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Induction of Polyploidy in Citrus Rootstocks through *In Vitro* Colchicine Treatment of Seed-Derived Explants

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Abstract: Polyploidy, frequently observed in citrus species, aids in achieving better adaptation to environmental stresses. In this context, the current work aims to develop stable tetraploids in citrus rootstock cultivars, viz., Rough lemon, Rangpur lime and Alemow, through *in vitro* colchicine treatments. Seed-derived explants were obtained by culturing sterile seeds on MS basal media. Seedlings with a size of 5–8 mm (hypocotyl) were exposed to colchicine treatment. After treatment, the surviving seedlings were minigrafted onto six-month-old rootstock for better growth. Colchicine concentrations of 0.1%, 0.2%, 0.3% and control for durations of 16 or 24 h were tested with respect to the induction of polyploidisation. Treatment with 0.1% colchicine for 24 h resulted in high rates of mutation for polyploidisation and showed the highest tetraploid induction percentage (18.3%) in all the rootstock cultivars. High colchicine concentration and long exposure time decreased the survival of the observed seedlings. Flow cytometry and cytological methods were used for confirmation of autotetraploidy in the analysed samples. The surviving seedlings were identified on the basis of morphological and cytological variables, such as leaf area and stomata size, which significantly increased with increasing ploidy level. The proposed method was found to be an effective way to induce the polyploidy in Rangpur lime, Rough lemon and Alemow rootstocks.

Keywords: polyploidy; citrus; rootstocks; tetraploidy; flow cytometry; cytology

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1. Introduction

Polyploidy is a common occurrence in many plants, including fruit crops, where it has played an essential role in their evolution. During their development, nearly all angiosperms go through at least one round of complete genome replication [1,2]. Polyploidy is characterised by the presence of more than two sets of chromosomes. Polyploids can be allotetraploid or autotetraploid, arising from sexual reproduction via 2n gametes with a diploid set of chromosomes acquired from each of its parents or duplication of the somatic chromosomes of single diploid species, respectively [3–5]. The approach of colchicine-induced mutation in ploidy levels was used in some fruit crops, viz., banana [6], mulberry [7] and *Citrus* sp. [8]. Colchicine ($C_{22}H_{25}NO_6$) is one of the chemical mutagens most widely used by plant breeders for chromosome duplication, and inhibits the production of spindle threads during mitotic division. Flow cytometry is a useful tool in polyploidy induction studies, and permits the measurement of the fluorescence of large numbers of stained nuclei within seconds [9].

Rootstocks have a crucial role in citrus production, as they can overcome limiting factors such as climate, poor soil conditions, and biotic/abiotic stresses. Rough lemon (*Citrus jambhiri* Lush.), Rangpur lime (*Citrus limonia* Osbeck) and Alemow (*Citrus macrophylla* Wester) are the most predominantly used citrus rootstocks in India. These rootstocks have an impact upon various plant and fruit quality attributes, such as drought tolerance, fruit size, fruit organoleptic quality, susceptibility to cold stress, and disease resistance [10].

Rough lemon is more drought tolerant and has vigorous tree growth [3]. Fruits harvested on lemon-type rootstocks are not of high quality, but the size is large, with a thick, rough and often pebbled rind. Juice content is low in total soluble solids and contains more water compared to the juice of fruits from trees grafted upon sour orange (*Citrus aurantium* L.). *Citrus jambhiri* Lush. (Rough lemon) is not tolerant to flooding conditions, and so suffers quickly from root rot and foot rot due to *Phytophthora* sp.-induced lesions. It has been revealed that Rangpur lime rootstock induces high yield in sweet orange varieties when they are grafted upon it [11]. Additionally, it is susceptible to foot rot, blight disease and cold stress. Furthermore, the Rangpur lime rootstock induces vigorous canopy, larger size fruit and better juice quality characteristics than the fruits produced on rough lemon [11]. *Citrus jambhiri* Lush. and *Citrus limonia* Osbeck are also most susceptible to cold stress conditions.

Up until the 1900s, citrus breeders were trying to find new citrus rootstocks. Citrus rootstock research has mostly been focused on Citrus Tristeza Virus (CTV) tolerance, alkalinity, salt, drought and cold stress tolerance, and a positive impact on tree size, high fruit yield and quality. So far, troyer citrange, carrizo citrange, Swingle citrumelo, C32 citrange and C35 citrange, have all been successfully implemented in various citrus production areas [10].

In 1950, Dr Bill Bitters observed that Alemow citrus rootstock was more tolerant to root rot and foot rot caused by *Phytophthora* sp. and tolerant to high boron (B) concentration, while at the same time being susceptible to cold stress, CTV, and citrus blight. Alemow budded varieties have vigorous tree growth and higher fruit yield. Rough lemon, Rangpur lime, and Alemow were selected and implemented for their tolerance against CTV and higher fruit yield attribute, but their use is limited by their susceptibility to cold stress. Additionally, these rootstocks have vigorous growth habits, and hence are not suitable for high-density plantations.

Tetraploid rootstocks, both autotetraploid and allotetraploid, have been proven to reduce tree size in general [12]. Several studies have demonstrated that tetraploidisation in citriculture is an effective method for improving abiotic stress tolerances due to phenotypic variation generated by polyploidy [13] such as drought [3], boron toxicity [14], salinity stress [14], chromium toxicity [15], and chilling stress [16]. The polyploid plant material used in studies, comparing abiotic stress tolerances of diploids and tetraploids, was mainly obtained through spontaneous selections from nurseries [3,17] or somatic hybridisation studies [18]. Tetraploid genotypes were utilised as rootstocks for citrus varieties in 1958, after spontaneous tetraploidy in citrus rootstocks was discovered [19]. Presently, there are only a small number of reports on colchicine induction of tetraploidy in citrus rootstocks. Previous reports have revealed autotetraploid induction using various concentrations of colchicine from the ovules of carrizo citranges, Yuma Ponterosa lemon, and Sacaton citrumelo citrus rootstocks [20].

In this context, in the present study, the authors demonstrate an economically viable and technologically feasible method towards the development of stable tetraploids in three commercial citrus rootstocks (Rangpur lime, Rough lemon, Alemow), which are popular among citrus farmers, via colchicine-treated meristematically active seeds.

2. Materials and Methods

2.1. Plant Materials

Fruits were collected from mature trees of Rangpur lime, Rough lime, and Alemow grown in the farm of the ICAR-Central Citrus Research Institute, Nagpur (India) and seeds were extracted. The extracted seeds were disinfected with 0.7% sodium hypochlorite solution along with several drops of tween-20 wetting agent for 10 min followed by rinsing 2–3 times with autoclaved distilled water. Sterilised seeds were cultured onto seed germination Murashige and Skoog (MS) basal medium under sterile conditions; inoculated seeds were maintained at 26 ± 1 °C, 16 h photoperiod and 44.46 µ mol m^{-2} s^{-1} light intensity for 12–14 days until the hypocotyl emerged from the cotyledons and reached a

size of 5–8 mm. At this stage of the active meristematic state, germinated seeds were ready to be treated with colchicine.

2.2. Colchicine Treatments

This investigation was based on a two-way factorial design consisting of three different colchicine concentrations viz 0.1%, 0.2% and 0.3% (*w*/*v*) under two distinct exposure periods. Colchicine stock solution was prepared by dissolving colchicine in dimethylsulfoxide (DMSO) followed by the addition of sterile water to bring the final concentration to 1 g/mL. Germinated seeds were placed in a Petri plate containing liquid MS basal salt media with final colchicine concentrations of 0.1, 0.2, 0.3% (*w*/*g*) and control (without colchicine). The petri plate was sealed and wrapped. Culture was kept in a dark chamber maintained at 26 ± 1 °C with colchicine treatment periods of 16 or 24 h. Afterwards, the seeds were removed from the solution and cultured on MS seed media, under dark conditions for two (2) weeks in order to facilitate seedling elongation. Later, the light conditions were shifted under 16 h light/8 h dark for further growth. Variegated and strong seedlings were selected for minigrafting on 6-month-old rootstock. Grown up seedlings with new emerging leaves were analysed with respect to their ploidy level via flow cytometry.

2.3. Ploidyanalysis

Ploidy was analysed using a flow cytometer (Partec Gmbh, Munster, Germany), by estimating the volume and florescence of isolated nuclei. The ploidy is presented in the form of a histogram of integral fluorescence with the peaks depicting the ploidy level of the respective sample.

The cells were labelled with fluorescent (4′, 6-diamidino-2-phenylindole dihydrochloride) staining; the individual cells or particles were illuminated by the excitation light and the fluorescent light intensity, which is proportional to DNA content, was measured and analysed in order to depict the respective number of chromosomes and estimated the ploidy level of the samples. The run sample was analysed in a UV-LED with light emission at 365 nm, and more than 5000 nuclei were assessed in each sample. Histograms were constructed using CyView software (Partec Gmbh, Germany) [8].

2.4. Chromosome Counting

The protoplast dropping method of chromosome preparation, developed by Kesara Anamthawat-Johnsson, (2013) was used with some modifications. The protoplast dropping method is a technique used for the preparation of high-quality chromosome spreads from plant cells. As citrus chromosomes are small in size, i.e., 2–4 µm with low mitotic index [21], this protocol resolved the problem of spreading and visualisation of chromosome in citrus crop and improved the quality of chromosome spreads, unlike the routinely used traditional squash preparation methodology.

In the protoplast dropping method, citrus tissue was treated with enzymes (mix of cellulase/pectinase) to remove the cell wall and to release the protoplasts. The protoplasts were then collected and dropped onto a microscope slide, and were allowed to burst and release their chromosomes. The chromosomes were then stained and visualised under a microscope.

2.5. Stomata Morphology

Stomata analysis was carried out with the help of moulds made from transparent nail paint. Thin coatings were applied to the abaxial surface of developing leaves from plants with proven tetraploidy. With the help of transparent sticky tape, the dried nail polish coating on the leaves was removed and transferred to a glass slide. The analysis was carried out using a Leica phase contrast microscope (magnifications of 40 and 100×) with a video camera attached to a computer. Stomata frequency per 500 µm/sq and stomata length were measured [22].

2.6. Statistical Analysis

The results related to the effect of various colchicine treatments on percentage of variation in plant morphology and seedling height were analysed using a two-way ANOVA design with cultivars, three colchicine concentrations and two different exposure times, and 50 explants per treatment (except for control, with 50), with four replications. Stomata morphometry (stomata length, density) was analysed using *t*-test ($p < 0.05$).

3. Results

Colchicine treatments significantly reduced the survival rates of seed derived explants, of all citrus rootstocks at all doses and exposure times. The higher the colchicine concentration and the higher the exposure, the lower the survival rate. Rootstock seedling survival across all colchicine treatments was the highest for Rangpur lime (39.61%), followed by Alemow, having 36.19%, and Rough lemon, with 35.18%. The recorded seedling survival rates for control treatments without any colchicine exposures were 69.53% and 70.14%, respectively. All citrus rootstock cultivars showed a survival percentage of around 40% at 0.1% (*w*/*v*) /16 h exposure treatment. Statistically significant treatment differences were found for the survival rate of the seedlings (Table 1).

Table 1. Percentage survival of citrus rootstock seedlings 2 weeks after treatment with three colchicine concentrations and two exposure periods.

Cultivar	T-1 (Control 16 h)	T-2 (Control 24 h)	T-3 (0.1%/16 h)	T-4 (0.1%/24 h)	T-5 (0.2%/16 h)	T-6 (0.2%/24 h)	T-7 (0.3%/16 h)	T-8 (0.3%/24 h)	Mean 2
Rangpur lime	89.35 (71.00)	88.32 (70.06)	48.35 (44.05)	37.25 (37.66)	25.52 (37.66)	16.4 (30.33)	14.2 (23.82)	9.45 (22.10)	41.10 (39.611)
Alemow	87.37 (69.28)	85.27 (67.46)	33.25 (35.19)	27.37 (31.50)	21.4 (27.54)	15.45 (23.12)	11.55 (19.82)	7.3 (15.58)	36.12 (36.190)
Rough lemon	88.32 (70.13)	89.35 (71.07)	39.35 (38.83)	22.32 (28.14)	17.2 (24.44)	12.45 (20.54)	8.3 (16.55)	4.27 (11.72)	35.19 (35.181)
Mean 1	88.34 (70.140)	87.64 (69.531)	40.31 (39.363)	28.98 (32.438)	21.37 (27.441)	14.76 (22.497)	11.33 (19.493)	7.00 (15.048)	
	CD	C	T	I					
	0.05	0.96 *	1.57 *	2.73 *					
	0.01	1.28 **	2.09 **	3.63 **					

Numbers in parentheses are arc sine-transformed means of four replicate batches. T = Treatment; C = Cultivar; I = Interaction (Treatment × Cultivar). ** Highly significant ($p \leq 0.01$), * significant ($p \leq 0.05$). Mean 1: Mean cumulative response of each treatment to all the cultivars. Mean 2: Mean cumulative response of each cultivar to all the treatments.

The higher the concentration [0.3% (*w*/*v*)] and the longer the exposure time (24 h), the more stunted the growth and the higher the seedling mortality. Three weeks after treatment, the results revealed that the control treatment had the maximum mean plant height value (6.30 cm), while the colchicine treatment [0.3% (*w*/*v*)/24 h] had the lowest mean plant height (0.40 cm). Average heights (mean of two exposures) of the control seedlings at 1 month after treatment were 5.58, 6.35, and 6.39 cm for rootstocks of Rangpur lime, Alemow and Rough lemon, respectively. All the treatments differed significantly with respect to the plant height parameter (Table 2). The colchicine treatment of 0.1% (*w*/*v*) for 24 h. produced a greater number of putative tetraploids and mixoploids with variegated plant morphology. In terms of morphological variation among the three studied citrus rootstocks, Rangpur lime showed the highest variation percentage (18.3%) followed by Alemow (15.27%) and Rough lemon (11.2%). Statistically significant values were obtained for the variation percentage in plant morphology (Table 3). The results for the number of tetraploids and mixoploids obtained with each treatment (Table 4) indicated the cumulative number of tetraploids observed (six) was greater with 0.1%/24 h exposure time for all three rootstocks, followed by 0.2%/24 h exposure time (three) and 0.2%/16 h (two). The maximum numbers of tetraploids were observed in Rangpur lime (four) and Alemow (four) followed by Rough lemon (three). Higher ploidy levels (six pentaploids, four of Rangpur

lime and two of Rough lemon) were observed at 0.2% colchicine concentration with a 24 h exposure time followed by 0.3% 16 h exposure time (two pentaploids of Rangpur lime) (Table 4). The maximum number of mixoploids was observed in Alemow at 0.1%/24 h exposure time (four) followed by 0.2%/16 h in Rangpur lime (two), 0.2%/24 h (two) and 0.3%/16 h (two) in Rough lemon. The colchicine treatment of 0.1%/24 h exposure time yielded an increased number of mixoploids (Table 4).

Table 2. Average height of surviving citrus rootstock seedlings 1 month after treatment with three colchicine concentrations and two exposure periods.

Cultivar	T-1 (Control 16 h)	T-2 (Control 24 h)	T-3 (0.1%/16 h)	T-4 (0.1%/24 h)	T-5 (0.2%/16 h)	T-6 (0.2%/24 h)	T-7 (0.3%/16 h)	T-8 (0.3%/24 h)	Mean 2
Rangpur lime	5.67	5.5	1.62	1.6	0.98	0.92	0.84	0.57	2.214
Alemow	6.8	5.9	2.51	1.7	1.2	0.8	0.67	0.52	2.513
Rough lemon	6.42	6.37	1.56	1.2	0.85	0.62	0.58	0.4	2.252
Mean 1	6.300	5.925	1.900	1.500	1.010	0.784	0.697	0.497	
	CD	C	T	I					
	0.05	0.07 *	0.13 *	0.22 *					
	0.01	0.10 **	0.17 **	0.29 **					

T = Treatment; C = Cultivar; I = Interaction (Treatment × Cultivar). ** Highly significant ($p \leq 0.01$), * significant ($p \leq 0.05$). Mean 1: Mean cumulative response of each treatment to all the cultivars. Mean 2: Mean cumulative response of each cultivar to all the treatments.

Table 3. Percentage of variation of citrus rootstock seedlings before minigrafting with three colchicine concentrations and two exposure periods.

Cultivar	T-1 (Control 16 h)	T-2 (Control 24 h)	T-3 (0.1%/16 h)	T-4 (0.1%/24 h)	T-5 (0.2%/16 h)	T-6 (0.2%/24 h)	T-7 (0.3%/16 h)	T-8 (0.3%/24 h)	Mean 2
Rangpur lime	0.00	0.00	6.17 (14.26)	18.3 (25.28)	12.2 (20.40)	9.32 (17.66)	4.2 (11.78)	1.85 (7.66)	6.50 (12.13)
Alemow	0.00	0.00	4.25 (11.70)	15.27 (22.95)	11.25 (19.51)	8.05 (16.08)	4.25 (11.66)	0	5.37 (10.24)
Rough lemon	0.00	0.00	5.3 (13.24)	11.2 (19.51)	6.55 (14.80)	2.15 (7.22)	11.52 (7.02)	0	4.59 (7.72)
Mean 1	0.00	0.00	5.24 (13.07)	14.92 (22.58)	10.00 (18.24)	6.50 (13.65)	6.56 (10.15)	0.61 (2.55)	
	CD	C	T	I					
	0.05	0.90 *	1.48 *	2.56 *					
	0.01	1.20 **	1.96 **	3.40 **					

Numbers in parentheses are arc sine-transformed means of four replicate batches. T = Treatment; C = Cultivar; I = Interaction (Treatment × Cultivar). ** Highly significant ($p \leq 0.01$), * significant ($p \leq 0.05$). Mean 1: Mean cumulative response of each treatment to all the cultivars. Mean 2: Mean cumulative response of each cultivar to all the treatments.

Statistical analysis indicated significant differences among rootstock cultivars, colchicine concentrations and exposure times with respect to parameters like survival rate, height of plants, and variation percent at the $p < 0.05$ level (https://ccari.icar.gov.in/wasp/index.php, accessed on 21 May 2023).

Furthermore, only 4.27 to 14.2% of plants were recovered from seed-derived explants treated with 0.3% (w/v) colchicine. Meanwhile Whereas, 0.1% (w/v) colchicine treatment for 16 and 24 h, on the other hand, resulted in survival rates of roughly 22.32 to 48.35% [23], a lethal dose with 25–50 percent lethality resulted in the highest mutation rate.

Table 4. Polyploid production of three citrus rootstock cultivars with colchicine at three concentrations and two exposure periods.

Treatment	Number of Field Transferred Confirmed Tetraploids (4n)	Number of Field Transferred Confirmed Pentaploids (5n)	Number of Field Transferred Confirmed Mixoploid Number
		Rangpur lime	
Control 16 h.	--	--	--
Control 24 h.	--	--	--
0.1%/16 h.	--	--	--
0.1%/24 h.	3	--	--
0.2%/16 h.	1	--	1 (3n + 4n) + 1 (3n + 6n)
0.2%/24 h.	--	2	--
0.3%/16 h.	--	2	--
0.3%/24 h.	--	--	--
		Alemow	
Control 16 h.	--	--	--
Control 24 h.	--	--	--
0.1%/16 h.	--	--	--
0.1%/24 h.	2	--	4 (2n + 4n)
0.2%/16 h.	1	--	--
0.2%/24 h.	1	--	--
0.3%/16 h.	--	--	--
0.3%/24 h.	--	--	--
		Rough lemon	
Control 16 h.	--	--	--
Control 24 h.	--	--	--
0.1%/16 h.	--	--	--
0.1%/24 h.	1	--	--
0.2%/16 h.	--	--	--
0.2%/24 h.	2	2	2 (2n + 4n)
0.3%/16 h.	--	--	2 (2n + 4n)
0.3%/24 h.	--	--	1 (2n + 5n)
Total	11	6	11

3.1. Characterisation of Tetraploid Rootstock Cultivars by Flow Cytometry

The results revealed that rootstock obtained from both Alemow and Rough lemon was of the mixoploid category, with both diploid and tetraploid sectors (Figure 1), while Rough lemon had an additional pentaploid category (Figure 2). In Rangpur lime, four (4) main classes of plants were identified as diploid, mixoploid (chimeras with tetraploid sectors) and pentaploid (Figure 3).

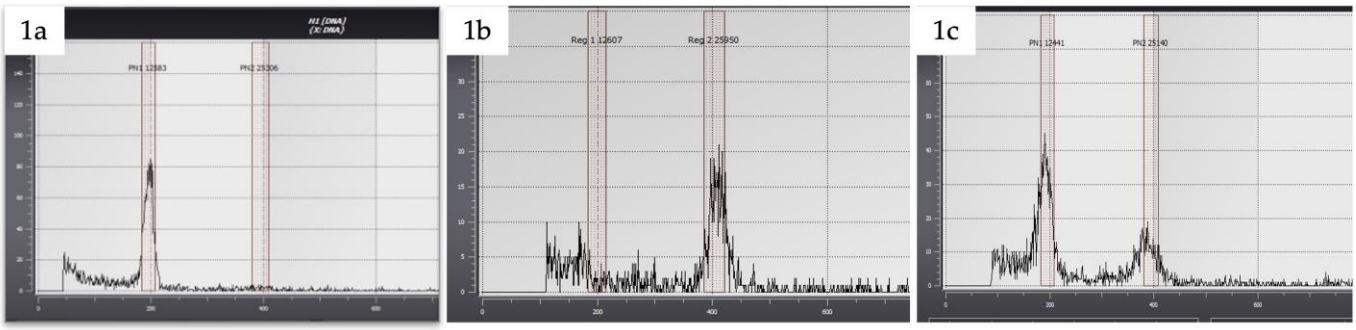

Figure 1. Ploidy analysis results in Alemow: diploid control (**1a**); tetraploid (**1b**); mixoploid (2n + 4n) (**1c**).

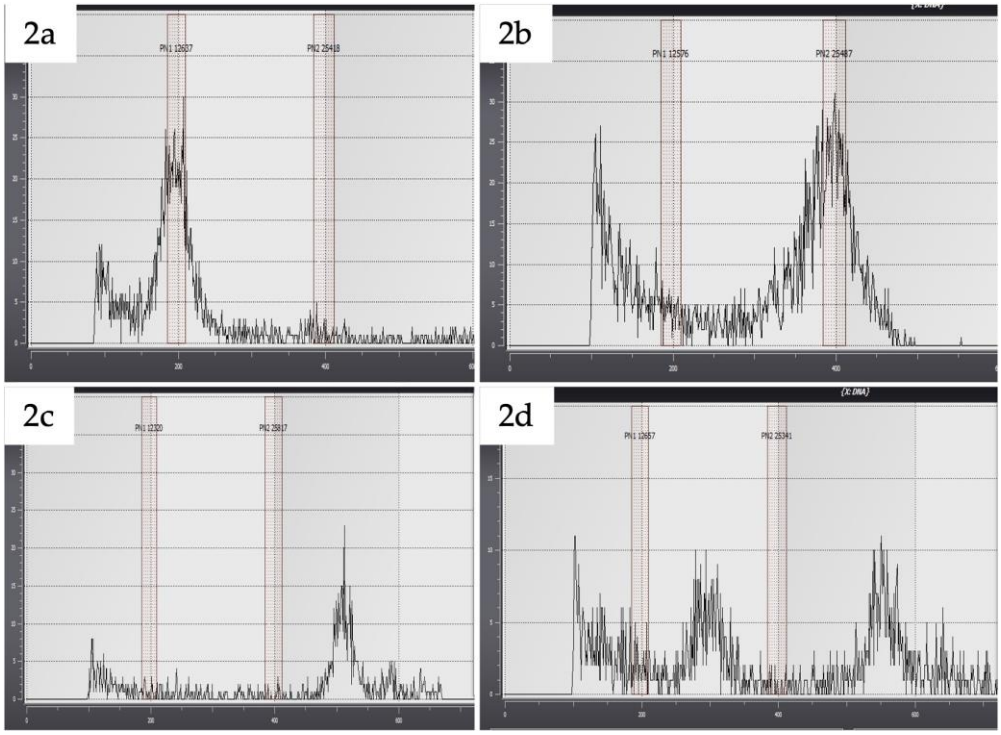

Figure 2. Ploidy analysis results in Rangpur lime: diploid control (**2a**); tetraploid (**2b**); pentaploid (**2c**); mixoploid (3n + 6n) (**2d**).

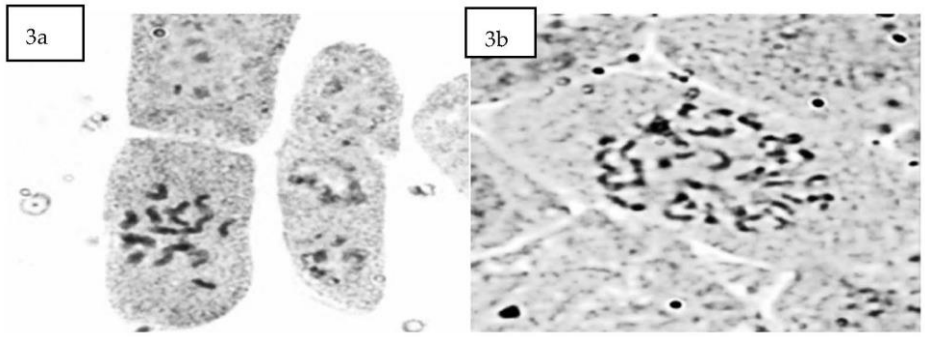

Figure 3. Processed metaphase cell of Rough lemon (*Citrus jambhairi* Lush.) sample: (**3a**) diploid chromosomes count is (2n = 2x = 18); (**3b**) tetraploid chromosomes count is (2n = 4x = 36).

The fluorescence intensity X-mean of the sample was used to assess the ploidy level, with the main peak of diploid plants having a lower fluorescence intensity compared to that of tetraploid plants. The coefficient of variation (c.v) was a measure of the spread of the data, with a lower c.v indicating less variability in the fluorescence intensity. The results indicated that the fluorescence intensities of the main peaks of diploid and tetraploid Rangpur lime plants were at 1.94 (c.v) and 3.57 (c.v), respectively. In Rough lemon plants, the main peaks of diploid and tetraploid were at 1.69 (c.v) and 3.55 (c.v), respectively. In diploid Alemow plants, the main peak was at 3.18 (c.v), and in triploid plants the main peak was at 5.07 (c.v), which was about twice of control sample [24].

Overall, the study provided important insights on the generation or creation of diverse citrus polyploids, which could have important research implications on growth and cultivation patterns as well as on citrus breeding towards cultivar development.

3.2. Tetraploid Characterisation by Cytology

Chromosome number count was carried out using the enzyme digestion and protoplast drop technique to reconfirm the results obtained (shown in Figure 3) by flow

cytometry. Flow cytometry certified the tetraploid used as the sample. The control plants were 2n = 2x = 18 (Figure 4a), and tetraploid plants 2n = 4x = 36 (Figure 4b); mixoploid (Figure 4c) plants were not taken into consideration for analysis.

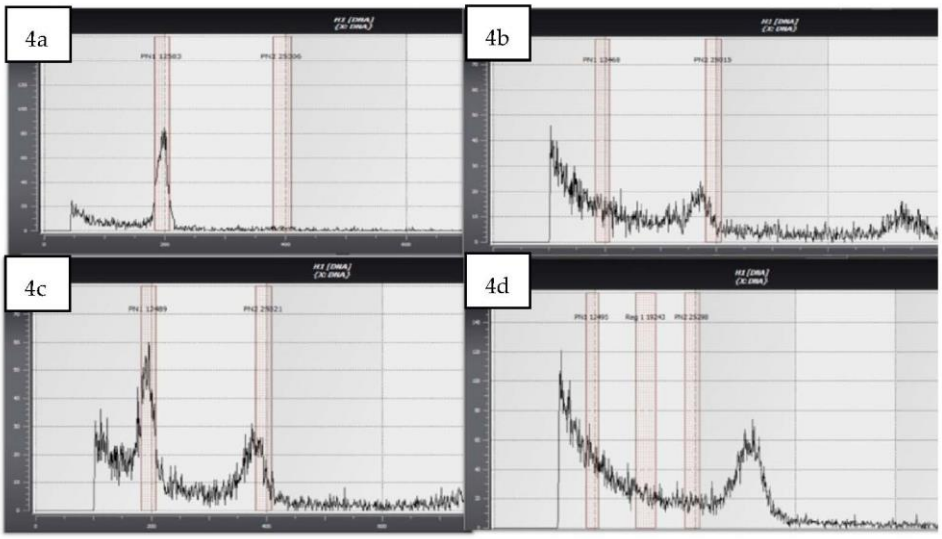

Figure 4. Ploidy analysis results in Rough lemon via flow cytometric technique diploid control (**4a**), tetraploid (**4b**), mixoploid (2n + 4n) (**4c**), pentaploid (**4d**).

3.3. Stomatal Analysis

The results of the morphometric analysis of the stomata (guard cells) indicated significant differences between diploid and tetraploid plants in terms of the number of guard cells and the length of the stomata. Tetraploid plants had lower numbers of guard cells per 500 μm/sq^2 leaf area compared to diploid leaf samples and around a 32% reduction in the number of guard cells. The average lengths of stomata in diploid and tetraploid samples were 38.44 μm and 54.70 μm, respectively, and the observed stomata length in tetraploid samples was 42.29% more extended when compared to the diploid sample. The T-test results, with a significance level of $p > 0.01$, indicated that the differences observed between diploid and tetraploid samples were statistically significant (Figure 5) (Table 5).

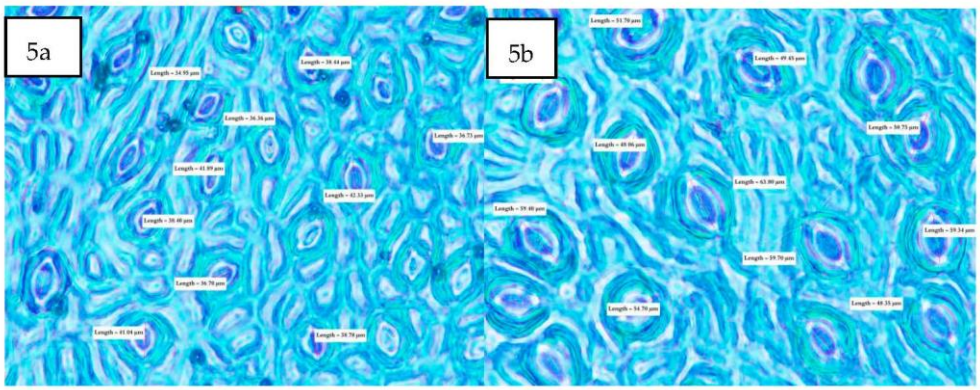

Figure 5. Stomata morphometric analysis in control (**5a**), and tetraploid sample of Rough lemon (**5b**) for stomata frequency per 500 μm/sq^2 and length of stomata.

Table 5. Morphometric analysis of stomata in tetraploid and control samples of Rough lemon.

	Tetraploid Stomatal	Diploid Stomatal
Stomatal count	11	10
Avg	54.44	38.56
SD	5.31	2.49
Variance	28.23	6.24
T-Statistic	8.614	
T-Table (0.05) *	2.093	
T-Table (0.01) **	2.861	

** Highly significant ($p \leq 0.01$), * significant ($p \leq 0.05$).

3.4. Plant Morphology Study

Autotetraploid plants were characterised by plant morphological features like the existence of round leaves with a dark green colour (Figure 6), shorter internodes and petioles, and thicker stems and roots, and even large-sized flowers (Figure 7), demonstrating a phenomenon widely known as the gigas effect (Figures 8–10). Thick petals and large-sized stigma were observed in tetraploid flowers of the Alemow rootstock cultivar under field conditions. The average flower diameter found in diploid plants was 3.5 cm, whereas the average length recorded in tetraploid flowers was 4.5 cm (Figure 7).

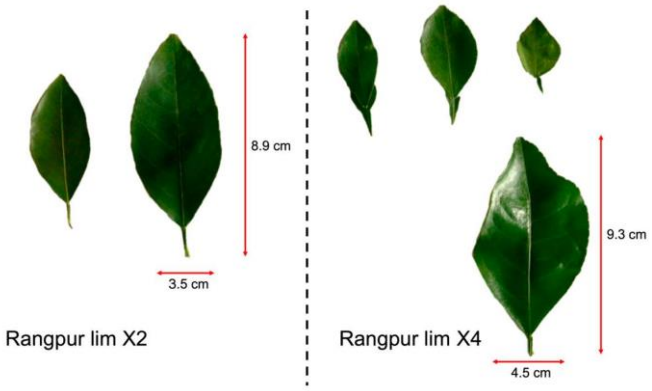

Figure 6. Leaf phenotypic features of diploid and tetraploid plants of Rangpur lime. Round leaves, dark green colour, and higher leaf area from control plant like.

Figure 7. Leaf phenotypic features of diploid and tetraploid plants of Rangpur lime. Round leaves, dark green colour, and higher leaf area than the control plant.

Figure 8. Control diploid plants of Rough lemon (**8a**). Tetraploid plants of Rough lemon (**8b**) exhibit distinctly shorter internodes.

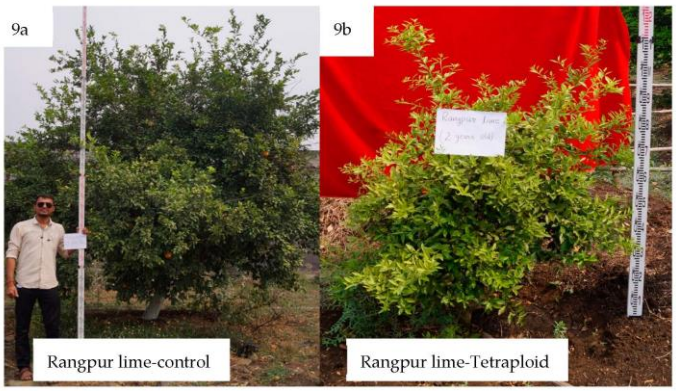

Figure 9. Control diploid plant of Rangpur lime (**9a**) and tetraploid plant of Rangpur lime (**9b**) exhibiting a dwarf character.

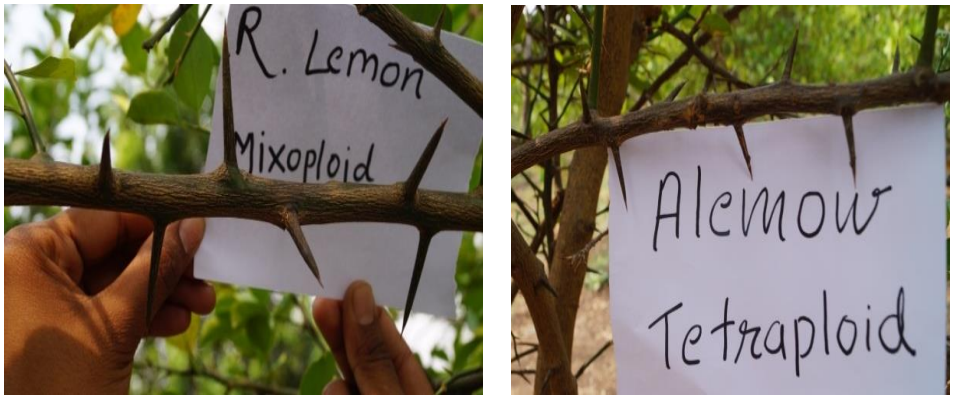

Figure 10. Thorny characters of Rough lemon—mixoploid—and Alemow—Tetraploid.

The results for plant morphology in colchicine-induced polypolids of the three different rootstocks studied revealed that the maximum plant height was recorded in all the field-transferred rootstocks for diploid seedlings, followed by mixoploids and tetraploids.

Significantly higher leaf width values were obtained in tetraploid rootstocks of Rough lemon and Alemow, whereas the maximum leaf thickness was recorded in mixoploids of Rough lemon and Alemow. In the case of Rangpur lime, the maximum leaf width and thickness were observed in diploids followed tetraploids. Plant height was significantly higher in diploids of all three rootstocks, followed by mixoploids and tetraploids. Therefore, the desirable morphological trait of dwarfness was more prominent in the case of tetraploids of all the studied rootstocks. Higher values for thorn thickness were recorded in the field-transferred tetraploids of both the Rough lemon and Alemow rootstocks. The maximum

length of thorns was noticed in polyploids of Rough lemon and Alemow compared to diploids (Tables 6–8).

Table 6. Plant morphology variation in colchicine-induced polyploids of Rough lemon.

Rough Lemon		Plant Height	Leaf Length	Leaf Width	Leaf Thickness	Thorn Length	Thorn Thickness
Tetraploid		3.525 b	98.50 a	55.00 a	1.975 c	34.50 a	5.00
Mixoploid		3.825 b	100.0 a	50.75 c	2.150 a	35.50 a	4.25
Diploid		4.500 a	93.50 b	53.25 b	1.050 b	31.50 b	3.75
CD	1%	2.65 **	2.09 **	0.16 **	0.79 **	NS	NS
	5%	1.84 *	1.46 *	0.11 *	0.55 *	2.96 *	NS

** Highly significant ($p \leq 0.01$), * significant ($p \leq 0.05$), NS = non-significant. If the difference between the two mean values of any particular parameter is it higher than CD, then it considered statistically significant. 'a' indicates highest value followed by 'b' & 'c' in treatment mean value.

Table 7. Plant morphology variation in colchicine-induced polyploids of Alemow.

Alemow		Plant Height	Leaf Length	Leaf Width	Leaf Thickness	Thorn Length	Thorn Thickness
Tetraploid		2.650 b	113.0 a	53.75 a	2.050 a	45.00 a	7.500 a
Mixoploid		2.825 b	109.5 ab	46.50 b	2.250 b	38.50 b	6.250 b
Diploid		4.275 a	98.50 b	52.00 ab	1.025 c	28.25 c	4.500 c
CD	1%	NS	NS	0.25 **	0.79 **	3.88 **	1.27 **
	5%	11.36 *	5.78 *	0.17 *	0.55 *	2.70 *	0.88 *

** Highly significant ($p \leq 0.01$), * significant ($p \leq 0.05$), NS = non-significant. If the difference between the two mean values of any particular parameter is it higher than CD, then it considered statistically significant. 'a' indicates highest value followed by 'b' & 'c' in treatment mean value, further were 'ab' together are mentioned it means they are closely related value to 'a'.

Table 8. Plant morphology variation in colchicine-induced polyploids of Rangpur lime.

Rangpur Lime		Plant Height	Leaf Length	Leaf Width	Leaf Thickness	Thorn Length	Thorn Thickness
Tetraploid		1.075 b	70.75 b	31.50 a	0.625 b	12.00 b	1.125 b
Diploid		4.025 a	104.5 a	46.75 b	1.350 a	35.25 a	4.250 a
CD	1%	9.55 **	NS	3.34 **	0.49 **	5.10 **	0.96 **
	5%	6.30 *	0.52 *	2.20 *	1.07 *	3.36 *	0.63 *

** Highly significant ($p \leq 0.01$), * significant ($p \leq 0.05$), NS = non-significant. If the difference between the two mean values of any particular parameter is it higher than CD, then it considered statistically significant. 'a' indicates highest value followed by 'b' & 'c' in treatment mean value.

4. Discussion

In this study, polyploidy was induced in seed-derived explants of apomictic citrus cultivars by treating them with colchicine at various concentrations and for different exposure times. Colchicine was applied to meristematically active seed-derived explants of Rough lemon, Rangpur lime, Alemow rootstock cultivars during the formative stage. This method has previously been used in other *Citrus* species, such as mandarins [25], pummelos [18], and other plant species, such as pomegranates [25,26], and the most commonly used MS basal media resulted in high germination rates in citrus [27]. It has been stated that germination success is influenced by a variety of parameters, including genotypes, culture media, phytohormones, and culture conditions [28]. As previously stated, the most important parameters in the development of polyploids are colchicine concentration and treatment/factors periods [25,29,30]. The efficacy of developing stable polyploids is dependent on the balance between colchicine concentration and exposure time [31]. The survival rate was low in this investigation when the colchicine concentration and exposure period were increased. All colchicine doses used in this study caused polyploidisation. Generally, higher concentration and longer exposure period often result in decreased survival rate in the treated tissue [9,32,33]. This low survival rate is because of

the higher colchicine concentrations and longer exposure time hampering seedling growth, causing hyperploidy, browning, and necrosis in the meristematic tissue, and ultimately the death of the seedling [34]. Higher mortality in the treated rootstock seedlings was observed due to the toxicity of the colchicine chemicals. Decreased seedling survival was observed with increasing colchicine concentration, as well as with longer exposure to the colchicine. The seedling surviving percentage was around 40% at the lowest colchicine concentration of 0.1%. The survival rate dropped to 4.27 to 14.2% at a colchicine concentration of 0.3%. When the colchicine exposure period was increased from 16 to 24 h, for each concentration, the survival rate dropped to almost half. These results revealed that the survival rate was inversely proportional to the colchicine concentration and exposure time.

The survival rate of colchicine-treated explants provided important information concerning the determination of the optimum colchicine dose and exposure period. Plants recovered at the highest colchicine concentration with long exposure times [0.3% (*w*/*v*)/16 h and 0.2%/24 h] had reduced survival rate; however, plants recovered at this concentration were able to produce higher ploidy levels, such as pentaploids [under 0.2% and 0.3% (*w*/*v*)]. Variations in polyploidy recovery were observed among the different citrus rootstock cultivars. In Rangpur lime, four tetraploids and two mixoploids, in Alemow, four tetraploids and four mixoploids, and in Rough lemon, three tetraploids and five mixoploids were observed. When the targeted tissue is multicellular, the occurrence of mixoploids is commonly found. This explains why only a few colchicine-exposed cells were mutagenized. From these partially mutated meristems, a mixture of tetraploid and diploid tissue was observed, which later differentiated to form the plant organs [8].

Previous reports have also stated that the genotype factor can affect the efficiency of polyploidy induction [35]. In the current study, the efficiency of polyploidy induction was affected by the genotype, and the authors found that Rangpur lime and Rough lemon rootstock were the most responsive for polyploid induction, followed by Alemow. The higher ratio between mixoploid and tetraploid *in vitro* plant recovery in citrus rootstocks suggested the difficulty of colchicine penetration into the meristematic region. It was observed that the seedlings derived from the colchicine-treated seeds were stunted in height compared to the control seedlings, which is in agreement with previous findings in cotton [36]. A reduction in the growth rate of the treated seedlings was observed in all three rootstock cultivars in all colchicine treatments, which is in agreement with the previous report [8].

Accurate and timely identification of polyploids can shorten the culture period and enhance the effectiveness of polyploid breeding. Polyploidy was determined using multiple approaches, including morphological traits, flow cytometry (FCM), and chromosomal counts, for better screening and confirmation of ploidy [37]. Due to chromosome doubling, polyploidy plants have distinct exterior morphological traits from diploids, primarily in the shape and size of roots, stems, leaves, flowers, and fruits. The ovary diameter, male flower petals and anther diameter, leaf length and leaf width ratio are all good indicators of ploidy level in citrus. Although morphological parameters are frequently utilised as the first primary selection criteria for polyploids, they are not always accurate [38]. Plants were selected based on morphological features indicative of induced tetraploids (increased width-to-length leaf ratios, thicker stems, higher number of chloroplasts per guard cell, and larger stomata) in *Lagerstroemia indica*, and then validated by FCM [39]. Only half of the morphologically screened tetraploid plants were verified to be tetraploids [39]. FCM analysis can be performed upon various types of tissues and cell layers in order to rapidly determine ploidy in a large number of plants [40]. This allows the early analysis of polyploid plants, saving both effort and time [41,42]. FCM is a rapid and efficient method for analysing a large number of samples, but it requires accurate samples and expensive equipment. Chromosome counting is the most accurate method, but it is time consuming and cumbersome [43]. Even though chromosome counting using the cytological technique is accurate, it is cumbersome and time consuming; therefore, use of FCM is more

common, because of the rapid delivery of results and lower time consumption, with the only limitation being the higher cost of the equipment [40].

Hence, in the present study, the authors used a combination of cytology and flow cytometry techniques for double confirmation of the induced polyploidy in the field-transferred samples.

This study provides important information linked to the factors/treatments affecting polyploidy induction in citrus rootstock cultivars, which can enhance the efficiency of the production of polyploid/tetraploid plants.

5. Conclusions

In the present investigation, an economically viable and technologically feasible method of inducing tetraploidy is described in which the meristematically active seeds of three commercial citrus rootstocks are treated with various concentrations of colchicines for different exposure times. Stable tetraploids were successfully produced from all three of the studied citrus rootstocks and were confirmed by flow cytometry, chromosomal counting, and also by morphological evaluation. This method facilitated the treatment of large numbers of seeds at the same time, and limited the safety risks associated with the handling of toxic colchicine chemicals.

The development of tetraploid citrus rootstocks can lead to early precocity in new orchards compared to in orchards raised using traditional practices. The successful generation of stable tetraploid rootstocks can induce better scion performance in terms of yield, fruit quality and resistance to various biotic and abiotic stresses.

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