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Effect of colchicine induced tetraploids of ginger (*Zingiber officinale* Roscoe) on cytology, rhizome morphology, and essential oil content

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ABSTRACT

Ginger (*Zingiber officinale* Roscoe; 2 n = 22) is an important spice crop recognized for its medicinal benefits and pharmacological activities. The crop is said to have a narrow genetic base in the cultivated gene pool due to hinderance in sexual recombination. Polyploid induction is an efficient strategy to develop new cultivars in vegetatively propagated horticultural crops. In this study, successful induction of tetraploids (2 n = 44) in ginger 'IISR Rejatha' was achieved through colchicine treatment and the tetraploid plants were subsequently characterized. The 21 putative polyploids were identified by altered morphology, particularly plant height, leaf size, pseudostem height and thickness through colchicine treatment. The chromosome counts confirmed polyploidization in two of the tetraploid lines (2 n = 4x = 44), while that of the control diploid plants was 2 n = 2x = 22. Further, the ploidy levels of diploid and tetraploids were confirmed by flow cytometric analysis. The morphological characterization of the induced tetraploids revealed significant differences in leaf and pseudostem characters. Significantly larger rhizome diameter (3.26 cm) and higher yield per plant (418.65 g/plant) was recorded in Tetraploid 2 compared with Tetraploid 1 (2.88 cm, 320.81 g/plant) and diploid (2.00 cm, 280.33 g/plant). Overall, the colchicine induced tetraploids in ginger have larger rhizomes and greater source of essential oil.

1. Introduction

Ginger (*Zingiber officinale* Roscoe) is commercially cultivated in almost all tropical and subtropical countries and India, China, Nigeria, Indonesia, Bangladesh, Thailand, Philippines, and Jamaica are the major global producers (Parthasarathy et al., 2012). It is an important spice that has a long history of medicinal use in India, China, and South East Asia for conditions such as headaches, nausea, rheumatism, and colds (Grant and Lutz, 2000; Parthasarathy et al., 2012). Antiemetic, antiulcer, anti-inflammatory, antioxidant, antiplatelet and anticancer are the major pharmacological activities possessed by ginger (Fuhrman et al., 2000; Nicoll and Henein, 2009; Shukla and Singh, 2007; Rohini and Srikumar, 2019; Kausar et al., 2021). There is a potential for developing newer genotypes and value-added products in ginger with enhanced medicinal properties.

Ginger is a diploid with 2n = 22 as the most frequently reported chromosome number (Ramachandran, 1969; Purseglove et al., 1981; Nair, 2016). The major crop improvement goals in this vegetatively propagated crop are resistance to rhizome rot, bacterial wilt, high essential oil, bold rhizome and less fiber, besides high yield (Ravindran et al., 2005). Conventional breeding in ginger is prevented due to the lack of seed set which is being attributed to the structural abnormalities in the genome such as inversion and translocation heterozygosity (Nair, 2016). Polyploidy breeding is one of the frequently used methods in vegetatively propagated horticultural crops and colchicine remains the prominent antimitotic agent in the induction of polyploids (Eng and Ho, 2019). It induces polyploidy by inhibiting the spindle fiber formation during cell division resulting in the prevention of polar movement of chromosomes which ultimately results in the production of polyploid cells (Jordan and Wilson, 1998).

Induction of tetraploids of ginger using the colchicine has been successfully reported in a few genotypes (Adaniya and Shirai, 2001; Smith et al., 2004; Kun-Hua et al., 2011; Zhou et al., 2020). The rhizome vigor due to tetraploidy can be harnessed successfully in ginger for developing genotypes with bold rhizomes suitable for the processing industry. Also, the enhanced pollen fertility in tetraploid ginger may be effective in stimulation of seed set (Nair, 2016). Earlier investigations have outlined the variation between different varieties of ginger and

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growing conditions (Wohlmuth et al., 2006; Bailey-Shaw et al., 2008). The autotetraploid line in ginger yielded large rhizomes of premium grade confectionary ginger (Smith et al., 2004). Thus, development of tetraploids of different genotypes in ginger are feasible for commercial and industrial production. The successful polyploidy induction for enhanced production of essential oil and secondary metabolites have been reported in *Artemisia annua* (Lin et al., 2011), *Echinacea purpurea* (Abdoli et al., 2013), *Thymus persicus* (Tavan et al., 2015) and *Citrus limon* (Bhuvaneswari et al., 2020). However, polyploidy induced by colchicine in ginger 'IISR Rejatha' and subsequent impact of secondary metabolites has not yet been studied. Therefore, in the present study, an efficient *in vivo* artificial tetraploid induction protocol in ginger 'IISR Rejatha' was developed. Additionally, morphological characterization along with essential oil profile of the induced tetraploids and its diploids were investigated.

2. Materials and methods

2.1. Induction of polyploidy

Rhizomes of ginger 'IISR Rejatha' (2 n = 22) were obtained from National Active Germplasm Site, ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, India. The rhizomes were washed thoroughly in distilled water to remove soil debris and stored. The emerging buds with active cell division were excised from the rhizomes and then transferred to a Petri dish containing a layer of filter paper to remove excess surface water. A borehole of 2 mm diameter and 4 mm deep, opposite to the apical meristem of each bud, was made using a cork-borer and plugged with absorbent cotton.

The 5 × 2 factorial experiment was carryout with four replications. Thirty emerging buds per treatment were submerged in different concentrations of an aqueous solution of colchicine (0.025%, 0.05%, 0.075%, and 0.1%) at two different time durations (24 h and 48 h). The colchicine solutions were supplemented with 2% (w/v) dimethyl sulphoxide. Alternatively, the emerging buds were submerged in distilled water for control treatment. The treated rhizome buds were rinsed thrice in distilled water and then planted in a plug-tray containing potting mixture (3 parts of topsoil: 1part sand: 1 part vermicompost). The trays were maintained under the naturally ventilated polyhouse. The putative tetraploids (first generation) from different colchicine treatments were grouped based on leaf size, pseudostem height and thickness, recorded after 90 days of planting.

2.2. Ploidy determination

2.2.1. Ploidy determination by chromosome count

The rhizomes of putative tetraploids (second generation) were planted in sand trays and watered regularly. On sprouting of rhizomes, five metaphase plates with good chromosome spread from 2 to 3 slides were used for chromosome number analysis of each putative tetraploids following the method of Nair (2016). Further, 25 mitotic metaphase plates from five different root tips were counted to confirm the chromosome numbers of identified tetraploids.

2.2.2. Ploidy determination by flow cytometry

The stability of identified polyploids (third generation) was analysed in the next generation using flow cytometry. Briefly, small part of tender leaf tissue from the reference standard ('IISR Rejatha', 2 n = 22) and unknown samples (two tetraploid lines) were finely chopped with a fine pair of scissors in 1 mL hypotonic propidium iodide lysis buffer (Krishan, 1975; buffer with minor modification) containing sodium citrate tribasic dehydrate (Sigma-Aldrich), 2 mg/mL RNase A (Sigma-Aldrich), 50 μg/mL PI (Sigma-Aldrich), and 0.3% (v/v) Tween-20 (Sigma-Aldrich). To decrease the effects of cytosolic compounds on propidium iodide fluorescence, β-mercaptoethanol (1%) was also added to the buffer. After chopping, the suspension was filtered through a 10-μm cell strainer (CellTrics, Sysmex) and collected in 1.5 mL eppendorf tubes. After 15 min incubation, the suspension of isolated nuclei was run on a Cytoflex flow cytometer (Beckman Coulter). For ploidy analysis, reference control was used as an external standard.

Reference controls and unknown samples were acquired using a Cytoflex flow cytometer (Beckman Coulter) equipped with 488 nm laser and 585/42 Band Pass filter. For each sample, around 500–2000 nuclei were collected and analyzed. Detector gain was set using diploid control and the same gain settings were used to run the samples. The results were displayed as one-parameter histograms with G_0/G_1 peak on a linear scale. The fluorescence intensity (median value) of G_0/G_1 was recorded for reference control and samples. The CV of G_0/G_1 peaks were less than 5% for reference controls and samples. The fluorescence intensity data was analysed using FCS Express Software (DENOVO software, USA). The ploidy level of the samples was determined using the formula: Sample ploidy = reference ploidy × (median value of sample G_0/G_1 peak)/(median value of standard G_0/G_1 peak).

2.3. Morphological characteristics

The identified two polyploids and 'IISR Rejatha' were further propagated asexually using rhizomes of the third generation to obtain sufficient rhizomes for field planting. The rhizomes (fourth generation) were harvested in January and were planted for characterization of tetraploid lines (fifth generation). The experiment (three genotypes evaluated in RBD with eight replications) was carried out at ICAR-Indian Institute of Spices Research (ICAR-IISR), Kozhikode, Kerala, India. The geographical coordinates of the experimental location is 11°17′N, 75°50′E and 60 m MSL. All the three genotypes were planted in open field raised beds during the first week of June with eight replications (3 m^2/replication/genotype) and harvested during mid-January. The recommended package of practices of ICAR-IISR was followed for raising the crop (Jayashree et al., 2015). Morphological characters of plants were visually observed at the end of grand growth phase *i.e.* 150 days after planting, and described. The characterization of the putative tetraploids focused on plant height, number of pseudostems, pseudostem diameter, number of leaves, leaf length, leaf girth and rhizome diameter were measured as per the DUS guidelines for ginger (PPV&FRA, 2007). The yield per plant (fresh) was recorded at harvest from five randomly selected plants per replication. Fresh ginger was washed thoroughly and hand peeled. The dry rhizome yield was recorded after oven drying 17 h at 55 °C.

2.4. Essential oil

The rhizomes of the two tetraploid lines and 'IISR Rejatha' were harvested in January and were used to determine the volatile oil content (eight replications). The volatile oil content was extracted from dried rhizomes using the Clevenger method (ASTA, 1997). Twenty grams of rhizome powder was transferred to a short neck round bottom flask containing 500 mL water. The apparatus was set in the Clevenger apparatus and boiled for three hours. This was allowed to stand until the oil layer was clear. The volume of the oil collected was recorded (nearest 0.02 mL).

Essential oil (%) = [Amount of oil collected (mL) /Weight of sample (g)] x 100.

2.5. Statistical analysis

Data on the percentage of sprouting was subjected to analysis of variance (ANOVA) using Microsoft Excel and means were separated using Duncan's multiple range test at $p = 0.05$. The angular transformation of the data was done before analysis and original data was retained for presentation. The statistical analysis of morphological and rhizome characters was carried out using analysis of variance (ANOVA) to determine the significance of each parameter. The results were

subjected to Duncan's multiple range test (DMRT), and differences were considered significant at P value of < 1%.

3. Results and discussion

3.1. Polyploidy induction

Colchicine began to play an important role in plant breeding after the report of Blakeslee and Avery (1937) that this alkaloid can double chromosome number in plants. In plants, the colchicine concentration and duration of treatment are inter-dependent and most vital in polyploidization. Generally, the colchicine concentration ranges from 0.05% to 1.0% and the duration of treatment from hours to weeks, depending on the methods of application (Eng and Ho, 2019). Also, the survival rate of vegetative buds is inversely related to the treatment time and concentration of colchicine (Huy et al., 2019). In the present study, the impact of different concentrations of colchicine on sprouting (%) is presented in Table 1. The ANOVA (Supplementary data, Table S1) indicated significant differences between colchicine concentration and duration on the sprouting of rhizome buds (P < 0.01). The sprouting per cent was proportional to the concentration of colchicine and the treatment time. The control at 24 h and 48 h recorded maximum sprouting of 99.0% and 97.7%, respectively. The sprouting of colchicine treated rhizome buds ranged from 70.4% to 92.5%, whereas the highest sprouting was found at the lowest colchicine concentration of 0.025%. Increasing the colchicine concentrations generally led to a decrease in sprouting of rhizome buds of ginger 'IISR Rejatha'. Colchicine concentration of 0.2%, 24 h sprouting rate was only up to 79.3%. In line with the present results, higher concentration and longer duration of colchicine treatment resulted in lesser sprouting (Ramachandran and Nair, 1992; Smith et al., 2004). Also, colchicine treated rhizome buds sprout slowly during the early stages of planting, and this was positively correlated with treatment duration and concentration. The slow sprouting due to colchicine is secondary to its toxicity, therefore a recovery time for ginger buds was needed. However, this initial slow growth was not noticed in the further generations. This result is similar to that of the polyploidy induction study of ginger (Zhou et al., 2020); *Hedychium muluense* (Sakhanokho et al., 2009) and *Populus* (Xu et al., 2018). The obtained tetraploids grew slower than the control plants in the early stage, but the growth rate was significantly on par after 2–3 cycles. Zhou et at. (2020) also reported that the first visible effect of colchicine was the delayed growth rate of explants compared to untreated buds.

In ginger, the widely reported chromosome number is 2 n = 22 (Ramachandran, 1969; Ratnambal, 1979; Rai et al., 1997; Eksomtramage et al., 2001; Wang et al., 2014; Nair, 2016). Liu et al. (2007) reported plant morphological characters are indicators of ploidy and used for screening of *Platanus acerifolia*. This method is much robust than screening polyploids suing stomatal characters. Therefore, in the first-generation plants raised from different colchicine treatments, plant morphological traits such as leaf size (length and width), pseudostem height and thickness were used to pre-screen tetraploids (data not shown). The chromosome numbers of 21 such shortlisted plants were screened by chromosome count using a root-tip squash. Among the 21 plants, the chromosome number of two plants was 2 n = 44 (tetraploid). Both these plants (0.1/48/3 and 0.1/48/5) were derived from the buds treated with 0.1% colchicine for 48 h. Analysis of 25 cells from five root tips of these two tetraploid lines showed no variation in tetraploid chromosome number of 2 n = 44 (Fig. 1 B, C). The stability of the two identified tetraploids was determined in two consecutive generations by further chromosome number analysis and confirmed as 2 n = 44 for both the polyploids. The results of the present study also showed that except for these two lines, most lines recorded 2 n = 22 (Fig. 1A) (Table 2). No spontaneous polyploidization occurred because all control plants were diploids, but polyploidy was induced in colchicine treated rhizome buds. Using colchicine, successful induction of stable tetraploid lines was reported in ginger cultivars 'Maran' and 'Mananthody' (Ramachandran, 1982). Later, induced tetraploids of ginger using colchicine were reported by Adaniya and Shirai (2001) and Kun-Hua et al. (2011). A tetraploid line 'Buderim Gold' from the local cultivar was developed in Australia and released for commercial cultivation (Smith et al., 2004). After three vegetative cycles of tetraploid lines, the ploidy level of these tetraploid lines was confirmed using flow cytometry analysis (Fig. 1 D,E, F).

Table 1
Effect of different colchicine concentrations on polyploidy induction in ginger.

Colchicine treatment		Sprouting rate (%)
Concentration (%)	Duration (h)	
Control (0)	24	99.0 ± 1.00
	48	97.7 ± 0.58
0.025	24	92.5 ± 0.44
	48	90.9 ± 0.71
0.05	24	90.2 ± 0.46
	48	88.5 ± 0.12
0.1	24	88.1 ± 0.35
	48	85.4 ± 0.60
0.2	24	79.3 ± 0.67
	48	70.4 ± 0.30

Data represents means±standard deviations of four independent replications (n = 120).

3.2. Variations in induced tetraploids

Many morphological effects have been obtained from induced polyploidization. In the present study, the morphological features of tetraploid lines such as plant height, pseudostem, leaf and rhizome characters were evaluated and compared with diploid control plants. The ANOVA (Supplementary data, Table S2; Table 3) indicated significant differences between individual tetraploid lines and their diploids regarding plant height, number of tillers, leaf length, and leaf width. The plant height of the Tetraploid 2 (78.95 cm) was significantly taller than that of the Tetraploid 1 (71.32 cm) and diploid plants (63.74 cm). The tetraploid lines had fewer but significantly thicker shoots and possessed all the polyploid characteristics. Induced tetraploids usually have larger leaves compared to their diploid counterparts (Liu et al., 2007). Consistent with previous study, the tetraploid plants recorded significantly broader leaves compared with the diploid parent. The differences in leaf number, length and width were significant 150 days after planting. The length and width of diploid leaves were about 21.73 cm and 2.01 cm, respectively, whereas two tetraploids recorded 22.64 cm and 2.53 cm; 22.40 cm and 2.96 cm, respectively. These morphological changes in tetraploid lines led to vigorous growth, which was translated into an increase in rhizome size. Dhooghe et al. (2011) reported increased cell size in polyploid genotypes which resulted in improved traits compared with their diploid progenitor and may have greater benefit for commercialization and cultivation. Zhou et al. (2020) also found that tetraploids were larger than their diploids for leaf length, leaf width, leaf thickness, and stem diameter.

Rhizome morphologies of tetraploids in comparison with a diploid ('IISR Rejatha') is presented in Fig. 2 A-C and Table S3. The fresh rhizome yield per plant was significantly higher in two tetraploid lines (320.81 g and 418.65 g, respectively) than their diploid counterpart (280.33 g) (Table 4). The dry rhizome yield per plant also followed a similar trend with maximum in Tetraploid 2 (89.91 g). The tetraploid lines had significantly larger rhizomes (rhizome thickness of 2.88 cm and 3.26 cm) (Fig. 2 E, F), although 'IISR Rejatha' had a significantly smaller rhizome (2.00 cm) (Fig. 2, D). The vigorous nature of polyploids of horticultural crops were reported earlier by Tal (1980), Zhang et al. (2010), and Tan et al. (2019). Consistent with present results, similar observations have also been reported by earlier workers in ginger tetraploids (Ratnambal and Nair, 1982; Ramachandran and Nair, 1992;

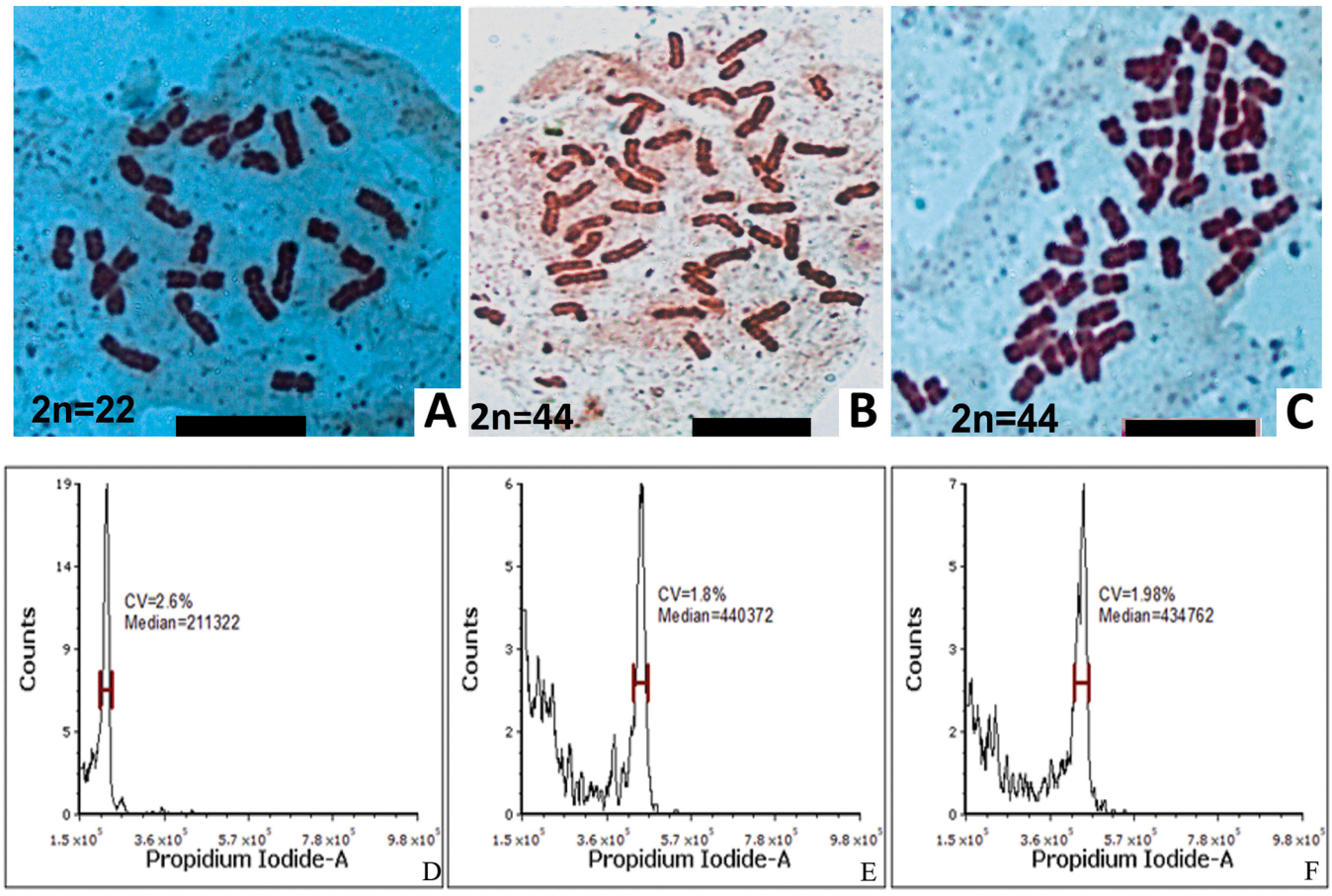

Fig. 1. A. Mitotic metaphase plate of 'IISR Rejatha' showing 2 n = 22; B. Induced polyploid 0.1/48/3 showing 2 n = 44; C. Induced polyploid 0.1/48/5 showing 2 n = 44. Bars represent 10 μm in A-C; Flow cytometry histogram of nuclei of 'IISR Rejatha' (D), Tetraploid 1(E), Tetraploid 2 (F).

Table 2
Chromosome number analysis of ginger plants derived from colchicine treated buds of cv. IISR Rejatha.

Identity	No. of cells counted	Chromosome number (2n)
0.025/24/1	10	22
0.025/48/2	10	22
0.025/48/3	10	22
0.050/24/1	10	22
0.050/24/2	10	22
0.050/48/4	10	22
0.050/48/5	10	22
0.050/48/7	10	22
0.050/48/10	10	22
0.1/24/1	10	22
0.1/24/2	10	22
0.1/24/4	10	22
0.1/24/6	10	22
0.1/24/7	10	22
0.1/24/8	10	22
0.1/48/2	10	22
0.1/48/3	25	44
0.1/48/5	25	44
0.1/48/9	10	22
0.2/48/1	10	22
IISR Rejatha	10	22

Adaniya and Shirai, 2001; Kun-Hua, 2011). The most significant results of polyploids were addition of extra gene copies which results in bigger cells and vigor (Sattler et al., 2016). A higher yielding tetraploid line in ginger with bold rhizomes that are preferred for processing industries (Smith and Hamill, 2002; Smith et al., 2004).

3.3. Comparison of essential oil

The content of essential oil in each tetraploid line is shown in Table 4. The essential oil content of the two tetraploid lines (1.50% and 1.61%) was higher than the diploid parent (1.43%). The highest essential oil content was recorded in Tetraploid 2. In polyploids, multiplication of genome causes higher level of essential oil and secondary metabolites. Among the induced autotetraploids, doubling of the genomic content of cells advocates functionalized genome multiplications, improved protein synthesis and thus augmented secondary metabolite biosynthesis (Gantait and Mukherjee, 2021).

4. Conclusion

Successful *in vivo* protocol for induction of tetraploid lines in ginger 'IISR Rejatha' has been established using colchicine. This study confirmed the duplication of tetraploid nuclear content through chromosome count and flow cytometry. The induced tetraploids showed improved morphological attributes of vigorous growth, increased leaf biomass, yield, and bolder rhizomes that could broaden the genetic base. Among the two tetraploids, Tetraploid 2 was promising in terms of rhizome size, fresh yield, dry yield and oil content. All the above characters of the identified tetraploid lines would enhance commercial significance for yield and therefore the processing industry.

CRediT authorship contribution statement

D. Prasath: Conceptualization, Investigation, Methodology, Data analysis, Writing – original draft. **R. R. Nair:** Validation, Writing –

Table 3
Morphological characteristics of diploid and tetraploid plants.

	Plant height (cm)	Number of tillers	Pseudostem diameter (cm)	No. of leaves	Leaf length (cm)	Leaf width (cm)
Diploid (IISR Rejatha)	63.74 ± 2.11c	11.33 ± 0.90a	2.24 ± 0.16c	82.88 ± 3.04c	21.73 ± 0.46b	2.01 ± 0.13c
Tetraploid 1 (0.1/48/3)	71.32 ± 0.52b	11.17 ± 0.36ab	2.84 ± 0.12b	95.63 ± 3.58b	22.64 ± 0.38a	2.53 ± 0.27b
Tetraploid 2 (0.1/48/5)	78.95 ± 1.23a	10.49 ± 0.53b	3.20 ± 0.20a	99.75 ± 2.12a	22.40 ± 1.08ab	2.96 ± 0.10a

Different letters indicate significant differences detected using DMRT.

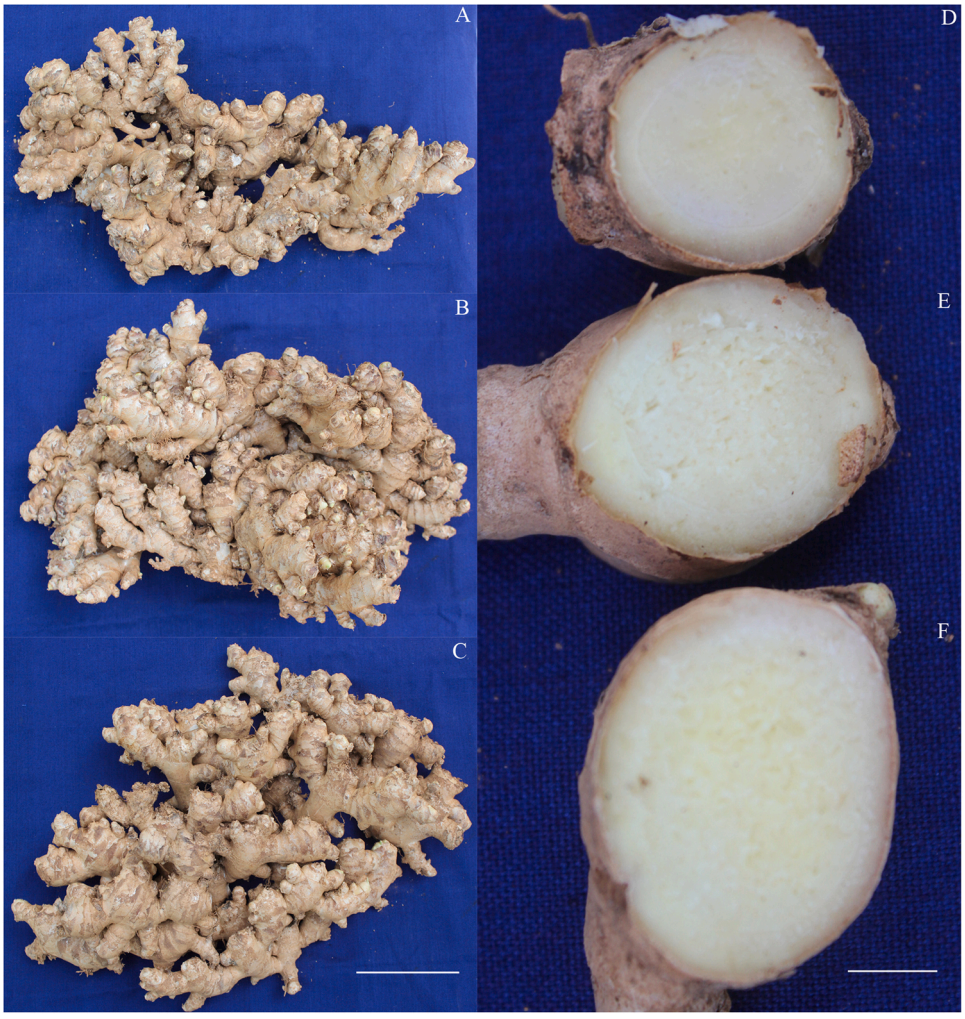

Fig. 2. A. normal rhizome of 'IISR Rejatha'; B (0.1/48/3) & C (0.1/48/5) induced polyploids from colchicine treated rhizome; Bars represent 7 cm in A-C; D. A normal rhizome of 'IISR Rejatha'; E (0.1/48/3) & F (0.1/48/5) induced polyploids from colchicine treated rhizome; Bars represent 1 cm in D-F.

Table 4
Rhizome characteristics and essential oil of diploid and tetraploid plants.

	Rhizome diameter (cm)	Rhizome yield per plant (fresh) (g)	Rhizome yield per plant (dry) (g)	Essential oil (%)
Diploid (IISR Rejatha)	2.00 ± 0.11c	280.33 ± 5.02c	64.88 ± 1.48c	1.43 ± 0.03c
Tetraploid 1 (0.1/48/3)	2.88 ± 0.15b	320.81 ± 7.35b	71.52 ± 1.76b	1.50 ± 0.05b
Tetraploid 2 (0.1/48/5)	3.26 ± 0.16a	418.65 ± 6.45a	89.91 ± 1.81a	1.61 ± 0.04a

Different letters indicate significant differences detected using DMRT.

review & editing. **P. A. Babu**: Investigation, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jarmap.2022.100422.

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