



# Colchicine-mediated in vitro polyploidization in gerbera hybrid

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## Abstract

An efficient in vitro protocol for high-frequency polyploidization for the first time in gerbera hybrid (BGC-2019-01) was developed in the present study. Two-week-old in vitro-developed shoots (tips) were treated individually with 0.1%, 0.25% and 0.5% (w/v) colchicine solutions for 4, 6, 8, and 12 h. The colchicine-treated shoot tips were then inoculated on Murashige and Skoog (MS) medium fortified with 1.5 mg/l *meta*-Topolin for multiple shoot proliferation and later transferred into 1.5 mg/l indole-3-acetic acid-fortified MS medium for rooting of shoots. The ploidy levels of the colchicine-treated and regenerated plantlets along with the non-treated ones were confirmed via flow cytometry analysis and metaphasic chromosome count. The highest frequency of tetraploid plantlets (50%) were obtained when shoot tips were treated with 0.1% colchicine for 4 h. Morphological observations revealed that induced tetraploid plantlets exhibited delayed fresh shoot initiation, fewer but longer shoots, as well as fewer but broader leaves. Likewise, the study of stomata revealed that in comparison to their diploid counterparts, the tetraploid plantlets exhibited less frequent yet significantly larger stomata, and higher number of chloroplasts. The tetraploids were recorded with significantly higher chlorophyll, carotenoid, and anthocyanin content during the photosynthetic pigment analyses. During ex vitro acclimatization and field growth, the tetraploid plants exhibited delayed proliferation but with higher vigor and thickened broad leaves. The genetic uniformity among the diploid and the tetraploid plants was confirmed using conserved DNA-derived polymorphism (CDDP), directed amplification of minisatellite-region DNA (DAMD), inter simple sequence repeats (ISSR), and start codon targeted (SCoT) polymorphism marker systems. The tetraploids developed in the present study would be of immense importance for the genetic improvement of gerbera as far as its ornamental values are concerned.

**Keywords** Anti-mitotic agent · Cut flower · Cytology · Flow cytometry · Genetic fidelity · Molecular marker · Photosynthetic pigments · Polyploidy

## Introduction

*Gerbera jamesonii* Bolus ex Hooker f., belongs to the Asteraceae family ( $2n = 2x = 50$ ). It is native to South Africa and Asia, and is listed among the top five in-demand cut flowers in the global floricultural trade. The present cultivars that are used for cut and pot flowers are the results of an interspecific hybridization program (*Gerbera jamesonii* × *Gerbera*

*viridifolia*) that was initiated for the genetic improvement in gerbera (Cardoso and Teixeira da Silva 2013). Conventionally, gerbera is known to be propagated via seeds as well as by vegetative methods (with division of clumps and cuttings) (Gantait et al. 2011). Seed-based propagation results in undesirable variations among the offsprings (as an outcome of cross-pollination), exhibits highly heterozygous nature, and also takes longer time for initiation of flowering. Hence, seed propagation is not widely popular for commercial cultivation. Although propagation via division of clumps is the most commonly followed method of propagation, it is facing drawbacks because of slower rate of multiplication and high susceptibility to soil-borne diseases (Cardoso and Teixeira da Silva 2013). Therefore, researchers established in vitro micropropagation protocols of gerbera plants with the aim of rapid multiplication of large numbers of true-to-type and

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pathogen-free hybrids in recent past (Ibrahim and Yassin 2020, 2021; Mahanta et al. 2023).

The present floriculture industry, as per the prevailing demand, is shifting towards large-scale production of plantlets and variations that add unique ornamental value(s). For commercial cut-flowers like gerbera, such variants with enhanced ornamental value can be achieved via *in vitro* polyploid induction (Gantait et al. 2011). Induction of polyploidy via the application of anti-mitotic agents is the fastest method, and colchicine is the most commonly used anti-mitotic agent (Gantait et al. 2011; Manzoor et al. 2018; Khalili et al. 2020; Niazian and Nalouisi 2020). Polyploids were reported to outperform their diploid parents in most plant species. In gerbera, enhancing the ornamental values like an increased size of flowers, longer stalks, and extended vase-life can be beneficial in fetching higher market value (Gantait et al. 2011; Khalili et al. 2020; Bhattarai et al. 2021). Artificial induction of polyploidy under *in vitro* system can result in quicker induction of variation (Manzoor et al. 2018; Niazian and Nalouisi 2020) and enhance the ornamental characters in gerbera; nonetheless, the success of such *in vitro* polyploidization proved to be genotype-dependent. To date, there are merely four reports on five different gerbera genotypes with distinct colchicine treatment conditions (Tosca et al. 1995; Li et al. 2009; Gantait et al. 2011; Khalili et al. 2020); however, there is no such report on the hybrid (Dana Ellen × Brilliance; BGC-2019-01).

Genetic fidelity assessment of the *in vitro* regenerants has become essential to confirm the genetic stability of the plantlets produced via tissue culture. Inter simple sequence repeats (ISSR) marker system has proved to be simple, effective and reproducible tool for evaluation of genetic uniformity of plants. Other than ISSR, start codon targeted (SCoT) polymorphism, and conserved DNA-derived polymorphism (CDDP) are newly developed marker system for assessing genetic diversity. They are usually dominant, reproducible and are reported to be more informative marker system (Saidi et al. 2020). Apart from the above marker system, directed amplification of minisatellite-region DNA (DAMD) are reported to be quick, reproducible, highly polymorphic and are randomly distributed in the genome resulting in an equally efficient marker system for assessing genetic diversity (Tiwari et al. 2015). To date, there is no report on the use of CDDP, DAMD, ISSR, and SCoT primers for assessing genetic fidelity of *in vitro* diploid and autopolyploid regenerants of gerbera.

Keeping this backdrop in view, the present study aimed to develop a protocol, for the first time on gerbera hybrid BGC-2019-01, for induction of polyploidy using colchicine, to confirm the ploidy level(s) via flow cytometry, cytological assessment and genetic assessment and to evaluate the polyploids based on associated

morpho-anatomical and biochemical attributes in comparison to their diploid counterparts.

## Materials and methods

### Initiation of multiple shoot culture

For the establishment of multiple shoot culture, shoot tips of gerbera hybrid (collection no. BGC-2019-01) were collected from a 3-month-old field-grown plant and were disinfected (following Gantait and Mahanta 2022). The explants were trimmed (~1 cm) and transferred to Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 1.5 mg/l *meta*-Topolin for multiple shoot proliferation (Mahanta et al. 2023).

### Colchicine treatment of shoot tips

Two-week-old actively growing shoot tips were selected from multiple shoot cultures, then trimmed (~1 cm), dipped in 0.1, 0.25, and 0.5% (*w/v*) colchicine (98.5% Extra pure, Loba Chemie Pvt. Ltd., India) solution for 4, 6, 8 and 12 h under a laminar air-flow chamber. After each specified time, the shoot tips were washed with autoclaved water, dried on blotting paper, and inoculated in nutrient media (as specified above) for survival and growth during the next 2 weeks. Shoot tips without colchicine treatment served as control. Proliferated shoots were transferred into 1.5 mg/l indole-3-acetic acid-fortified MS medium for rooting.

### Culture conditions

MS medium (HiMedia, India; Code PT101) supplemented with *meta*-Topolin was used with 0.7% (*w/v*) agar as a gelling agent. All the cultures were maintained at under  $25 \pm 2$  °C temperature, 60% relative humidity and 16h photoperiod using cool white fluorescent tubes with a photosynthetic photon flux density of 50  $\mu\text{mol}/\text{m}^2/\text{s}$ .

### Flow cytometry analysis

Leaf samples from control and colchicine-treated-cum-regenerated plantlets were collected to assess the ploidy level via flow cytometry analysis. For the preparation of nuclei suspensions, ~2 cm<sup>2</sup> leaf tissues were chopped in 5 ml of modified Galbraith's extraction buffer (Galbraith et al. 1983). Then nuclear suspensions were filtered using double-layered 30  $\mu\text{m}$  nylon filters and mixed with 50  $\mu\text{g}/\text{ml}$  of propidium iodide plus 50  $\mu\text{g}/\text{ml}$  RNase solution, after which they were placed on ice in dark for 20 min. The nuclei suspensions were analyzed using flow cytometer (BD LSRFortessa™ Cell analyzer, BD Biosciences, USA) with ~10,000 nuclei per run.

## Metaphase chromosome count

Fresh roots (1–1.5 cm long) from control and colchicine-treated-cum-regenerated plantlets were collected and pre-treated for 5 h with saturated  $p$ -dichloro-benzene ( $p$ DB) solution; then the roots were transferred to fixative solution (glacial acetic acid: ethanol; 1:3  $v/v$ ) for 24 h. Next, the roots were transferred to 70% ethanol (preservative). Individual roots were collected and treated with 9 drops of 2% aceto-orcein and one drop of 1 N HCl for 20 min and briefly warmed under flame (followed by Halder and Ghosh 2021). Then the root tips were squashed on slide using thumb pressure on the cover slip and then observed under an Auxioscope (Carl Zeiss) using Axiovision Rel 4.0 software for counting metaphasic chromosomes.

## Growth and development of plantlets

The tetraploids, confirmed via flow cytometry analysis and chromosome count were subcultured further for comparative study of the growth and development (along with the diploids) with respect to the number of shoots, number of leaves, size of leaves ( $\text{cm}^2$ ) and length of the shoots (cm) (measured using mm graph sheet).

## Study of frequency and size of stomata

To have a comparative study of the size and frequency of the stomata of diploid and tetraploid, the epidermis from the abaxial leaf surface was peeled and mounted in glycerol after staining with safranin following the Clarke (1960) and Speckman et al. (1965). The length and width of stomata and the number of chloroplasts (in guard cells) were measured under the microscope (EVOS<sup>®</sup> FL microscope, Life technologies<sup>™</sup>, USA).

## Photosynthetic pigment analyses

Some key photosynthetic pigments such as chlorophyll, carotenoid, and anthocyanin were analyzed from leaves of both tetraploid plantlets and their diploid counterparts using the methods described by Sadasivam and Manickam (1992).

**Chlorophyll:** 0.1 g of leaf samples were crushed in 10 ml of 80% acetone followed by extraction of the solution using Whatman No. 1 paper and absorbance was recorded at 645 and 663 nm using a spectrometer (Eppendorf BioSpectrometer<sup>®</sup> kinetic, Germany).

$$\text{Total chlorophyll (mg/g)} = 20.2(A_{645}) + 8.02(A_{663}) \times \text{volume}/1000 \times \text{wt. of sample.}$$

**Anthocyanin:** 10 g of leaf sample was crushed with 10 ml of ethanolic HCl. The solution was filtered using Whatman

No. 1 paper and the volume was made up to 100 ml to be stored in dark for 2 h followed by recording OD at 535 nm.

$$\text{Total anthocyanin (mg/100 g)} = \text{OD} \times \text{dilution} \times \text{volume made up} \times 100/\text{wt. of sample} \times e.$$

where,  $e = 98.2$  (absorbance of a solution containing 1 mg/ml anthocyanin).

**Carotenoid:** 5 g of leaf sample was crushed in acetone and then filtered using Whatman No. 1 paper. Then it was transferred to a separating funnel containing 20 ml of petroleum ether with gentle mixing. Then, 20 ml of 5% sodium sulfate, followed by 20 ml of petroleum ether was added, forming a clear supernatant. The lower phase was re-extracted with additional petroleum ether and then absorbance was recorded at 452 nm with petroleum ether as blank.

$$\text{Total carotenoid (mg/100 g)} = 3.857 \times \text{OD} \times \text{volume made up} \times \text{dilution} \times 100/\text{wt. of sample} \times 1000$$

## Acclimatization and ex vitro evaluation of plants

The in vitro regenerated diploid and tetraploid plantlets with well-developed shoots and roots were transferred to a mixture of sand, soil, tea leaves extract and cow urine (1:1:1:1;  $v/v$ ) for primary acclimatization (following Gan-tait et al. 2011). The complete set up was covered with a transparent polythene sheet with adequate aeration facility. Covering the plantlets with polythene sheet and intermittent spraying of water ensure maintenance of relative humidity in the microenvironment created for the plantlets. After 4 weeks, the plants were transferred to earthen pots containing a mixture of sand and soil in equal proportion (1:1;  $v/v$ ) for secondary acclimatization.

Throughout the acclimatization process, the growth and development of the plants was recorded to evaluate the ex vitro performance of the diploids and tetraploids. The observations such as plant height, number of shoots, number of leaves, leaf length, leaf width and leaf area were mainly recorded after 1 month, 4 months and 7 months of acclimatization. Plant height was measured from the ground to the tip of the plant and number of shoots and leaves were recorded for both diploid and tetraploid plants. The length, width, and area of the fully matured leaves of both diploid and tetraploid were recorded with the help of mm graph sheet.

## Genetic fidelity assessment

Genomic DNA of two diploid and four tetraploid plants (randomly selected) were isolated from first leaf tissue collected from 7 month-old plant using DNA extraction kit (GCC Biotech, Kolkata, India). A total of 20 reproducible primers (5 CDDP, 5 DAMD, 5 ISSR and 5 SCoT) were used to confirm the genetic uniformity of the diploid and the induced tetraploid plants. PCR amplification of the extracted DNAs were carried out by preparing a PCR mix containing 12.5 µl of master mix, 9.5 µl of sterile water, 1 µl of required primer, and 2 µl of working DNA sample. They were then kept in thermal cycler for amplification.

After amplification of the products, gel electrophoresis was carried out in 1.5% (w/v) agarose gel for separation of bands according to their size and a 100 bp ladder was used for scoring of the separated bands accordingly. The well resolved bands were scored and genetic fidelity between diploid and tetraploid plant DNA samples was determined.

## Statistical analysis

The experiments were carried out in a completely randomized design in which each experiment was performed five times with 20 explants (or plantlet) per treatment. The collected data were statistically analyzed for one-way analysis of variance and the significant variation among the treatment data (mean ± SE) was calculated employing Tukey's test and student *t*-test at  $P=0.05$  level in SPSS (version 20.0, SPSS Inc., Chicago, IL, USA) software.

## Results and discussion

### Colchicine treatment and survival of shoots

From the data recorded, it was evident that irrespective of the treatment duration, survival frequency decreases with an increase in the colchicine concentration and lowest survival was recorded in 0.5–1% colchicine with 12-h exposure. High survival percentage was recorded in 0.1% colchicine with any given treatment duration (Table 1) after the control (Fig. 2a), with the highest survival rate being obtained in 0.1% colchicine at 4-h treatment followed by 0.1% colchicine at 6-h, 8-h and 12-h treatments (Fig. 2b). The longer duration treatments (presumably because of excess colchicine accumulation) found to produce toxicity ensuing in lower survival of the treated shoot tips. Likewise, the high survival rate of gerbera shoot tips in lower concentration of colchicine in comparison to higher concentration was also reported earlier

**Table 1** Effect of different concentrations and duration of in vitro colchicine treatment on survival and ploidy level in gerbera

Duration (h)	Colchicine conc. (w/v) (%)	Survival %	Ploidy level (% frequency)		
			Diploid	Mixoploid	Tetraploid
4	0.1	70 ab	10 b	10 b	50 a
	0.25	40 bcd	20 b	10 b	10 b
	0.5	20 cd	10 b	10 b	0 b
6	0.1	60 abc	30 b	10 b	20 b
	0.25	40 bcd	0 c	30 a	10 b
	0.5	20 cd	10 b	10 b	0 b
8	0.1	50 bcd	10 b	30 a	10 b
	0.25	20 cd	10 b	10 b	0 b
	0.5	10 d	0 c	10 b	0 b
12	0.1	50 bcd	20 b	30 a	0 b
	0.25	10 d	0 c	10 b	0 b
	0.5	10 d	0 c	10 b	0 b
Control	0	100 a	100 a	0 b	0 b

Data represent mean of five replicates with 20 samples (shoot tip explants) per treatment

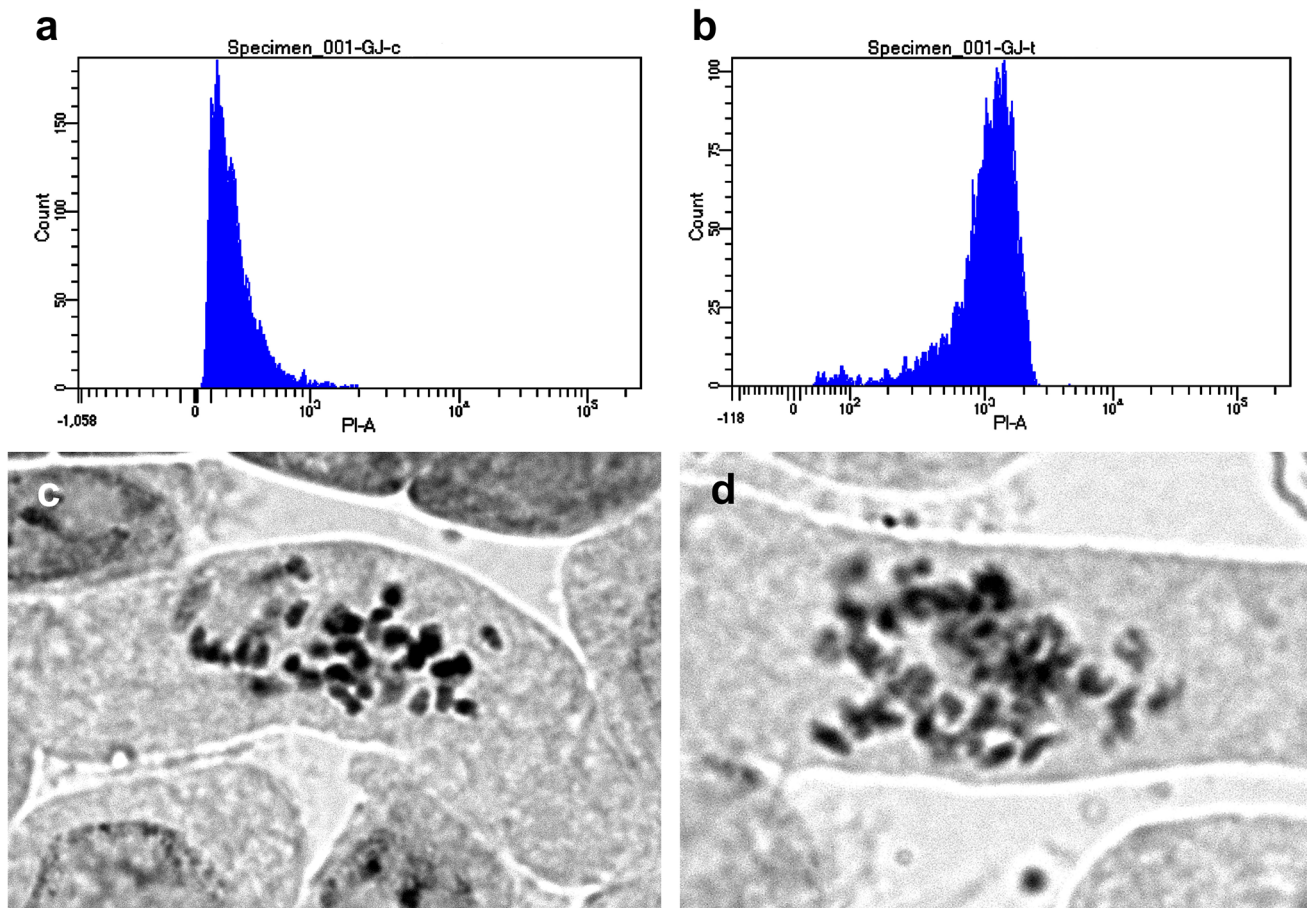
Data for each column with different alphabets are significantly different according to Tukey's test at  $P=0.05$

(Gantait et al. 2011). Similar results were obtained under in vivo conditions when 0.1% colchicine was applied on gerbera for 4 times resulting in induction of tetraploids with a maximum survival rate (Bhattarai et al. 2021).

### Ploidy status of colchicine-treated shoots via flow cytometry and chromosome count

The ploidy levels of all the colchicine-treated and the non-treated (diploid) plantlets were confirmed by flow cytometry analysis and via chromosome counting method. From the flow cytometric analysis results, the peak position of the tetraploids (Fig. 1b) was recorded to be almost doubled when compared to the nuclear DNA content of the diploid plantlets (Fig. 1a). Highest percentage (50%) of tetraploids was identified from 0.1% colchicine at 4-h treatment followed by 0.1% colchicine at 6-h (20%) and 8-h treatments (10%). However, no tetraploids were obtained in the longest treatment duration i.e., 12 h in the present study but the maximum mixoploids (30%) were induced in 0.1% colchicine at 8-h, 0.1% at 12-h, and 0.25% at 6-h treatments (Table 1). The tetraploids were also confirmed via counting of the chromosomes at the metaphase cell division stage. The root tip cells of tetraploid plantlets exhibited doubled number of chromosomes ( $2n=4x=100$ ) than that of the diploid ( $2n=2x=50$ ) counterparts (Fig. 1c, d). Similar results were reported when gerbera (cv. 'Cabana' and 'Dalma') shoot tips were treated in 0.1% colchicine for 48 h, indicating an efficient dose for induction of maximum tetraploids (Li et al. 2009). Flow





**Fig. 1** Flow cytometry- and metaphasic chromosome count-based assessments of colchicine-treated and non-treated gerbera (*Gerbera jamesonii* Bolus ex Hooker f.) plantlets showing their ploidy levels. Histograms (of DNA content) of relative log-transformed

fluorescence intensities of **a** non-treated plantlet and **b** colchicine-treated (tetraploid) plantlet; metaphasic chromosome number of **c** non-treated plantlet ( $2n=2x=50$ ) and **d** colchicine-treated (tetraploid) plantlet ( $2n=4x=100$ )

cytometry has been one of the most reliable methods used by multiple researchers in gerbera to confirm the ploidy level of plants (Gantait et al. 2011; Bhattarai et al. 2021). As an alternative to flow cytometry, chromosome count had been proven to be the conventional and accurate method for confirmation of the ploidy level of a plant but that required adequate dispersion of the chromosomes for precise counting. As the substitute of flow cytometry, confirmation of diploid and tetraploids in gerbera (cv. Mini Red) was reported via the direct chromosome counting method as well (Khalili et al. 2020).

### Growth of plantlets, their leaf and stomata characters

Significant differences were observed from the data recorded on the growth parameters of diploid and induced tetraploid

plantlets (Table 2). It was recorded that tetraploid plantlets took a comparatively longer duration for fresh shoot initiation than the diploid ones. The tetraploid plantlets produced significantly fewer shoots than the diploid plantlets yet the tetraploid plantlets recorded higher shoot length when compared to the diploid ones (Fig. 2c). Significant differences were observed in the leaf shape, size and color of the tetraploid and the diploid plantlets (Fig. 2d). The tetraploids exhibited darker and larger leaves while the diploids showed comparatively lighter and smaller leaves. The darker leaves in tetraploids might be the result of higher chlorophyll content, and larger leaves were a result of the increase in the cell and organ size in tetraploids called the ‘Gigas’ phenomenon. The most common effect of polyploidy in plants is the expansion in cell size, which is brought on by the greater number of gene copies. As a result, polyploid individuals could have bigger roots, leaves, tubercles, fruits, flowers, and

**Table 2** Comparative analysis of in vitro growth indices and plant pigments between diploid and tetraploid plantlets of gerbera (*Gerbera jamesonii* Bolus ex Hooker f.)

Days to new shoot induction	Number of shoots	Shoot length (mm)	No. of leaves	Leaf length (mm)	Leaf width (mm)	Leaf area (mm <sup>2</sup> )	Stomata frequency	Stomata length (µm)	Stomata width (µm)	No. of chloroplasts	Chlorophyll (mg/g)	Carotenoid (mg/100 g)	Anthocyanin (mg/100 g)
Diploid	7.1 ± 0.56	28.1 ± 0.70	17.7 ± 1.61	7.01 ± 0.12	4.73 ± 0.12	60.17 ± 0.46	14.4 ± 0.47	28.9 ± 0.27	23.7 ± 0.68	10.7 ± 0.44	0.79 ± 0.04	3.77 ± 0.06	4.55 ± 0.02
Tetraploid	3.9 ± 0.37	35.9 ± 0.97	11.0 ± 1.06	11.42 ± 0.18	7.39 ± 0.15	130.9 ± 0.56	8.2 ± 0.32	58.0 ± 0.41	44.1 ± 0.48	31.2 ± 0.48	1.47 ± 0.02	4.93 ± 0.02	7.35 ± 0.05
T value	4.77**	6.55**	3.47**	21.00**	13.3**	98.23**	10.87**	59.44**	24.57**	31.06**	22.67**	145**	46.66**

\*Significant at 5% probability level

\*\*Significant at 1% probability level (using *t*-test); Data represent mean ± standard error of five replicates with 20 samples per treatment

seeds than their diploid counterparts (Sattler et al. 2016). Similar results were reported in induced gerbera tetraploids when compared to the diploid ones (Gantait et al. 2011).

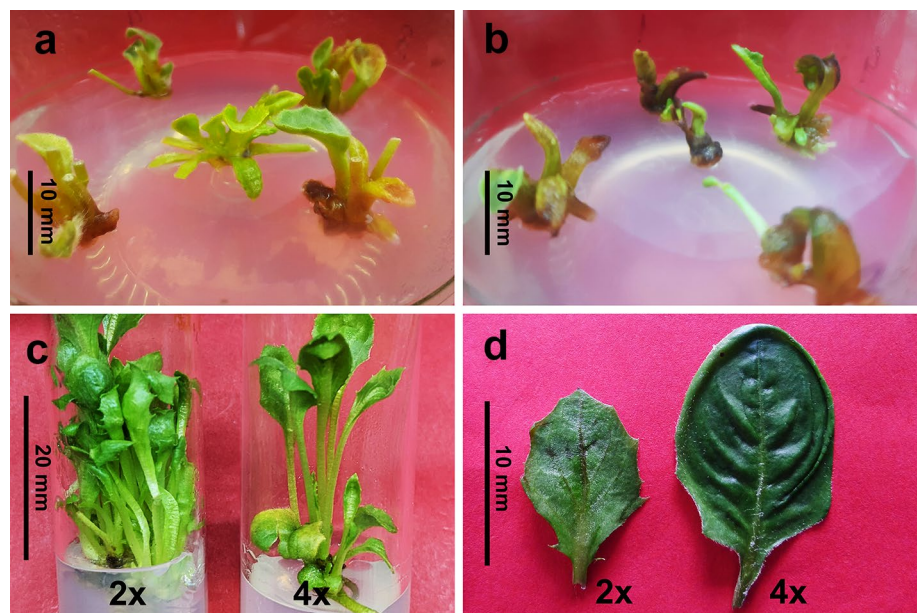
The study of stomata also proved to be an efficient approach to supplement the results of flow cytometry analysis and chromosome counting. The leaves of tetraploid plantlets were reported to exhibit larger yet fewer stomata on the abaxial surface than that of their diploid counterparts (Fig. 3a) when observed under each microscopic field. The length and width of the stomata were recorded to be doubled in tetraploids than that of the diploid ones (Fig. 3b). The number of chloroplasts in the guard cells of the tetraploid stomata was also recorded to be almost three times higher as compared to the diploid plantlets (Table 2). Similar results were reported in other cultivars and hybrid (cv. Scilla, cv. Mini Red, and *Gerbera hybrida*) of gerbera wherein the tetraploids showed longer plant height with broad and darker leaves, and stomata were almost double in size of that of the diploid ones with a greater number of chloroplasts in the guard cells (Gantait et al. 2011; Khalili et al. 2020; Bhattarai et al. 2021).

### Photosynthetic pigment analyses

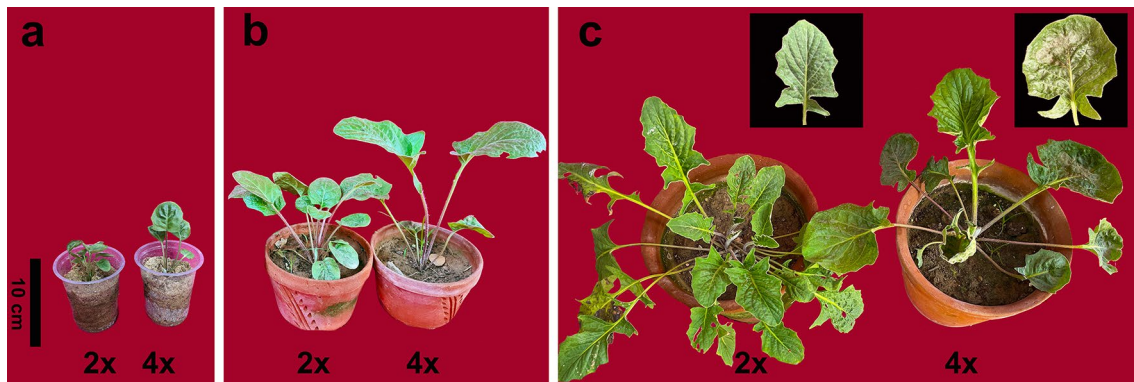
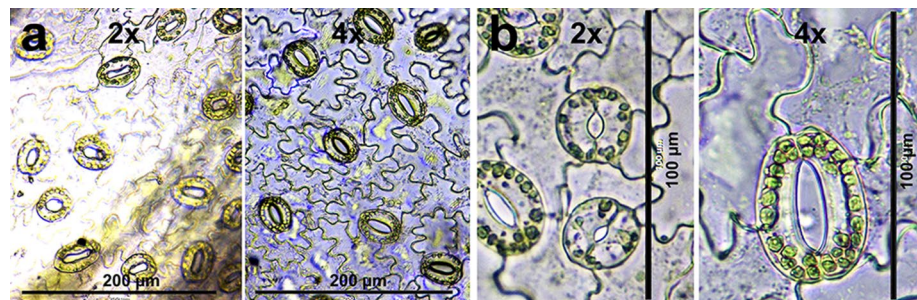
Upon analysis of various pigments like chlorophyll, anthocyanin, and carotenoid, which help in the growth and development of plantlets by being involved in photosynthesis, it was recorded that concentrations of all three pigments were significantly increased in the case of tetraploid plantlets (Table 2). Chlorophyll content was recorded to be doubled in the case of tetraploids when compared to the diploid ones, while a significant increase was observed in the case of carotenoid and anthocyanin contents in comparison to those of the diploid plantlets. Chlorophyll and carotenoid are directly involved in the process of photosynthesis. Their enhanced concentration may result in the enhancement in the photosynthetic activity. Likewise, an increase in anthocyanin content may be responsible for the development of more intense color in tetraploids as compared to the diploid ones. The results of the present study were comparable with the report obtained in *Lilium* species (Wang et al. 2021) wherein an increase in chlorophyll and carotenoid content was recorded in tetraploids when compared to their diploid counterparts. Photosynthetic pigments are significantly related to polyploidization, and the contents of such pigments are primarily driven by polyploidization (Münzbergová and Haisel 2019).



**Fig. 2** Comparative survival and growth of colchicine-treated (tetraploid) and non-treated gerbera (*Gerbera jamesonii* Bolus ex Hooker f.) plantlets. Survival of **a** non-treated shoot tips and **b** colchicine-treated shoot tips; morphological variations of non-treated (diploid) (2x) and colchicine-treated (tetraploid) (4x) plantlets in terms of **c** shoot multiplication and proliferation, and **d** leaf shape and size



**Fig. 3** Comparative stomata features of colchicine-treated (tetraploid) and non-treated (diploid) gerbera (*Gerbera jamesonii* Bolus ex Hooker f.). Stomata frequency of **a** diploid (left) and tetraploid (right) plantlets; stomata size and chloroplast count of **b** diploid (left) and tetraploid (right) plantlets



**Fig. 4** Comparative ex vitro growth and development of colchicine-treated (tetraploid) and non-treated (diploid) gerbera (*Gerbera jamesonii* Bolus ex Hooker f.) plants. **a** After 1 month of growth, **b** after

4 months of growth, **c** after 7 months of growth (inset, comparative morphology of leaves)

### Acclimatization and ex vitro morphological performance

The ex vitro performance of diploid and tetraploid plants were evaluated on the basis of morphological characteristics

like plant height, number of shoots, leaves, leaf length and width, and leaf area. From the observations, it was evident that tetraploids outperformed their diploid counterparts in terms of plant height and leaf parameters (leaf length, width and area) during entire growth phase (Fig. 4). Nonetheless,

**Table 3** Comparative analysis of ex vitro growth indices between diploid and tetraploid plants of gerbera (*Gerbera jamesonii* Bolus ex Hooker f.) at different growth stages

	1-month					4-month					7-month							
	Plant height (cm)	No. of shoots	No. of leaves	Leaf length (cm)	Leaf width (cm)	Leaf area (cm <sup>2</sup> )	Plant height (cm)	No. of shoots	No. of leaves	Leaf length (cm)	Leaf width (cm)	Leaf area (cm <sup>2</sup> )	Plant height (cm)	No. of shoots	No. of leaves	Leaf length (cm)	Leaf width (cm)	Leaf area (cm <sup>2</sup> )
Diploid	4.5±0.3	3.0±0.2	9.8±0.5	1.1±0.1	0.7±0.1	0.9±0.1	10.6±0.3	3.4±0.2	10.8±0.3	3.9±0.2	2.4±0.3	7.9±0.4	10.6±0.3	3.4±0.2	10.8±0.3	6.4±0.5	4.1±0.4	22.9±0.5
Tetra-	8.6±0.4	1.3±0.1	4.6±0.4	2.7±0.2	1.7±0.1	4.3±0.1	20.2±0.7	2.1±0.1	5.4±0.2	6.8±0.3	4.5±0.4	18.5±0.7	20.2±0.7	2.1±0.3	5.4±0.4	10.1±0.3	8.2±0.5	69.1±0.7
ploid																		
T value	9.31**	7.08**	8.52**	7.67**	6.67**	54.33**	12.63**	7.64**	14.59**	9.58**	5.83**	75.5**	12.63**	7.64**	14.59**	10.82**	20.50**	164.89**

\*Significant at 5% probability level

\*\*Significant at 1% probability level (using *t*-test); Data represent mean ± standard error of five replicates with 20 samples per treatment

the multiplication of shoots and number of leaves were recorded to be comparatively high in case of diploids (Table 3). After 1 month of acclimatization (Fig. 4a), height of the diploid plants was recorded to be 4.5 cm while tetraploid plants were recorded to be 8.6 cm high. Number of shoots and leaves were recorded to be 3 and ~10 per plant, respectively, in case of diploids while in tetraploids these were recorded to be 1.3 and 4.6, respectively. Average leaf area of fully matured leaf in diploid plant was recorded to be 0.9 cm<sup>2</sup> while in case of tetraploid plant it was recorded to be 1.7 cm<sup>2</sup>, nearly doubled the leaf area of the diploid plant. When the plants were compared at their 4-month and 7-month stages (Fig. 4b, c), the height along with the number of shoots and leaves were recorded to be comparable indicating the growth of the plant in terms of canopy area (only) following 4-month of acclimatization. At 7-month stage, the diploid plants attained a height a 10.6 cm while tetraploid plants were 20.2 cm. Approximately, 3.4 number of shoots per plant were observed in diploid plants as compared to 2.1 shoots per plant in case of tetraploid plants. Number of leaves were recorded to be nearly 11 in diploid and 5.4 in tetraploid plants. The fully matured leaf recorded 6.4 cm of length and 4.1 cm in width in diploid with a leaf area of 22.9 cm<sup>2</sup>. In case of tetraploid plants the leaf length was measured to be 10.1 cm with a width of 8.2 cm and an area of 69.1 cm<sup>2</sup>. The size and thickness of leaf is observed to be increased with darker in color and thicker stem in tetraploids when compared to the diploid ones. Increase in the size and thickness of leaf of tetraploids was found to be in accordance with the reports of Gantait et al. (2011), Khalili et al. (2020) and Bhattarai et al. (2021) in *Gerbera jamesonii* and was also reported in other plant species like *Paulownia tomentosa* (Tang et al. 2010), *Lagerstroemia indica* L. (Zhang et al. 2010). The reduced rate of multiplication in tetraploid plants was in accordance with the result obtained in *Punica granatum* by Shao et al. (2003) and was also reported in *Gerbera jamesonii* by Gantait et al. (2011) and Khalili et al. (2020). The reduced multiplication rate of autopolyploid plants may be attributed to the reduced rate of cell division (Stebbins 1984).

### Genetic fidelity assessment

Genetic fidelity of diploid and tetraploid plants were assessed using each 5 of CDDP, DAMD, ISSR and SCoT primers. A total of 1392 scorable and reproducible bands were obtained from all the 4 marker systems used within a size range of 160–3000 bp (Table 4). Total of 438 bands were obtained

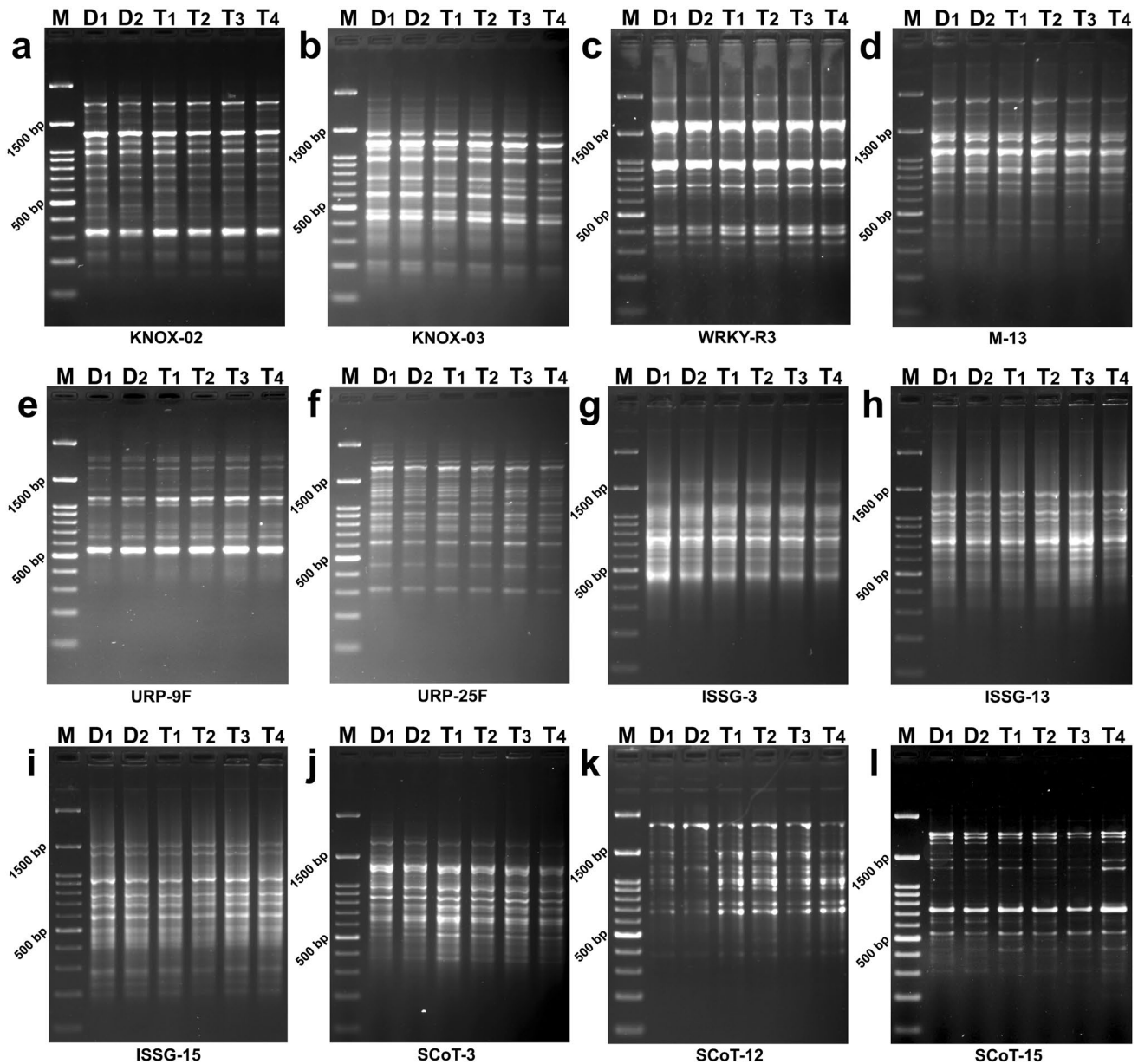


**Table 4** Conserved DNA-derived polymorphism (CDDP), directed amplification of minisatellite-region DNA (DAMD), inter simple sequence repeats (ISSR), and start codon targeted (SCoT) polymorphism primers, used for genetic stability assessment of diploid and tetraploid gerbera (*Gerbera jamesonii* Bolus ex Hooker f.) plants, their sequences with number and size range of amplified fragments

Primer(s)	Sequence (5'–3')	$T_m$ (°C)	No. of scorable bands per primer per sample	Total number of bands	Size range (bp)
<b>CDDP</b>					
KNOX-01	AAGGGSAAAGCTSCCSAAG	55	13	78	260–2800
KNOX-02	CACTGGTGGGAGCTSCAC	55	15	90	160–2250
KNOX-03	AAGCGSCACTGGAAGCC	55	16	96	190–2600
WRKY-R3	GCASGTGTGCTCGCC	55	13	78	290–2900
ERF2	GCSGAGATCCGSGACC	55	16	96	240–2900
<b>DAMD</b>					
M13	GAGGGTGGCGGCTCT	55	15	90	310–2800
URP2R	CCCAGCAACTGATCGCACAC	55	9	54	520–2700
URP4R	AGGACTCGATAACAGGCTCC	52	14	84	410–3000
URP9F	ATGTGTGCGATCAGTTGCTG	52	10	60	550–2400
URP25F	GATGTGTTCTTGGAGCCTGT	52	17	102	310–2600
<b>ISSR</b>					
ISSG-1	GGAGGAGGAGGAGGA	52	6	36	480–1100
ISSG-3	GTCGTCGTCGTCGTC	53	7	42	500–1400
ISSG-5	AGAGAGAGAGAGAGAGT	47	7	42	250–900
ISSG-13	ACACACACACACACACT	51	10	60	480–1450
ISSG-15	CACACACACACACAGT	53	7	42	600–1600
<b>SCoT</b>					
SCoT-3	CAACAATGGCTACCACCG	52	12	72	380–2200
SCoT-12	ACGACATGGCGACCAACG	57	12	72	230–1450
SCoT-15	ACGACATGGCGACCGCGA	60	12	72	310–2500
SCoT-18	ACCATGGCTACCACCGCC	60	11	66	300–2300
SCoT-19	ACCATGGCTACCACCGGC	60	10	60	410–2800
Total			232	1392	160–3000

with 73 scorable bands per primer per sample from 5 CDDP primers with a size range of 160 to 2900 bp. From DAMD primers, total of 390 bands were obtained ranging from 310 to 3000 bp (Fig. 5). Among the DAMD primers, the minimum number of bands i.e., 9 scorable bands per primer per sample (5'CCCAGCAACTGATCGCACAC3') and the maximum of 17 scorable bands per primer per sample (5'GATGTGTTCTTGGAGCCTGT3') were scored. Total of 222 bands were obtained from the 5 ISSR primers used with the minimum number of 6 scorable bands per primer per sample [5'GGAGGAGGAGGAGGA3'] with a size range of 480–1100 bp. From the SCoT primers, a total of 342 bands were obtained within a size range of 230–2800 bp. Using SCoT primers, the minimum of 10 scorable bands per primer per sample (5'ACCATGGCTACCACCGGC3') and the maximum of 12 scorable bands per primer per sample (5'CAA

3'; 5'ACGACATGGCGACCGCGA3') were obtained during genetic fidelity assessment among diploid and tetraploid plants. The genetic fidelity assessment confirmed that the colchicine-induced tetraploids were genetically similar i.e., there is no change in the DNA sequence after colchicine treatment and only the content of the DNA is doubled as compared to the non-treated ones. This is a pioneer report in assessing the genetic fidelity of diploid and tetraploid plants. Reports are available confirming the genetic fidelity of in vitro regenerated plantlets in various plant species using different marker systems. DAMD and SCoT primers were reported to be used for confirming genetic uniformity of in vitro regenerated plantlets of *Dendrobium heterocarpum* Wall. ex Lindl. (Longchar and Deb 2022). CDDP primers have been reported to be used for genetic diversity analysis in anthurium (Saidi et al. 2018) and gerbera (Saidi et al. 2020).



**Fig. 5** Agarose gel electrophoresis of amplified fragments of tetraploid plants ( $T_1$ – $T_4$ ) and the diploid plants ( $D_1$ – $D_2$ ) of gerbera (*Gerbera jamesonii* Bolus ex Hooker f.) showing monomorphic bands generated from conserved DNA-derived polymorphism (CDDP) **a** KNOX-02, **b** KNOX-03, **c** WRKY-R3, directed amplification of mini-

satellite-region DNA (DAMD) **d** M13, **e** URP9F, **f** URP25F, inter simple sequence repeats (ISSR) **g** ISSG-3, **h** ISSG-13, **i** ISSG-15, and start codon targeted (SCoT) polymorphism **j** SCoT-3, **k** SCoT-12, **l** SCoT-15 primers. Lane M represents 100 bp ladder

## Conclusion

Polyploidy has been an important evolutionary event that is employed by plant breeders towards the improvement of commercial traits of the plants. Polyploidy is reported to

increase the size of the cells, and DNA content, ultimately increasing the size of the organs that is called the ‘Gigas’ effect. The resulting gerbera tetraploids in the present study were observed to have larger and darker leaves, larger stomata with a higher number of chloroplasts in the guard cells,

and with longer plant height when compared to the diploid ones. There was also a significant increase in the content of the plant pigments in tetraploids that can enhance the photosynthetic products and potential flower color development. These characteristics may lead to the enhancement of ornamental features such as longer stalk and larger flower, thereby increasing the commercial value of this gerbera hybrid.

**Author contributions** SG and SB contributed to the study conception and design. MM and SG performed the material preparation, experimentations, data collection and analysis. MM and SG prepared the first draft of the manuscript. SG, SS, RS and SB commented and edited on previous versions of the manuscript. All authors read and approved the final manuscript.

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**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Code availability** Not applicable.

## Declarations

**Conflict of interest** The authors have no relevant financial or non-financial interests to disclose.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Consent to participate** Not applicable.

**Consent for publication** All the authors gave their consent for publication of the results.

## References

- Bhattarai K, Kareem A, Deng Z (2021) In vivo induction and characterization of polyploids in gerbera daisy. *Sci Hortic* 282:110054
- Cardoso JC, Teixeira da Silva JA (2013) Gerbera micropropagation. *Biotechnol Adv* 31:1344–1357
- Clarke J (1960) Preparation of leaf epidermis for topographic study. *Stain Technol* 35:35–39
- Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* 220:1049–1051
- Gantait S, Mahanta M (2022) Hyperhydricity-induced changes among in vitro regenerants of gerbera. *S Afr J Bot* 146:496–501
- Gantait S, Mandal N, Bhattacharyya S, Das PK (2011) Induction and identification of tetraploids using in vitro colchicine treatment of *Gerbera jamesonii* Bolus cv. Sciella. *Plant Cell Tissue Organ Cult* 106:485–493
- Halder T, Ghosh B (2021) Cytological, genetical and phytochemically stable meta-Topolin (mT)-induced mass propagation of underutilized *Physalis minima* L. for production of withaferin A. *Biocatal Agric Biotechnol* 33:102012
- Ibrahim MA, Yassin MM (2020) Direct shoot regeneration by in vitro culture of the gerbera (*Gerbera jamesonii* Bolus) capitulum explant. *Plant Cell Biotechnol Mol Biol* 21:1–9
- Ibrahim MA, Yassin MM (2021) Adventitious shoot proliferation from callus of *Gerbera jamesonii* young leaf explant. *DYSONA-Appl Sci* 2:47–52
- Khalili S, Niazian M, Arab M, Norouzi M (2020) In vitro chromosome doubling of African daisy, *Gerbera jamesonii* Bolus cv. Mini Red. *The Nucleus* 63:59–65
- Li H, Yan B, Zhang T, Jiang Y, Zhang H, Yu L, Li S (2009) Preliminary studies on polyploidy mutation of cut flower *Gerbera jamesonii* Bolus. *Acta Hortic Sin* 36:605–610
- Longchar TB, Deb CR (2022) Optimization of in vitro propagation protocol of *Dendrobium heterocarpum* Wall. ex. Lindl. and clonal genetic fidelity assessment of the regenerates: An orchid of horticultural and medicinal importance. *S Afr J Bot* 149:67–78
- Mahanta M, Gantait S, Mukherjee E, Bhattacharyya S (2023) meta-Topolin-induced mass propagation, acclimatization and cytogenetic fidelity assessment of gerbera (*Gerbera jamesonii* Bolus ex Hooker f.). *S Afr J Bot* 153:236–245
- Manzoor A, Ahmad T, Bashir MA, Baig MMQ, Quresh AA, Shah MKN, Hafiz IA (2018) Induction and identification of colchicine induced polyploidy in *Gladiolus grandiflorus* ‘White Prosperity’. *Folia Hortic* 30:307–319
- Münzbergová Z, Haisel D (2019) Effects of polyploidization on the contents of photosynthetic pigments are largely population-specific. *Photosynth Res* 140:289–299
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Niazian M, Nalousi AM (2020) Artificial polyploidy induction for improvement of ornamental and medicinal plants. *Plant Cell Tissue Organ Cult* 142:447–469
- Sadasivam S, Manickam A (1992) *Biochemical methods for agricultural sciences*. Wiley Eastern Limited
- Saidi A, Daneshvar Z, Hajibarat Z (2018) Comparison of genetic variation of *Anthurium andraeanum* cultivars using SCoT, CDDP and RAPD markers. *Plant Tissue Cult Biotech* 28:171–182
- Saidi A, Hajkazemian M, Emami SN (2020) Evaluation of genetic diversity in gerbera genotypes revealed using SCoT and CDDP markers. *Pol J Nat Sci* 35:21–34
- Sattler MC, Carvalho CR, Clarindo WR (2016) The polyploidy and its key role in plant breeding. *Planta* 243:281–296
- Shao JZ, Chen CL, Deng XX (2003) In vitro induction of tetraploid in pomegranate (*Punica granatum*). *Plant Cell Tissue Organ Cult* 75:241–246
- Speckman GJ, Post J, Dijkstra M (1965) The length of stomata as an indicator for polyploidy in rye grass. *Euphytica* 14:225–230
- Stebbins GL (1984) Polyploidy and the distributed of arctic-alpine flora: a new evidence and new approaches. *Bot Hely* 94:1–13
- Tang ZQ, Chen DL, Song ZJ, He YC, Cai DT (2010) In vitro induction and identification of tetraploid plants of *Paulownia tomentosa*. *Plant Cell Tissue Organ Cult* 102:213–220
- Tiwari V, Mahar KS, Singh N, Meena B, Nair KN, Datt B, Rana TS (2015) Genetic variability and population structure of *Bergenia ciliata* (Saxifragaceae) in the Western Himalaya inferred from DAMD and ISSR markers. *Biochem Syst Ecol* 60:165–170



- Tosca A, Pandolfi R, Citterio SA, Fasoli A, Sgorbati S (1995) Determination by flow cytometry of the chromosome doubling capacity of colchicine and oryzalin in gynogenetic haploids of gerbera. *Plant Cell Rep* 14:455–458
- Wang LJ, Cao QZ, Zhang XQ, Jia GX (2021) Effects of polyploidization on photosynthetic characteristics in three *Lilium* species. *Sci Hortic* 284:110098
- Zhang QY, Luo FX, Liu L, Guo FC (2010) In vitro induction of tetraploids in crape myrtle (*Lagerstoemia indica* L.). *Plant Cell Tissue Organ Cult* 101:41–47

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