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In vivo polyploidy induction in *Dendrobium crumenatum* through colchicine treatment

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Abstract

Polyploidy induction plays a significant role in hybridizing and improving orchids (Orchidaceae). Induction of polyploidy can help to restore fertility by doubling of chromosomes and thus leading to the creation of allotetraploids which can help breeders develop improved hybrids and novel types by contributing beautiful floral or growth characteristics unobtainable from the diploid forms. The objective of the present study was to investigate the effective colchicine concentration and duration of exposure for the polyploidy induction in *Dendrobium crumenatum* via *in vivo* method, thus improving the flower characteristics. For the *in vivo* induction of polyploidy, *D. crumenatum* plantlets were chosen and subjected to eight different colchicine treatments. Treatments were based on colchicine concentration (0.05 and 0.1%) and the duration of exposure of plantlets to these doses of colchicine (24, 48, 72 and 96 h). The morphological characters, like shoot length, diameter of pseudobulb, number of leaves and width of leaves showed treatment mean values greater than their corresponding control plantlets. After analyzing the histogram peaks of *in vivo* treated samples, it was observed that the highest tetraploid induction (50%) was achieved with the treatment of 0.05% colchicine for 96 hours. Moreover, the maximum mixoploids (66.67%) were obtained from two different treatments; first, with 0.05% colchicine for 72 hours and second, with 0.05% colchicine for 48 hours. These results suggest that longer treatment duration of 96 hours with 0.05% colchicine leads to higher tetraploid induction while shorter durations of 72 and 48 hours with the same concentration of colchicine are more effective for producing mixoploids. Stomatal observations exhibited a lower stomatal density, but increased stomatal size in polyploids than diploids.

Key words: *In vivo*, polyploidy, *Dendrobium crumenatum*, antimitotic agent, colchicine, flow cytometry

Introduction

Orchids belong to Orchidaceae which is one of the largest and highly evolved family accounting for almost 10% of flowering plants. It comprises about 35,000 species belonging to 850 genera. Orchids have a high economic value both in commercial horticulture, as potted plants or cut flowers, and in traditional Chinese medicine (Chase *et al.*, 2015; Grosso *et al.*, 2017).

Among various orchids, Dendrobium has become increasingly popular due to its floriferous flower sprays, year-round availability, wide range of colours, sizes, shapes and long flowering life of several weeks to months. There are several methods for improving a crop, that includes; conventional, nonconventional and advanced breeding techniques and polyploidy breeding is one among them. Induction of polyploidy has been an important method for improvement of orchids to create increasing interest and demand in the floriculture market. The improvement of characters like colour, flower shape, longevity, fragrance, and the creation of novel variations in Dendrobium orchids through polyploidy can increase its commercial value and help produce outstanding hybrids. In light of this, the present investigation is formulated to induce polyploidy in *Dendrobium crumenatum* through *in vivo* technique and its subsequent analysis using flow cytometry.

Dendrobium crumenatum is a common epiphytic orchid, which is commonly known as dove orchid or pigeon orchid or sparrow orchid (Leong and Wee, 2013). The flower is small, white color with the lip having a bright yellow disc and has a strong fragrance (Meesawat and Kanchanapoom, 2007). This orchid species has many peculiar features of flowering, for instance, gregarious flowering (Holttum, 1964; Bernier *et al.*,1993). The flowers bloom exactly 9 days after a rainfall event. All plants in a certain area start to flower at the same time and are at the same stage of floral development throughout the year, but the blooms last for only a full calendar day (Holttum, 1964; Goh *et al.*, 1982). With the expected outcome of polyploidy induction, artificial induction of polyploidy using antimitotic agent, colchicine was carried out to generate *D. crumenatum* orchid flowers with improved size, better floral characteristics and longer shelf life.

Polyploidization has an important role in the hybridization, improvement and production of premium species and varieties in the orchid floriculture industry (Miguel and Leonhardt, 2011; Huy *et al.*, 2019). Polyploid orchids had thicker stems, thicker and wider leaves, and higher-quality flowers with intensified color patterns (Miguel and Leonhardt, 2011).

Chromosome doubling is an acknowledged mechanism to obtain different ploidy levels in plants, and it is usually achieved by chemical treatments using anti-microtubule agents such as colchicine or oryzalin. The most commonly used chemical agent for the induction of polyploidy is colchicine. The method is referred as colchiploidization and the induced polyploids can be called as colchiploids. This method of artificial induction of

polyploidy by using colchicine was developed by Blakeslee and Avery (1937). Ploidy levels could be easily determined by flow cytometry technique (Allum *et al.*, 2007). In this context, the objective of the present study was to determine the effective concentration of colchicine and the appropriate duration of treatment for polyploid induction and thus improving the flower character.

Materials and methods

The investigation, *in vivo* induction of polyploidy was carried out in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani during 2019-21.

Plant material: For the induction of polyploidy, the plant material chosen was 8-12 cm long *D. crumenatum* plantlets and the antimitotic agent used for the study was colchicine.

Polyploidization: The plantlets were subjected to eight different colchicine treatments. The treatments were based on colchicine concentration (0.05%, 0.1%) and the duration of exposure of plantlets to these doses of colchicine (24, 48, 72, 96 h) (Vichiato *et al.* (2007). Colchicine solution for the treatment (0.05% and 0.1%) was prepared using sterile distilled water. The plantlets for the experiment were subjected to different treatments by completely immersing it in colchicine solution. Then, these were kept in dark till the end of their respective treatment duration. Constant air bubbling was achieved with domestic aquarium aerating pumps in order to prevent the damage caused by oxygen depletion. After each treatment, the treated plants were thoroughly washed in running water, followed by washing in sterile distilled water, to remove the excess of colchicine. The plants were then properly planted in black plastic pots using coconut husk chips, charcoal, brick pieces as substrate. The design for the study was CRD (completely randomized design) and for each treatment, 6 replications were carried out. The control for each treatment of the experiment were plantlets which were subjected to same conditions but without colchicine. Management and fertilizer application were undertaken as per Package of Practices Recommendations of Kerala Agricultural University (KAU, 2016).

Survival rate and response: The observations, percentage survival of plantlets after colchicine treatment (observation taken immediately after potting of the treated plantlets) and percentage of plants showing response after colchicine treatment (observation recorded after 2 weeks of colchicine treatment) were recorded.

Vegetative characters: The observations, plant height (cm), shoot diameter (cm), pseudobulb height (cm), pseudobulb diameter (cm), number of leaves, length of leaves (cm), width of leaves (cm) of the treated and control plantlets were recorded after 6 months of treatment.

Statistical analysis: The observations recorded for vegetative characters were subjected to Analysis of Variance (ANOVA) and correlation analysis. Statistical analysis of the data was performed using the software, GRAPES (Gopinath *et al.*, 2020) provided by Kerala Agricultural University. Treatment means were compared by calculating the critical difference (CD) at 5 per cent level of significance.

Flow cytometry analysis: For evaluating the change in ploidy level of *D. crumenatum*, flow cytometry analysis was performed for the treated plantlets at Rajiv Gandhi Institute for Biotechnology, Thiruvananthapuram For the sample preparation for loading onto FACS machine (BD FACSAria II) for flow cytometry analysis, leaves were collected from plants after 6 months since colchicine treatment. The flow cytometry analysis was performed based on the procedure of Dolezel *et al.* (2007).

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 *D. crumenatum*, which is the control (diploid). Leaf sample (30mg) was carefully weighed by avoiding the midrib and placed in the centre of plastic petridish. 1 mL of ice-cold Otto I buffer was added to one side of the petri dish. The leafbit was chopped into very fine slices. Homogenate was mixed by pipetting up and down for several times (air bubbles were avoided). It was then incubated for 15 minutes in ice (4^{o}C). The homogenate was filtered using a 40 µm cell strainer into a labelled Eppendorf tube (2mL), so that the sample volume was above 500 µL. The tubes were centrifuged at 150g (g- relative centrifugal force) for 5 minutes at 4^{o}C. The nuclei were resuspended by gentle shaking and 100 µL fresh ice cold Otto I buffer was added and stored at 4^{o}C. After that, Otto II buffer was added to the nuclear suspension. 60 µL of RNase and PI was added, as the requirement is 50 µL mL^{-1}. It was incubated for 5 minutes and filtration using 40 µm cell strainer was done onto a labelled FACS tube and tubes were wrapped with an aluminium foil. While loading onto FACS machine (BD FACSAria II) of Rajiv Gandhi Institute for Biotechnology, Poojapura, the aluminium foil wrapped around the tube was removed. 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.

Stomatal observations: The plants which exhibited a change in ploidy level were carefully observed for stomatal observations/ parameters. For noting stomatal observations, clear fingernail polish was applied to the abaxial side of the leaf for 2x2 cm square. After the nail polish had dried, it was carefully removed using needle and fine tip forcep and was mounted onto a dry microscopic slide (Cramer, 1999). The stomatal frequency and size of diploid and tetraploids were compared and analyzed.

Results and discussion

The present study was carried out to develop polyploid *D. crumenatum* orchids which would subsequently contribute for improvement in floral characteristics and shelf life. The maximum survival percentage (100%) were noticed in the treatments (0.05% colchicine, 24 h), (0.10% colchicine, 24 h) and (0.01% colchicine, 48 h). The minimum survival percentage (66.67%) were recorded in the treatments (0.05% colchicine, 96 h) and (0.10% colchicine, 96 h). Control plantlets which were subjected to same conditions, but without colchicine treatment recorded 100 % (for 24 h, 48 h, 72 h) and 83.33 % (96 h) survival percentage.

The highest percentage of plants showing response after

colchicine treatment (83.33%) were noticed in treatments (0.05% colchicine, 24 h) and (0.05 % colchicine, 48 h). The lowest percentage of plants showing response after colchicine treatment (16.67 %) was noticed in treatment (0.10% colchicine, 96 h). Control plantlets which were subjected to same conditions, but without colchicine treatment recorded 100 % (for 24 and 48 h) and 83.33 % (72 and 96 h) survival percentage (Table 1).

Table 1. Percentage survival and percentage of *D. crumenatum* plantlets showing response after colchicine treatment via *in vivo* method of induction

Treatments	Plants survival (%)		Plants showing response (%)	
	Treated	Control	Treated	Control
T_1 (0.05%, 24 h)	100	100	83.33	100
T_2 (0.10%, 24 h)	100	100	66.67	100
T_3 (0.05%, 48 h)	100	100	83.33	100
T_4 (0.10%, 48 h)	83.33	100	50	100
T_5 (0.05%, 72 h)	83.33	100	66.67	83.33
T_6 (0.10%, 72 h)	83.33	100	50	83.33
T_7 (0.05%, 96 h)	66.67	83.33	33.34	83.33
T_8 (0.10%, 96 h)	66.67	83.33	16.67	83.33

When *D. crumenatum* plantlets were subjected to colchicine treatment, a decrease in the survival percentage was observed when the observation was taken immediately after potting of the treated plantlets. It was observed that as the duration for exposure towards colchicine treatment increased, there was a reduction in the survival percentage of plantlets. Similar conclusions were also reported by Silva *et al.*, 2000; Atichart and Bunnag, 2007. This might be due to the toxic impact of high concentration of colchicine to plant cell, which causes the blockage of spindle fiber development and modifying the differentiation process contributing to plant death eventually as reported by Petchang, 2010 and Blasco *et al.*, 2015. In contradiction to the present study, Vichiato *et al.* (2007) observed 100 per cent survival in all the treatments for polyploidy induction via *in vivo* technique in *Dendrobium nobile*. The control for the experiment which was maintained in the same experimental condition but without colchicine also showed a difference from 100 per cent survival percentage in few cases. This indicated that the apart from the toxicity due to colchicine, the complete immersion of plantlets in water has got a significant impact on the survival of the plantlets, when the duration of immersion period is increased.

On performing statistical analysis, it was concluded that significant variations were observed among all the treatments. (Table 2) The characters length of shoot, diameter of pseudobulb, number of leaves and width of leaf showed an increased mean value than their corresponding control means. The mean values of the characters, diameter of shoot and length of leaves noted lower mean values than their control. The mean value for the character height of pseudobulb were found to be same for treated and control plantlets. Similar effect of colchicine in increasing the plant height was also reported by Vichiato *et al.* (2014). Vichiato *et al.* (2014) and Pham *et al.* (2019) observed a decreased mean value for shoot diameter for diploids when compared to the tetraploids. Zakizadeh *et al.* (2020) observed an increased mean value for the characters, pseudobulb height and diameter, leaf length and leaf width, incase of tetraploids than diploids. Mohammadi *et al.* (2021) reported an improvement in number of leaves and leaf width of tetraploids than diploids. Thus, it was concluded from the present study that, the deviations of the observations of treated plants from control were mainly due to impact of antimitotic agent (colchicine) on the treated material (Fig. 1). On performing correlation analysis, significant positive genotypic correlation was observed between the characters; length of shoot and height of pseudobulb, diameter of shoot and width of leaf, length of pseudobulb and number of leaves, number of leaves and length of leaf, width of leaf and number of leaves (Table 3).

Flow cytometry histogram of treated and control samples: Single major peak P2 was obtained at channel number 50 (X axis) indicating diploid condition (2n=38) when the control or untreated samples were analyzed (Fig. 2a.). Two peaks P2 and P3 were obtained at channel number 50 and 100 (X axis) indicating mixoploidy with bothdiploid and tetraploid condition. (Fig. 2b.). Single major peak P3 was obtained at channel number 100 (X axis) indicating the tetraploid condition (2n=76) (Fig. 2c.).

In the *in vivo* colchicine treated *D. crumenatum* samples, the

Table 2. Mean performance of vegetative characters in colchicine treated *Dendrobium crumentaum plants* via *in vivo* method of induction

Treatment	Shoot length (cm)		Shoot diameter (cm)		Height of pseudobulb (cm)		Diameter of pseudobulb(cm)		Number of leaves		Length of leaf (cm)		Width of leaf (cm)	
	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control
T_1	15.16	12.5	0.20	0.20	7.2	7	0.65	0.57	5.33	4	9.7	9.5	2.43	1.6
T_2	13	12.5	0.203	0.20	6.73	7	0.70	0.57	5.33	4	10.06	9.5	2.56	1.6
T_3	17.8	14	0.206	0.22	7.06	7.2	0.64	0.65	3.67	5	5.73	8.9	2.36	1.5
T_4	13.5	14	0.226	0.22	7.03	7.2	0.75	0.65	3.67	5	7.63	8.9	2.06	1.5
T_5	15.66	14	0.183	0.21	6.86	6.6	0.73	0.62	3	3	9.86	9.4	2.2	1.5
T_6	17.5	14	0.206	0.21	6.76	6.6	0.74	0.62	3.33	3	8	9.4	2.33	1.5
T_7	13.83	13.5	0.233	0.20	6.66	6.8	1.08	0.72	3.33	3	9.43	9	2.46	1.7
T_8	14.66	13.5	0.18	0.20	6.86	6.8	0.95	0.72	2.67	3	8.86	9	1.63	1.7
Mean	15.141	13.5	0.205	0.207	6.900	6.9	0.783	0.64	3.791	3.75	8.662	9.2	2.258	1.575
SE	0.462		0.01		0.111		0.017		0.373		0.076		0.075	
CD (5%)	1.385		0.029		0.331		0.052		1.117		0.229		0.223	

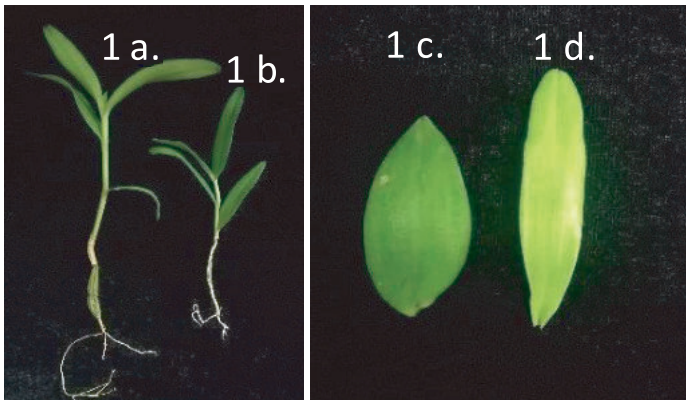

Fig. 1. *Dendrobium crumentum*: Plantlets- a. treated b. control; Leaves- c. treated d. control

maximum tetraploids (50%) were obtained from the colchicine concentration of 0.05% for the treatment duration 96 h. This result is comparable to the previous findings by Sanguthai *et al.* (1973), Watrous and Wimber (1988), Silva *et al.* (2000). In the present study, it was observed that the mixoploid induction was found to be more common than the tetraploid induction. It was also noted that, for the same exposure time *i.e.*, 96 h and colchicine concentration 0.10%, only mixoploids were obtained. In a similar study conducted by Vichiato *et al.* (2007) in *Dendrobium nobile*, highest number of tetraploids (29.17%) were obtained by immersing the plants in 0.1% colchicine solution for 96 h. (Table 4)

The stomata of tetraploids showed an increased size than the diploids whereas the stomatal density of tetraploids was found to be lower than that if diploids (Table 5) Silva *et al.* (2000) reported that on comparing diploid relatives with polyploid plants, polyploid plants displayed larger stomata and lower stomatal density and also observed that the number of chloroplasts per guard cell to be higher. Such stomata features have been efficiently used to distinguish the polyploid regenerants of several plant species in orchids.

Table 3. Genotypic correlation of vegetative characters in colchicine treated *Dendrobium crumenatum* via *in vivo* method ofinduction

	X_1	X_2	X_3	X_4	X_5	X_6
X_1	1.000					
X_2	-0.298*	1.000				
X_3	0.308*	-0.107	1.000			
X_4	-0.415*	0.285	-0.810**	1.000		
X_5	-0.312*	0.106	0.411*	-0.585**	1.000	
X_6	-0.329*	-0.469**	-0.369*	-0.032	0.315*	1.000
X_7	0.042	0.517**	-0.072	-0.325*	0.744**	0.073

*Significant at 5 per cent level **Significant at 1 per cent level X_1: Length of shoot, X_2: Diameter of shoot, X_3: Pseudobulb height, X_4 : Pseudobulb diameter, X_5: Number of leaves, X_6: Leaf length, X_7: Leaf width

Table 4. Flow cytometry analysis of *in vivo* colchicine treated *Dendrobium crumenatum* samples

Treatments	Mixoploid (%)	Tetraploid (%)
T_1	25	0
T_2	25	25
T_3	66.67	0
T_4	50	25
T_5	66.67	33.34
T_6	33.34	33.34
T_7	0	50
T_8	50	0

Table 5. Stomatal Analysis of *Dendrobium crumenatum* leaf samples

	Stomatal frequency (in 0.02mm^2)
Diploid	5.667
Tetraploid	3.33

With the present investigation, successful polyploids of *D. crumenatum* were obtained and which could later contribute for the ultimate objective like improved size, floral characteristics and shelf life. This indeed can be observed only when the plant reaches the flowering stage. While through the present study, it has been concluded that the most effective treatment for

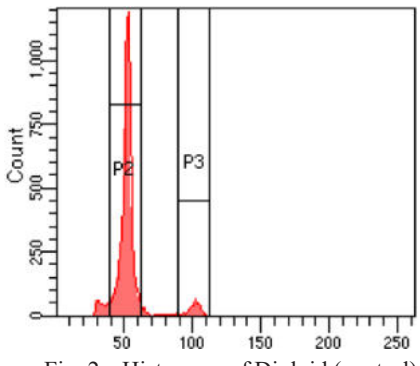

Fig. 2a. Histogram of Diploid (control)

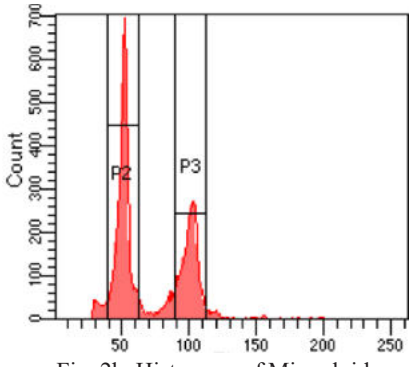

Fig. 2b. Histogram of Mixoploid

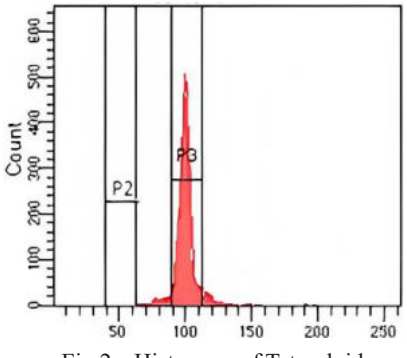

Fig.2c. Histogram of Tetraploid

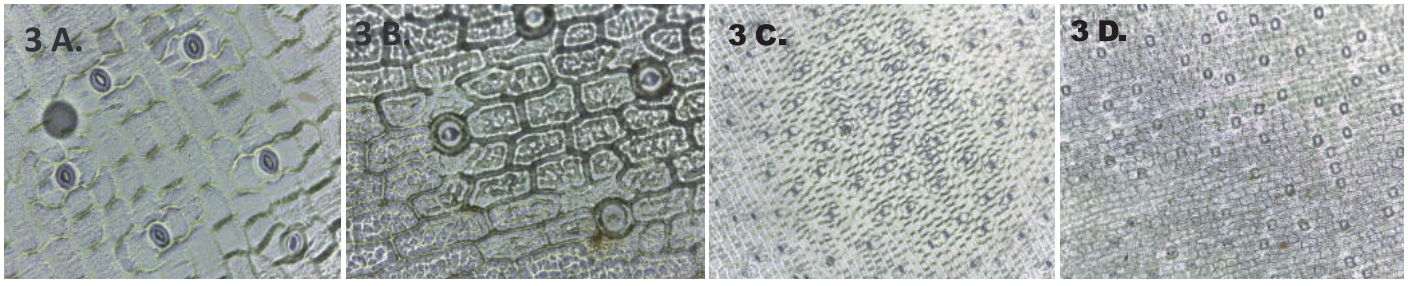

Fig 3. *Dendrobium crumenatum*: Stomata (400X magnification) A. Diploid B. Tetraploid, Stomata (100Xmagnification) C. Diploid D. Tetraploid

polyploidy induction in *D. crumenatum* via *in vivo* method is 0.05% colchicine for the treatment duration of 96 h.

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