

RESEARCH ARTICLE

In vivo induction and identification of heteroploids in ginger

Silpa SG¹, Sreekala GS^{1*}, Deepa S Nair¹, Roy Stephen², Swapna Alex³, Suhara Beevy S⁴, Beena Thomas⁵

¹Department of Plantation, Spices, Medicinal and Aromatic Crops, College of Agriculture, Kerala Agricultural University, Vellayani, Thiruvananthapuram 695 522, Kerala, India

²Department of Plant Physiology, College of Agriculture, Kerala Agricultural University, Vellayani, Thiruvananthapuram 695 522, Kerala, India

³Department of Biotechnology and Molecular Biology, College of Agriculture, Kerala Agricultural University, Vellayani, Thiruvananthapuram 695 522, Kerala, India

⁴Department of Botany, University of Kerala, Kariavattom Campus, Thiruvananthapuram 695 581, Kerala, India

⁵Department of Plant Breeding and Genetics, College of Agriculture, Kerala Agricultural University, Vellayani, Thiruvananthapuram 695 522, Kerala, India

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Abstract

Ginger, a commercially important spice, is propagated vegetatively and genetic variations among clones are limited. The study aimed to induce heteroploidy in ginger to create genetic variations that could potentially enhance production, improve quality and disease and insect resistance was undertaken at College of Agriculture, Vellayani, Kerala. Eight superior ginger genotypes were selected and treated with colchicine (0.1%). *In vivo* induction using colchicine resulted in twenty suspected heteroploids. These genotypes were field planted and observed for morphologic, cytologic and yield characters. The morphological characters like plant height and number of leaves per plant were significantly higher in the treated genotypes compared to the control. The chromosome number counting revealed the plants T_1S_5 , T_5S_1 , T_8S_1 and T_8S_4 to be heteroploids with chromosome numbers 27, 68, 24 and 30 respectively. The histogram peak of the colchicine treated plants T_1S_5 , T_8S_1 and T_8S_4 obtained in both channels 50 and 200, confirming heteroploidy. Among the four heteroploids developed, the heteroploid plant, T_8S_1 was found to be highly promising with respect to plant height, number of leaves plant⁻¹, fresh rhizome yield plant⁻¹ and dry rhizome yield per plant. The study suggested that induced heteroploidy in ginger plants using colchicine can lead to genetic variations with potentially beneficial traits for cultivation.

Keywords

colchicine; ginger; heteroploids; polyploidy

Introduction

Ginger (*Zingiber officinale* Rosc. 2n=22), a member of the Zingiberaceae family, is a significant spice crop valued for its pharmacological and therapeutic properties and is rich in secondary metabolites (1). It has a special combination of qualities, including antioxidant, aphrodisiac, antibacterial and anti-inflammatory effects. In European medicine, ginger was a component of most pharmaceutical preparations and it was one of the most highly prized of all mild carminatives (2).

In addition to bold rhizomes, resistance to diseases including bacterial wilt and rhizome rot, reduced fibre, high essential oil and yield are major goals for crop improvement in ginger (2). Clonal selection is mostly used in breeding ginger because it displays a high level of sterility due to chromosomal aberrations such as inversions and translocations and is

vegetatively propagated, resulting in reduced diversity (3-5). As ginger lacks natural seed set and variability, crop development techniques like hybridization and selection are ineffective. Therefore, the earliest crop improvement programs concentrated on mutation breeding using ethyl methyl sulfonate (EMS) and gamma rays. Consequently, low yielding mutants were isolated and the impact of the mutagen treatment disappeared in later generations (6).

Ploidy has been essential to both systematic classification and evolution. Compared to their diploid relatives, polyploids exhibit more vigour and superior performance. Morphology is influenced by ploidy level and polyploid plants are found in both horticultural and agricultural crops because they frequently have better morphological traits than their diploid counterparts (7). A previous study reported that it may be possible to produce improved tetraploid varieties of ginger from some of the diploid clones (8). Induction of autotetraploidy tried at Kerala Agricultural University in Himachal Pradesh, Maran, Nadia and Rio de Janeiro cultivars of ginger produced 2 autotetraploids which recorded higher rhizome yield and stomatal size and lower stomatal frequency than the corresponding diploids (9).

Chromosome number counting and flow cytometry analysis are regarded as direct methods of ploidy confirmation while indirect methods include morphological and stomatal characterization. Colchicine is used as an effective anti-mitotic agent for the induction of polyploidy and the ploidy confirmation can be done using flow cytometry analysis. Flow cytometry (FCM) is so far the most widely used method for determining the amount of nuclear DNA in plants. It makes it possible to quickly measure the fluorescence of several stained nuclei. Because of the relationship between ploidy and nuclear DNA content, the assay can be used to identify mixoploidy, determine ploidy level, and, in some cases, identify aneuploidy (10). FCM is the current method of choice since it is dependable, simple, and fast. Since the procedure typically yields samples from a few tens of milligrams of plant tissues, it is frequently termed non-destructive and appropriate for small-scale research.

In India, so far no polyploid variety has been reported for cultivation in ginger. Hence, an experiment was formulated with the objective to develop heteroploids *in vivo* from ginger using colchicine for superior yield and quality.

Materials and Methods

Eight genotypes were selected for developing heteroploids. Of this, four were the promising varieties (Athira and Aswathy released from Kerala Agricultural University and IISR Varada and IISR Mahima released from ICAR-Indian Institute of Spices Research). Remaining four genotypes (Genotype 1, 2, 3 and 4) were maintained in the Department of Plantation, Spices, Medicinal and Aromatic Crops. The rhizomes were properly cleansed to remove soil particles before being stored. The experiment was conducted in the Department of Plantation, Spices, Medicinal and Aromatic

Crops, College of Agriculture, Vellayani, Trivandrum, Kerala, India. For analysis, single bud rhizomes with a prominent bud were taken. The experiment thus had 16 treatments involving the eight genotypes and their corresponding control plants.

Induction of ploidy

The concentration of colchicine taken was 0.1 %. A hole of 3 mm diameter and depth was made close to the sprouting bud using a needle and 1 ml of colchicine solution was applied into the hole. The treatment was done between 6 am - 9 am and the period of treatment was 4 hr and repeated for 2 consecutive days. The rhizome bits treated by colchicine were covered by cotton soaked in colchicine of corresponding percentage over the axillary bud and kept overnight on the second day after the treatment. Thereafter, each treatment was washed in sterile water and air dried (9).

The protrays (50 celled) were filled with coir pith compost containing nursery material and farm yard manure (3:1), enriched with Plant Growth Promoting Rhizobacteria or *Trichoderma* 10 g/kg of mixture. The ginger sprouts were planted in protrays and partial shade was provided. The germinated plantlets were planted in polybags between one and half and 2 months of planting. The polybags were filled with soil, coir pith compost and farm yard manure in 2:1:1 ratio and *Trichoderma* (10 g/kg) was added to the media.

Experimental design

The first experiment aimed at developing heteroploids *in vivo* was analysed using Completely Randomised Design (CRD) consisting of 16 treatments and the results were interpreted at 1 % probability level using Grapes software, KAU (11). The heteroploids derived from *in vivo* method were field planted and evaluated for morphological and cytological parameters in the second generation and the ploidy level was confirmed by flow cytometry. The design of the experiment was RBD consisting of 16 treatments and 3 replications with 2 plants per replication. The plot size was 1x1 m and the crop duration was 9 months, which was carried out between January 2020 and October 2020. The experimental plot was prepared by ploughing followed by bed preparation in the garden of the Department of Plantation, Spices, Medicinal and Aromatic Crops, College of Agriculture, Vellayani. The treated rhizome bits were planted at a spacing of 20 cm × 20 cm. The experimental site is located at 8°28'28"N latitude and 76°57'47"E longitude and at an altitude of 28 m above mean sea level. The soil of the experimental location was red loam belonging to the Vellayani series and texturally classified as sandy clay loam. The mean relative humidity, minimum temperature, maximum temperature and rainfall were highest for the months of August (93.20 %), March (24.40 °C), March (33.40 °C) and May (12.60 mm) respectively during the field trial. The heteroploids thus screened were selected based on yield and quality parameters.

Estimation of morphological characters

Morphological characterization of the treated plants and the suspected plants were done in both the first and second seasons. Plant height, number of tillers and number of leaves per plant of both diploids and treated plants were

estimated at 90, 120 and 180 days after planting (DAP). The height of the plant was expressed in centimetre and measured from the plant's base to the tip of the main shoot's young, completely opened leaf. The number of aerial shoots and leaves produced by each observational plant was counted and mean expressed.

Estimation of anatomical characters

Anatomical parameters like stomatal frequency, length, breadth, epidermal cell number and chloroplast number were only recorded in the first season.

The leaves were collected only after exposure to enough sunlight, preferably in the noon. Using the nail varnish procedure, three samples of epidermal cells were taken. Number of stomata was counted in both control and treated plants and divided with the area to obtain frequency of stomata. The length and width of stomata was measured from five cells selected at random and stomatal size was compared. In a similar manner, the number of cells per millimetre of leaf in both the treated and control plants was used to determine the epidermal cell size. To record stomatal measurements, four leaves from the same section of the diploid and treated plants were selected. Three leaf sections from each plant were examined for chloroplast counts. Using a scalpel, the upper mesophyll tissues were removed, leaving the bottom epidermis. The number of chloroplasts in each of the 3 leaf samples was determined by counting 5 pairs of guard cells. An image analyzer (Leica) was used to measure the stomatal characteristics at 40 \times and 100 \times magnification (12).

Chromosome counting to confirm ploidy

Plants suspected as polyploids based on morphological and anatomical characters in the first season were subjected to cytological study to confirm the chromosome number. Root tips for analysis were collected from the rhizomes planted in pots on initiation of root emergence. Between 11:00 and 11:30 a.m., actively growing root tips of 5 - 10 mm length were collected and chromosome counting was done during the mitotic metaphase stages using a Leica DMRB (Leica, Germany) microscope with a 100 \times objective on three metaphase plates having good chromosome counts (13).

Ploidy confirmation by flow cytometry

A single step protocol was done to prepare nuclear suspensions. A small amount of the leaf of ginger plant (typically 60 mg) was placed in the center of a plastic Petri dish. Around 1 ml of ice-cold Tris.MgCl₂ buffer (nuclei isolation buffer) was added to this (14). A sharp, disposable scalpel was used to quickly chop the ginger leaf tissue in the buffer. The homogenate was filtered through a 42- μ m nylon mesh into a labeled sample tube. The filtrate was visually inspected to ascertain that it was free of any particles that could cause instrument clogging. Stock solution of a DNA fluorochrome was added and the mixture was gently agitated. DNA fluorochrome employed was Propidium Iodide (PI). PI stock solution was prepared at a concentration of 1 mg mL⁻¹. Preparation was carried out using double distilled water and was filtered using 0.22 μ m filter. The finished product was stored at -22 °C in 1 mL aliquots. In a similar manner, 1 mg mL⁻¹ of RNase stock

solution was prepared. To inactivate DNases, the solution was heated at 90 °C for 15 min during preparation. PI was typically used at 50 μ g mL⁻¹ simultaneously with RNase at 50 μ g mL⁻¹. Before analysis, the sample was incubated on ice for a few minutes to an hour, shaking it periodically. The nuclear DNA content was later examined (10). The FACS machine (BD FACSAria II) analysis was done at Rajiv Gandhi Centre for Biotechnology, Poojapura, Thiruvananthapuram.

The untreated ginger leaf samples were used as the standard (control or diploid) for standardizing the initial weight of the leaf sample to be taken and the rpm of the centrifugation process in order to achieve a suitable histogram. The diploid peak of the control sample was carefully noted. A flow cytometry histogram was acquired for every loaded sample. By contrasting the peaks seen in the treated samples and the control (diploid) samples, the number of chromosomes was deduced. The flow cytometry histogram represents Propidium Iodide- Area in the X axis and count or the number of cells that have taken up the propidium iodide dye in the Y axis. The number of cells corresponding to the ploidy of the ginger genotypes was obtained from the flow histogram.

Estimation of yield parameters

The number of days taken for yellowing and drying of the plant was noted as the maturity period. Harvesting was done 8 months after planting after yellowing and drying of the plants. Yield of each plant in terms of fresh weight was recorded and expressed as (g plant⁻¹). The rhizomes and roots were washed and allowed to dry in hot air oven at 70+ 5°C until constant weight was obtained to obtain dry rhizome and dry root weight (g plant⁻¹) respectively.

Results

Evaluation of first generation of plants

The colchicine treated genotypes grew slow initially and exceeded the control genotypes at later stages of growth. At 180 DAP, plant height, number of tillers and number of leaves per plant of treated plants surpassed diploid plants (Table 1). As a result, all the morphological characters recorded were higher in the control genotypes up to 180 DAP. Treated plant T₈ recorded the maximum plant height of 61.32 cm at 180 DAP. The mean plant height also showed significant difference between the treated and control genotypes which was 58.52 cm and 53.14 cm respectively at 180 DAP. At 180 DAP, the maximum number of tillers was recorded in treated plant T₈(8.17) followed by T₅ (8.00) while the maximum number of leaves was also recorded in treated plant T₈ (75.63) followed by T₆ (74.60) although no-significant.

All the anatomical parameters recorded varied significantly among the different treated and control genotypes (Table 2). Stomatal frequency was found to be lower in the different genotypes of treated plants, the least being recorded in T₅ (54.82 mm⁻²). The maximum stomatal length of 133.74 μ m and stomatal breadth of 90.97 μ m was found in the treated plant, T₅. When compared to the control, the treated plants had a higher number of chloroplasts, with T₁ having the highest number (19.10 mm⁻²).

Table 1. Effect of colchicine on the growth characters of ginger genotypes at 90, 120 and 180 DAP

Treatments and control	Plant height (cm)			Number of tillers			Number of leaves		
	90 DAP	120 DAP	180 DAP	90 DAP	120 DAP	180 DAP	90 DAP	120 DAP	180 DAP
T ₁	27.85 ^c	35.45 ^{bc}	59.04 ^{ab}	3.19 ^{bcd}	5.33	7.45	12.10 ^b	23.57 ^d	72.00
T ₂	26.73 ^c	39.05 ^{bc}	59.54 ^{ab}	2.60 ^{cde}	4.40	6.60	11.40 ^b	22.80 ^d	67.80
T ₃	28.73 ^c	36.58 ^{bc}	60.26 ^{ab}	2.29 ^{de}	4.71	7.36	11.93 ^b	26.36 ^{bcd}	71.75
T ₄	28.61 ^c	35.67 ^{bc}	59.16 ^{ab}	1.94 ^e	4.59	7.10	9.56 ^b	24.13 ^{cd}	72.30
T ₅	30.90 ^c	40.05 ^b	60.30 ^{ab}	3.00 ^{bcd}	5.09	8.00	11.88 ^b	27.25 ^{abcd}	71.30
T ₆	29.12 ^c	33.52 ^c	51.44 ^{de}	2.10 ^{de}	4.33	7.00	10.00 ^b	22.33 ^d	74.60
T ₇	29.50 ^c	33.08 ^c	57.67 ^{abc}	2.33 ^{cde}	3.67	7.67	8.00 ^b	24.33 ^{bcd}	70.20
T ₈	30.46 ^c	38.31 ^{bc}	61.32 ^a	3.17 ^{bcd}	5.18	8.17	12.75 ^b	27.11 ^{abcd}	75.63
C ₁	41.13 ^{ab}	47.41 ^a	56.17 ^{bcd}	3.60 ^{abc}	6.00	6.80	24.40 ^a	30.40 ^{ab}	70.00
C ₂	42.10 ^{ab}	46.77 ^a	53.95 ^{cde}	3.00 ^{bcd}	5.40	6.80	21.20 ^a	31.20 ^{ab}	66.40
C ₃	44.85 ^a	50.37 ^a	55.66 ^{bcd}	4.20 ^{ab}	6.20	7.00	23.40 ^a	30.60 ^{ab}	70.00
C ₄	39.28 ^b	49.09 ^a	53.69 ^{cde}	4.40 ^a	5.40	6.40	24.20 ^a	28.50 ^{abcd}	69.20
C ₅	40.35 ^b	47.27 ^a	51.92 ^{de}	4.40 ^a	6.40	7.40	21.20 ^a	33.00 ^a	69.00
C ₆	40.10 ^b	45.40 ^a	49.80 ^e	2.80 ^{cde}	5.80	6.60	24.20 ^a	29.40 ^{abc}	70.20
C ₇	41.86 ^{ab}	47.77 ^a	51.66 ^{de}	3.00 ^{bcd}	5.40	6.40	24.80 ^a	29.00 ^{abc}	68.80
C ₈	44.64 ^a	50.44 ^a	53.06 ^{cde}	3.40 ^{abcd}	6.20	7.80	24.80 ^a	30.20 ^{ab}	69.20
CD (0.05)	3.80	5.04	4.84	1.09	NS	NS	5.79	6.25	NS
SEm (±)	0.71	0.90	0.85	0.24	0.32	0.34	0.92	1.18	2.25

Table 2. Effect of colchicine on the anatomical parameters of ginger genotypes treated *in vivo* in the first generation

Treatments	Stomatal frequency (mm ⁻²)	Stomatal size		Chloroplast number (mm ⁻²)	Epidermal cell number (mm ⁻²)
		Stomatal length (μm)	Stomatal breadth (μm)		
T ₁	83.25 ^d	90.45 ^{bc}	79.83 ^{ab}	19.10 ^a	25.10 ^c
T ₂	83.25 ^d	87.61 ^{bcd}	86.43 ^{ab}	13.00 ^{bc}	32.20 ^b
T ₃	100.51 ^c	68.91 ^{cde}	67.59 ^{bc}	17.20 ^{ab}	29.80 ^b
T ₄	102.54 ^{bc}	60.79 ^{cde}	57.93 ^c	15.71 ^{ab}	31.00 ^b
T ₅	54.82 ^e	133.74 ^a	90.97 ^a	17.88 ^a	24.80 ^c
T ₆	107.61 ^{abc}	32.97 ^e	30.67 ^d	17.60 ^{ab}	29.00 ^{bc}
T ₇	106.60 ^{abc}	40.62 ^{de}	37.84 ^d	18.20 ^a	28.20 ^{bc}
T ₈	83.25 ^d	103.97 ^{ab}	78.08 ^{ab}	18.77 ^a	23.80 ^c
C ₁	113.71 ^a	32.70 ^e	23.60 ^d	8.20 ^d	60.60 ^a
C ₂	109.64 ^{ab}	30.78 ^e	24.13 ^d	9.40 ^{cd}	61.80 ^a
C ₃	111.67 ^a	31.79 ^e	23.88 ^d	10.40 ^{cd}	59.80 ^a
C ₄	111.67 ^a	31.93 ^e	23.68 ^d	7.00 ^d	60.60 ^a
C ₅	113.71 ^a	30.55 ^e	23.41 ^d	10.40 ^{cd}	61.20 ^a
C ₆	110.66 ^{ab}	30.70 ^e	23.18 ^d	9.40 ^{cd}	61.80 ^a
C ₇	107.61 ^{abc}	30.75 ^e	23.09 ^d	11.00 ^{cd}	60.00 ^a
C ₈	111.67 ^a	31.92 ^e	23.13 ^d	8.00 ^d	61.80 ^a
CD (0.05)	8.02	32.96	18.67	4.77	5.33
SEm (±)	2.84	9.42	5.14	1.25	1.34

2). Epidermal cell number was found to be higher for the control plants compared to treated genotypes and among treated plants, treatment T₈ recorded the minimum epidermal cell number (23.80 mm⁻²).

The chromosome number counting was done using aceto-orcein stain using root tips on the suspected ginger plants which is presented in Table 3. The analysis revealed that the colchicine treated plant T₁S₅ of treatment T₁ had a chromosome number of 2n = 27 while the diploid recorded chromosome number of 2n = 22. The colchicine treated plant T₅S₁ of treatment T₅ recorded a chromosome number of 2n = 68 and the corresponding diploid had a chromosome number of 2n = 22. Two colchicine treated plants of treatment T₈ (T₈S₁ and T₈S₄) recorded chromosome number of 2n = 24 and 2n = 30 respectively while the diploid

recorded chromosome number is 2n = 22. All the remaining colchicine treated plants recorded diploid chromosome number of 2n = 22.

Evaluation of second generation of plants

From the data obtained in the first year around 20 plants which were suspected to be heteroploids were carried over to the next generation for field planting to study their stability. This was necessary to ascertain the ploidy. The suspected heteroploids were screened based on morphological, cytological and yield characters in the second year. The 20 suspected heteroploids included 3 treated plants of T₁ (Athira), T₂ (Aswathy), T₃ (IISR Varada), T₄ (IISR Mahima), T₅ (Genotype 1), T₈ (Genotype 4) and one each from T₆ (Genotype 2) and T₇ (Genotype 3).

The treated genotypes recorded significantly higher

Table 3. Effect of colchicine on the cytological parameter of ginger genotypes treated *in vivo*

Treatments	Chromosome number recorded in the twenty suspected heteroploid plants		
	Suspected heteroploid plants		
T ₁	S ₁ 22	S ₅ 27	S ₁₀ 22
T ₂	S ₁ 22	S ₂ 22	S ₃ 22
T ₃	S ₁ 22	S ₂ 22	S ₃ 22
T ₄	S ₁ 22	S ₂ 22	S ₃ 22
T ₅	S ₁ 68	S ₂ 22	S ₃ 22
T ₆	S ₁ 22		
T ₇	S ₁ 22		
T ₈	S ₁ 24	S ₃ 22	S ₄ 30

* S- Suspected plant

values for morphological characters like plant height and number of leaves per plant at 90, 120 and 180 DAP. The maximum plant height at 90 DAP was recorded in treatment T₅ (55.52 cm) which was on par with T₁ (54.27 cm), T₈ (53.01 cm), T₂ (51.13 cm) and T₄ (50.50 cm). The plant height of control plants at 90 DAP was maximum in C₈ (44.25 cm), which was on par with all other control genotypes. At 120 DAP, the maximum plant height was recorded in treated plant T₁ (69.75 cm) which was on par with the plant height of all other treated genotypes. The maximum plant height among control plants was recorded in C₅ (50.85 cm) which was on par with all other control genotypes. At 180 DAP the maximum plant height was recorded in treated plant of T₁ (80.78 cm) which was on par with the plant height of all treated genotypes and control genotype C₇ (73.30 cm).

Control genotype C₄ recorded a plant height of 68.48 cm which was on par with C₈ (67.12 cm), C₆ (65.37 cm), C₂ (64.78 cm) and C₁ (60.58 cm).

Significant difference in number of tillers were recorded only at 90 and 120 DAP. The significantly higher number of tillers at 90 DAP was produced by treatments T₈ and T₅ while at 120 DAP, the number of tillers produced by the treatment T₁ was significantly higher. At 120 DAP, the maximum number of tillers was produced by the treated plant T₁ (9.44) which was on par with all other treated genotypes except T₃ (8.00). The significantly higher number of leaves at 180 DAP was also recorded in treatment T₁ (110.00) (Table 4).

The chromosome number counting confirmed the

Table 4. Effect of colchicine on the plant height, number of tillers and number of leaves of heteroploid ginger genotypes

Treatments and control	Plant height (cm)			Number of tillers			Number of leaves		
	90 DAP	120 DAP	180 DAP	90 DAP	120 DAP	180 DAP	90 DAP	120 DAP	180 DAP
T ₁	54.27 ^a	69.75 ^a	80.78 ^a	5.70 ^{ab}	9.44 ^a	10.90	30.30 ^{ab}	53.00 ^a	110.00 ^a
T ₂	51.13 ^{abc}	68.70 ^a	73.48 ^{abcde}	5.67 ^{ab}	8.67 ^{ab}	9.33	29.67 ^{ab}	51.67 ^{ab}	95.67 ^b
T ₃	48.75 ^{bcd}	66.17 ^{ab}	77.25 ^{ab}	5.33 ^{abc}	8.00 ^b	9.67	27.67 ^{abc}	49.00 ^{ab}	97.67 ^a
T ₄	50.50 ^{abc}	67.63 ^a	76.39 ^{abc}	5.67 ^{ab}	8.67 ^{ab}	8.00	27.00 ^{bcd}	49.33 ^{ab}	100.00 ^a
T ₅	55.52 ^a	66.79 ^{ab}	75.98 ^{abc}	6.00 ^a	9.33 ^a	10.00	31.00 ^a	50.00 ^{ab}	103.33 ^a
T ₆	49.18 ^{bcd}	64.38 ^{ab}	75.00 ^{abcd}	5.33 ^{abc}	8.33 ^{ab}	9.00	29.00 ^{ab}	51.00 ^{ab}	99.00 ^a
T ₇	47.58 ^{cde}	64.44 ^{ab}	74.93 ^{abcd}	5.33 ^{abc}	8.67 ^{ab}	8.00	28.67 ^{ab}	51.33 ^{ab}	92.67 ^b
T ₈	53.01 ^{ab}	69.43 ^a	80.33 ^a	6.00 ^a	9.33 ^a	10.00	29.33 ^{ab}	54.67 ^a	100.00 ^a
C ₁	42.00 ^f	49.12 ^c	60.58 ^{ghi}	4.67 ^{abc}	6.00 ^c	7.00	22.33 ^e	30.00 ^d	73.67 ^{cd}
C ₂	42.00 ^f	49.17 ^c	64.78 ^{fghi}	4.00 ^c	5.67 ^c	7.67	24.00 ^{de}	32.00 ^{cd}	68.33 ^d
C ₃	43.42 ^{ef}	49.50 ^c	58.10 ⁱ	4.33 ^{bc}	6.00 ^c	8.00	22.67 ^e	32.67 ^{cd}	73.67 ^{cd}
C ₄	42.50 ^{ef}	49.43 ^c	68.48 ^{cdefg}	4.33 ^{bc}	5.67 ^c	8.00	22.33 ^e	33.67 ^{cd}	72.00 ^{cd}
C ₅	42.22 ^f	50.85 ^c	59.17 ^{hi}	4.00 ^c	6.67 ^c	8.67	25.00 ^{cde}	37.67 ^c	71.33 ^d
C ₆	39.22 ^f	48.82 ^c	65.37 ^{fghi}	4.33 ^{bc}	5.67 ^c	9.33	23.67 ^{de}	33.00 ^{cd}	72.00 ^{cd}
C ₇	43.33 ^f	49.19 ^c	73.30 ^{abcde}	4.33 ^{bc}	6.33 ^c	9.00	23.00 ^e	33.67 ^{cd}	73.67 ^{cd}
C ₈	44.25 ^{def}	49.95 ^c	67.12 ^{defgh}	4.00 ^c	6.00 ^c	8.00	24.00 ^{de}	29.00 ^d	73.67 ^{cd}
CD (0.05)	5.31	5.58	8.21	1.46	1.28	NS	3.53	6.17	12.45
SEm (±)	1.84	1.93	2.84	0.51	0.44	0.65	1.22	2.14	4.31

plants T₁S₅, T₅S₁, T₈S₁ and T₈S₄ to be heteroploids with chromosome numbers 27, 68, 24 and 30 respectively. The histogram peak of the colchicine treated plants T₁S₅, T₈S₁ and T₈S₄ were obtained in both channels 50 and 200, confirming heteroploidy. The histogram peak of the treated plant genotype, T₅S₁ was obtained only in channel 200 also confirming heteroploidy.

The significantly highest fresh rhizome yield was recorded in control plant C₇ (315.17 g plant⁻¹) which was on par with colchicine treated plants of T₅ (305.00 g plant⁻¹), T₈ (301.88 g plant⁻¹), T₄ (295.00 g plant⁻¹), T₁ (290.47 g plant⁻¹), T₃ (258.17 g plant⁻¹), control genotypes of C₅ (248.67 g plant⁻¹), C₆ (237.00 g plant⁻¹) and C₈ (236.67 g plant⁻¹). The maximum dry rhizome yield was recorded in control plant of C₇ (59.42 g plant⁻¹) which was on par with treated plant T₈ (58.80 g plant⁻¹), T₄ (57.39 g plant⁻¹), T₁ (56.27 g plant⁻¹), control plant C₅ (50.21 g plant⁻¹), treated genotypes T₃ (50.07 g plant⁻¹), T₅ (48.86 g plant⁻¹), T₂ (45.00 g plant⁻¹), control genotypes C₆ (48.18 g plant⁻¹) and C₈ (47.03 g plant⁻¹).

The highest fresh rhizome yield per plot was recorded in control plant C₇ (1885.50 g plot⁻¹) followed by treated plant T₅ (1825.00 g plot⁻¹), T₈ (1815.65 g plot⁻¹) and T₄ (1775.00 g plot⁻¹). The maximum dry rhizome yield per plot was recorded in control plant C₇ (358.25 g plot⁻¹) followed by treated plant of T₈ (359.39 g plot⁻¹) T₄ (344.17 g plot⁻¹) and T₁ (329.80 g plot⁻¹). The significantly higher dry root yield was recorded in treated plant T₅ (4.35 g plant⁻¹) (Table 5).

Table 5. Effect of colchicine on yield parameters of suspected ginger plants treated *in vivo* in the second year

Treatments	Maturity period (days)	Fresh rhizome yield (g/plant)	Fresh rhizome yield (g/plot)	Dry rhizome yield (g/plant)	Dry rhizome yield (g/plot)	Dry root yield (g/plant)
T ₁	234	290.47 ^{abc}	1741.40	56.27 ^{ab}	329.80	4.10 ^{ab}
T ₂	240	226.92 ^{bcd}	1360.75	45.00 ^{abcde}	259.99	2.57 ^{cde}
T ₃	235	258.17 ^{abcde}	1552.50	50.07 ^{abc}	294.21	2.47 ^{cde}
T ₄	234	295.00 ^{abc}	1775.00	57.39 ^{ab}	344.17	2.55 ^{cde}
T ₅	238	305.00 ^{ab}	1825.00	48.86 ^{abcd}	291.59	4.35 ^a
T ₆	236	193.33 ^e	1170.00	38.25 ^{cde}	238.74	2.76 ^{bcd}
T ₇	237	209.00 ^{de}	1257.00	42.84 ^{bcd}	263.53	3.57 ^{abcd}
T ₈	240	301.88 ^{ab}	1815.65	58.80 ^a	359.39	4.03 ^{ab}
C ₁	245	187.03 ^e	1131.10	35.77 ^e	211.31	2.27 ^{de}
C ₂	240	200.00 ^{de}	1190.00	38.66 ^{cde}	229.99	2.04 ^e
C ₃	245	211.67 ^{de}	1275.00	41.98 ^{bcd}	245.93	2.16 ^e
C ₄	236	196.67 ^e	1182.00	37.95 ^{de}	221.85	1.76 ^e
C ₅	240	248.67 ^{abcde}	1496.00	50.21 ^{abc}	300.62	2.13 ^e
C ₆	244	237.00 ^{abcde}	1411.00	48.18 ^{abcd}	292.55	2.10 ^e
C ₇	230	315.17 ^a	1885.50	59.42 ^a	358.25	3.04 ^{abcde}
C ₈	243	236.67 ^{abcde}	1410.00	47.03 ^{abcd}	283.10	2.39 ^{cde}
CD (0.05)	NS	82.69	4.07	15.12	0.47	1.38
SEm (±)	0.55	28.63	1.35	5.24	0.15	0.48

Characterization of the heteroploids in the second year

Morphological characters

The plant height recorded in the heteroploid plants and their corresponding control plants showed significant variation at 90, 120 and 180 DAP (Table 6). The maximum plant height at 90 and 120 DAP was recorded in the heteroploid plant T₈S₄ which was 58.75 cm and 69.94 cm respectively while the maximum plant height at 180 DAP was recorded in the heteroploid plant T₈S₁ (81.18 cm). At 180 DAP, the maximum number of tillers were produced in the heteroploid plant T₁S₅ (11.00) and was on par with T₈S₁ (10.50), T₈S₄ (10.00) and T₅S₁ (9.50). The maximum number of leaves at 120 and 180 DAP was produced in the heteroploid plant T₈S₁ (54.50 and 111.00 respectively) while its corresponding diploid recorded 30.00 and 78.00 number of leaves respectively.

Yield characters

The maximum fresh rhizome yield per plant was recorded in the heteroploid plant T₈S₁ (300.00 g/plant) which was on par with T₁S₅ (285.40 g/plant), T₈S₄ (282.50 g/plant), T₅S₁ (267.50 g/plant) and control plant C₅ (249.00 g/plant). There was significant difference in dry rhizome yield among the different heteroploids and control plants and the maximum dry rhizome yield per plant was recorded in heteroploid plant T₈S₁ (57.30 g/plant).

Table 6. Morphological characters, yield parameters and chromosome number of heteroploids in the second year

Heteroploids and control	Plant height (cm)			Number of tillers			Number of leaves			Fresh rhizome yield (g/plant)	Dry rhizome yield (g/plant)	Dry root yield per plant	Chromosome number
	90 DAP	120 DAP	180 DAP	90 DAP	120 DAP	180 DAP	90 DAP	120 DAP	180 DAP				
T ₁ S ₅	52.56 ^b	69.38 ^a	81.07 ^a	5.50	9.50 ^a	11.00 ^a	31.00 ^a	50.50 ^a	110.00 ^a	285.40 ^b	56.02 ^{ab}	4.89	27
C ₁	41.00 ^d	49.61 ^b	60.22 ^d	4.50	6.00 ^b	7.50 ^d	23.00 ^c	31.00 ^c	78.50 ^c	190.00 ^c	35.83 ^d	3.00	22
T ₅ S ₁	54.68 ^b	70.25 ^a	78.20 ^b	5.50	9.00 ^a	9.50 ^{bc}	30.50 ^a	50.50 ^a	100.00 ^b	267.50 ^b	47.85 ^c	2.57	68
C ₅	42.61 ^{cd}	50.35 ^b	58.09 ^d	4.50	6.50 ^b	8.50 ^{cd}	25.50 ^b	42.00 ^b	77.50 ^c	249.00 ^b	49.25 ^{bc}	3.65	22
T ₈ S ₁	54.77 ^b	69.00 ^a	81.18 ^a	6.00	9.50 ^a	10.50 ^{ab}	29.50 ^a	54.50 ^a	111.00 ^a	300.00 ^a	57.30 ^a	2.83	24
T ₈ S ₄	58.75 ^a	69.94 ^a	80.62 ^a	6.00	9.00 ^a	10.00 ^{abc}	29.50 ^a	53.00 ^a	94.50 ^b	282.50 ^b	53.00 ^{abc}	3.15	30
C ₈	43.75 ^c	49.40 ^b	67.56 ^c	4.50	6.50 ^b	9.00 ^{cd}	25.00 ^{bc}	30.00 ^c	78.00 ^c	244.00 ^b	48.00 ^c	3.21	22
CD (0.05)	2.22	2.61	2.16	NS	0.93	1.92	2.39	6.58	9.05	53.37	7.30	NS	
SEm (±)	0.64	0.76	0.63	0.58	0.27	0.56	0.69	1.90	2.61	15.42	2.11	0.47	

Discussion

Colchicine, an alkaloid substance obtained from *Colchicum autumnale* L., is the most commonly utilized antimitotic agent to induce polyploidy in numerous species. Colchicine inhibits spindle formation by binding to microscopic proteins (15). It has been observed in *Musa acuminata* that the lethal effect of colchicine interferes with the germination process and viability by interfering with the enzymes involved in the process of germination due to its toxic effect (16). A colchicine concentration of 0.1 % was used in this study in reference to previous studies conducted by in ginger wherein she obtained 2 autotetraploid ginger plants using colchicine (9). The autotetraploid plants obtained by her were characterized by slower initial growth, increased leaf area, stomatal size, epidermal cell size and a very high pollen fertility over the control. She also obtained stability in rhizome yield in both the generations.

The morphological and cytological features of treated plants showed variations on comparison with their diploid counterparts. Therefore, the morphological features like plant height, number of tillers, number of leaves and the anatomical features like stomatal count and size and chloroplast number of the treated plants were critically examined to select putative heteroploids to be carried over to the next generation. Around 20 such plants were identified and planted in the second generation along with a few other selected plants which showed better performance along with their corresponding diploid counterparts. Screening of plants on the basis of morphological and cytological features have been discussed in some other studies in ginger (17, 18). A study reported stomatal size to be an important indicator to detect polyploids (19). It concluded that there is a significant correlation between ploidy of plants and their stomatal number, size and chloroplast number (20). The efficiency of stomata size in distinguishing plants with varying ploidy levels has also been employed in many other plant species (16).

In this study the suspected plants that were carried over to the next generation showed an initial slow growth that later surpassed the control plants at 180 DAP which might be attributed to the smaller amounts of growth hormones present at the initial stage (21). This lethal effect of colchicine has been discussed in previous studies by in *Vicia faba* and in ginger (18, 22). The suspected plants showed improved morphological features after 180 DAP.

There was an increase in plant height, number of tillers and number of leaves in the suspected plants. Plant morphological characters are reported to be better indicators of ploidy than screening polyploids using stomatal characters (23). Leaf morphological features have also been used in previous studies conducted in *Platanus acerifolia* to pre-screen tetraploids (23). Enhanced morphological features have been reported in previous studies conducted by in ginger; in parsley (17, 24, 25). Gigas characters in induced polyploids of ginger including an increase in size of plant parts like leaves and the rhizome was reported by (8).

Other anatomical characters like the stomatal frequency decreased (Figs. 5 and 6) while the stomatal size was found to increase in the suspected plants (Figs. 1 to 4). A study reported that the stomata of polyploids are larger and exist at a lower density than diploids (26). Similar reports have been recorded in many other plants such as *Anise hyssop* (12). The chloroplast number was also higher in treated plants compared to the control like the stomatal size (Figs. 7 and 8) while the epidermal size reduced.

The suspected heteroploids along with their diploid counterparts were planted in the second generation in field for evaluation of the stability of these genotypes. The performance of treated genotypes in the second generation were better than the control genotypes. The slow sprouting of colchicine treated rhizomes in the first generation was not noticed in the second generation. Ginger buds require some recuperation time since the toxicity of colchicine causes sluggish sprouting. This initial delayed growth was not noticed in the subsequent generations as also reported by (18). Similar observations were also made in *Hedychium* (27). The plant height of the treated genotypes was better than their corresponding control at all stages of observation. There was significant difference in plant height between the different treated and control genotypes at 90, 120 and 180 DAP (Table 26). Treatments T₁, T₅ and T₈ were confirmed to be heteroploids. Also observed that the treated plants grew vigorously as the control plants (28). Similar observations on plant height of tetraploids were also recorded by in parsley (25). Colchicine treated genotypes produced a greater number of leaves than control genotypes and the results were also significant at all stages of observation. Thicker and dark green leaves were also reported in tetraploids of *Ocimum basilicum* (20). Also reported that polyploidy resulted in increased cell size in genotypes which will in turn benefit their commercialization

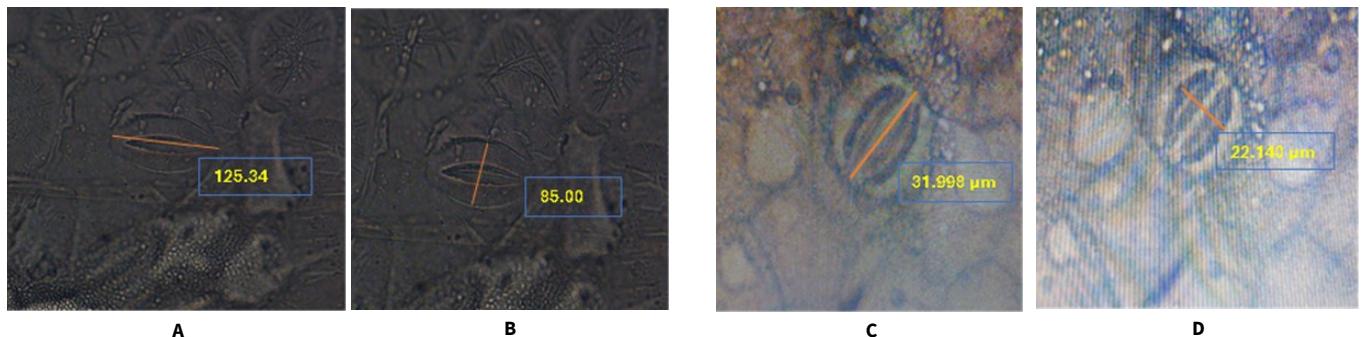
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Fig. 1. (A) Stomatal length and (B) breadth of suspected heteroploid plant T_1S_5 (C) Stomatal length and (D) breadth of control C_1 .

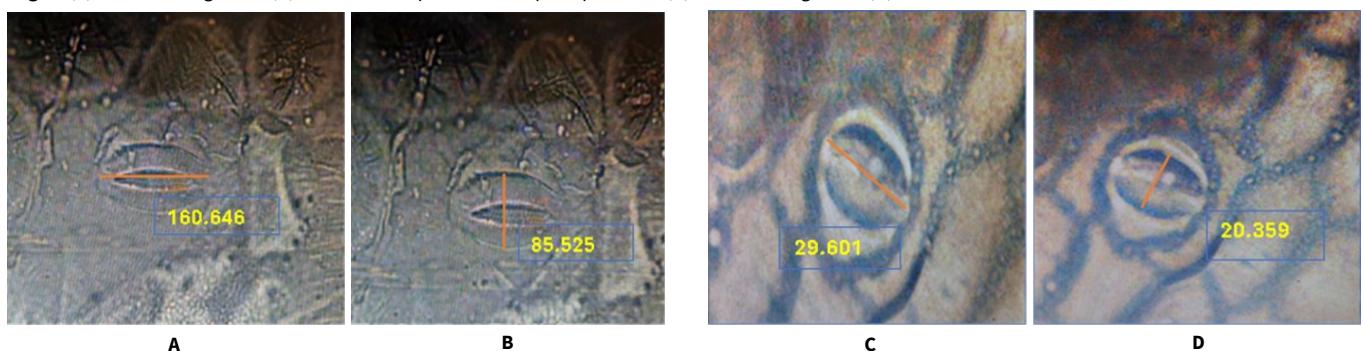


Fig. 2. (A) Stomatal length and (B) breadth of suspected heteroploid plant T_5S_1 (C) Stomatal length and (D) breadth of control C_5 .

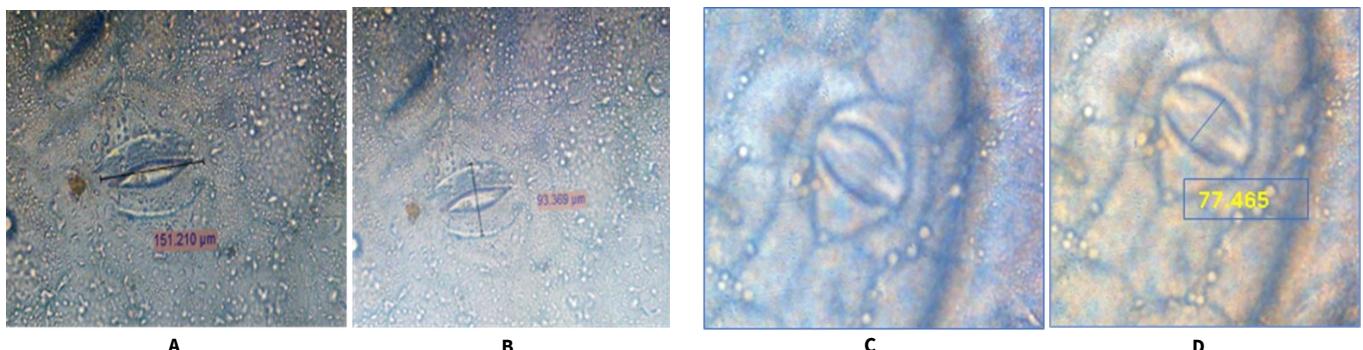


Fig. 3. Stomatal length and breadth of suspected heteroploid plants (A) & (B) T_8S_1 and (C) & (D) T_8S_4 .

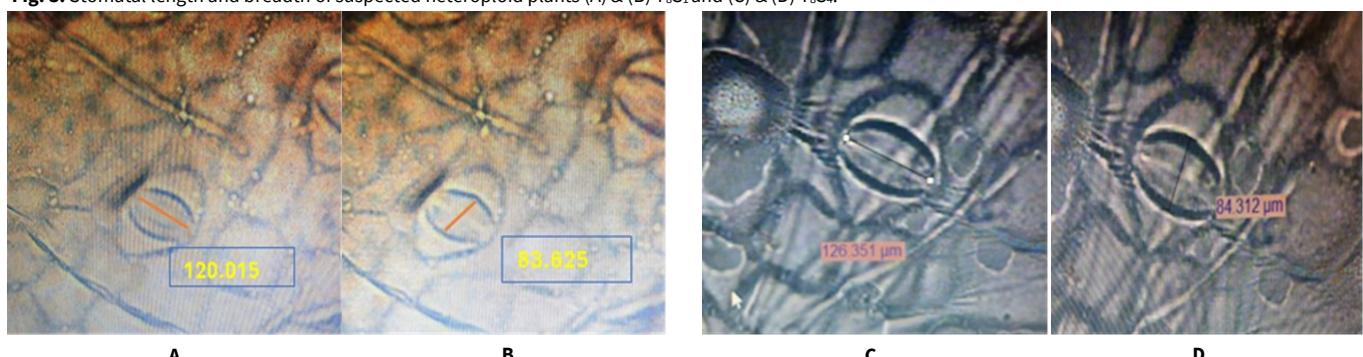


Fig. 4. Stomatal length and breadth of suspected heteroploid plants (A) & (B) T_1S_1 and (C) & (D) T_1S_{10} .

and cultivation (18).

The anti-mitotic properties of the chromosome doubling agent, colchicine, results in the disruption of microtubules to produce polyploids or colchipooids. The widely reported chromosome number of this rhizomatous vegetative plant is $2n = 22$ with a basic chromosome number of $x = 11$ (29). Among the twenty suspected plants four were heteroploids as confirmed by cytological study. The four heteroploid plants (T_1S_5 , T_5S_1 , T_8S_1 and T_8S_4) record-

-ed chromosome numbers of 27, 68, 24 and 30 respectively (Figs. 9 and 10).

The remaining 16 suspected heteroploids recorded somatic chromosome number of $2n = 22$. (13) reported an aneuploid somatic chromosome number of $2n = 24$ in an accession number 147 and chromosome numbers of $2n = 22$, $2n = 30$, $2n = 34$ and $2n = 42$ in another accession number 195. Natural occurrence of mixoploid ginger has also been reported by previous studies (30, 13).

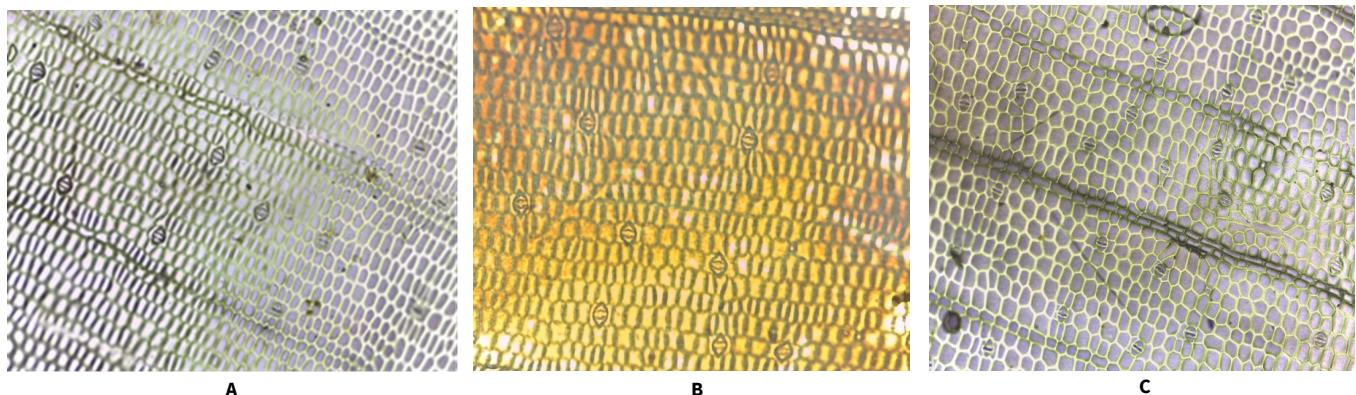
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Fig. 5. Stomatal number seen in suspected heteroploid plants (A) T₅S₁ (B) T₁S₅ and (C) diploid plant.

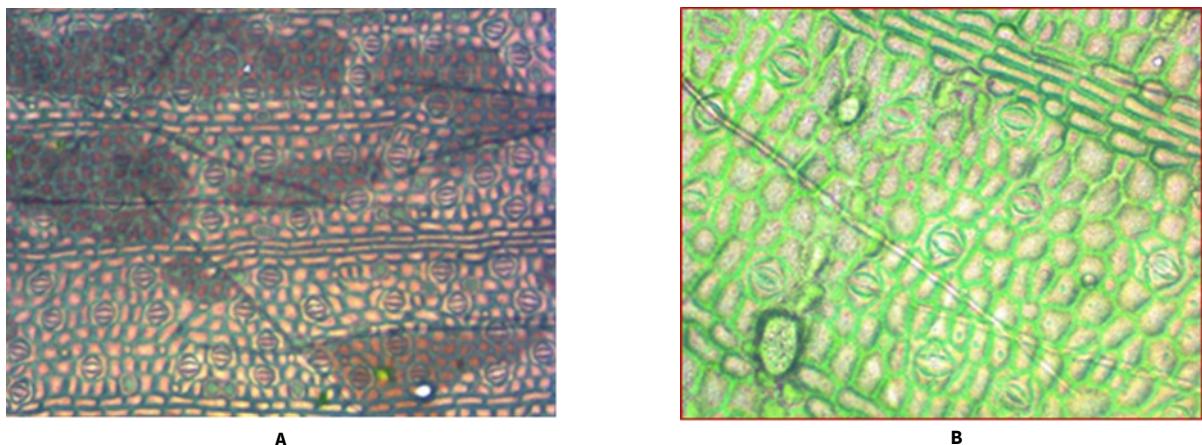


Fig. 6. Stomatal number seen in suspected heteroploid plant (A) T₈S₄ and (B) diploid plant.

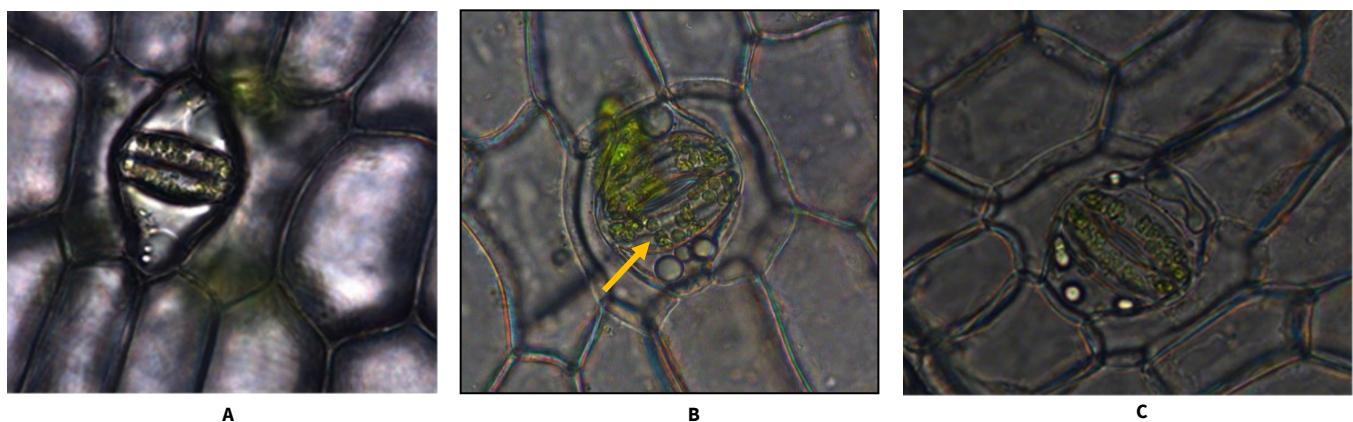


Fig. 7. Chloroplast number in suspected heteroploid plants (A) T₈S₁(20) (B) T₅S₁(21) and (C) T₈S₄(15).

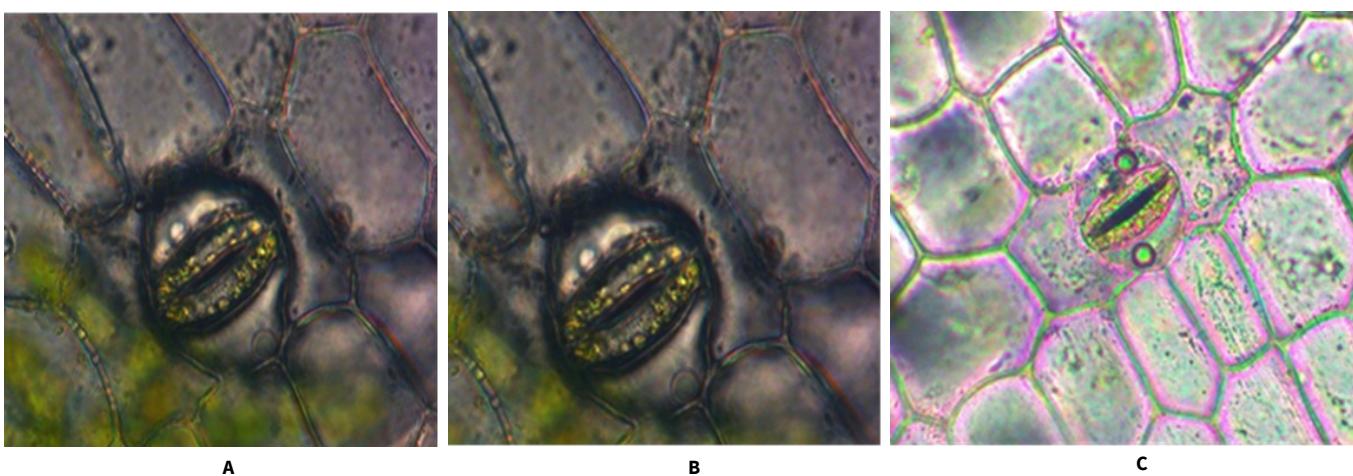


Fig. 8. Chloroplast number in the diploid plants.

A somatic chromosome number of $2n = 24$ has been reported previously in a ginger cultivar (31).

FCM has been used to identify polyploid ginger plants induced through chromosomal doubling of diploid material. The peak position of the G_1 nuclei of a standard ginger plant was compared with the peak position of the G_1 nuclei of the known sample to estimate the ploidy level. The ploidy level of diploid and colchicine treated genotypes was satisfactorily ascertained using flow cytometry as depicted by representatives of histograms showing diploids (2x) (Fig. 11) and heteroploids. Given that younger tissues with lower concentrations of starch and other metabolites were utilized in the investigation compared to older tissues, the trustworthiness of the data was indicated by the coefficients

chromosome counting could only confirm chromosome numbers of $2n=27$ in T_1S_5 , $2n=24$ in T_8S_1 and $2n=30$ in T_8S_4 .

The phenomenon of heteroploidy was frequently observed in our investigation, and FCM was quite efficient in recognizing these cases. It is equally efficient in detecting a huge number of cells in a relatively short span of time. Antimitotic chemicals may not always have the ability to reach all of a plant's meristems, let alone those that are actively dividing, which could lead to the emergence of diploids (33). According to previous study the antimitotic chemical can only penetrate to the deepest meristematic layer after an extended application time during which the cells previously affected might have been killed which can also result in mixoploids (34). Also, that, the meristematic

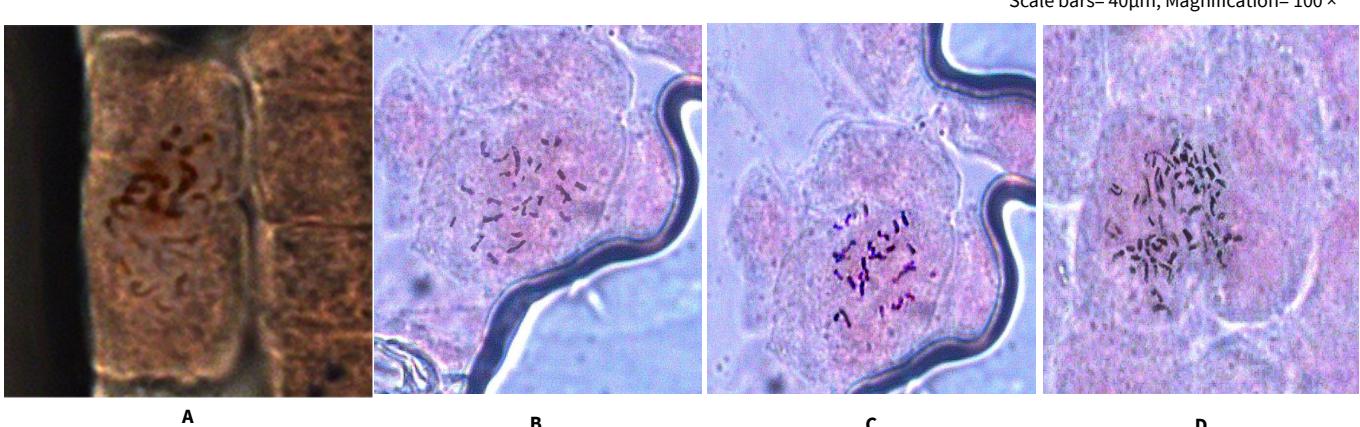


Fig. 9. (A) & (B) Chromosome number $2n = 22$ and $2n = 27$ recorded in the control T_1 and heteroploid plant of T_1S_5 obtained *in vivo* (C) & (D) Chromosome number $2n = 22$ and $2n = 68$ recorded in the control T_5 and heteroploid plant of T_5S_1 obtained *in vivo*

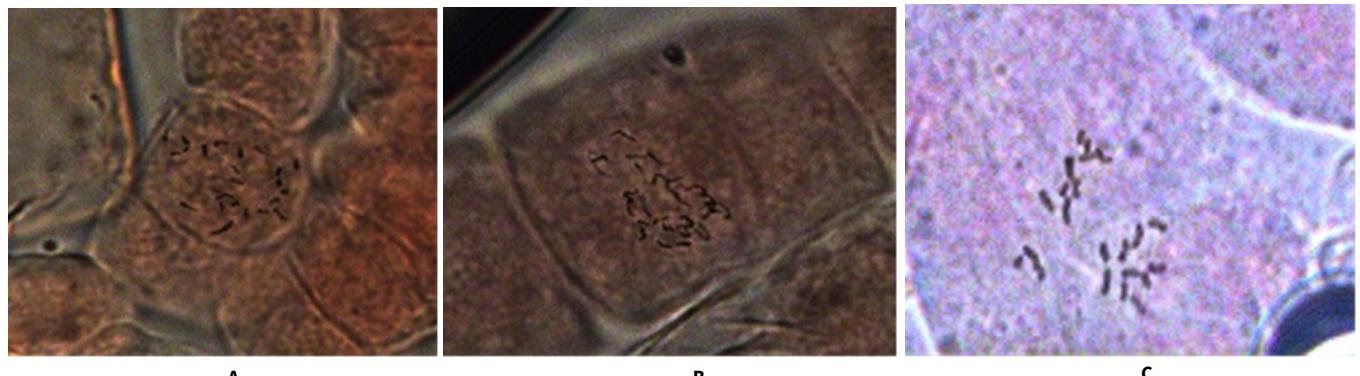


Fig. 10. (A) Chromosome number $2n = 24$ in heteroploid T_8S_1 (B) $2n = 30$ in heteroploid T_8S_4 produced *in vivo* and (C) $2n = 22$ in the control C_8 .

of variation, which were generally less than 2.0 (32). Out of the 20 suspected heteroploids only one fourth of them were confirmed to be heteroploids, confirming the greater reliability of FCM analysis (23).

Chromosome counting enables assessment of the results obtained through FCM. On comparison of the results of chromosome counting and flow cytometry histogram it can be interpreted that the plant, T_5S_1 is a heteroploid with chromosome number of $2n = 68$ as its histogram peak was obtained only in 200X (Fig. 12B). The remaining plants, T_1S_5 , T_8S_1 and T_8S_4 could be thought of as heteroploid-mixoploids (Figs. 12A, 13A and 13B) having both diploid and tetraploid or triploid or other subsequent ploidy levels as flow cytometry histogram produced peaks at P_2 population (diploid condition) and P_3 , P_4 and P_5 populations. But

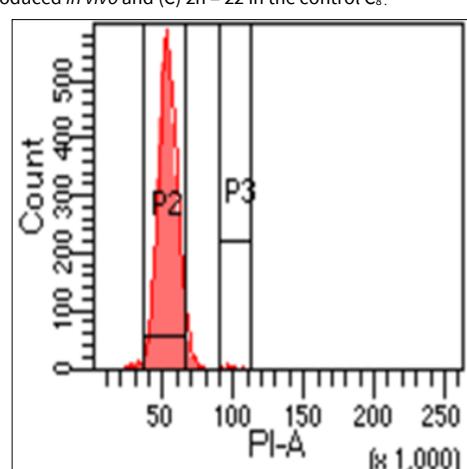


Fig. 11. Flow cytometry histogram of the ginger (standard) which is the diploid control.

cells cannot be all at the same time, at the same stage of division. Since all the control plants were diploids, no spontaneous polyploidization occurred but polyploidy was induced in colchicine treated genotypes. They obtained 4 types of polyploids in their study with *Hedychium muluense* (ornamental ginger) plants, which included triploid, tetraploid and mixoploid plants ($2x + 4x$ and $3x + 4x$). One study reported the occurrence of mixoploids having diploid-tetraploid cells in germplasm samples of ginger from China (30,35). Findings from earlier research reported that the progenies of tetraploids consisted of various portions of diploids and tetraploids in chickpea (36). They observed that this might be either due to the presence of both diploid and tetraploid shoots or due to the reversal of ploidy. They explained that the ploidy can be reversed due to accidental fusion of gametes during meiosis, which results in eight chromosomes generated by uneven chromosome separation at Anaphase I. A report suggested that the development of diploid cells in tetraploids during pre-meiosis stage could lead to inversion (37). Another rationale was that the development of multipolar spindles occurs in complement fractionation, which leads to a diploid condition (38).

Although we obtained heteroploid-mixoploids in the

study, it can be considered successful because mixoploid plants can later result in the development of autotetraploids. A study obtained an entirely autotetraploid population of *T. foenumgraecum* by the culture of seeds stemming from tetraploid branches of mixoploid plant (39). Tetraploid plants were obtained from mixoploid *Dioscorea zingiberensis* plants (40). Researchers were also able to separate diploid and tetraploid *Lolium perenne* L. from mixoploid ryegrass generated by treating germinating seeds with colchicine (41).

On the other hand, mixoploid plants are considered unstable as competition occurs between polyploid and original cells resulting in the elimination of the former. Thus, mixoploid status can be reversed to the original ploidy level. In some studies, deliberate efforts are made to eliminate mixoploids as they are often regarded as undesirable by products of polyploidization studies mainly through mechanical isolation of putative polyploids, using nodal segments for regeneration of shoots and by using apical buds for repeated subcultures (42). Another observation with mixoploids is that they do not keep their ploidy level in subsequent generations (43). Stable synthetic tetraploids have been earlier reported in previous studies in *Rhododendron* L. (44). It was observed that tetraploid plants

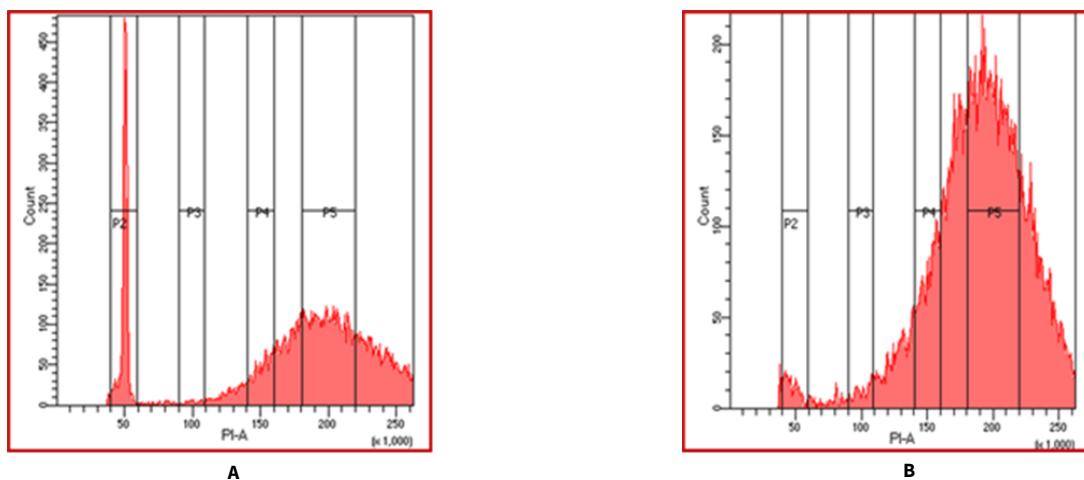


Fig. 12. Flow cytometry histogram of the heteroploid plants of ginger produced *in vivo* (A) (T_1S_5) having a chromosome number of $2n = 27$ (B) (T_5S_1) having a chromosome number of $2n = 68$.

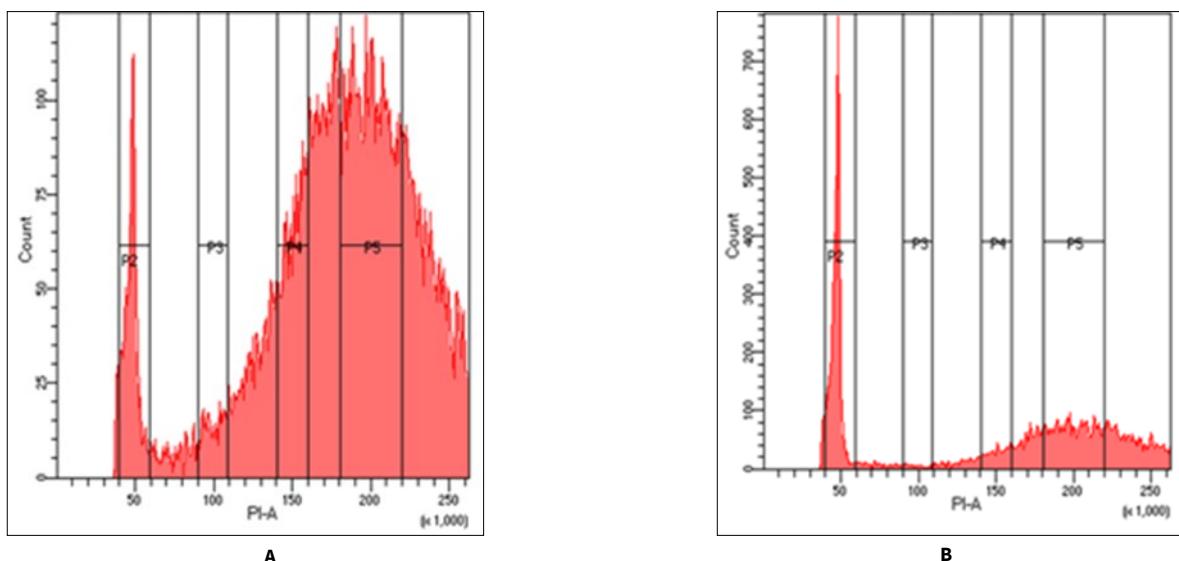


Fig. 13. Flow cytometry histogram of the heteroploid plants of ginger produced *in vivo* (A) (T_8S_1) having a chromosome number of $2n = 24$ (B) (T_8S_4) having a chromosome number of $2n = 30$.

Acacia mangium Willd. were later reclassified as diploids and mixoploids about 16 months later when the plants were transferred under field conditions showing their instability (45).

The maximum yields at harvest were obtained in control genotype, C₇, treated genotype T₅ and T₈. Similarly, dry rhizome yields were also higher in C₈, T₅ and T₈. Higher dry root yields were obtained in T₅, T₁ and T₈. The greater yield in control genotype, C₇ could be attributed to the larger and bold rhizomes. But induction of polyploidy was difficult in this genotype as the rhizome buds did not survive the lethality of the chemical and subsequently the sprouting percentage was very less. In *Cannabis sativa* polyploid plants were found to be lower and the changes were also hardly visible on comparison with diploids (46). An investigation recorded that although pollen fertility was higher in the tetraploids, pod set was higher in the diploids in chick pea (36).

Treated genotypes of T₅, T₈, T₄ and T₁ recorded fresh rhizome yields which were on par with control genotype, C₇. Induced tetraploids with larger rhizomes have been previously reported by (8). Higher rhizome yields of up to 0.90 times greater in induced tetraploid ginger than the diploid genotype was obtained by (17). An autotetraploid line in ginger plant which yielded larger rhizomes of top-quality confectionary ginger ideal for processing companies (24). A research identified 2 tetraploid lines in ginger with enhanced yield of 320.81 g and 418.65 g compared to diploid (280.33 g)(18). Similarly, dry rhizome yield was higher in the tetraploids (89.91 g). Polyploidy causes the addition of extra gene copies, which results in larger cell size and vigour as reported by (27).

There has also been reports which showed that tetraploids are inferior or sometimes equal to diploids with respect to some characters. Such observations had been made in green gram and black gram (47, 48). They indicated that tetraploids do not express gigantism in all the characters.

The four heteroploid plants that were obtained *in vivo* through colchicine treatment were compared with their respective control to assess which heteroploid performed better among all. Among the 5 heteroploids developed T_{8S1} was found to be highly promising with respect to plant height, number of leaves plant⁻¹, fresh rhizome yield plant⁻¹ and dry rhizome yield per plant. Vigorous nature of polyploids have also been reported in previous studies (49, 50).

Conclusion

Successful *in vivo* polyploidization for induction of heteroploids in ginger using colchicine has been established through the study. The genotypes Athira, Genotype 1 and Genotype 4 proved to be highly efficient for ploidy induction studies among the eight ginger genotypes tried. Two heteroploids could be produced from the Genotype 4. The induced heteroploids although showed poor growth and yield characters in the first generation which might be largely due to the effect of the antimitotic agent, however in the second-generation better characteristics for growth and

yield were recorded. Initial screening of putative polyploids by means of morphological characters and stomatal parameters followed by chromosome counting and flow cytometry were found to be the most suitable methods for confirming the ploidy level. Four heteroploids has been identified and the stability has been recorded.

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Authors' contributions

SSG conducted the experiments and wrote the manuscript. SGS and DSN aided in study conception, manuscript preparation and design of the experiment. RS, SA, SBS and BT contributed towards data collection, analysis and interpretation of the results. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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