In Vitro Androgenesis in Anther Cultures of *Crateva nurvala* Buch. Ham.

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ABSTRACT

The anthers of *Crateva nurvala*, at late uninucleate to early binucleate stages of microspore development, were cultured on Murashige and Skoog's basal medium (BM) supplemented with different concentrations of sucrose, 6-Benzylaminopurine (BAP, 0.5 or 1.0 mg l^{-1}) and/or 2,4-Dichlorophenoxyacetic acid (2,4-D, 0.5 mg l^{-1}). Among the media tested, high frequency of callusing was observed from the anthers cultured on BM adjuvated with 2,4-D alone or along with BAP. The calli were repeatedly sub-cultured on BM adjuvated with 1-6 mg l^{-1} BAP at intervals of one month each. Three shoots differentiated from two of the calli during the fifth sub-culture on the medium adjuvated with 3 mg l^{-1} BAP and one during the sixth passage on the medium fortified with 4 mg l^{-1}BAP. The regenerated shoots exhibited differences in their growth characteristics; especially the size of leaves; two being slow growing with smaller leaves. These shoots were individually multiplied using their nodal explants. Up to the fourth passage, cloned shoots maintained the original phenotype of the shoots from which they had originated. DNA content of the four clones was estimated by flow cytometry. Two of these clones had DNA content comparable to the diploid, while other two had near haploid DNA content, indicating the possible origin of these clones from microspores. This inference was further strengthened when mean guard cell size of the clones with near-haploid contents was found to be smaller than those of the mature tree and *in vitro* raised seedlings. Though, the ultimate aim of regenerating haploid plants of *C. nurvala* was not realized, flow cytometric and stomatal size analyses strongly suggested androgenic origin of two shoots.

Key words: Androgenesis, Anther culture, *Crateva nurvala*

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Introduction

The process of development of sporophyte from the male gametophyte is referred to as androgenesis (Kostoff, 1929, Maraschin, et al., 2005, Segui-Simarro, 2010). The possibility of such process was indicated way back in 1929, when among the progeny of a cross between *Nicotiana tabacum* var. *macrophylla* (3n=72, female) and *N. langsdorfii* (2n=18, male) a haploid seedling (n=9) with the characteristics of only the male parent was found (Kostoff, 1929). In the same year, Clausen and Lammerts (1929), obtained a haploid plant of *N. tabacum* (n=24) in a cross between *N. tabacum* as the male parent with *N. digluta* (2n=72) as the female parent. Since then, such embryo sac based androgenic haploids (in vivo development of a male-derived haploid embryo from a fertilized egg where the female nucleus is eliminated, Segui-Simarro 2010) of a few more species have been reported, albeit at very low frequencies (Babbar, 1982, Dunwell, 2010). The possibility of largescale production of androgenic haploids was opened up by the discovery of development of plants from the male gametophytes (microspores) in *in vitro* cultured anthers of *Datura innoxia* by Guha & Maheshwari (1964, 1966). This technique of production of haploids through anther or microspore culture has now been extended to hundreds of species (Dunwell, 2010). However, efforts to further apply it to hitherto unexplored taxa continue unabated.

The genetic improvement of plants based on conventional breeding methods depends heavily on utilization of pure lines (homozygous plants) raised through repeated selfing for many generations. In species having juvenile periods ranging from a few years to a few decades, as in trees, one cannot raise pure lines in one's life span by this method. This problem is further compounded if the species happens to be self-incompatible, thus precluding the possibility of selfing. Thus, investigations on the possibility of raising haploid

plants that can be diploidized to raise homozygous plants, through anther culture of tree species, are more fruitful in terms of saving both time and labour (Babbar & Gupta, 1986). However, compared to herbaceous taxa, anther culture studies on tree species are few (Babbar & Jain, 2000). Some of the examples of tree species in which such attempts have been successful with different degrees are *Carica papaya* (Tsay & Su, 1985), *Peltophorum pterocarpum* (Rao & De, 1987), *Citrus* spp. (Geraci & Starrantino, 1990), *Malus* spp. and *Prunus avium* (Höfer & Hanke, 1990), *Azadirachta indica* (Gautam et al., 1993, Chaturvedi et al., 2003), *Fragaria* × *ananassa* (Owen & Miller 1996), *Musa* sp. (Assani et al., 2003), *Madhuca indica* (Sarkar et al., 2004), *Citrus avium* (Chiancone et al., 2015) and *Cocos nucifera* (Perera & Vidhanaarachchi, 2021).

The present paper reports results of investigations undertaken on *Crateva nurvala* Buch. Ham. to explore the possibility of its haploid production through *in vitro* androgenesis. In an earlier study on anther culture of an allied species, *C. adansonii*, only diploid plants could be regenerated from the calli that originated from connective tissue of the cultured anthers (Tyagi and Kothari, 2005). *C. nurvala*, belonging to Capparidaceae, is an important medicinal plant. It is used in traditional systems of medicine, such as Ayurveda and Unani, as a high value therapeutic. It is effective in treatment of urinary disorders, kidney stones, prostate enlargement and bladder sensitivity. Lupeol, a pentacyclic triterpene bioactive principle isolated from its bark minimizes the deposition of stone-forming constituents in kidneys. It has also been reported to have anti-arthritic, hepatoprotective, and cardio-protective actions (Bopana & Saxena, 2008, Kumar et al., 2020).

Materials and Methods

Flowering in *C. nurvala* in Delhi starts in April. The flowers are bisexual and arranged in racemose type of inflorescence (Fig. 1 A, B). Number of stamens per bud varies from 20 to 26. The floral buds at different stages of development were collected from a *C. nurvala* tree, growing in North campus of University of Delhi. The stage of microspore development was correlated with the length of anthers (3-5 mm) by making squash preparations of the same in 1% acetocarmine and observing them under a compound microscope. Buds with anthers at late uninucleate to early binucleate stages of development were surface sterilized with 0.05% $HgCl_2$ for 15 minutes and were then rinsed 4-5 times with sterilized distilled water. Anthers (10

Figure 1 A, B: Inflorescence and solitary flower of *Crateva nurvala*. (A) Inflorescence with inset of a floral bud of the size ascertained to be containing microspores at desired stage of development. (B) A flower.

per culture tube) were cultured on Murashige and Skoog's (1962) basal medium (BM), supplemented with different concentrations of sucrose 2, 4, 6 and 8%, w/v), gelled with 0.8% agar (Qualigens, India), and adjuvated with 6-Benzylaminopurine (BAP, 0.5 or 1.0 mg^{-1}) and/or 2,4-Dichlrophenoxyacetic acid (2,4-D, 0.5 mg l^{-1}). For preparation of the medium, all the constituents except sucrose and agar were mixed. However, agar and sucrose were mixed together in a volume of distilled water that was about 40% of the final volume. In order to melt the agar, this mixture was incubated in an autoclave for 10 minutes at 0.5-0.6 kg cm^{-2}. The mixture of molten agar and sucrose was then mixed with other constituents of the medium and the final volume was raised to the desired level. This was followed by adjustment of pH to 5.8 by adding 0.1 M NaOH or 0.1 M HCl, as required. Twenty ml of the medium, when it was still in viscous liquid form, was dispensed per culture tube The culture tubes were plugged with non-adsorbent cotton wrapped in muslin cloth. The culture tubes containing the medium were sterilized by autoclaving at 1.05 kg cm^{-2} at 121 ^{o}C for 15 minutes. The anther cultures were initially incubated in dark for three weeks and thereafter, in continuous light (3.86 W m^{-2}) provided by cool daylight 40 W fluorescent tubes (Phillips, India) at 25±2 ^{o}C for three weeks more.

All the cultures were observed at weekly intervals for callusing or any other morphogenetic development. The callusing anthers were transferred to MS medium supplemented with 3% (w/v) sucrose and augmented with different concentrations of BAP (1, 2, 3, 4, 5 and 6.0 mg^{-1}), maintaining 12 cultures for each treatment during all the sub-culture passages. Subsequently, only the calli were repeatedly sub-cultured on the same medium after every 30 days.

For each treatment, 40 anthers were cultured and the experiments were repeated twice. However, the data for each treatment is based on the number of anthers left after discounting infected cultures. To see if the observed differences are statistically significant, pooled up data for each treatment was subjected to chi-square test with 0.05% probability.

For ascertaining the microspore origin of the regenerants in anther cultures, two methods were used. These were (a) flow cytometric analysis and (b) comparing the guard cell dimensions of leaves from mature tree, *in vitro* raised seedlings and anther-derived shoots. Flow cytometric analysis was conducted using the protocol as described by Arumugunathan and Earle (1991).

For determining the guard cell size, peel mounts of the leaves from different sources as described above were prepared. Since leaf tissue of *in vitro* raised plants was very soft, manual peeling was not possible. Therefore, peels were prepared by dissolving the mesophyll tissue in diluted nitric acid. The leaves of each sample were taken separately in culture tubes and warmed in nitric acid till mesophyll tissue dissolved. For *in vitro* leaves, 40% nitric acid was used, while for leaves from mature tree, 60% nitric acid was more effective. The peels after acid treatment were washed 3-4 times with distilled water by slow pipetting. Subsequently, the peels were deacidified with KOH solution (one pellet in 25 ml of distilled water). Thereafter, these were left overnight in 1% (w/v) safranin for staining and then washed with water to remove extra stain. The peels from upper epidermis that lacked stomata were discarded. Lower epidermal peels having stomata were mounted in a drop of glycerine on a clean glass slide. For measuring the stomatal size, ocular calibrated beforehand with a stage micrometer was used. The experiment was repeated thrice.

Results

After one week of incubation in dark, anthers cultured on the media supplemented with 2,4-D alone or in combination with BAP swelled considerably. However, no swelling of anthers was observed on the basal medium or the same supplemented with BAP alone. By the end of fourth week, green compact calli developed from the filamentous end and soft friable callus from the entire length of ruptured anthers (Fig. 2A). After transfer to light, an increase in callusing was observed. The highest percentage of callusing was on the medium supplemented with 2,4-D and 2% sucrose. The increase in sucrose concentration in the medium affected the callusing response adversely. However, a high percentage of anthers callused on the media supplemented with both 2,4-D and BAP. Although, the values of callusing anthers for different treatments were numerically different, the differences were not statistically significant (Table 1).

For differentiation, callusing anthers were transferred to MS medium containing 3% sucrose and augmented with 1.0 to 6.0 mg l^{-1} BAP. No significant qualitative change, was observed during the course of first sub-

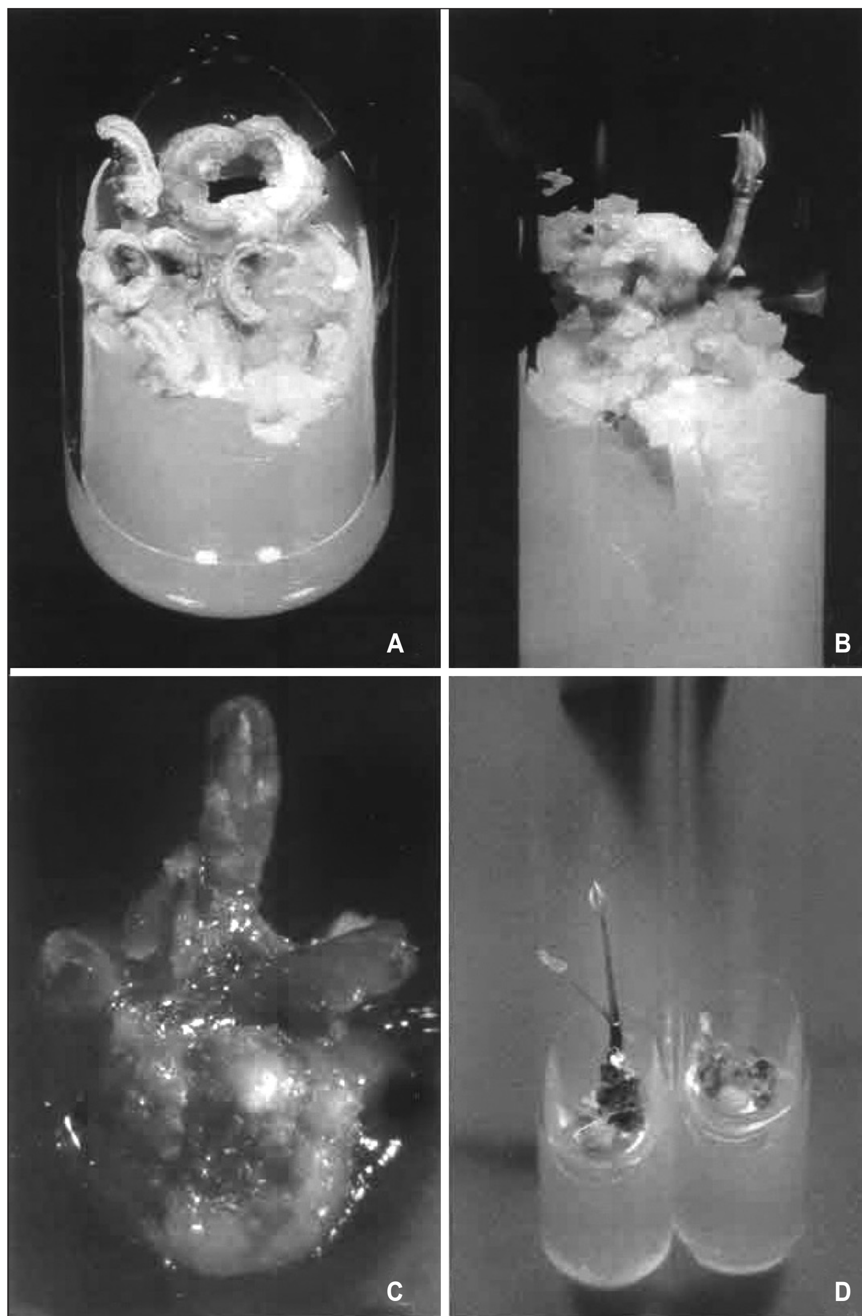

Figure 2 A-D: Anther culture of *Crateva nurvala*. (A) Development of calli from anthers cultured for five weeks on MS + 2,4-D, 0.5 mg ml^{-1}. (B) Development of a shoot from callus cultured on BAP, 3.0 mg m l^{-1} after six passages on the same medium, each lasting one month. (C) Multiple shoot buds from the nodal explant of an anther callus-derived shoot after three weeks of culture on the medium adjuvated with 0.5 mg ml^{-1} BAP. (D) Two types of shoots multiplied from the original anther callus derived shoots.

culture except in some cases calli became compact and green. The calli were sub-cultured on the same medium after monthly intervals. Only after fifth sub-culture, three shoots developed on the medium supplemented with 4.0 mg l^{-1} BAP and one shoot on the medium containing 3 mg l^{-1} BAP (Fig. 2B). Thus, out of a total of 2520 anthers cultured on different media, only 0.16% regenerated shoots. Since the number of differentiated shoots was very low, these were multiplied on MS medium containing 3% sucrose and supplemented with 0.5 mg l^{-1} BAP (Fig. 2C). The shoots multiplied and maintained the growth characteristics similar to the original four shoots, hereafter referred to as clones one to four. These clones exhibited differences in

Table 1. Response of *Crateva nurvala* anthers cultured on MS basal medium (BM) containing different concentrations of sucrose, BAP and/or 2,4-D for 30 days. Values followed by same superscript(s) are not statistically significant (P = 0.05%).

Culture medium	Number of anthers	% Callusing anthers	Relative amount of callus
BM + 2% sucrose	110	0^{c}	0
BM + 4% sucrose	110	0^{c}	0
BM + 6% sucrose	110	0^{c}	0
BM + 8% sucrose	100	0^{c}	0
BM + 2% sucrose + 0.5 mg l^{-1} BAP	110	0^{c}	0
BM + 4% sucrose + 0.5 mg l^{-1} BAP	110	0^{c}	0
BM + 6% sucrose + 0.5 mg l^{-1} BAP	100	0^{c}	0
BM + 8% sucrose + 0.5 mg l^{-1} BAP	100	0^{c}	0
BM + 2% sucrose + 1.0 mg l^{-1} BAP	110	0.91^{c}	++
BM + 4% sucrose + 1.0 mg l^{-1} BAP	110	0^{c}	0
BM + 6% sucrose + 1.0 mg l^{-1} BAP	100	0^{c}	0
BM + 8% sucrose + 1.0 mg l^{-1} BAP	100	0^{c}	0
BM + 2% sucrose + 0.5 mg l^{-1} 2,4-D	110	91.81^{a}	++
BM + 4% sucrose + 0.5 mg l^{-1} 2,4-D	110	80.0^{ab}	+++
BM + 6% sucrose + 0.5 mg l^{-1} 2,4-D	90	76.67^{ab}	++
BM + 8% sucrose + 0.5 mg l^{-1} 2,4-D	100	69.0^{ab}	++
BM + 2% sucrose + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} 2,4-D	110	89.09^{ab}	++
BM + 4% sucrose + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} 2,4-D	110	90.0^{ab}	+++
BM + 6% sucrose + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} 2,4-D	90	84.44^{ab}	+++
BM + 2% sucrose + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} 2,4-D	110	84.5^{ab}	+++
BM + 2% sucrose + 1.0 mg l^{-1} BAP + 0.5 mg l^{-1} 2,4-D	110	83.63^{ab}	+++
BM + 4% sucrose + 1.0 mg l^{-1} BAP + 0.5 mg l^{-1} 2,4-D	110	88.18^{ab}	+++
BM + 6% sucrose + 1.0 mg l^{-1} BAP + 0.5 mg l^{-1} 2,4-D	110	80.0^{ab}	+++
BM + 8% sucrose + 1.0 mg l^{-1} BAP + 0.5 mg l^{-1} 2,4-D	90	66.7^{ab}	++++

their growth and morphology (Fig. 2D). Compared to clones one and three, shoots of clones two and four were stunted in growth up to third passage. By the eighth passage, shoots of clone two and four had also attained growth comparable to the other two clones.

To determine the ploidy of anther-derived shoots, flow cytometric analysis of four clones in their second and fourth passages was conducted. Leaves from the shoots regenerated from microshoots that were earlier developed from the seedling explants sub-cultured and were being repeated as described earlier Walia et al., 2003) served as control. This analysis revealed that out of four clones, 'C' values of two clones (2 and 4) were between 1C and 2C (control), whereas the other two clones (1 and 3) had values closer to that of the control (Table 2).

Owing to the inconclusive results obtained from flow cytometry and inability to do cytological analysis due to non-availability of roots, average stomatal sizes of the four clones were determined. For this peel mounts from leaves of four anther-derived shoots, *in vitro* raised seedlings and those of mature tree were examined (Fig. 3 A-F). The latter two served as controls.

The distribution of stomata in peel mounts was scanty in some parts, while in a few regions they occurred in clusters. Because of the non-uniform distribution of stomata, stomatal frequencies were not recorded. The dimensions of stomata were measured in terms of length and width of guard cells. Corresponding with the flow cytometric data, stomatal sizes in clones 2 and 4 were smaller than the controls, while the other two clones 3 and 4 had stomatal sizes comparable to that of controls (Table 2).

Discussion

In *C. nurvala*, anthers cultured on media supplemented with 2,4-D alone or in combination with BAP produced calli. The requirement of both auxin and cytokinin for elicitation of response has been reported in other genera as well. Some of such examples are *Hevea brasiliensis* (Chen et al., 1982), *Carica papaya* (Tsay & Su, 1985), *Malus domestica* and *Prunus avium* (Hoeffer & Henke, 1990), *Azadirachta indica* (Gautam et al.,

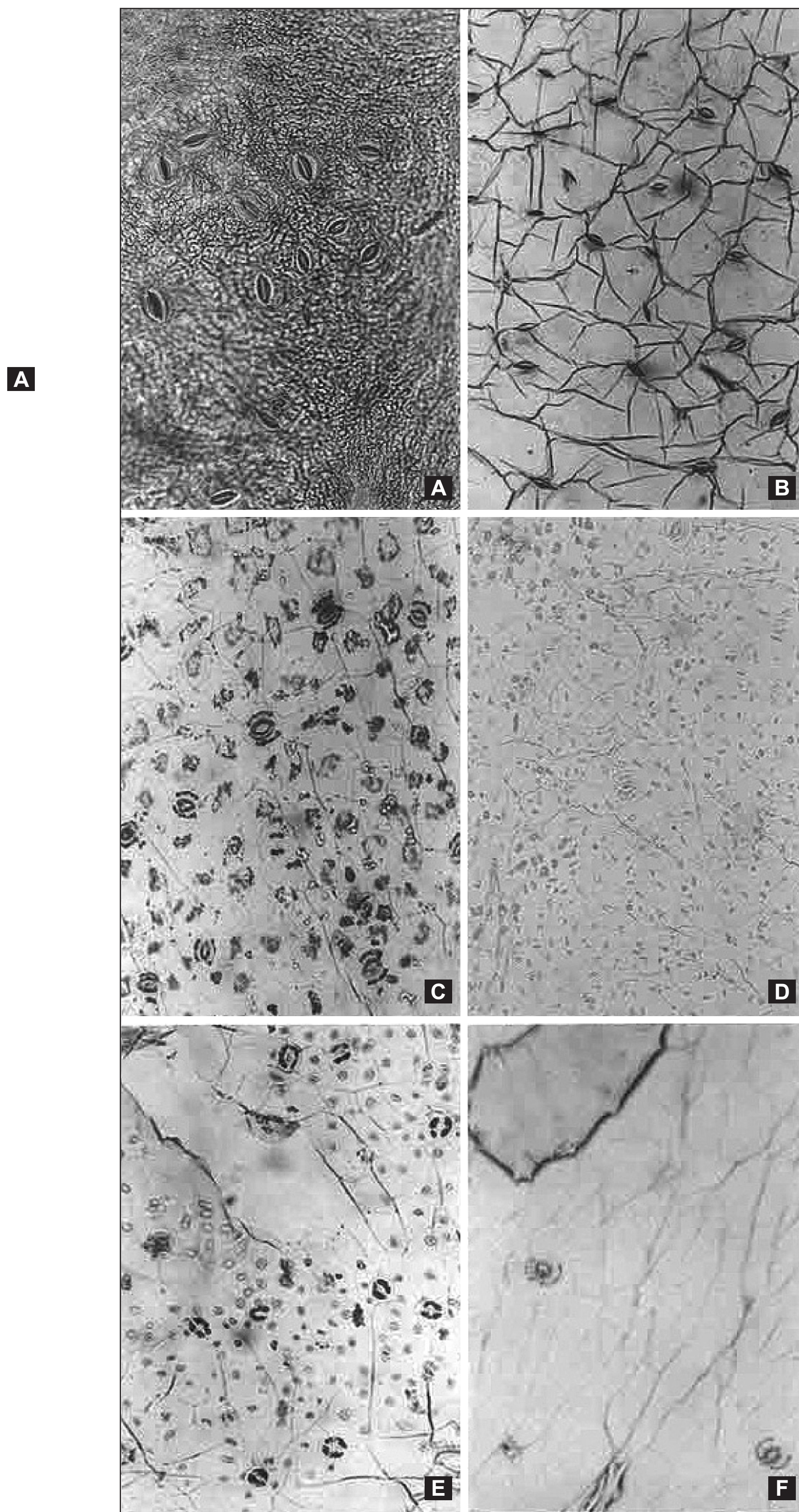

Figure 3: A-F: Peel mounts of leaves of *Crateva nurvala* from (A) mature tree, (B) *in vitro* grown seedling, (C-F) clones 1-4 of anther-derived shoots, respectively.

Table 2. Stomatal dimensions (mean ± standard deviation), mean flow cytometric (FCM) peak values and % DNA contents of leaves derived from different sources, including four anther derived shoots' clones of *Crateva nurvala* (in relation to that of the mature tree taken as 100%).

Source of leaves	Length of stomata (µm)	Width of guard cells (µm)	Mean peak FCM value	Relative DNA content (%)
Mature tree (control 1)	38.25±7.03	8.56±2.22	84.84	100
In vitro seedling/microshoot	32.01±5.90	8.41±2.59	76.05	89.6
Clone 1 shoot	28.08±4.81	8.67±1.94	76.84	90.6
Clone 2 shoot	24.5±6.36	6.68±2.92	59.05	69.6
Clone 3 shoot	32.43±5.52	9.88±2.9	80.03	94.3
Clone 4 shoot	24.82±5.07	6.93±3.02	56.09	66.1

1993, Chaturvedi et al., 2003), *Fragaria x ananassa* (Owen & Miller, 1996), and *Musa* sp. (Assani et al., 2003). For subsequent development, callusing anthers were transferred to the medium containing only BAP (1.0-6.0 mg l^{-1}). During sub-cultures, which were after every one month, only four shoots developed. Thus, the response was sporadic. However, as two of these shoots differed in their growth characteristics especially in their length and the size of leaves at corresponding stages of development of the other two, we decided to investigate these further. Therefore, the shoots were multiplied as four separate clones by cultivating their nodal explants on shoot regeneration medium following the protocol earlier developed in our laboratory (Walia et al., 2003). Shoots emanating from these original four clones maintained their phenotype similar to those of the shoots from which these originated for three multiplication cycles. By the eighth passage even the shoots having their origin from the two exhibiting stunted growth became comparable to other two clones. This could have happened because of the spontaneous doubling of the chromosomes, which is well known to happen in repeated sub-cultures as is exemplified by microspore-derived plants of cabbage and broccoli (Yuan et al., 2015). However, this explanation would be valid only if the shoots with stunted growth were presumably haploid.

To check the above presumption, regenerated shoots were analysed for their ploidy status. The most suitable and direct method of ascertaining the chromosome numbers by root tip squash preparation could not be used because of the non-availability of roots. Therefore, ploidy determination was attempted by two methods, which were one involving direct estimation of genome size through flow cytometry and the second an indirect method based on the comparison of stomatal (guard cell) size, commonly used for this purpose (Dunwell, 2010). By flow cytometry nuclear DNA content is estimated, which directly correlates with ploidy of the plant (Sharma et al., 1983, Sree Ramulu & Digkhuis, 1986, Martin & Widholm, 1996.

In the present study, flow cytometric analyses revealed ploidy of the two clones comparable to the controls (diploid, 2n = 26, see Singhal et al., 1985) and two were near haploids. Variations in genomic content of the plants regenerated from anthers have been commonly recorded. These variations arise due to change in ploidies in culture, chromosomal rearrangements or changes at sub-microscopic level, or could be pre-existing among the microspores (Babbar & Kalia, 1998). In this context, it would be worth mentioning that 8.4% of the observed PMCs of *C. nurvala* were reported to contain 1-3 supernumerary B-chromosomes (Singhal et al., 1985). These B chromosomes are a major source of intraspecific variation in nuclear DNA amounts in numerous species of plants (Jones et al., 2008), and could well have been in microspore population of *C. nurvala* too. The other reason could have been meiotic abnormalities during microsporogenesis, which affect genetic constitution and pollen viability (Kaur & Singhal, 2019). Incidentally, Singhal et al. (1985) have already reported 30-35% pollen sterility *C. nurvala*, which could have been because of impaired segregation of chromosomes during male meiosis.

As an indicator of ploidy, guard cell size/stomatal size is used frequently, especially for regenerants derived from anther culture (Babbar & Kalia, 1998, Dunwell, 2010). Some of the taxa in which this feature has been used for ploidy determination of anther-derived plants are *Solanum nigrum* (Harn, 1972), *Pelargonium roseum* (Tokumasu & Kato, 1979), *Oryza sativa* (Chen et al., 1986), *Hordeum vulgare* (Borrino & Powell, 1988), *Brassica oleracea* var. *botrytis* (Ockendon, 1988), *Anthurium andraeanum* (Winarto et al., 2010) and *Brassica napus* (Soroka, 2013). The results of guard cell measurements in the leaves of four clones

substantiated the flow cytometric inferences as the two clones having DNA contents comparable to the control also had similar sized guard cells. The other two clones having DNA contents lesser than the controls had guard cells smaller than those of the controls. Thus, the results of both methods used for determination of ploidy status indicated the microspore origin of the regenerated shoots through androgenesis.

To conclude, though the attempts to raise haploid plants of *C. nurvala* were not successful, these cannot be considered totally futile as the observations are indicative of induction of androgenesis. The regeneration of haploid plants from microspores of *C. nurvala* would require further extension and extensive improvement in the methods used in the present study.

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