compare Tables 2 and 3). The calli from 13-week-old cultures grown on MS + 0.5 μ*M* 2,4-D showed regeneration in 75% of the cultures with an average of 4.5 shoots per culture (Table 2). Compared to this, the maximum regeneration frequency shown by calli from MS + 1 μ*M* 2,4-D + 10 μ*M* Kn was only 25% (Table 3).

The calli obtained from culture on MS medium with 0.5 μ*M* 2,4-D exhibited a decline in regeneration potential with age, and after 28 weeks they did not show any regeneration on MS medium with 5 μ*M* BAP. In the presence of a higher BAP concentration (7.5 μ*M*) even older calli showed some regeneration (Table 2).

Interestingly, calli from culture on MS + 1 μ*M* 2,4-D + 10 μ*M* Kn, which showed very poor regeneration initially, not only maintained their regeneration potential for a longer time, but at 36 weeks of age showed significantly higher regeneration than 13- or 28-week-old calli. However, even these calli totally lost their regeneration potential after 36 weeks (Table 3).

On the basis of these results, we therefore suggest that calli may be initially multiplied on MS medium supplemented with 0.5 μ*M* 2,4-D and subsequently transferred to MS medium with 1 μ*M* 2,4-D and 10 μ*M* Kn. The optimum concentration of BAP for younger calli in the shoot regeneration medium is 5 μ*M*.

On regeneration media, the calli first turned brown, but after 4 weeks green nodular structures appeared all over the calli. After another 2 weeks distinct green shoot buds appeared on the nodular portions of calli. In 8-

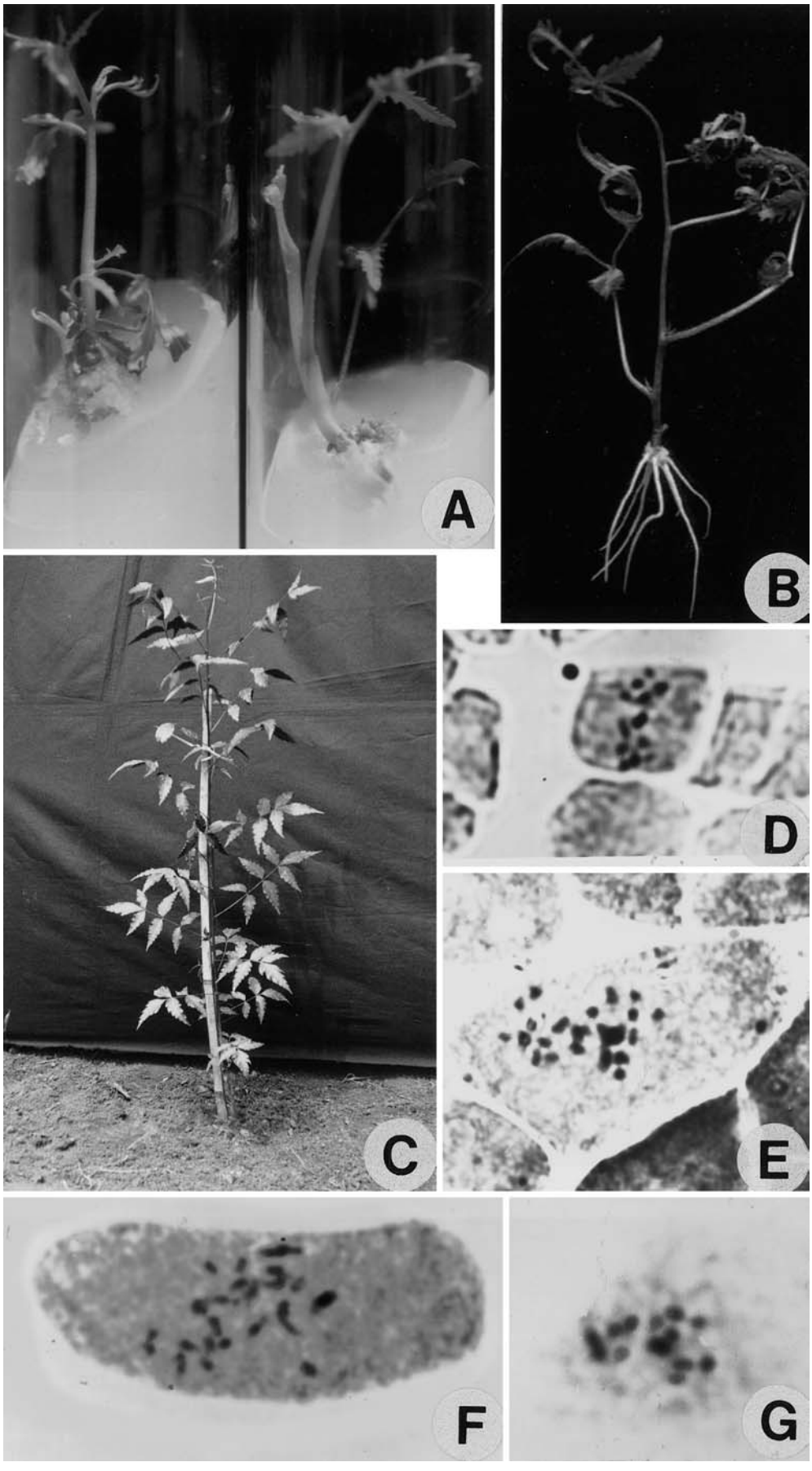

Fig. 2 **A** Individual shoots were transferred to MS + 0.5 μ*M* BAP. After 8 weeks, the shoots elongated (×1.7). **B** A shoot from **A**, rooted on 1/4-strength MS + 0.5 μ*M* IBA (×1.6). **C** Three-year-old anther-derived haploid plant growing in field (×0.1). **D** Shoot-tip cell of an anther-derived plant, showing the haploid number of chromosomes (2*n*=*x*=12) (×2,100). **E** Root-tip cell of another anther-derived plant, showing 2*n*-2=22 (aneuploid) (×680). **F** Root-tip cell of a seedling, showing 2*n*=2*x*=24 (×1,460). **G** Meiotic metaphase-I in pollen mother cell showing *n*=*x*=12 (×4,200)

week-old cultures more than half of the callus had turned green and bore distinct shoots (Fig. 1D). The regeneration potential of the callus was completely lost after 17 months.

Histological studies

Serial sections of the regenerating calli revealed the presence of nests of vascular elements surrounded by compact cambium-like cells (Fig. 1E). Shoot buds differentiated from these vascularised nodules and grew out through the disintegrating surrounding cells (Fig. 1F).

Shoot elongation, multiplication, rooting and transplantation

The shoots that regenerated from anther callus did not grow to be longer than 1 cm on the regeneration medium. Therefore, these shoots were detached from the callus and transferred individually to MS medium with 0.5 μ*M* BAP for elongation. On this medium 77% of the cultures developed 5-cm-long shoots, with each shoot having an average of 4.5 nodes after 8 weeks (Fig. 2A).

The shoots were multiplied, rooted and hardened by following the procedure as described by Chaturvedi et al. (in preparation). The shoots were multiplied through axillary shoot proliferation on MS medium with 1 μ*M* BAP and 250 mg l^{-1} CH at a rate of seven- to eightfold every 8 weeks. At each subculture, that axillary shoot which had grown fairly long was cut into single node segments and transferred to fresh medium for further multiplication. The number of propagules obtained at the end of a multiplication cycle was taken to be the rate of shoot multiplication. After 8 weeks on multiplication medium more than 90% of the cultures developed shoots that were on average 8 cm long, each with seven to eight nodes.

Terminal 4-cm-long portions of shoots from 8-week-old cultures on MS + 1 μ*M* BAP + 250 mg l^{-1} CH were used for rooting. The remaining shoots were cut into single-node segments and utilised for further multiplication. For rooting, 1/4-strength MS medium supplemented with 0.5 μ*M* IBA was tested. On this medium, root initiation occurred within 3 weeks, and after 4 weeks 80% of the shoots had formed five roots (Fig. 2B). Transplantation survival of the micropropagated plants was more than 83% (Fig. 2C).

Cytology

The ploidy level determined from 15 plants regenerated from anther callus showed that 60% of the plants had the haploid number of chromosomes ($2n=x=12$) (Fig. 2D), 20% were diploids ($2n=2x=24$) and the remaining 20% were aneuploids ($2n-2=22$) (Fig. 2E).

Those plants growing in soil were checked for ploidy confirmation at intervals; they showed the haploid number even after 3 years. These field-grown haploid plants are now more than 2 m tall.

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

Discussion

Obtaining homozygous lines of woody perennials by the conventional method of recurrent inbreeding is impractical because of the highly heterozygous nature and long generation cycle of these plants as well as inherent inbreeding depression (Baldursson and Ahuja 1996). On the other hand, homozygous plants can be obtained in a single generation by diploidisation of haploids. About 40 years ago, Guha and Maheshwari (1964) demonstrated the totipotency of microspore cells of *Datura*, which led to the emergence of a very efficient technique for haploid production in large numbers. To date, this technique has been successfully applied to over 134 species distributed within 25 families (Bhojwani and Razdan 1996). It has been the most popular approach to haploid production.

Despite considerable efforts, androgenesis in woody plants is still difficult, and there is to date no report of the in vitro production of haploids in neem, possibly because in anther cultures of most woody plants, including neem (present study), both pollen embryogenesis/callusing and callusing of the anther-wall tissue occurs simultaneously. This poses the problem of distinguishing between calli of gametophytic and sporophytic origins. One possibility to circumvent this problem is to culture isolated microspores. However, so far there is only a single successful report of androgenesis in microspore cultures of a woody species (Höfer et al 1999). Pelletier and Ilami (1972) introduced the concept of "Wall Factor", according to which the somatic tissues of the anther play an important role in the induction of sporophytic divisions in pollen (Bhojwani and Razdan 1996). In poplar (Zhang et al 1979) and rubber (Chen 1986), most of the calli that emerged after 20–25 days of anther culture was of sporophytic origin, whereas the small calli that emerged after 30–50 days of culture were derived from microspores.

Similarly, in the present study, histological sections revealed that in 4-week-old cultures the anther-wall cells had started dividing while the microspores appeared to be unchanged. However, in 8-week-old cultures the anther locules were filled with the callus. Sustained growth of callus in recurrent subcultures occurred on MS medium supplemented with 2,4-D and Kn. These calli differ-

entiated shoots when transferred to a medium containing only BAP, with 5 μ*M* BAP the optimum concentration for young calli, while older calli showed the best regeneration at 7.5 μ*M* BAP. The composition of the callus maintenance medium had a definite effect on the regeneration potential of calli. Calli maintained on medium containing 2,4-D exhibited good regeneration initially, but calli multiplied on a medium containing 1 μ*M* 2,4-D and 10 μ*M* Kn retained the regeneration potential for a longer period. In the present study, 60% of the plants regenerated from anther cultures of neem were haploid, suggesting their androgenic nature. These haploid plants have been established in the field.

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