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Studies on *in vitro* chromosome doubling of haploid derived through androgenesis in marigold

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ABSTRACT

A local strain of *Tagetes patula* L. was used for *in vitro* androgenesis with floret size ranging from 2 to 4.5 mm. One haploid from anther derived regenerants was isolated and confirmed by cytological analysis and chloroplast counting. The isolated haploid was multiplied and subjected to colchicine treatment (0.005%, 0.01%, 0.02% for 36 and 48 hours) using nodal segment explants under *in vitro* conditions. The results revealed that treatment T_1- Colchicine (0.005%) for 36 hours exposure was good for parameters like per cent survival (81.22%), per cent diploidization (48.00%), leaf length (3.77 cm), leaf width (2.49 cm), number of shoots per plant (9.89) followed by treatment T_2- Colchicine (0.005%) for 48 hours exposure for parameters like per cent survival (69.67%), per cent diploidization (37.22%), leaf length (3.56 cm), leaf width (1.97 cm), number of shoots per plant (8.67). Cytological analysis and chloroplast counting in stomatal guard cells were done to determine ploidy levels in colchicine-treated haploid plantlets. Cytological analysis of 38 plants revealed that 26 (68.42%) were tetraploids and 12 (31.57%) were polyploids. Whereas through chloroplast counting, 38 plants were screened, out of which 26 plants (68.42%) were tetraploids with a mean of 16 chloroplasts in the guard cells and 12 plants (31.57%) were polyploids with a mean of 18 to 22 chloroplasts in the guard cells.

Key words: *Tagetes patula* L., Doubled haploids, Ploidy analysis, Colchicine, Choloroplast.

INTRODUCTION

Marigold (*Tagetes* spp.) is a popular ornamental crop belonging to the *Asteraceae* family. Marigold flowers are extensively used as loose flowers for making garlands, religious offerings, social functions and other purposes like pigment and essential oil extraction. It is also widely grown for display purposes in garden beds and pots. A total of 55 species are reported in the genus *Tagetes*, out of which African marigold (*Tagetes erecta* L.) and French marigold (*Tagetes patula* L.) are of commercial importance (Hernandez and Ham, 3).

Biotechnology is one of the most powerful tools for plant breeding. Among these tissue culture techniques, haploid and doubled haploid production can be extremely valuable in crop improvement programmes for many important crops. Haploid induction through anther or ovule culture is the only way to achieve homozygosity in self-incompatible species, dioecious species and species that suffer from inbreeding depression due to self-pollination in major *Asteraceae* crops like chrysanthemum (Wang *et al*., 15). Doubled haploids (DHs) refer to the lines obtained by doubling the chromosome number of a haploid line using antimitotic agents. DH production can occur spontaneously and artificially using chromosome-doubling agents like colchicine, trifluralin, oryzalin, etc. DHs are important in crop improvement programmes as they can be utilized as parental lines in hybridization programmes. Besides, DHs have other benefits, such as direct release as new varieties (Veilleux, 14), separation of recessive gene mutants, relation to genetic mapping, genomics, gene expression, and reverse breeding (Wijnker and de Jong, 16). Using DHs developed through tissue culture drastically reduces the time for precise and efficient selection of desirable traits.

Marigold is a highly cross-pollinated crop that generally takes 5 to 6 generations to develop an inbred line from the heterozygous population. Homozygous parental lines are indispensable for the success of any hybrid development programme. Hence, anther culture is an approach which can considerably boost the breeding programme by providing homozygous lines after a single *in vitro* culture step. It avoids the time-consuming process of developing inbred lines by selfing over years and generations. To boost the F_1 hybrid development programme in Marigold, the utility of DHs is more as they can be used as homozygous parental lines in the breeding programme (Han *et al*., 2).

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MATERIALS AND METHODS

A local strain of *Tagetes patula* L. with a bud length of 2.0 to 2.5 cm and a floret size of 3.0 to 3.5 mm was found best as it observed maximum early uninucleate (38%), late uninucleate (45%) and early binucleate (14%) stages, which were highly suitable for culturing anthers. Nodal segments of *in vitro* multiplied haploids were subjected to chromosome doubling using colchicine (Hi Media Private Limited, New Delhi). The stock solution was prepared by dissolving 1 g of colchicine in 100 ml of autoclaved double distilled water (1%). Then, the stock solution was diluted with sterile distilled water to prepare the working stock. MS medium was prepared by supplementing sucrose (3%), solidifying with agar-agar (0.8%) and without adding growth regulators. Five hundred (500) ml of medium was prepared separately in four 1000 ml conical flasks. The contents were transferred to a vertical autoclave and sterilized at 121°C for 20 minutes at 15 lbs/inch^2 pressure. After autoclaving, flasks were transferred to a laminar airflow chamber, and four different colchicine concentrations (0, 0.005, 0.01 and 0.02%) were separately added by using 0.22 µm syringe filters. One haploid was isolated and was confirmed through direct and indirect screening methods. It was further multiplied and maintained. For doubling the chromosome number of these plants, nodal segments of 2 cm (approx.) length were cut and inoculated in already prepared colchicine medium in three different concentrations (0.005, 0.01 and 0.02%) along with control. The shoots were incubated in two different durations (36 and 48 hours) of each concentration (Table 1). The treated shoots were transferred to MS medium supplemented with sucrose (3%) without adding growth regulators. After two sub-cultures, surviving healthy shoots were transferred to rooting media containing Half MS media, 0.8% agar-agar, $AgNO_3$ (2.5 ml/l), Adenine sulphate (0.016 g/l), sucrose (60 g/l), and IBA (0.5 mg/l). Ten days after treatment, healthy, actively grown roots were used for cytological studies, whereas *in vitro* grown leaves were taken for chloroplast studies. The methods to determine ploidy level were direct and indirect. In the direct screening method, chromosome counting was done at mitotic metaphase. The root tip squash technique was used with a few modifications (Rajalakshmi and Joseph, 10; Zhang *et al*.,17). In the indirect screening method, chloroplast number was counted in stomata guard cells. The third leaf tip of the *in vitro* raised plants was collected and used for counting chloroplast in the stomata guard cell. Leaves from the regenerants were kept in 3:1 ethanol and glacial acetic acid solution for 4-6 hours at room temperature. The central portion of these leaves (0.5 cm^2) was used, and the abaxial side was selected for the observation. Then, each separated leaf piece was placed in a drop of potassium iodide or acetocarmine solution on a clean glass slide, placed cover slip on the leaf and examined at 100x magnification using a compound microscope (Nikon ECLIPSE 50 *I*). Stomatal chloroplasts in 25 individual guard cells were counted from regenerated plants, and the average mean values were compared with the respective donor mother plants. The experiment was designed in complete randomized design (CRD),

Table 1. Effect of colchicine treatment on per cent survival, per cent diploidization, leaf length, leaf width and number of shoots per plant of *in vitro* multiplied haploids.

Treatment (s)	Per cent survival	*Percent diploidization	Leaf length (cm)	Leaf width (cm)	Number of shoots per plant
Control-T0	100.00 ± 0.00 (90.00)	0.00 ± 0.00 (0.00)	1.54 ± 0.13	0.78 ± 0.07	3.22 ± 0.28
Colchicine (0.005%) for 36 hrs -(T1)	81.22 ± 0.81 (64.40)	48.00 ± 0.80 (43.87)	3.77 ± 0.14	2.49 ± 012	9.89 ± 0.63
Colchicine (0.005%) for 48 hrs -(T2)	69.67 ± 1.33 (56.66)	37.22 ± 1.05 (37.60)	3.56 ± 0.14	1.97 ± 0.16	8.67 ± 0.50
Colchicine (0.01%) for 36 hrs - (T3)	49.44 ± 0.84 (44.70)	32.78 ± 0.85 (34.93)	3.03 ± 0.10	1.61 ± 0.09	7.78 ± 0.40
Colchicine (0.01%) for 48 hrs -(T4)	23.22 ± 0.64 (28.81)	12.78 ± 0.57 (20.92)	2.98 ± 0.08	1.54 ± 0.10	7.00 ± 0.37
Colchicine (0.02%) for 36 hrs - (T5)	13.22 ± 0.62 (21.29)	6.00 ± 0.47 (14.10)	2.63 ± 0.09	1.51 ± 0.11	6.33 ± 0.33
Colchicine (0.02%) for 48 hrs -(T6)	4.56 ± 0.18 (12.31)	0.33 ± 0.17 (1.91)	2.48 ± 0.11	1.41 ± 0.09	5.11 ± 0.31
Mean	48.76	19.59	2.86	1.62	6.86
S.E (m)	0.75	0.66	0.12	0.11	0.42
CD (5%)	2.14	1.87	0.33	0.31	1.19

Figures in parenthesis are arcsine transformed values

*Ploidy analysis through chromosomal and chloroplast counting

and data were analyzed using statistical software SPSS. For data that represent percentages, the arcsine transformation was used (Cohen *et al*., 1)

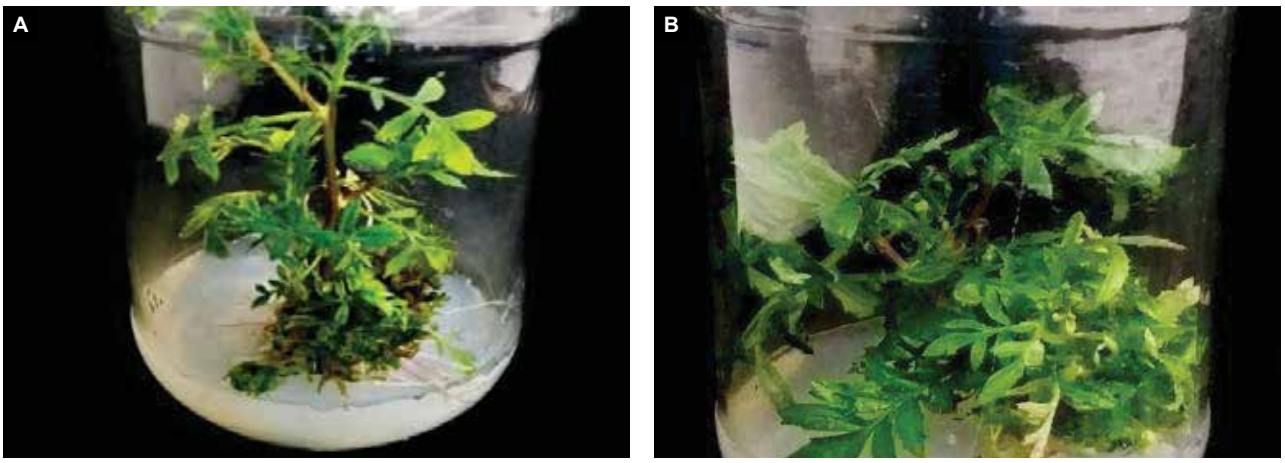
RESULTS AND DISCUSSION

The results revealed that the survival percentage of haploid nodal segments treated with colchicine was significantly lower than the control. It is also evident from the data that increasing the concentration of colchicine and duration time decreased the survival of treated haploid shoots (Table 1). Among the different colchicine treatments, the maximum per cent survival was found in treatment T_1 (81.22%) followed by treatment T_2 (69.67%). The treatment (T_1) was statistically significant among all the treatments. Among the different colchicine treatments, the maximum diploid frequency was found in treatment T_1 (48.00%), followed by treatment T_2 (37.22%). Maximum leaf length was observed in treatment T_1 (3.77 cm), followed by treatment T_2 (3.56 cm). Maximum leaf width was observed in treatment T_1 (2.49 cm), followed by treatment T_2 (1.97 cm). The maximum number of shoots per plant (9.89) was observed in treatment T_1, followed by treatment T_2 (8.67). In control, 100 per cent survival was observed with leaf length (1.54 cm), leaf width (0.78 cm), and shoot number (3.22). The treatment (T_1) was statistically significant among all the treatments for all the parameters (Fig.1. A-B & Fig. 2. A-B). Previously, Kurimella *et al.* (6) reported maximum survival of haploid marigold plants treated with colchicine @ 0.001% for 15 and 30 hours, and they also observed maximum chromosome doubling of colchicine-treated haploid plants at 0.01% concentration.

T_1: Colchicine 0.005% (36 hours)

T_2: Colchicine 0.005% (48 hours)

Fig. 1. Shoot initiation (A, B) in colchicine-treated haploid plants in French marigold (*Tagetes patula* L.).



T_1: Colchicine 0.005% (36 hours)

T_2: Colchicine 0.005% (48 hours)

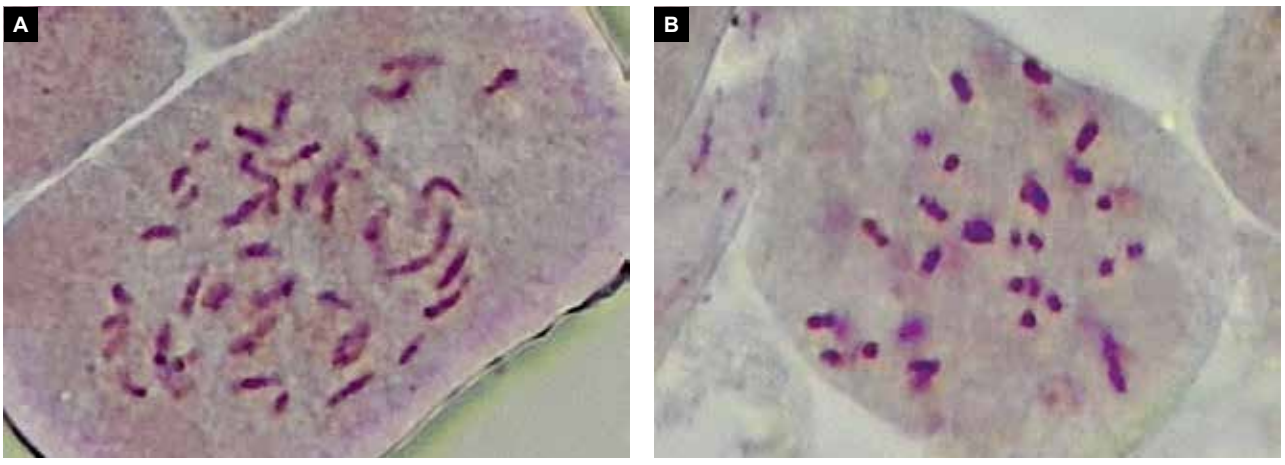
Fig. 2. Shoot growth (A, B) in colchicine-treated haploid plants in French marigold (*Tagetes patula* L.).

Sajjad *et al*. (11) also reported that the induction rate of multiple shoots increased with the increase in colchicine concentration, but very high colchicine concentration had a negative effect on shoot proliferation in African marigold. High colchicine concentration has a lethal impact, which leads to much stress on plant cells and causes death of explant. Similar to our findings, Liu *et al*. (7) reported that in *Platanus acerifolia* tetraploid plants, the leaf petioles tended to be slightly shorter and broader than the diploids, and the leaves were also thicker, broader, and darker green in appearance compared to diploids. Similarly, Todorova *et al.*(12) reported that doubled haploid lines in sunflowers, when treated with a colchicine solution of 0.15% concentration for five hours, were found to be fertile in nature. Gynogenic pot gerbera haploid plants were treated with 0.05% colchicine for different time durations, *i.e*. 2, 3, or 6 days and the occurrence of diploid plants was highest (34.1%) in two days of treatment, followed by three days (24.2%). They also reported that as the duration of colchicine treatment increased, there was a gradual decrease in plant survival. which is in accordance with present findings.

Cytological analysis and chloroplast counting in stomatal guard cells were carried out to determine ploidy levels in colchicine-treated haploid plantlets. Among anther derived doubled haploid plantlets, 38 plants were chosen randomly for cytological analysis and ploidy level determination (Table 2). Out of the 38 plants, 26 plants (68.42%) were found to be tetraploids (2n = 4x = 48), while 12 plants (31.57%) were found to be polyploids with ploidy level 4x to 6x (Fig. 3. A-B). The number of chloroplasts present in the stomatal guard cells of the same 38 plants was studied. Results showed that the tetraploids had 16 chloroplasts in their guard cells, and the polyploids had 18 to 22 chloroplasts in their guard cells. (Fig. 4. A-B). Similarly, Kumar *et al.*(4) reported that among the 56 plants tested in *Tagetes patula* cv. Pusa Arpita, 37 plants had a chromosome number of 48, similar to their tetraploid donor mother plant (2n = 4x = 48); 11 plants were found to be polyploids having chromosome number more than 48. Lu and Bridgen (8) reported that 41% of treated plants were found to be tetraploid in ploidy analysis through chromosome counting, and after one year, 87.5% of the treated tetraploids were found stable. Our research findings are in close agreement with Kumar *et al.* (5), who reported the number of chloroplast present in the stomatal guard cell of the 56 individual plants studied in marigolds. It was found that the tetraploid donor parent had 16.3 chloroplasts in their guard cells, and the polyploids had 23.7. Vanlaere *et al*.(13) reported

Table 2. Cytological analysis and chloroplast counting in stomatal guard cells to determine ploidy level in colchicine-treated haploid regenerants of French marigold (*Tagetes patula* L.).

Technique	Plants (No.) screened for ploidy analysis	Ploidy level	
		Tetraploid (4x)	Polyploid (>4x-6x)
Chromosomal count in root tip cells	38	26 (68.42%)	12 (31.57%)
Chloroplast per pair of stomatal guard cells	38	26 (68.42%)	12 (31.57%)



Tetraploid (2n=4x=48) Triploid (2n=3x=36)

Fig. 3. Cytological screening of colchicine treated haploid plants in French marigold (*Tagetes patula* L.).

Tetraploid (chloroplast number=16) Polyploid (chloroplast number=18-22)

Fig. 4. Chloroplast number in stomatal guard cell of colchicine treated haploid plants in French marigold (*Tagetes patula* L.).

that stomatal density was negatively correlated with increased ploidy level.

AUTHORS' CONTRIBUTION

Conceptualization (SP, KPS,N), Designing of experiment (SP, KPS,N), Contribution of research materials (KPS,SP,N, LS,NM), Execution of lab experiments (VC,SP),Preparation of manuscript (VC, SP)

DECLARATION

The authors declare no conflict of interest.

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REFERENCES:

1. Cohen, J., Cohen, P., West, S. G., and Aiken, L. S. 2003. *Applied Multiple Regression/ Correlation Analysis for the Behavioral Sciences.* Lawrence Erlbaum Associates Publishers, USA 691p.

2. Han, D. S., Niimi, Y. and Nakano, M. 1997. Regeneration of haploid plants from anther cultures of the Asiatic hybrid lily 'Connecticut King'. *Plant Cell Tissue Organ Cult.* **47**: 153-58.

3. Hernandez, G. and Ham, M. L. 2007. Marigold biotechnology: Tissue culture and genetic transformation. *Transgenic Plant J.* **1**:169-74.

4. Kumar, K. R., Singh, K. P., Bhatia, R., Raju, D. V. S. and Panwar, S. 2019. Optimising protocol for successful development of haploids in marigold (*Tagetes* spp.) through *in vitro* androgenesis. *Plant Cell Tissue Organ Cult.* **138**: 11-28.

5. Kumar, K. R., Singh, K. P., Raju, D. V. S., Bhatia, R., and Panwar, S. 2020. Maternal haploid induction in African marigold (*Tagetes erecta* L.) through *in vitro* culture of un-fertilized ovules. *Plant Cell Tissue Organ Cult.* **143**:549-64.

6. Kurimella, R. K., Bhatia, R., Singh, K. P., and Panwar, S. 2021. Production of haploids and doubled haploids in marigold (*Tagetes spp*.) using anther culture. **In**: *Doubled Haploid Technology* . Humana, New York, pp. 271-87.

7. Liu, G., Li, Z. and Bao, M. 2007.Colchicine induced chromosome doubling in *Platanus acerifolia* and its effect on plant morphology. *Euphytica*, **157**:145-54.

8. Lu, C. and Bridgen, M. P. 1997. Chromosome doubling and fertility study of *Alstroemeria aurea* x *A. caryophyllaea*., *Euphytica*, **94**:75-81.

9. Miyoshi, K. and Asakura, N. 1996. Callus induction, regeneration of haploid plants and chromosome doubling in ovule cultures of pot gerbera (*Gerbera jamesonii*). *Plant Cell Rep.* **16**: 1-5.

10. Rajalakshmi, R. and Joseph, J. 2004. Karyomorphometrical analysis and chemical

polymorphism in *Tagetes erecta* and *Tagetes patula*. *Philipp. J. Sci.* **133**:135.

11. Sajjad, Y. Jaskani, M. J., Mehmood, A., Ahmad, I. and Abbas, H. 2013. Effect of colchicine on *in vitro* polyploidy induction in African marigold (*Tagetes erecta*). *Pak. J Bot.* **45**: 1255-58.

12. Todorova, M., Ivanov, P., Shindrova, P., Christov, M. and Ivanova, I. 1997. Doubled haploid production of sunflower (*Helianthus annuus* L.) through irradiated pollen- induced parthenogenesis. *Euphytica*, **97**:249-54.

13. Vanlaere, K., Franca, S. C., Vansteenkiste, H., Van Huylenbroeck, J., Steppe, K. and Van Labeke, M. C. 2011. Influence of ploidy level on morphology, growth and drought susceptibility in *Spathiphyllum wallisii*, *Acta Physiol. Plant.* **33**:1149-56.

14. Veilleux, R E. 1994. Development of new cultivars via anther culture. *HortScience,* **29**: 1238-41.

15. Wang, H., Dong, B., Jiang, J., Fang, W., Guan, Z., Liao, Y., Chen, S. and Chen, F. 2014. Characterization of *in vitro* haploid and doubled haploid *Chrysanthemum morifolium* plants via unfertilized ovule culture for phenotypical traits and DNA methylation pattern. *Front. Plant Sci.* **5**:1-10.

16. Wijnker, E. and de Jong, H. 2008. Managing meiotic recombination in plant breeding. *Trends in Plant Sci.* **13**: 640-46.

17. Zhang, P., Zeng, L., Su, Y., Gong, X. and Wang, X. 2011. Karyotype studies on *Tagetes erecta* L. and *Tagetes patula* L. *African J. Biotechnol.* **10**:16138- 44.

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