The Pharma Innovation Journal 2023; SP-12(9): 1258-1268

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; SP-12(9): 1258-1268

www.thepharmajournal.com
Received: 02-07-2023
Accepted: 12-08-2023

Silpa SG
Department of Plantation Crops and Spices, College of Agriculture, Kerala Agricultural University, Trivandrum, Kerala, India

Sreekala GS
Department of Plantation Crops and Spices, College of Agriculture, Kerala Agricultural University, Trivandrum, Kerala, India

Deepa S Nair
Department of Plantation Crops and Spices, College of Agriculture, Kerala Agricultural University, Trivandrum, Kerala, India

Beena Thomas
Department of Plant Breeding and Genetics, College of Agriculture, Kerala Agricultural University, Trivandrum, Kerala, India

Corresponding Author:
Silpa SG
Department of Plantation Crops and Spices, College of Agriculture, Kerala Agricultural University, Trivandrum, Kerala, India

In vitro induction and identification of heteroploids in ginger

Silpa SG, Sreekala GS, Deepa S Nair and Beena Thomas

Abstract
Ginger is a significant commercial crop grown for its flavour, pungency, and scent. Ginger being propagated exclusively by vegetative means, the number of clones available is limited. Induced polyploidy is used by some plant breeders for improving yield, quality and to impart pest and disease resistance. Hence, the present investigation is an attempt to induce heteroploidy in eight ginger genotypes using colchicine *in vitro*. Single bud rhizomes were treated with 0.2% colchicine and morphological, yield and cytological observations were recorded in both treated and control diploid plants. At 60 days, the treated plants recorded very less survival percentage compared to the control ginger genotypes. The maximum survival percentage among colchicine treated plants was in H_8 (5%) followed by H_1 (4%) and H_5 (3%). The treated plant of genotype 8 produced by *in vitro* method had a chromosome number of 2n=24 which was also a heteroploid confirmed through chromosome counting and flow cytometry analysis. The corresponding diploid had a chromosome number of 2n=22.

Keywords: Ginger, colchicine, polyploidy, heteroploids, mixoploids

1. Introduction
Ginger (*Zingiber officinale* Rosc) belonging to the family Zingiberaceae, is usually consumed as a dietary condiment throughout the world [35]. Ginger is being widely used for the treatment of various ailments like nausea, common colds, arthritis and migraines for thousands of years. The pharmacological and medicinal properties of ginger have been extensively reviewed [32]. In European medicine, ginger was a component of most pharmaceutical preparations and it was one of the most highly prized of all mild carminatives [19, 26].
India is among leading producers of ginger in the world and during 2018-19 the country produced 1844000 MT of ginger from an area of 172000 hectares [17]. Ginger is cultivated in most of the states in India. However, 65% of the nation's overall production is collectively done by states like Gujarat, Karnataka, Orissa, Assam, Meghalaya, and Arunachal Pradesh. Ginger is propagated by seed rhizomes which are carefully preserved without any infection.
The important crop improvement objectives in ginger are resistance to diseases like bacterial wilt and rhizome rot besides bold rhizome, less fibre, high essential oil and yield [26]. Ginger has a narrow genetic base and breeding is limited mostly to clonal selection. This is due to the fact that ginger exhibits high sterility [10, 25] due to chromosomal aberrations such as inversions and translocations [23, 24] and is propagated by vegetative means creating less variability. Therefore, in order to produce variability, alternative breeding techniques like polyploidy breeding are needed.
Ploidy has been crucial to evolution and orderly classification [13]. Polyploids are more vigorous and perform better than their diploid relatives. Ploidy level affects morphology, and polyploid plants are found in both horticultural and agricultural crops because they frequently have better morphological traits than their diploid counterparts [33]. Tetraploid ginger has noticeably broader and greener leaves than diploid ginger, better height as well as significantly fewer but thicker shoots and a larger rhizome mass after harvest. This was demonstrated by [38]. Natural polyploidy has played an important role in the evolution of spices like black pepper, vanilla, turmeric, small cardamom, saffron etc. Polyploidy breeding using colchicine in ginger is mostly done *in vitro*. Colchicine is administered directly via syringe injection [15], by covering the roots of seedlings in paper towels or applied by cotton plug method on shoot apices. The *in vitro* system of regeneration is less time consuming and has a higher rate of ploidy conversion.
It may be possible to produce improved tetraploid varieties of ginger from some of the diploid clones [22]. The tetraploid of the ginger variety used in this study showed a significant increase

in yield of rhizomes. Induction of autotetraploidy tried in Kerala Agricultural University in Himachal Pradesh, Maran, Nadia and Rio de Janeiro cultivars of ginger using three concentrations of colchicine (0.10, 0.25 and 0.40 per cent) and two methods of application (injection and hole method) produced two autotetraploids which recorded higher rhizome yield, higher and lower stomatal size and frequency respectively than the corresponding diploids [31].

Higher pollen fertility (27.4 to 74.2%) and germinability (4.8 to 12.9%) in tetraploid ginger have been reported by [1] when compared with diploid ginger. *In vitro* polyploid induction studied in ginger by [12] revealed autotetraploids with high essential oil content, about 23% higher than that of the control. Studies by [28] in ornamental ginger concluded that when embryogenic callus was treated with 60 mM oryzalin, the maximum tetraploid induction frequency (15%) was achieved.

Flow cytometry (FCM) using DNA-selective fluorochrome is now the most popular technique for determining the amount of nuclear DNA in plants. The assay can be used to determine ploidy level, detect mixoploidy, and, in some circumstances, aneuploidy due to the link between ploidy and nuclear DNA content [3]. FCM is now the preferred method since it is quick, easy, and dependable. The procedure is often non-destructive and appropriate for the study of small individuals because samples are typically prepared from only a few tens of milligrams of plant tissues.

2. Materials and Methods

The experiments conducted at Department of Plantation Crops and Spices, College of Agriculture, Vellayani during 2018 identified four promising genotypes of ginger for yield. Athira and Aswathy are two high yielding varieties of ginger released by KAU from Maran and Rio de Janeiro with an average yield of 21 and 23 t/ha respectively. IISR Varada and IISR Mahima are also high yielding varieties of ginger yielding 22.66 and 23.2 t/ha respectively. An experiment was formulated to produce heteroploids *in vitro* from Athira, Aswathy, Varada, Mahima and the promising four ginger genotypes evaluated in the Department of Plantation Crops and Spices. Thus, a total of eight genotypes and their respective control plants were evaluated. H_1 to H_8 denote the colchicine treated genotypes and C_1 to C_8 denote their respective control plants. Hundred rhizome bits of each treatment was used in the experiment and single plant observation was undertaken.

2.1 Treatments

A total of sixteen treatments including eight respective controls were tried in Completely Randomized Design.

H_1- Athira
H_2- Aswathy
H_3- IISR Varada
H_4- IISR Mahima
H_5- Genotype 1
H_6- Genotype 2
H_7- Genotype 3
H_8- Genotype 4
HC_1- Control parent Athira
HC_2- Control parent Aswathy
HC_3- Control parent IISR Varada
HC_4- Control parent IISR Mahima
HC_5- Control parent Genotype 1
HC_6- Control parent Genotype 2
HC_7- Control parent Genotype 3
HC_8- Control parent Genotype 4

The heteroploids were compared with the respective parents and IISR Varada was kept as standard check. The plantlets obtained after treatment were hardened in primary and secondary nurseries and planted in grow bags. The *in vitro* plantlets of the above untreated ginger cultivars were also maintained in grow bags. The morphological, physiological and cytological observations were carried out.

2.2 Surface sterilization of explant

Rhizomes of *Zingiber officinale* Rosc. Genotypes were collected and stored under shade for sprouting. The sprouted apical buds were cut from the rhizome and used as the explant. The apical buds along with a portion of the rhizome were first washed in detergent solution for 10-15 min. After several washes in water the rhizome portion was excised and the apical buds were treated with 0.4% bavistin fungicide for 30 minutes followed by treatment with tetracycline (500 ppm) for 30 minutes. Tetracycline preparation consists of addition of about 1.5 g of K-cycline (Agricultural grade) into 30ml of sterile water. After subsequent treatments with bavistin and tetracycline the apical buds were washed thoroughly with distilled water. The buds were then transferred to autoclaved petri dishes and taken to the laminar air flow chamber. Using sterilized forceps and blade several outer layers of leaves were removed from the apical buds. From these surface-sterilized buds, shoot tips (about 6.0 mm in height) after removing two or three younger leaf primordia were washed with 70% alcohol for 30 seconds followed by surface sterilization using mercuric chloride 0.1% for 1 minute. After each treatment with mercuric chloride explants were washed with sterile water and the process of dipping in mercuric chloride and washing was repeated 3 times.

2.3 Preparation of stock solution and media

Stock solutions (Stock A, B, C, D, E & F) of macronutrients, micronutrients, vitamins and plant growth regulators (BA and NAA) were prepared and stored in refrigerated conditions. Each compound was dissolved separately in double distilled water. Components are added one at a time and dissolved and the volume made up to 100 ml in a volumetric flask.

Cytokinins and auxins were the two important plant growth regulators used in different concentrations and combinations for bud proliferation, shoot multiplication and *in vitro* rooting etc. The concentration of Benzyl adenine (BA) and Naphthalene acetic acid (NAA) used in the experiment was 2 ppm and 0.05 ppm respectively. The volume of these hormones to be added was calculated based on the volume of media prepared. Stock solution of BA was prepared by weighing the required quantity of BA and dissolving in 1 N NaOH or 1 N HCl and the volume made up to 100 ml with double distilled water. Stock solution of NAA was prepared by weighing the required quantity of NAA and dissolving in 95% ethyl alcohol and the volume made up to 100 ml with double distilled water.

Murashige and Skoog medium was used as the basal media for the experiment. For the preparation of 1 L MS media, required quantity of stock solutions were pipetted out one by one to a beaker containing 600-800 ml distilled water. Then sucrose (30 g L^{-1}), Myoinositol (100 mg L^{-1}), and MS supplement (3.55 g L^{-1}) were weighed and dissolved in it. The pH of the medium was adjusted to 5.7-5.8 by using 1 N NaOH or 1 N HCl. After adjusting the pH, volume was made

up to 1 L. Agar (6.5 - 7 g L^{-1}) was added and melted in microwave oven. Then the prepared medium was poured into clean, autoclaved culture bottles and autoclaved for 20 minutes at 15 lbs. pressure at 121˚C. The media were allowed to cool and kept at room temperature for one week and examined for any type of contamination on it.

2.4 Colchicine solution preparation and induction

Colchicine solution was prepared at a concentration of 0.2% (w/v). The required quantity of colchicine was dissolved in 10 ml of ethyl alcohol followed by dilution with 90ml of distilled water. The prepared colchicine solution was added to MS media under LAF conditions using filter sterilization to avoid contamination. Nylon filter was used for this purpose. The media was kept under check for a week for any signs of contamination and later used for inoculation.

Sterilized rhizome buds were first inoculated in liquid MS medium containing 2.0 ppm BA, 0.05 ppm NAA, and colchicine at concentration of 0.2% (w/v) [1]. The liquid media was kept with rhizome buds for eight days. The rhizome buds were taken out on the 9th day and washed three times with sterile distilled water. The buds were checked thoroughly for contamination and degraded buds were discarded. The medium without colchicine served as the control (0 day). All cultures were incubated at 16 hrs light and 8 hrs dark photoperiod (cool, white fluorescent light) and 25±2˚C temperature. The cultures were observed daily. The washed rhizome buds were inoculated into solid MS media supplemented with 2.0 ppm BA for bud proliferation and *in vitro* rooting. After six weeks of culture establishment, the survived explants of both treated and untreated samples were transferred to fresh MS medium supplemented with 2.0 mgL^{-1} BAP and 0.05 mg L^{-1} NAA for shoot multiplication. The cultures were maintained by regular sub culturing on fresh medium. Rooted treated and untreated samples with shoots about 4-5 cm in length were removed from the culture bottles and the roots were washed under running tap water to remove agar. Then the plantlets were transferred to sterile poly pots (small plastic cups) containing pre-soaked coir dust and washed sand (1:1) maintained inside the growth chamber made by the polythene sheet. After acclimatization of plantlets, they were transplanted to pots containing the mixture composed of sand, red soil and cattle manure (1:1:1). Morphological, physiological and cytological observations were recorded up to 6 months after planting.

2.5 Evaluation of the plants produced *in vitro*

The morphological, cytological and physiological parameters were examined

2.5.1 Estimation of morphological parameters

The total numbers of days to sprouting and survival percentage were estimated at the beginning stages for diploids and treated plants. Survival percentage was estimated as the number of seedlings that survived till transplanting out of the total number of rhizomes that sprouted. Plant height, number of tillers per plant, number of leaves per plant and leaf area 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 centimeter 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. The length of the upper fourth leaf of the main shoot was measured from the base of the petiole to the highest tip of the leaf and expressed in centimeters for the purpose of measuring leaf area. The main shoot's top fourth leaf's width was also measured using a scale and stated in centimeters at its broadest point. The following formula was then used to estimate leaf area.

Leaf Area = k x Leaf length x Leaf width – 0.7607

Where, k= 0.6695 and was expressed in cm^2 [11].

2.5.2 Estimation of physiological parameters

Physiological parameters like chlorophyll content, photosynthetic rate, transpiration rate, stomatal conductance and leaf temperature were recorded at 6 months after planting. UV-VIS spectrophotometer (ELICO, SL21 UV-VIS spectrophotometer) was used to measure the chlorophyll content. Leaf bits (0.1g) collected from both control and treated plants were washed in distilled water and used for the estimation of chlorophyll. Pigments were extracted from leaf bits by using acetone: DMSO (1:1) mixture. The leaf sample was incubated in acetone: DMSO solution overnight in the dark condition. The colored solution was decanted into measuring cylinder and made up to 10ml. The absorbance was recorded at 663 and 645 nm using UV-visible spectrophotometer. Chlorophyll a, chlorophyll b and total chlorophyll were estimated and expressed in mg/g of fresh sample. The formula for calculating chlorophyll a, b and total chlorophyll is given below,

Chlorophyll a = [{12.7 (A663) - 2.69 (A645)} x volume/ (weightx1000)]

Chlorophyll b = [{22.9 (A645) - 4.68 (A663)} x volume/ (weightx1000)]

Total chlorophyll = [{20.2 (A645) + 8.01 (A663)} x volume/ (weightx1000)]

Photosynthetic rate, transpiration rate, stomatal conductance and leaf temperature were measured using IRGA (Infrared Gas Analyzer) and expressed as µmolCO_2/m^2/s, mmol H_2O/m^2/s, mmol/m^2/s and ˚C respectively.

2.5.3 Estimation of cytological characters

Cytological parameters like chromosome number, stomatal frequency, length, breadth, epidermal cell number and chloroplast number were recorded. Flow cytometry analysis was also done to confirm the ploidy.

The survived plants were subjected to cytological study to confirm the chromosome number. Root tips for analysis were collected from the rhizomes planted in portrays on initiation of root emergence. Between 11:00 and 11:00 a.m., actively growing root tips of 5 to 10 mm length were collected and pretreated at 4-5 °C for 4 hours with a 1:1 mixture of 2 mM 8-hydroxyquinoline and saturated paradichlorobenzene solution. The root tips were properly washed in double distilled water before being fixed for 24 hours in a 3:1 solution of ethyl alcohol and acetic acid. The hydrolysis of the fixed root tips in 5 M HCl at 0 °C for 4 min, staining in 2% acetoorcein for 4 h, and subsequent crushing in 45% acetic acid were all performed on them. On temporary slides, chromosome counts were counted and examined during the mitotic metaphase stages using a Leica DMRB (Leica, Germany) microscope

with a 100× objective on three metaphase plates having good chromosome counts [16].

For stomatal counting, 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. A small patch of the leaf's abaxial side was painted with a fine coat of clear nail polish and allowed to dry. Then, it was taken out with a pair of fine tip forceps. A microscope slide was mounted with the polish strips. It was mounted with a drop of water and observed under microscope. 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. Similarly, epidermal cell size was obtained by counting the number of cells per mm^2 of leaf in both control and treated plants. Four leaves were chosen from the same part of diploid and treated plants for recording stomatal measurements. Three leaf sections from each plant were examined for chloroplast counts. Using a knife, the upper mesophyll tissues were removed, leaving the bottom epidermis. A drop of water was used to stabilize this spot on the slide. This section was placed on a slide with a drop of water, covered with a coverslip, and examined under a microscope. In each of the three leaf samples, the chloroplasts were counted from five pairs of guard cells. An image analyser (Leica) was used to measure the stomatal characteristics at 40X and 100X magnification [8, 37].

2.5.4 Ploidy confirmation by flow cytometry

Nuclear suspensions were prepared according to the single step protocol. A small amount of plant tissue (usually 60 mg) was positioned in the middle of a plastic petri dish. This was then mixed with 1 ml of ice-cold Tris.$MgCl_2$ buffer for nuclei isolation [27]. Tris.$MgCl_2$ buffer was prepared using 200 mM Tris, 4 Mm $MgCl_2.6H_2O$, 0.5% (v/ v) Titron X-100, pH 7.5 [20]. The homogenate was mixed well by pipetting up and down for several times while avoiding air bubbles. After being filtered through 42-µm nylon mesh the homogenate was poured into a labeled sample tube. To avoid instrument clogging the filtrate was checked visually to ascertain that it is free of any particles. Stock solution of a DNA fluorochrome was added and gently shaken. DNA fluorochrome used was Propidium Iodide (PI). At a concentration of 1 mg ml^{-1}, PI stock solution was prepared. Preparation was carried out using double distilled water and was filtered using 0.22 µm filter and stored at -22 °C in 1 mL aliquots. Refreezing again after thawing was avoided. Similarly, 1 mg ml^{-1} concentration of RNase stock solution was prepared. During preparation the solution was heated at 90 °C for 15 minutes to inactivate DNases. PI and RNase were typically used at 50 µg ml^{-1} simultaneously. It is necessary to utilize RNase and PI simultaneously since PI binds to dsRNA as well. Before analysis, the sample was incubated on ice (a few minutes to 1 h), with occasional shaking. Later nuclear DNA content was analyzed [3]. The FACS machine (BD FACSAria II) analysis was done at Rajiv Gandhi Centre for Biotechnology, Poojapura.

The standardization regarding the initial weight of the leaf sample to be taken and the rpm of the centrifugation process for obtaining proper histogram was carried out using the untreated samples of *Zingiber officinale* L., which is the control (diploid). The diploid peak of the control sample was carefully noted. Flow cytometry histogram was obtained for each of the sample loaded. Chromosome number was interpreted by comparing the peaks obtained in control (diploid) and the treated samples.

2.5.5 Estimation of yield parameters

After the plants turned yellow and dried, rhizomes were gathered. Yield of each plant in terms of fresh weight of rhizome was recorded (g/plant)

2.6 Statistical analysis

The data on numerous observations obtained during the period of study was subjected to statistical analysis using Grapes software, KAU [9] and the results were interpreted at 1% probability level. Experiments were analysed using Completely Randomised Design (CRD) consisting of 16 treatments.

3. Results

3.1 Effect of colchicine on morphological characters

The maximum number of days for sprouting (46.50) was taken by the treatment H_8 which was significantly on par with all other treated genotypes. The least number of days for sprouting (11.40) was taken by the check variety IISR Varada which was significantly on par with all other control genotypes. The mean number of days taken for sprouting in treated genotypes was 41.54 significantly different from control genotypes (14.23). The survival percentage in control plants ranged from 21% in C_1 to 11% in C_6. While 21 plants of C_1 survived at 60 days only 2 plants of H_2, H_3, H_4, H_6 and H_7 survived among treated plants (Table 1).

There was significant difference in plant height between the different treated and control plants at 90, 120 and 180 DAP. Among treated plants, H_6 recorded the maximum plant height (13.06 cm) which was significantly on par with all other treated genotypes at 90 DAP (Table 2). At 120 DAP, the maximum plant height among treated plants was recorded in H_7 (29.79 cm). Among control plants, the maximum height was recorded by C_4 (35.68 cm) significantly on par with all other control genotypes. At 180 DAP, treated plants recorded lesser plant heights and the maximum was recorded in H_4 (38.76 cm) and the least in H_6 (29.84 cm). The mean plant height in treated genotypes was 33.41 cm which was significantly different from the mean plant height of control genotypes (39.73 cm) at 180 DAP.

There was no significant difference in the number of tillers produced by the different treated and control plants at 90 and 120 DAP. At 90 DAP, the mean number of tillers produced in treated genotypes 1.52 significantly different from the mean number of tillers produced in control genotypes (2.00). Similarly, at 120 DAP, variations were significant between the mean number of tillers produced in treated and control genotypes which was 2.04 and 2.63 respectively. At 180 DAP there was significant difference in the number of tillers produced by different treated and control plants (Table 3). Among treated plants, the maximum number of tillers, 3.50 was recorded in H_2 significantly on par with H_5 (3.33), H_3 and H_4 (3.00), H_8 (2.80) and H_1 (2.75). At 180 DAP, the mean number of tillers produced in treated genotypes was 2.75 significantly different from the mean number of tillers produced in control genotypes (3.45).

There was significant difference in the number of leaves produced by the different treated and control plants at 90, 120 and 180 DAP and control plants produced a greater number of leaves compared to treated plants at all stages of observation

(Table 4). At 90 DAP, among the colchicine treated plants, the highest number of leaves was recorded in H_4 (5.00) significantly on par with all other treated genotypes. Among control, C_5 recorded the maximum number of leaves (15.33) which was significantly on par with all other control genotypes. At 120 DAP, treated plants produced lesser number of leaves ranging from 7.67 in H_1 to 10.00 in H_8, which were significantly on par to each other. At 180 DAP, among treated plants, the maximum number of leaves were recorded in H_7 (18.50) followed by H_2 (18) which was significantly on par with all other treated genotypes. At 180 DAP, the mean number of leaves produced in treated genotypes was 16.40 significantly different from the mean number of tillers produced in control genotypes (23.75).

The leaf length and breadth were recorded at 90, 120 and 180 DAP and the leaf area were calculated. There was significant difference in leaf length and leaf area at 90 DAP between the different ginger genotypes. At 90 DAP, among treated plants maximum leaf length was recorded in H_3 (7.88 cm) significantly on par with H_1 (7.59 cm), H_2 (7.03 cm), H_4 (6.81 cm) and H_5 (6.80 cm) (Table 5). Among treated plants the maximum leaf area recorded was in H_3 (4.77 cm^2) significantly on par with all other treated genotypes except H_8 (2.57 cm^2). The maximum leaf area of 9.35 cm^2 was recorded in control plant C_5 significantly on par with C_2 (8.38 cm^2) and C_1 (7.86 cm^2). Similarly, at 120 DAP, there was significant difference in leaf length and leaf area between the different treated and control plants. The maximum leaf length among treated plants was recorded in H_1 (11.68 cm) significantly on par with all other treated genotypes except H_8 (8.60 cm). Among treated plants, the leaf area was maximum recorded in H_1 (8.80 cm^2) significantly on par with all other treated genotypes. At 180 DAP, no significant difference was recorded between the different colchicine treated and diploid plants in leaf length, breadth and leaf area. The mean leaf length and area recorded in treated plants was 15.54 cm and 14.13 cm^2 respectively which was significantly different from the mean leaf length and area recorded in control genotypes, 18.41 cm and 18.43 cm^2 respectively.

3.2 Effect of colchicine on physiological characters

There was significant difference in chlorophyll b content between the different treated and control plants. The maximum chlorophyll b content was recorded in treated plant H_3 (1.32 mg/g) significantly on par with H_5 (1.10 mg/g), control plants C_5 (1.06 mg/g), C_6 (0.99 mg/g), C_1 (0.91 mg/g) and treated plant H_1 (0.90 mg/g) (Table 6). There was no significant difference in photosynthetic and transpiration rate between the different treated and control genotypes. There was significant difference in stomatal conductance between the different treated and control plants. The maximum stomatal conductance was recorded in treated plant H_2 (13.50 mmol/m^2/s) significantly on par with control plant C_8 (13.33 mmol/m^2/s), C_2 (13.00 mmol/m^2/s), C_1 (12.33 mmol/m^2/s) and treated plant H_1 (10.67 mmol/m^2/s). There was significant difference in mean stomatal conductance between the treated and control genotypes which was 8.04 mmol/m^2/s and 8.66 mmol/m^2/s respectively. The leaf temperature in colchicine treated and control ginger plants ranged from 27.2 to 27.4 °C. The maximum leaf temperature among treated genotypes was recorded in H_4 (27.4 °C) which was significantly on par with H_2, H_4, H_6 and H_8 (27.3 °C). The maximum leaf temperature among control genotypes was recorded in C_3 and C_6 (27.4 °C) which was significantly on par with C_2, C_4, C_5 and C_7 (27.3 °C).

3.3 Effect of colchicine on cytological characters

The chromosome number counting was done using aceto-orcein on the survived plants. The treated plant of genotype 8 produced by *in vitro* method had a chromosome number of 2n=24 which was also a heteroploid confirmed through chromosome counting and flow cytometry analysis. The corresponding diploid had a chromosome number of 2n=22 (Fig. 1).

There was no significant difference in stomatal frequency and stomatal size between the different treated and control plants (Table 7). The variations were significant between treated and control genotypes in the case of mean stomatal frequency, length and breadth. The mean stomatal frequency in treated genotypes was 107.34 mm^{-2} significantly different from the control genotypes (111.25 mm^{-2}). The mean stomatal length and breadth recorded in treated genotypes was 36.53 µm and 31.13 µm respectively significantly different from the control genotypes which was 31.34 µm and 23.25 µm respectively. There was no significant difference in chloroplast number between the different treated and control plants. The mean chloroplast number in treated genotypes was 9.64 mm^{-2} significantly different from control genotypes (10.62 mm^{-2}). There was no significant difference in epidermal cell size between the different treated and control plants but significant variation was recorded between the mean epidermal cell size between the treated and control genotypes which was 53.20 and 60.73 mm^{-2} respectively.

3.4 Flow cytometry analysis to confirm the ploidy

The flow cytometry histogram was generated using FACS machine to determine the heteroploid among survived plants. The histogram peak of the colchicine treated plant H_8S_1 was obtained in both 50X and 200X, confirming heteroploidy. The histogram peak of the corresponding diploid plants was obtained in 50X. 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 (Fig. 2). The number of cells corresponding to the ploidy of the ginger genotypes was obtained from the flow histogram.

3.5 Effect of colchicine on yield characters

There was no significant difference in yield between the different treated and control plants. The maximum yield was recorded in control plant C_5 (72.67 g/plant) followed by 67.50 g/plant in treated plant H_4. The mean fresh rhizome yield recorded in treated genotypes was 47.81 g/plant significantly different from control genotypes (51.34 g/plant).

4. Discussion

Colchicine treated plants took a greater number of days for sprouting than control plants. Colchicine treatment produced significant difference in the number of days taken for sprouting between treated and control plants. Less growth hormone and lowered activity of metabolites [14] reduced rate of cell division [5] cause slower growth rate in polyploid plants. The first visible effect of colchicine was the delayed growth of explants as reported by [29]. These results are also in agreement with some other previous studies which reported that induced polyploid plants grew at a slower rate compared to the control plants [30, 40]. The treated buds took 10-15 days for bud initiation compared to 3-4 days in untreated explants

as also recorded by [29] in *Zingiber officinale* Roscoe 'Fengtou' ginger.

The highest reduction in survival was at 60 days indicating that the survival data up to the 60th day need to be recorded to understand the lethality of the chemical colchicine. Reduction in survival rate of colchicine treated plants is an indication of the lethal effect of colchicine. Longer treatment durations and higher antimitotic chemical concentrations decreased the survival rate while increasing tetraploid induction. Spindle inhibitors may produce physiological disturbances that lower survival rates by slowing down cell division [34, 36]. Similar comparable outcomes were observed in *Aframomum* [39], *Mentha* [7] and *Ocimum* [18]. Control plants showed better growth rate at 90, 120 and 180 DAP. Initial slow growth rate might be due to reduced rate of cell division, lower amount of growth hormone or lower rate of metabolic activities. Stunted growth rate was observed in colchicine treated seedlings of ginger [31, 42].

In ginger, the widely reported chromosome number is 2n=22 [16, 23]. Heteroploidy is the condition of having cells with chromosome number different from the normal 2n compliment. Natural occurrence of mixoploid ginger has been reported by [16]. The chromosome number counting of the suspected plants were carried out during the entire season to confirm the ploidy level. In the present study, a heteroploid with a chromosome number of 24 is obtained. The stomatal frequency was recorded lesser while the size was recorded higher for the treatment H_8. The stomatal frequency recorded in treatment H_8 was 99.48 number/mm^2 while the stomatal length and breadth recorded was 43.91 and 44.41 µm respectively. One plant among the five survived plants of the treatment H_8 was found to be a heteroploid. There was no significant difference in stomatal frequency, length and breadth between the different treated and control genotypes. [28] Reported that the stomatal frequency was 1.5 and 1.8 times higher in diploid *Hedychium muluense* plants than that observed in triploid and tetraploid plants respectively. Stomatal frequency and ploidy are inversely related as confirmed in earlier studies by [2].

To determine how polyploidization has modified the features of the polyploid, stomatal morphology can be considered as a reliable factor due to its uniform shape, ubiquity and homology across distantly related relatives [4]. Apart from being relatively easy and faster method, this makes stomatal study reliable and repeatable when different leaf samples are used [41]. Polyploidy induction produces bigger stomatal size and lesser density at the leaf abaxial surface of different species like *Impatiens walleriana* [6] and *Dendrobium officinale* [21].

The chloroplast number was found to be higher in the treatment H_8 but no significant difference was observed between the different colchicine treated and control plants. This indicates positive correlation between chloroplast numbers and ploidy as also reported in previous studies by [2]. The epidermal cell number recorded in the treatment H_8 was 44.60 number/mm^2 while it ranged from 47 to 60 number/mm^2 in other treated genotypes.

The fresh rhizome yield was less in treated genotypes compared to control plants and no significant difference was recorded. The rhizome yield in the treatment H_8 was 53.20 g/plant compared to 41.70 g/plant in the corresponding control.

Table 1: Sprouting percentage and heteroploidy induction rate of *in vitro* treated ginger genotypes

Treatments	Days to sprouting	Sprouting percentage (%)	Survival percentage (%) 60 days after planting	Maturity period (days)
H_1	41.17^a	17	4	155^{bc}
H_2	42.50^a	9	2	173^{abc}
H_3	39.00^a	14	2	170^{abc}
H_4	39.83^a	12	2	180^{abc}
H_5	38.00^a	21	3	165^{abc}
H_6	42.50^a	7	2	155^{bc}
H_7	43.17^a	6	2	160^{abc}
H_8	46.50^a	19	5	164^{abc}
C_1	12.00^b	40	21	195^{abc}
C_2	13.00^b	49	19	155^{bc}
C_3	15.00^b	46	19	148^{d}
C_4	12.00^b	40	12	160^{abc}
C_5	17.00^b	42	15	206^{ab}
C_6	17.00^b	40	11	181^{abc}
C_7	16.00^b	40	13	210^{a}
C_8	12.00^b	40	14	200^{ab}
IISR Varada	11.40^b	42	12	200^{ab}
CD (0.05)	10.85			54.53
S.Em (±)	2.54			3.45
Treated mean	41.54^a			165.21^b
Control mean	14.23^b			181.84^a
CD (0.05)	0.20			0.17
S.Em (±)	0.03			0.03

H_1 to H_8- Treated genotypes; C_1 to C_8- Control genotypes

Table 2: Effect of colchicine on plant height (cm) of *in vitro* treated ginger genotypes at 90, 120 and 180 days after planting

Treatments	90 days	120 days	180 days
H_1	11.81^{d}	24.52^{cd}	33.59^{cde}
H_2	11.67^{d}	20.42^{d}	34.23^{bcde}
H_3	11.98^{d}	27.66^{bcd}	32.10^{de}
H_4	12.16^{d}	28.13^{bcd}	38.76^{abc}
H_5	10.34^{d}	25.50^{cd}	32.68^{de}
H_6	13.06^{d}	24.82^{cd}	29.84^{e}
H_7	10.52^{d}	29.79^{abc}	32.08^{de}
H_8	12.14^{d}	29.34^{bc}	34.02^{de}
C_1	27.64^{ab}	35.14^{ab}	41.93^{a}
C_2	30.34^{a}	34.24^{ab}	38.99^{ab}
C_3	25.83^{bc}	32.72^{ab}	39.11^{ab}
C_4	23.34^{bc}	35.68^{a}	39.20^{ab}
C_5	25.03^{bc}	34.93^{ab}	41.69^{a}
C_6	22.18^{c}	34.44^{ab}	41.62^{a}
C_7	22.30^{c}	32.71^{ab}	39.71^{ab}
C_8	24.73^{bc}	29.98^{abc}	35.74^{bcd}
IISR Varada	24.60^{bc}	32.25^{ab}	35.50^{bcd}
CD (0.05)	6.25	6.77	6.45
S.Em (±)	1.54	1.72	1.60
Treated mean	11.71^{b}	26.24^{b}	33.41^{b}
Control mean	25.14^{a}	33.71^{a}	39.73^{a}
CD (0.05)	0.15	0.16	0.11
S.Em (±)	0.02	0.03	0.01

*****H_1 to H_8- Treated genotypes; C_1 to C_8- Control genotypes

Table 3: Effect of colchicine on number of tillers of *in vitro* treated ginger genotypes at 90, 120 and 180 days after planting

Treatments	90 days	120 days	180 days
H_1	1.25	2.00	2.75^{bc}
H_2	1.00	2.00	3.50^{ab}
H_3	1.50	2.00	3.00^{abc}
H_4	2.50	2.50	3.00^{abc}
H_5	2.33	2.67	3.33^{ab}
H_6	1.00	2.00	2.00^{c}
H_7	1.00	1.50	2.00^{c}
H_8	1.80	2.00	2.80^{bc}
C_1	2.00	3.00	3.33^{ab}
C_2	2.00	2.33	3.33^{ab}
C_3	1.67	2.67	3.67^{a}
C_4	2.33	2.67	3.33^{ab}
C_5	2.33	3.00	3.66^{a}
C_6	1.67	2.33	3.67^{a}
C_7	1.33	2.33	3.33^{ab}
C_8	2.68	3.00	3.67^{a}
IISR Varada	2.00	2.30	3.60^{a}
CD (0.05)	NS	NS	1.07
S.Em (±)	0.36	0.38	0.29
Treated mean	1.52^{b}	2.04^{b}	2.75^{b}
Control mean	2.00^{a}	2.63^{a}	3.45^{a}
CD (0.05)	0.09	0.22	0.27
S.Em (±)	0.01	0.04	0.04

*****H_1 to H_8- Treated genotypes; C_1 to C_8- Control genotypes

Table 4: Effect of colchicine on number of leaves of *in vitro* treated ginger genotypes at 90, 120 and 180 days after planting

Treatments	90 days	120 days	180 days
H_1	5.00^{d}	7.67^{c}	16.00^{e}
H_2	4.50^{d}	8.50^{c}	18.00^{e}
H_3	4.50^{d}	9.50^{c}	16.00^{e}
H_4	5.00^{d}	8.00^{c}	16.50^{e}
H_5	4.67^{d}	9.00^{c}	16.00^{e}
H_6	4.00^{d}	9.00^{c}	16.00^{e}
H_7	4.50^{d}	9.00^{c}	18.50^{de}
H_8	4.80^{d}	10.00^{c}	17.40^{e}
C_1	11.33^{c}	17.33^{ab}	24.00^{ab}
C_2	12.00^{bc}	17.33^{ab}	25.67^{a}
C_3	13.00^{abc}	15.00^{b}	24.00^{ab}
C_4	14.33^{abc}	17.00^{ab}	24.33^{ab}
C_5	15.33^{a}	17.00^{ab}	24.33^{ab}
C_6	14.67^{ab}	19.67^{a}	24.00^{ab}
C_7	13.00^{abc}	19.00^{a}	23.00^{bc}
C_8	13.00^{abc}	19.67^{a}	21.00^{cd}
IISR Varada	12.00^{bc}	18.50^{abc}	21.00^{cd}
CD (0.05)	3.69	3.81	2.94
S.Em (±)	1.14	1.28	0.91
Treated mean	4.61^{b}	8.82^{b}	16.40^{b}
Control mean	13.31^{a}	17.73^{a}	23.75^{a}
CD (0.05)	0.06	0.13	1.73
S.Em (±)	0.01	0.02	0.30

*****H_1 to H_8- Treated genotypes; C_1 to C_8- Control genotypes

Table 5: Effect of colchicine on leaf area of *in vitro* treated ginger genotypes at 90, 120 and 180 days after planting

Treatments	90 days			120 days			180 days		
	Leaf length (cm)	Leaf breadth (cm)	Leaf Area (cm^2)	Leaf length (cm)	Leaf breadth (cm)	Leaf Area (cm^2)	Leaf length (cm)	Leaf breadth (cm)	Leaf Area (cm^2)
H_1	7.59^{cde}	1.08	4.70^{ef}	11.68^{cde}	1.23	8.80^{cd}	18.94	1.33	15.97
H_2	7.03^{cde}	1.10	4.44^{efg}	10.35^{cdef}	1.15	7.20^{d}	15.88	1.36	13.69
H_3	7.88^{cd}	1.05	4.77^{ef}	10.38^{cdef}	1.33	8.40^{cd}	14.93	1.48	13.97
H_4	6.81^{def}	1.10	4.29^{efg}	9.77^{ef}	1.27	7.49^{d}	15.86	1.56	15.79
H_5	6.80^{def}	1.21	4.40^{ef}	10.08^{def}	1.24	7.93^{d}	15.35	1.39	13.49
H_6	5.50^{fg}	1.00	2.92^{fg}	10.26^{cdef}	1.22	7.50^{d}	14.35	1.48	13.52
H_7	5.35^{fg}	1.00	2.82^{fg}	9.90^{def}	1.35	8.18^{cd}	13.95	1.45	12.78
H_8	4.44^{g}	1.11	2.57^{g}	8.60^{f}	1.42	7.47^{d}	15.40	1.44	14.14
C_1	10.25^{b}	1.26	7.86^{ab}	14.79^{a}	1.44	13.53^{a}	20.14	1.64	21.32
C_2	10.72^{b}	1.28	8.38^{ab}	15.37^{a}	1.40	13.60^{a}	19.50	1.51	18.97
C_3	10.94^{b}	1.08	7.14^{bcd}	14.25^{ab}	1.34	12.00^{ab}	17.03	1.61	17.59
C_4	10.69^{b}	1.13	7.32^{bc}	14.06^{ab}	1.38	12.22^{ab}	16.79	1.64	17.65
C_5	12.43^{a}	1.22	9.35^{a}	14.27^{ab}	1.38	12.38^{ab}	17.58	1.52	17.10
C_6	8.37^{c}	1.13	5.54^{cde}	12.38^{bc}	1.41	10.86^{abc}	17.23	1.60	17.67
C_7	7.30^{cde}	1.21	5.26^{de}	12.07^{bcd}	1.30	9.94^{bcd}	19.30	1.59	19.78
C_8	6.33^{ef}	1.22	4.46^{efg}	11.78^{cde}	1.38	10.16^{bcd}	19.80	1.37	17.48
IISR Varada	8.10^{c}	1.00	4.66^{ef}	13.50^{ab}	1.35	11.44^{ab}	16.50	1.65	17.47
CD (0.05)	1.48	NS	1.99	2.25	NS	3.08	NS	NS	NS
S.Em (±)	1.15	1.49	1.05	1.40	0.95	1.25	0.90	0.46	1.02
Treated mean	6.41^{b}	1.04	3.83^{b}	10.11^{b}	1.28	7.84^{b}	15.54^{b}	1.43	14.13^{b}
Control mean	9.62^{a}	1.15	6.91^{a}	13.61^{a}	1.38	11.82^{a}	18.41^{a}	1.56	18.43^{a}
CD (0.05)	0.08	NS	0.13	0.06	NS	0.16	0.17	NS	0.17
S.Em (±)	0.01	0.04	0.02	0.01	0.04	0.03	0.03	0.02	0.03

*****H_1 to H_8- Treated genotypes; C_1 to C_8- Control genotypes

Table 6: Effect of colchicine on physiological parameters of *in vitro* treated ginger genotypes at 6 months after planting

Treatments	Chlorophyll content (mg/g)			Photosynthetic rate ($\mu molCO_2/m^2/s$)	Transpiration rate (mmol $H_2O/m^2/s$)	Stomatal conductance ($mmol/m^2/s$)	Leaf temperature (°C)
	Chlorophyll a	Chlorophyll b	Total				
H_1	2.26	0.90^{abcde}	3.16	4.50	0.38	10.67^{abc}	27.2^{b}
H_2	2.00	0.75^{bcde}	2.75	3.65	0.32	13.50^{a}	27.3^{a}
H_3	1.58	1.32^{a}	2.90	4.15	0.17	8.00^{cde}	27.2^{b}
H_4	1.83	0.71^{cde}	2.54	2.50	0.33	3.50^{f}	27.3^{a}
H_5	2.09	1.10^{abcd}	3.18	3.13	0.29	8.00^{cde}	27.4^{a}
H_6	1.96	0.81^{bcde}	2.77	2.25	0.14	7.50^{cde}	27.3^{a}
H_7	1.90	0.54^{efg}	2.44	5.25	0.17	3.50^{f}	27.2^{b}
H_8	1.93	0.85^{bcde}	2.77	3.10	0.19	10.00^{bcd}	27.3^{a}
C_1	2.00	0.91^{ab}	2.91	4.50	0.28	12.33^{ab}	27.2^{b}
C_2	2.07	0.82^{def}	2.88	3.13	0.19	13.00^{ab}	27.3^{a}
C_3	1.38	0.55^{fg}	1.93	3.53	0.14	8.00^{cde}	27.4^{a}
C_4	1.86	0.41^{abcd}	2.27	3.53	0.24	5.00^{ef}	27.3^{a}
C_5	1.41	1.06^{abc}	2.47	2.43	0.29	8.00^{cde}	27.3^{a}
C_6	1.40	0.99^{bcde}	2.39	3.13	0.21	7.00^{de}	27.4^{a}
C_7	2.20	0.85^{bcde}	3.04	4.70	0.13	3.00^{f}	27.3^{a}
C_8	2.90	0.49^{g}	3.39	3.73	0.34	13.33^{a}	27.2^{b}
IISR Varada	1.25	0.40^{abcd}	1.94	3.50	0.15	8.00^{cde}	27.2^{b}
CD (0.05)	NS	0.44	NS	NS	NS	3.19	0.13
S.Em (±)	1.13	0.12	0.24	1.09	0.24	1.29	0.10
Treated mean	1.91	0.81	2.78	3.53	0.18	8.04^{b}	27.28
Control mean	1.88	0.75	2.64	3.54	0.17	8.66^{a}	27.28
CD (0.05)	NS	NS	NS	NS	NS	0.15	NS
S.Em (±)	0.03	0.02	0.03	0.02	0.01	0.03	0.25

*****H_1 to H_8- Treated genotypes; C_1 to C_8- Control genotypes

Table 7: Effect of colchicine on cytological parameters and yield of *in vitro* treated ginger genotypes

Treatments	Stomatal frequency (number/mm^2)	Stomatal size		Chloroplast number (number/mm^2)	Epidermal cell number (number of cells/mm^2)	Fresh rhizome yield (g/plant)
		Stomatal length (µm)	Stomatal breadth (µm)			
H_1	113.24	32.56	26.75	9.67	58.67	64.03
H_2	108.80	38.62	26.81	10.00	60.00	37.50
H_3	101.52	38.51	34.17	8.00	59.00	55.00
H_4	119.29	33.37	28.88	7.00	53.50	67.50
H_5	106.55	30.99	32.27	8.67	52.70	41.67
H_6	106.60	32.18	28.50	8.00	52.00	29.00
H_7	101.52	32.56	27.68	8.50	47.00	35.00
H_8	99.48	43.91	44.41	14.60	44.60	53.20
C_1	111.67	32.70	23.60	11.33	59.00	61.70
C_2	110.41	30.78	24.13	11.00	61.70	45.83
C_3	112.94	31.79	23.88	11.00	59.34	36.33
C_4	107.87	31.93	23.68	10.00	58.77	53.00
C_5	115.48	30.55	23.41	11.67	61.30	72.67
C_6	111.67	30.70	23.18	11.00	62.00	61.70
C_7	109.14	30.75	23.09	9.00	62.00	38.30
C_8	112.94	31.92	23.13	10.33	62.00	41.70
IISR Varada	112.94	31.50	21.00	11.00	61.00	40.00
CD (0.05)	NS	NS	NS	NS	NS	NS
S.Em (±)	4.66	4.37	6.25	1.46	5.15	8.46
Treated mean	107.34^b	36.53^a	31.13^a	9.24^b	53.20^b	47.81^b
Control mean	111.25^a	31.34^b	23.25^b	10.62^a	60.73^a	51.34^a
CD (0.05)	1.09	0.21	1.08	0.17	0.87	0.17
S.Em (±)	0.18	0.03	0.18	0.03	0.14	0.03

*****H_1 to H_8- Treated genotypes; C_1 to C_8- Control genotypes

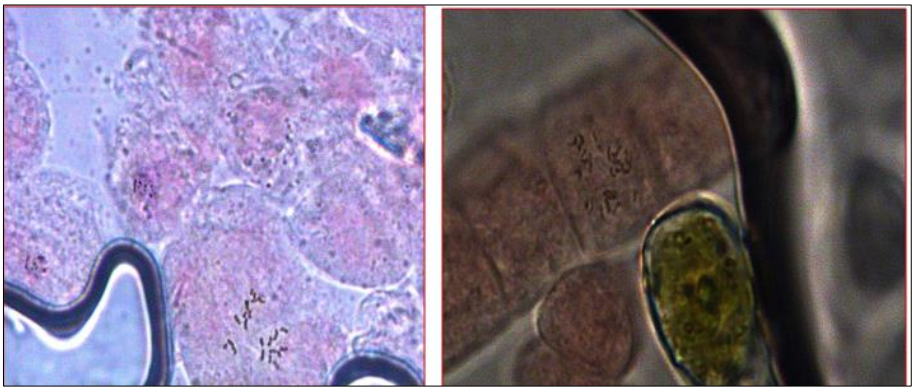

Fig 1: Chromosome number 2n=22 in the diploid plant of H_8, 2n=24 in the mixoploid plant of H_8 obtained *in vitro*

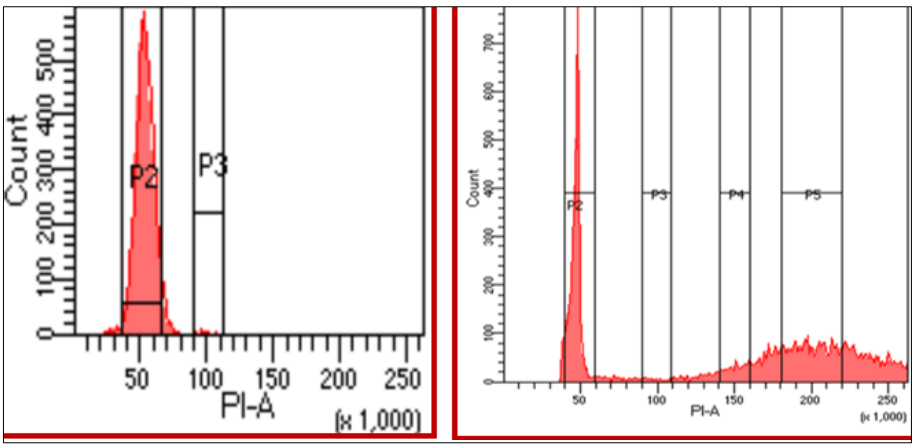

Fig 2: The histogram peak of diploid plant of H_8 obtained in 50X and the histogram peak of mixoploid plant of H_8 obtained in 200X

5. Conclusion

Colchicine, an alkaloid substance obtained from *Colchicum autumnale* L. is the most widely used antimitotic agent to induce polyploidy in many species. The morphological and cytological features of treated plants showed variations on comparison with their diploid counterparts. The survival rate of colchicine treated explants were very less although it was possible to obtain a heteroploid through this method. The

heteroploid was characterized by increased stomatal size and yield on comparison with other genotypes.

6. References

1. Adaniya S, Shirai D. *In vitro* induction of tetraploid ginger (*Zingiber officinale* Roscoe) and its pollen fertility and germinability. Sci. Hortic. 2001;88:277-287.
2. Beck SL, Dunlop RW, Fossey A. Stomatal length and frequency as a measure of ploidy level in black wattle, *Acacia mearnsii* (De Wild.). Bot. J Linn. Soc. 2003;141:177-181.
3. Dolezel J, Greilhuber J, Suda J. Estimation of nuclear DNA content in plants using flow cytometry. Nature 2007;2(9):2233-2244.
4. Doyle JJ, Coate JE. Polyploidy, the nucleotype, and novelty: the impact of genome doubling on the biology of the cell. International Journal of Plant Science. 2019;180(1):1-52.
5. Eigsti OJ. The pollen tube method for making comparisons of differences in mitotic rates between diploid and tetraploid. Genet. 1947;32(1):32-58.
6. Ghanbari MA, Jowkar A, Salehi H, Zarei M. Effects of polyploidization on petal characteristics and optical properties of *Impatiens walleriana* (Hook.). Plant Cell, Tissue and Organ Culture. 2019;138:299-310.
7. Ghani A, Neamati SH, Azizi M, Saharkhiz MJ, Farsi M. Artificial autotetraploidy induction possibility of two Iranian endemic mint (*Mentha mozaffarianii*) ecotypes. Not Sci. Biol. 2014;6(2):185-191.
8. Ghani A, Neamati SH, Azizi M, Saharkhiz MJ, Farsi M. Artificial autotetraploidy induction possibility of two Iranian endemic mint (*Mentha mozaffarianii*) ecotypes. Not Sci. Biol. 2014;6(2):185-191.
9. Gopinath PP, Parsad R, Joseph B, Adarsh VS. GRAPES: General Rshiny based analysis platform empowered by statistics; c2020. https://www.kaugrapes.com/home.version1.0.0.DOI.10.5281/zenodo.4923220.
10. Jayachandran BK, Vijayagopal PD. Attempts on breaking self-incompatibility in ginger (*Zingiber officinale* Rosc.). Agri. Res. J Kerala. 1979;17(3):256-257.
11. Joseph OP, Shipley JB, Racht EM, Slovis CM, Wrenn KD, Pepe PE, *et al*. Multicenter study of a portable, hand-size, colorimetric end-tidal carbon dioxide detection device. Ann. Emergency Med. 1992;21(5):518-523.
12. Kun-Hua W, Hua JM, Ping HH, Lin SG. Generation of autotetraploid plant of ginger (*Zingiber officinale* Rosc.) and its quality evaluation. Pharmacognosy Mag. 2011;7(27):200-206.
13. Lafuma L, Balkwill K, Imbert E, Verlaque R, Maurice S. Ploidy level and origin of the European invasive weed *Senecio inaequidens* (Asteraceae). Plant Syst. Evol. 2003;243(1-2):59-72.
14. Larsen P, Mintung S. Growth promoting and growth retarding substances in pollen from 2n and 3n apple varieties. Bot. Gaz. 1950;3(1):436-447.
15. Mishra BK, Pathak S, Sharma A, Trivedi PK, Shukla S. Modulated gene expression in newly synthesized autotetraploid of *Papaver somniferum* L. S. Afr. J Bot. 2010;76:447-452.
16. Nair RR. Variation in pollen fertility and chromosome number among germplasm collections of ginger (*Zingiber officinale* Rosc.) Int. J Cytol. Cytosystematics Cytogenet. 2016;69(1):73-81.
17. NHB [National Horticultural Board]. Horticulture crop 2nd advanced estimates for the year; c2019-20. [On-line]. Available: http://nhb.gov.in. [18 March 2023].
18. Omidbaigi R, Mirzaee M, Hassani ME, Moghadam SM. Induction and identification of polyploidy in basil (*Ocimum basilicum* L.) medicinal plant by colchicine treatment. Int J Plant Prod. 2010;4(2):87-98.
19. Parthasarathy VA, Srinivasan V, Nair RR, Zacharia TJ, Kumar A, Prasath, D. Ginger: Botany and Horticulture. Hortic. Rev. 2012;39:273-388.
20. Pfosser M, Amon A, Lelley T, Herberle-Bors E. Evaluation of sensitivity of flow cytometry in detecting aneuploidy in wheat using disomic and ditelosomic wheat-rye addition lines. Cytometry. 1995;21:387-393.
21. Pham P, Li Y, Guo H, Zeng R, Xie L, Zhang Z. Changes in morphological characteristics, regeneration ability, and polysaccharide content in tetraploid *Dendrobium officinale*. Hort Science. 2019;54(11):1879-1886.
22. Ramachandran K, Nair PNC. Induced tetraploids of ginger *(Zingiber officinale* Rosc). J Spices Aromat. Crops. 1992;1:39-42.
23. Ramachandran K. Chromosome numbers in Zingiberaceae. Cytologia 1969;34:213-221.
24. Ramachandran K. Polyploidy induced in ginger by colchicine treatment. Curr. Sci. 1982;51:288-289.
25. Ratnambal MJ. Cytogenetical studies in ginger (*Zingiber officinale* Rosc.) [Ph.D. thesis]. [Bombay]: University of Bombay; c1979.
26. Ravindran PN, Babu KN, Shiva KN. Botany and crop improvement of ginger. In: Ravindran. P.N., Nirmal Babu, K., editors. Ginger-The genus Zingiber. CRC Press, Boca Raton, FL; c2005. p. 15-85.
27. Sadhu A, Bhadra S, Bandyopadhyay M. Novel nuclei isolation buffer for flow cytometric genome size estimation of Zingiberaceae: A comparison with common isolation buffers. Ann. Bot. 2016;118:1057-1070.
28. Sakhanokho HF, Rajasekaran K, Kelley RV, Farjdj NI. Induced polyploidy in diploid ornamental ginger (*Hedychium muluense R. M.* Smith) using colchicine and oryzalin. Hort. Sci. 2009;44(7):1809-1814.
29. Zhou J, Guo F, Fu J, Xiao Y, Wu J. *In vitro* polyploid induction using colchicine for *Zingiber officinale* Roscoe cv. 'Fengtou' ginger. Plant Cell Tiss. Organ Cult. 2020;142:87-94.
30. Shao JZ, Chen CL, Deng XX. *In vitro* inducation of tetraploid in pomegranate (*Punica granatum*). Plant Cell Tiss. Organ Cult. 2003;75:241-246.
31. Sheeba PT. Induction of autotetraploidy in ginger. M.Sc. (Hort) thesis, Kerala Agricultural University; c1996. p. 73.
32. Shukla Y, Singh M. Cancer preventive properties of ginger: A brief review. Food Chem. Toxicol. 2007;45:683-690.
33. Smith MK, Hamill S, Gogel BJ, Severn-Ellis AA. Ginger (*Zingiber officinale*) autotetraploid with improved processing quality produced by an *in vitro* colchicine treatment. Aust. J Exp. Agric. 2004;44:1065-1072.
34. Stebbin GL. Polyploidy and the distribution of arctic-alpine flora: new evidence and new approaches. Bot. Helv. 1984;94:1-13.
35. Surh YJ, Park KK, Chun KS, Lee LJ, Lee E, Lee SS. Anti-tumor promoting activities of selected pungent phenolic substances present in ginger. J. Eniviron. Pathol. Toxicol. Oncol. 1999;18(2):131-9.

36. Swanson CP. Cytol. Cytogenetics. Hoboken (NJ): Prentice Hall; c1957.
37. Talebi SF, Saharkhiz MJ, Kermani MJ, Sharifi Y, Raouf fard F. Effect of different antimitotic agents on polyploid induction of anise hyssop (*Agastache foeniculum* L) Caryologia. 2017;70(2):184-193.
38. Wang ZM, Niu Y, Song M, Tang QL. Tetraploid of *Zingiber officinale* Roscoe. *in vitro* inducement and its morphology analysis. China Veg. 2010;4:41-46.
39. Wannakrairoj S, Wondyifraw T. *In vitro* chromosome doubling in korarima (*Aframomum corrorima* (Braun) P.C.M. Jansen) using colchicine and oryzalin. Kaset. J Nat. Sci. 2013;47(5):684-694.
40. Wright JW. Introduction of forest genetics. Academic Press, New York; c1976.
41. Xie X, Aguero CB, Wang Y, Walker MA. *In vitro* induction of tetraploids in Vitis x Muscadinia hybrids. Plant Cell, Tissue and Organ Culture. 2015;122:675-683.