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Induction and identification of colchicine induced polyploidy in *Gladiolus grandiflorus* 'White Prosperity'

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ABSTRACT

Gladiolus is one of the most important lucrative cut flower crops that is commercially cultivated worldwide due to its various spike forms, size, and shape and color combinations. In order to further increase the commercial and horticultural value by improving the ornamental traits of gladiolus 'White Prosperity', polyploidy was induced by soaking gladiolus corms in different colchicine concentrations (0.1%, 0.2% and 0.3%) for 24 h. Different colchicine concentrations had a little effect on sprouting and survival percentage but it significantly delayed the emergence of sprouts. About one third decreases in plant height along with reduction in number of leaves per plant, leaf area, length and width, chlorophyll content, diameter and number of cormlets per corm was observed in treated plants. Colchicine at 0.1% concentration improved the ornamental value of gladiolus by increasing vase life whereas colchicine at 0.3% was effective in increasing floret diameter. However, the colchicine treated plants exhibited delayed and reduced percentage of flowering corms. Pollen and stomatal study was done for the identification of polyploidy and it showed that both pollen and stomata size were increased while stomatal density and pollen fertility was significantly reduced in polyploid plants. Induction of polyploidy (mixoploids + octoploids) was achieved in all concentrations, however 0.2% and 0.3% concentrations of colchicine were effective for producing large number of polyploid plants. But at 0.1% concentration of colchicine, majority of plants did not show any change in their original ploidy level (tetraploid). These putative polyploids may be helpful for further improvement in ornamental and horticultural value of gladiolus.

Keywords: chromosome, diploid, pollen, stomata, tetraploid

INTRODUCTION

Gladiolus (*Gladiolus grandiflorus.* L.), is member of the Iridiaceae family and originated in South Africa that is cultivated worldwide due to its long elegant spikes with various shape, color and size of florets (Shaukat et al., 2013). It has high economic value for its widespread cut flower uses, ornamental plant in making bouquet, flower arrangements and for indoor decorations (Vasanthalumar et al., 2015). *Gladiolus* genus contains 180 species with ploidy level ranging from 2n=2x=30 to 2n=12x=180 (Larson, 2012). However, all the modern cultivars

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in this genus were developed from few wild species and are considered as tetraploids (Kole, 2011; Ohri, 2013).

Novelty in floral characteristics like flower color, shape, size, longest blooming period are the major preferences of consumers in gladiolus cut flower industry. Different breeding methods have been employed for genetic improvement of gladiolus cultivars. Induction of polyploidy is widely recognized as an effective technique among various breeding tools because it has broadened genetic base, development of breeding lines in a short time span, restores interspecific hybrid fertility and makes the viable crosses between different ploidy level genotypes (Pereira et al., 2014). It is a condition of having more than two sets of chromosomes (Tavan et al., 2015) and an important mechanism for plant evolution. Ability of polyploid plants to establish themselves in wide range of habitat and to survive in adverse environment makes them successful against their diploid ancestors due to presence of additional alleles which increase their heterozygousity (Alam et al., 2015). The most important results of polyploidy are increased in cell size due to the addition of extra gene copies. This effect of polyploidy is known as "gigas effect" (Sattler et al., 2015). It can be naturally or synthetically induced in plants by treating diploid cells with physical agents such as temperature chocks, X-rays and centrifugation or exposing plants tissue to mitotic inhibitor chemicals like colchicine, mercuric chloride, sulphanilamide, hexachloro-cyclohexane and veratrine (Kokate, 2011). In comparison to physical agents, the anti-mitotic chemicals are most commonly used for polyploidy induction, as these substances do not need any special equipment for application. Moreover, they are quite safe to handle in liquid medium (Lehrer et al., 2008). Colchicine is a chemical mutagen that is widely used for induction of polyploidy in various ornamental species like pelargonium (*Pelargonium graveolens*) (Jadrna et al., 2010), salvia (*Salvia hians*) (Grouh et al., 2011), Madgascar periwinkle (*Catharanthus roseus*) (Hosseini et al., 2013), orchid (*Dendrobium nobile*) (Vichiato et al., 2014), chrysanthemum (*Dendranthema indicum*) (He et al., 2016), Bougainvillea (*Bougainvillea glabra*) (Anitha et al., 2017), phlox (*Phlox drummondii*) (Dar et al., 2017) and swamp rosemallow (*Hibiscus moscheutos*) (Li and Ruter, 2017). Colchicine not only changes the chromosome number but also induces gene mutation in both seed and vegetatively propagated crops (Datta, 2009). It induces polyploidy by inhibiting the spindle fiber formation during cell division, chromosome gets multiplied but cell divisions do not occur, which results in production of polyploid cells (Rauf et al., 2006). It acts as point mutagen by changing the DNA nucleotide sequence, involving a single base or a base pair (Liu, 2012). Different plant parts like seed, apical meristems, flower buds and roots can be used to induce polyploidy (Sourour et al., 2014) however the best results have been obtained in a seed treatment. Success of polyploidy induction depends upon the colchicine application method, plant part used, species, concentration and duration of exposure. High concentration often leads to abnormalities in developing seedlings (Pirkooi et al., 2011).

Gladiolus is highly heterozygous crop which makes it an ideal plant material for polyploidy induction that results in the production of new forms in which one or few traits are improved without changing the whole genome. As gladiolus is lucrative crop in cut flower industry, so there is a need to create superior varieties within a short period of time through chromosome doubling because of continuous market demand. Therefore, the objective of present work was to induce polyploidy in gladiolus 'White Prosperity' through chemical mutagen colchicine. This work was aimed to develop diverse gladiolus breeding material having improved traits that could be used as parent material in future breeding programs.

MATERIAL AND METHODS

Experimental site

This research was conducted at research area of Department of Horticulture, PMAS-Arid Agriculture University, Rawalpindi, Pakistan from September 2015 to April 2016. This area lies between 34°N Latitude to 74°E Longitude. This is a semi- arid to sub-humid area where 80% rainfall occurs in monsoon season that continues from July to October (Rashid and Rasul, 2010).

Plant material and induction of polyploidy

Corms of gladiolus (*Gladiolus grandiflorus*) 'White Prosperity' with chromosome number 2n=4x=60 (Lim, 2012) were used for induction of polyploidy. Uniform sized corms of diameter 2.6 cm were purchased from Awan Seed Store, Islamabad, Pakistan. The non-dormant corms were soaked in 0.1%, 0.2% and 0.3% colchicine solution, while control corms were soaked in distilled water for 24 h time duration at room temperature. About 2-3 drops of DMSO (Dimethyl sulfoxide) were added

as a surfactant to colchicine solutions in order to increase colchicine penetration in cells. Corms were dipped in fungicide, copper oxychloride (0.8%) solution for 5 min. before planting in pots. Earthen pots (15 × 10 inch), planted with single corm, were filled with growing medium (Sand: Soil: FYM; 1:1:1), and were placed in a lath house covered with 40% green shading net. Irrigation frequency and weeding/hoeing were done as per requirements of the crop. This trial was carried out according to completely randomized design (CRD) having 4 treatments and 3 replications consisting of 16 corms in each replication. Data was analyzed by using one-way analysis of variance (ANOVA) through software "SPSS" and differences between means were compared by using Duncan's multiple range (DMR) test at 5% probability (Steel et al., 1997).

Morphological characterization

Morphological characters were compared between control and colchicine treated plants.

Vegetative parameters

Data was recorded for sprouting and survival of corms, number of sprouts per corms and number of days to sprouting. Plant height at flowering stage was measured from the base of a plant to the top of floral spike with measuring tape. Number of leaves per corm were calculated by counting, while the leaf area, leaf length and leaf width were calculated by using digital leaf area meter (AM-100, made ADC, UK) at flowering stage, whereas average leaf thickness was measured with vernier caliper. After harvesting, corm diameter was calculated with the help of vernier caliper and number of cormlets per corm was also counted manually.

Reproductive parameters

Days to spike initiation were counted from the corms planting date to the days when spikes started initiating, whereas days to open first floret was counted from the time of spike initiation to the days when first floret showed color. Floral spike length was measured from the base of the spike to the tip of a floral spike. Diameter of floret per spike was calculated by using measuring scale. Three florets were randomly selected per spike. Firstly, diameter of a single floret was measured in horizontal direction and then in vertical direction. Average of two measurements gives the floret diameter of a single floret. Number of florets was measured by counting total number of florets on a spike, while the thickness of harvested floral spike was measured

at the bottom of first floret with the help of vernier caliper. For inter floret length, average was taken by measuring distance between two florets on a spike.

Floral morphological variation was measured by visually observing differences in shape of florets. Percentage of flowering in each treatment was recorded by dividing total number of corms producing floral spike to total number of corms planted. Vase life was calculated when 50% florets on a floral spike were wilted. Freshly harvested spikes were placed in 2% sodium hypochlorite (NaOCl) solution in order to prevent the spikes from microbial contamination (Khezri et al., 2006). Fresh weight was measured on electronic balance right after the floral spikes were freshly harvested from the field. Floral spikes were dried in dry oven for 24 h at 65°C before taking their dry weight with the help of electronic balance.

Leaf and flower color

The leaf and flower color were measured with Chroma meter (Konica Minolta, CR-400) by using method of Zhang et al. (2014). Three color parameters (L*, a* and b*) were used to describe the color of object surface. L* represents brightness/lightness (higher values show the brighter color of surface). L* value varies from 0 to 100 as object surface color changes from black to white. Value a* is related to the redness and the greenness (negative value is for green color and positive for the red color of surface). b* is for yellowness and blueness index (negative value for blue and positive for the yellow color surface). Saturation or chrome value was calculated by formula $c = (a^2 + b^2)^{1/2}$ and hue angle h^o was calculated by equation $h^o = \arctan (b*/a*)$.

Micro-morphological parameters evaluation and ploidy estimation

Stomatal size and density

Stomatal characteristics were measured by the method of Omidbaigi et al. (2010). Well expanded, enlarged leaves at mature stage (flowering) were taken from both control and treated plants. Thick layer of transparent nail polish was applied to the small area of abaxial side of leaves and was left for drying. After that, the strip of dried nail polish having impression of leaf epidermis was removed through pointed tip forceps and placed on a microscopic slide. Stomatal size and density was observed under light microscope (Noif XSZ-107BN) at 400× magnification and stomatal size was measured by using ocular micrometer.

Pollen fertility

Pollen fertility status was measured by the method described by Oates et al. (2012). Pollen grains were collected from newly opened florets and placed on a microscopic slide. Pollen grains were stained with 1% aceto-carmine through micro-pipette and kept for drying for 15-20 min. at room temperature. Cover slip was placed after drying and pollen grain fertility and diameter was observed under light microscope (Noif XSZ-107BN) at 100x and 400x magnification. Normal shaped and well stained pink/red color pollen grains were scored as fertile while ruptured, collapsed and unstained pollen grains were considered as sterile. Pollen grain diameter was measured through ocular micrometer.

Biochemical analysis

Chlorophyll content

Chlorophyll content was measured by using the method of Makeen et al. (2007). Pigments were extracted by dissolving 100 mg of fresh mature leaf sample (at flowering stage) in 10 ml of 80% acetone and stored in dark for overnight at 4°C after mixing. Extraction solution was centrifuged at 5000 rpm for 10 min. and its absorbance was measured by spectrophotometer at 663 and 645 nm wavelengths. Amount of chlorophyll a, b and total was calculated

according to method described by Arnon (1949): a (mg g FW) = $11.64 \times (A_{663}) - 2.16 \times (A_{645})$, b (mg g FW) = $20.97 \times (A_{645}) - 3.94 \times (A_{663})$ and total (mg g FW) = $20.2 \times (A_{645}) + 8.02 \times (A_{663})$.

RESULTS

Impact of different concentrations of colchicine on sprouting and survival (%) is presented in Table 1. Results showed that there was no significant effect of different colchicine concentration on sprouting and survival of corms as there was only a slight decrease in sprouting and survival rate. Survival (%) ranges from 83.3% to 87.5% whereas sprouting (%) was 98% to 96% in all colchicine concentrations while the control plants sprouted and survived in 100%.

Vegetative response of control and colchicine treated plants of gladiolus 'White Prosperity' is summarized in Table 1. The first visible toxic effect of colchicine was delayed emergence of sprouts. Corms at 0.2-0.3% colchicine concentration took more days to sprout (15.8-16.8 days) as compared to control ones (8.3 days). Slight increase in number of sprouts per corm was observed with an increase in colchicine concentration. Significant increase in number of sprouts per corm at 0.2% (1.2 sprouts) and 0.3% (1.2 sprouts) was recorded as compared

Table 1. Vegetative parameters of control and colchicine treated plants of gladiolus 'White Prosperity'

Parameters		Control	Colchicine concentration (%)		
			0.1	0.2	0.3
Sprouting of corms (%)		100.0 a	98.0 a	98.0 a	96.0 a
Survival of corms (%)		100.0 a	83.3 a	84.2 a	87.5 a
Days to sprouting (No.)		8.3 ± 0.6 c	12.3 ± 1.2 b	15.8 ± 0.6 a	16.8 ±1.2 a
Sprouts per corm (No.)		1.0 ± 0.0 b	1.0 ± 0.0 b	1.2 ± 0.0 a	1.2 ± 0.0 a
Plant height (cm)		91.5 ± 6.6 a	67.4 ± 4.1 b	68.1 ± 2.5 b	65.0 ± 5.4 b
Leaves per plant		9.5 ± 0.4 a	6.4 ± 0.5 b	6.7 ± 0.3 b	7.0 ± 0.2 b
Leaf area (cm^2)		97.8 ± 8.8 a	43.2 ± 1.3 b	47.8 ± 3.1 b	42.3 ± 0.2 b
Leaf length (cm)		44.6 ± 1.5 a	23.2 ± 1.3 b	24.1 ± 1.0 b	26.0 ± 0.1 b
Leaf width (cm)		3.6 ± 0.0 a	1.9 ± 0.0 b	2.0 ± 0.1 b	1.9 ± 0.1 b
Leaf thickness (mm)		0.6 ± 0.0 b	0.7 ± 0.1 ab	0.7 ± 0.0 ab	0.8 ± 0.1 a
Corm diameter (cm)		8.8 ± 0.1 a	4.3 ± 0.1 b	4.2 ± 0.1 bc	3.94 ±0.0 c
Cormlets per corm (No.)		92.3 ± 3.7 a	32.0 ± 3.3 b	38.1 ± 9.3 b	48.7 ± 2.9 b
Leaf color**	L*	15.4 ± 0.5 b	26.7 ± 3.4 a	27.0 ± 1.1 a	23.8 ± 0.6 a
	a*	-9.6 ± 1.2 b	-2.9 ± 1.1 a	-4.9 ± 0.7 a	-2.9 ± 0.5 a
	b*	4.6 ± 0.6 b	6.4 ± 0.8 ab	7.2 ± 0.1 a	6.2 ± 0.5 ab
	c	16.3 ± 0.6 a	9.3 ± 2.6 b	8.8 ± 1.1 b	8.8 ± 0.4 b
	hº	169.2 ± 3.6 a	147.9 ± 10.5 a	143.1 ± 10.3 a	142.5 ± 12.9 a

*Mean values ± SE in lines followed by different letter(s) are significantly different at $p \leq 0.05$ according to Duncan's test. Mean n = 48 (16 plants × 3)
**L*= lightness, a* = redness/greenness, b* = yellowness/blueness, c = chrome and hº = hue angle

Figure 1. Impact of different concentrations of colchicine on leaves characteristics in gladiolus 'White Prosperity': wider, long and smooth surface leaves in control plants (a) while in colchicine treated plants leaves were shorter, thicker and had rough surface leaves at 0.1% (b), 0.2% (c) and 0.3% concentration (d)

to 0.1% colchicine concentration (1.0 sprout) and control (1.0 sprout).

Colchicine had inhibiting effect on growth of plant and significantly reduced the plant height in all concentrations. In treated plants, one third decrease in plant height ranging from 65.0-68.1 cm, was noticed as compared to non-treated plants (91.5 cm). Colchicine also exhibited inhibitory effect on leaves production as it reduced the number of leaves in all treated plants (6.4-7.0 leaves per plant) as compared to control ones (9.5 leaves per plant). Different concentrations of colchicine decreased the leaf area (43.2-47.8 cm^2), leaf length (23.2-26.0 cm) and leaf width (1.9-2.0 cm) whereas the control ones showed more leaf area (97.8 cm^2) with more leaf length (44.6 cm) and leaf width (3.6 cm). In treated plants at 0.3% colchicine, the leaf thickness was increased (0.8 mm) with comparison to control plants (0.6 mm). Moreover, leaves of treated plants had rougher surface whereas it was smooth in control plants (Fig. 1). In all colchicine treated plants, leaves had light green color as shown by increase in lightness/darkness (L*) value (23.8-27.0) and in control minimum L* value (15.4) represents dark color. Red/green index value (a*) was lower in control ones (-9.6) but it significantly increased in colchicine treatments, ranging from -2.9 to -4.9. As negative value of a* defines the green color thus low value of a* in control treatment represented leaves of darker green color, while in colchicine treatment increase in a* value depicted bright green color of leaves. Yellow/blue index value (b*) increased in colchicine 0.2% treatment (7.2) as compared to control plants (4.6). As the negative value of b* is used to represent the bluish color and positive value is for yellowish color. So, increase in value from control to colchicine treated plants gives an indication of change in color from

darker (blue) in control to brighter one (yellow) in different colchicine treated plants. Chroma value (c) in different colchicine treatments significantly decreased from 9.3 to 8.8 as compared to highest values in control (16.3) showing that leaves of highly saturated color. However, the hue angle (h^o), value did not show any difference between control and colchicine treated plants. Largest corm diameter was examined in control treatment. However, corm diameter (3.9-4.3 cm) was significantly reduced in different colchicine concentrations. Colchicine also adversely affected the multiplication rate of corms and reduced the number of cormlets per corm (32.0-46.9) at all concentrations in comparison to control plants.

Reproductive response of control and colchicine treated plants of gladiolus 'White Prosperity' is summarized in Table 2. Data showed that colchicine concentration 0.2 and 0.3% caused a significant delay in days required for floral spike initiation (167.7-180.7 days) and opening of first floret (180.3-191.3 days). While floral spike emerged early in 0.1% and control treatment (110.7 and 85.7 days) and took less number of days for first floret opening (129.7 and 107.7 days). Delay in flowering due to slow growth and development showed that after 100 days of treatment, colchicine treated plants (0.1%, 0.2% and 0.3%) were still in vegetative stage and continued producing leaves whereas in control plants reproductive growth had started and floral spikes were emerging from the leaf whorl (Fig. 2). Increase in colchicine concentration (0.2% and 0.3%) had a negative effect and it decreased floral spike thickness and number of florets per spike but floral spike length was reduced only in 0.2% concentration (Fig. 3). However maximum inter floret length per spike (3.9 cm) was recorded at 0.2% colchicine concentration while no significant

Table 2. Reproductive parameters of control and colchicine treated plants of gladiolus 'White Prosperity'

Parameters		Control	Colchicine concentration (%)		
			0.1	0.2	0.3
Days to spike initiation (No.)		85.7 ± 0.9 b	110.3 ± 25.4 b	167.7 ± 3.0 a	180.7 ± 17.9 a
Days to first floret opening (No.)		107.7 ± 1.8 b	129.7 ± 21.7 b	180.3 ± 3.3 a	191.3 ± 16.8 a
Floral spike length (cm)		74.7 ± 4.4 ab	86.3 ± 8.0 a	61.2 ± 4.3 b	70.3 ± 9.9 ab
Florets per spike (No.)		11.0 ± 0.2 ab	12.7± 0.3 a	10.7 ± 0.2 b	10.8 ± 0.6 b
Floral spike thickness (cm)		8.7 ± 0.4 ab	9.3 ± 0.7 a	7.1 ± 0.4 b	7.1 ± 1.0 b
Inter floret length (cm)		3.3 ± 0.1 b	3.5 ± 0.8 b	3.9 ± 0.1 a	3.5 ± 0.1 b
Floret diameter per spike (cm)		7.3 ± 0.1 b	7.1 ± 0.2 b	8.2 ± 0.3 ab	8.9 ± 0.7 a
Variation in floral morphology (%)		0.0 b	33.3 ab	66.7 a	0.0 b
Flowering corms (%)		35.4 a	8.3 b	12.5 ab	6.3 b
Vase life (No. of days)		9.3 ± 0.4 b	10.7 ± 0.3 a	8.0 ± 0.6 c	8.0 ± 0.0 c
Fresh weight (g)		62.0 ± 6.7 ab	69.0 ± 13.6 a	40.8 ± 2.7 b	47.7 ± 3.2 ab
Dry weight (g)		6.2 ± 0.7 ab	7.0 ± 1.5 a	3.3 ± 0.2 b	4.3 ± 0.3 ab
Flower color**	L*	76.1 ± 2.0 bc	71.7 ± 4.2 c	99.6 ± 6.7 a	89.8 ± 4.7 ab
	a*	-1.4 ± 0.2 a	-2.1 ± 0.3 a	-2.2 ± 0.9 a	-2.7 ± 0.3 a
	b*	0.8 ± 0.0 b	2.7 ± 0.4 a	3.1 ± 0.4 a	2.9 ± 0.1 a
	c	1.7 ± 0.1 b	3.4 ± 0.3 a	4.2 ± 0.3 a	3.6 ± 0.5 a
	h°	185.9 ± 11.5 a	150.6 ± 13.3 a	154.4 ± 22.8 a	164.4 ± 25.3 a

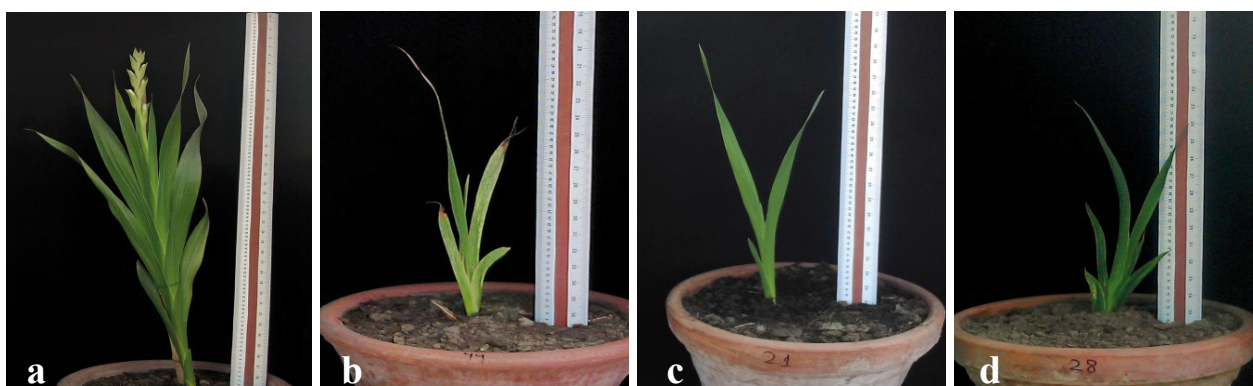Explanations: see Table 1

Figure 2. Impact of different concentrations of colchicine on plant growth of gladiolus 'White Prosperity' after 100 days: spike emergence was started in control plants (a) whereas in treated plants vegetative stage still going on at 0.1% (b), 0.2% (c) and 0.3% colchicine (d)
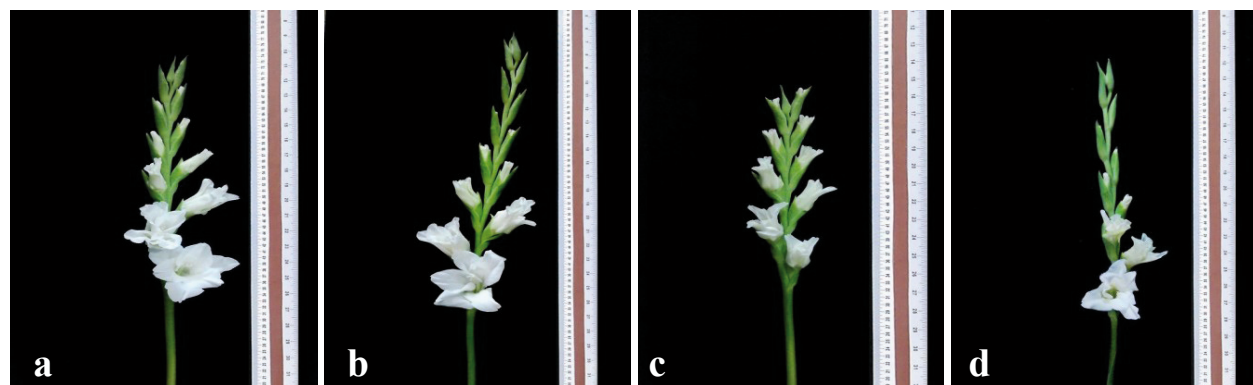

Figure 3. Impact of different concentrations of colchicine on floral spike length and number of florets per spike in gladiolus 'White Prosperity': spike length and number of florets per spike slightly increases at 0.1% colchicine (b) as compared to control (a) however floral spike length and number of florets per spike reduces at 0.2% (c) and 0.3% colchicine (d)

Figure 4. Impact of different concentrations of colchicine on floret diameter in gladiolus 'White Prosperity': floret diameter was less in control (a) and at 0.1% (b) but it was increased at 0.2% (c) and was maximum at 0.3% colchicine (d)
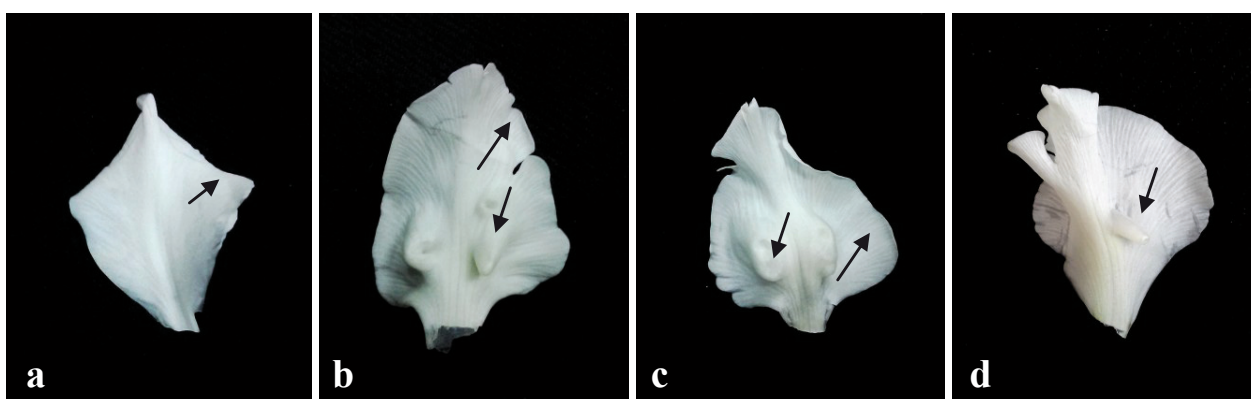

Figure 5. Morphological variation in flower petals of gladiolus 'White Prosperity': (arrows represent variation) petals had smooth edges and triangular shape in control plants (a) pointed outgrowth appeared on petal surface in treated plants along with elongated petal shape and serrated margins at 0.1% colchicine (b) flower produced oval shaped petals whose one end had ruffled edges at 0.2% colchicine (c) petals with pointed outgrowth were also produced at 0.2% colchicine (d)

change in inter floret length was observed at 0.1%, 0.3% colchicine and in control as well.

Plants treated with 0.3% colchicine concentration produced larger floret diameter (8.9 cm) as compared to smaller size (7.3 cm) of control and of colchicine 0.1% (7.1 cm) as shown in Figure 4. Although colchicine increased the floret diameter, while on the other hand, it also altered the flower morphology. Significant variations in floral morphology were observed only at 0.1% (33.3%) and 0.2% (66.7%) concentration. In control plants, flowers had petals with smooth edges and triangular shape but at 0.1% colchicine, flower petals became elongated with serrated margins along with pointed outgrowth on petals. At 0.2% colchicine, flowers produced oval shaped petals with ruffled edges at one side and pointed outgrowth on surface (Fig. 5). Flowering percentage in control plants was upto 34% but it was significantly reduced in 0.1% (8.3%) and 0.3% (6.3%) colchicine concentration. Effect of colchicine treatment on flower color was studied through different color parameters L*, a*, b*, c and h°. Brightness (L*) of flower color increased at 0.2% (99.6) and 0.3% colchicine concentration (89.8) whereas lightness value L* decreased at 0.1% colchicine concentration and in control. Yellow/ blue index (b*) value was minimum (0.8) in control but increased (2.7-3.1) in colchicine treatments. As the negative value of b* depict the bluish color and positive is used to define yellowish color. Thus, increase of b* value from control to all colchicine concentration represents the change of color from blue (dark color) in control to yellow (light color) in colchicine. Similarly, chroma values (c) were also increased in all colchicine treatments (3.4-4.2) whereas in control it was 1.7. These results showed that highly saturated color flowers were produced at all colchicine treated plants. Hue angle (h°), and red/ green index (a*) value did not significantly changed among control and different colchicine treatments.

Table 3. Micro-morphological parameters (stomata and pollen grain) evaluation for ploidy estimation

Parameters	Control	Colchicine concentration (%)		
		0.1	0.2	0.3
Pollen grain diameter (µm)	63.6 ± 3.5 b	74.6 ± 6.9 ab	90.8 ± 3.7 a	90.9 ± 5.3 a
Pollen grain fertility (%)	82.9 ± 1.0 a	82.0 ± 3.8 a	76.7 ± 2.1 ab	71.9 ± 2.6 b
Stomata length (µm)	22.6 ± 0.5 b	27.1 ± 1.5 a	27.1 ± 0.7 a	25.6 ± 0.4 a
Stomatal width (µm)	18.0 ± 0.9 b	21.1 ± 0.9 a	21.6 ± 1.0 a	21.2 ± 0.4 a
Stomatal density (mm^2)	18.8 ± 0.2 a	12.6 ± 1.0 bc	10.8 ± 0.3 c	13.3 ± 0.5 b

*Mean values ± SE in lines followed by different letter(s) are significantly different at $p \leq 0.05$ according to Duncan's test. Mean n = 48 (16 plants × 3)

Vase life of floral spike was maintained up to 9.3 days in control however it slightly increased at 0.1% colchicine concentration (10.7 days). Fresh weight and dry weight of floral spikes were significantly same at 0.1%, 0.3% colchicine and control, except 0.2% colchicine concentration where both decreased significantly.

Pollen grain diameter has direct correlation with a colchicine concentration and it increases with an increase in concentration. Minimum pollen grain diameter was recorded in control (63.6 µm) and 0.1% colchicine treated plants (74.6 µm) whereas it increased in 0.2-0.3% colchicine treated plants (90.8-90.9 µm) (Tab. 3, Fig. 6). Maximum pollen grain fertility (82.9%) was observed in control plants while it significantly reduced in 0.3% colchicine (71.9%) (Fig. 7). In colchicine treated plants, stomata had larger length (25.6-27.1 µm) and width (21.1-21.6 µm) but stomatal density was decreased (10.8-13.3 mm^2). An increase in stomatal
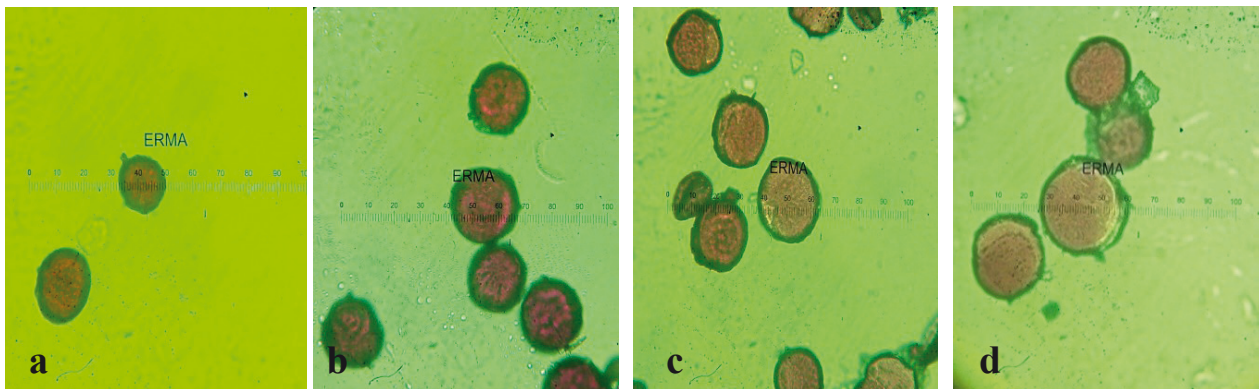

Figure 6. Impact of different concentrations of colchicine on pollen grain diameter (µm) in gladiolus 'White Prosperity': At 400× magnification, pollen grain diameter was minimum in control (a) whereas it increases at 0.1% (b), 0.2% (c) and 0.3% colchicine (d)
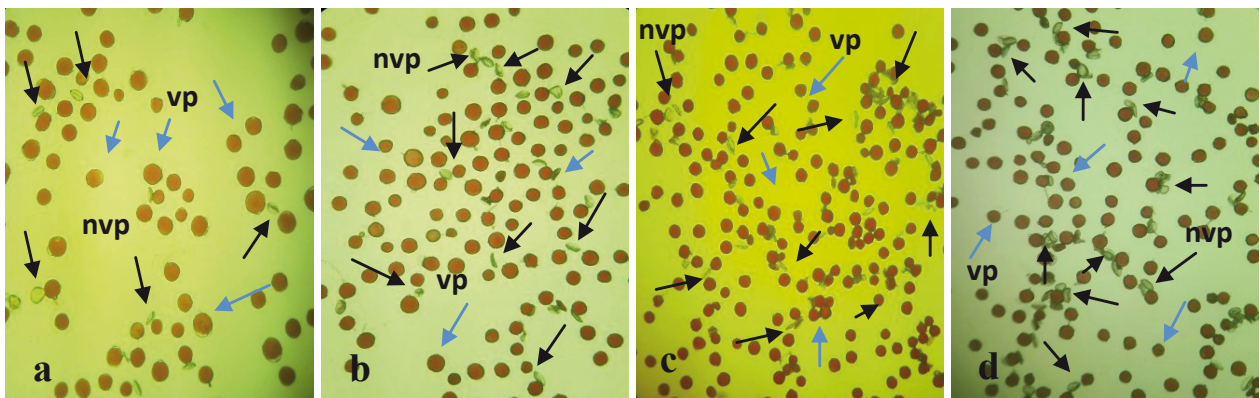

Figure 7. Impact of different concentrations of colchicine on pollen grain fertility percentage in gladiolus 'White Prosperity' (vp: viable pollens were of red circular shaped as shown by blue arrows whereas nvp: non- viable pollen was rupture and not fully stained as depicted by black arrows). At 100× magnification, pollen grains had high fertility in control plants (a) while it reduces at 0.1% (b), 0.2% (c) but significantly decreases at 0.3% colchicine (d)
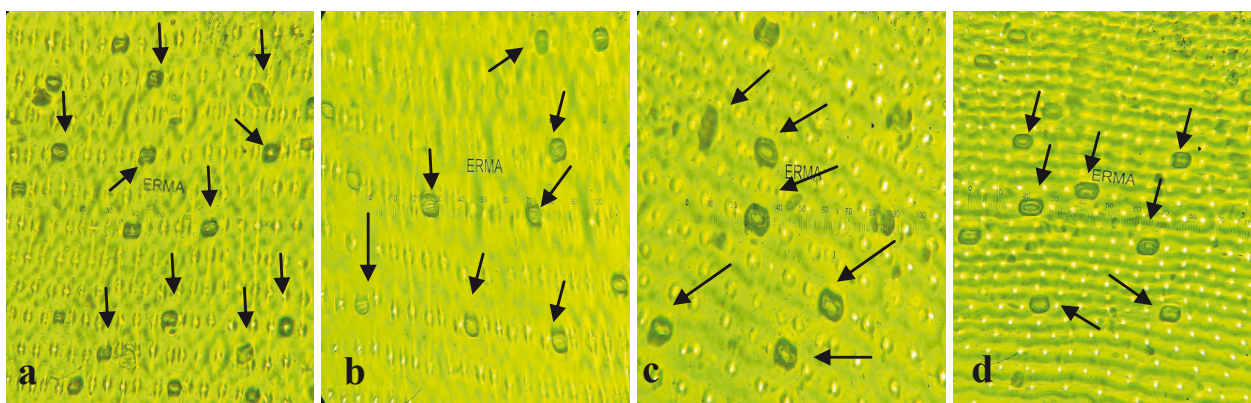
Figure 8. Impact of different concentrations of colchicine on stomatal characteristics in gladiolus 'White Prosperity' (Arrows represent stomata). At 400× magnification stomatal length and width was minimum but density was maximum in control plants (a) however stomatal length and width increases with decrease in stomatal density at 0.1% (b), 0.2% (c) and 0.3% colchicine (d)

density (18.8 mm^2) with reduced stomatal length (22.6 µm) and width (18.0 µm) were observed in control plants (Fig. 8).

Colchicine reduced the chlorophyll a (6.5-11.0 mg g FW), chlorophyll b (9.1-14.1 mg g FW) and total chlorophyll content (16.6-27.3 mg g FW) in comparison with control plants (Tab. 4).

Stomata and pollen grain study used for the identification of putative polyploids depicted that all colchicine concentrations were equally effective in the induction of octoploids (17-18%) (Tab. 5). However, generation of mixoploids plants was increased with an increased in colchicine concentration. Highest mixoploid plants (43%) were obtained at 0.3% colchicine concentration as compared to minimum rate of mixoploidy at 0.1% (31%) and 0.2% (38%) colchicine concentration. Colchicine concentration at 0.1% was too low to induce sufficient polyploidy as it did not change the

ploidy level of majority tetraploid plants (52%). But with an increase in colchicine concentrations, the percentage of tetraploid plants was reduced (44% and 40%) at 0.2% and 0.3% colchicine concentration respectively.

DISCUSSION

Results showed that colchicine at different concentrations did not significantly affect the sprouting and survival of gladiolus plants which might be due to the induction of plant defence mechanisms. Different defence mechanisms such as activation of specific ion channels, deposition of callose (polysaccharides) at cell wall, synthesis of reactive oxygen species (ROS), secondary metabolites, heat shock proteins (HSP), phytohormones (ABA, jasmonates, brassinosteriods) and regulating the expression of

Table 4. Comparison of chlorophyll content among control and colchicine treated plants in gladiolus 'White Prosperity'

Parameters	Control	Colchicine concentration (%)		
		0.1	0.2	0.3
Chlorophyll a (mg g FW)	16.6 ± 0.9 a	6.5 ± 0.9 c	10.2 ± 0.8 b	11.0 ± 0.6 b
Chlorophyll b (mg g FW)	22.6 ± 0.9 a	9.1 ± 1.2 c	13.0 ± 1.2 b	14.1 ± 1.0 b
Total (mg g FW)	41.1 ± 2.0 a	16.6 ± 2.3 c	23.9 ± 2.1 b	27.3 ± 1.7 b

Explanations: see Table 3

Table 5. Polyploidy induction (%)

Colchicine concentration (%)	Putative polyploids (%)		
	Tetraploid	Mixoploid	Octoploid
0.0 (Control)	100	0	0
0.1	52	31	17
0.2	44	38	18
0.3	40	43	17

genes makes the plant able to tolerate and survive against the phytotoxic chemical stress environment (Rejeb et al., 2014). A non- significant effect of colchicine on germination and survival percentage of black wattle (*Acacia mearnsii*) was observed when its seeds were grown on colchicine saturated filter papers (Harbard et al., 2012).

Gladiolus corms took a considerable number of days for sprouting at 0.2-0.3% colchicine concentration which might be due to the reduction in cell division rate. Haouala et al. (2009) noticed a slow pattern of germination process in colchicine treated sea trigonella (*Trigonella maritima*) seeds due to decrease in rate of division of meristematic cells and slow cell differentiation. Although there was a delayed sprouting process, however slight increase in number of sprouts per corm was observed at 0.2 and 0.3% colchicine concentration which might be due to the induction of polyploidy in cells by colchicine. In tetraploid plants of arabidopsis (*Arabidopsis thaliana*), enhanced growth was associated with increase in ploidy level. This increase doubled the DNA content for gene expression which in turn improves the metabolic activity of cells thus promotes the growth rate (Breuer et al., 2007). The positive effect of increasing ploidy on growth was also studied in colchicine induced double haploid plants of oilseed rape (*Brassica napus*) (Mohammadi et al., 2012).

Slow and stunted growth of gladiolus plants due to colchicine treatment was one of the successful indicators of polyploidy induction. It caused a one third decrease in plant height of gladiolus treated plants that may be due to physio-chemical disturbance of cell and induction of polyploidy. Colchicine treated plants of alocasia (*Alocasia macrorrhizos*) showed slow growth and smaller size plants than control because of disturbance of normal cell division by the penetration of colchicine into the apical meristems cells (Thao et al., 2003). Previous findings reported reduced plant height in cotton (*Gossypium arboreum*) and hairy vetch (*Vicia villosa*) probably resulting from reduced respiratory ratio, metabolic enzyme's activity and restriction in auxin supply to cell under different colchicine treatment (Rauf et al., 2006; Tulay and Unal, 2010). Colchicine significantly decreases the number of leaves per plant, leaf area, length and width but it increases leaf thickness in 0.3% concentrations. This may be due to reduction in cell division that retard the growth or it may occur due to hormonal imbalances. In colchicine treated chaste tree (*Vitex agnus-castus*), reduced leaf size was associated with

mutant membrane, abnormal development of a cell wall and difficulty in expansion or elongation of polyploid cells (Ari et al., 2015) whereas increase in mulberry (*Morus nigra*) leaves thickness could be due to increase in the spongy and palisade tissue layers in leaves of polyploid plants (Ramesh and Murthy, 2014). Development of light green color of gladiolus leaves in colchicine treated plants was attributed to reduction in green pigment synthesis, i.e. chlorophyll. Previous studies showed that reduced chlorophyll content in colchicine induced tetraploid plants of mat rush (*Juncus effusus*) occurred due to structural modifications such as disintegration in lamellar/thylakoid membrane of chloroplast which effect the synthesis of chlorophyll (Xu et al., 2010). Reduction in corm diameter and corm multiplication rate of treated plants is also one of the negative effects of colchicine treatment which could be the result of polyploidy induction. Decreased diameter of orchid (*Dendrobium nobile*) pseudobulbs as observed by Vichiato et al. (2014) was due to induction of polyploidy and doubling of cell genomic material. It causes increase in utilization of stored food during different growth stages and flowering. This increase in cell size of polyploid plants resulted in increase in transfer of stored food which ultimately affects the size of pseudo bulbs.

In this study, subsequent delayed in emergence of floral spike was observed with an increase in colchicine concentration. Delay in flowering is one of the common phenomena of colchicine treated plants due to doubling of chromosome that result in reduced cell growth and extended period of vegetative growth. In colchicine treated sesame (*Sesamum indicum*) plants, Anbarasan et al. (2014) observed that inhibition of floral hormones causes a delay in flowering. However late flowering in tetraploid orchid (*Dendrobium nobile*) plants could be associated with increased cell size and cell nuclear volume. As colchicine increases the cell size so these cells need more time and energy for a DNA duplication which reduced the rate of cell division (Vichiato et al., 2014). In brassica plants, (*Brassica campestris*) there was a delay of 11 days in 50% flowering in colchicine treated plants as compared to control plants (Kumar and Dwivedi, 2014). Colchicine at 0.1% concentration causes a noticeable change in floral characteristics. Treated plants were superior to control plants because of largest floret diameter, longest and thicker floral spike with increased number of florets. Colchicine improves the floral traits by inducing polyploidy

which increases the cell size and thus increases the size of organ (gigas effect). Increase in vase life upto 10.7 days at 0.1% colchicine concentration may be due to increase in floral spike length and thickness. As longest floral spike facilitated more uptake of solution that kept the flowers turgid and prolonged the vase life. Moreover, an increase in spike thickness prevented the spike bending and helped to store more nutrients for flowers. In colchicine treated siam tulip (*Curcuma sparganifolia* × *C. parviflora*) plants, delay in flower senescence might be caused by chromosome doubling that reduced mitotic division which slows the growth rate (Ketmaro et al., 2012). Increase in fresh and dry weight at 0.1% and 0.3% colchicine concentration also may be due to increase in floral spike length, thickness, floret diameter and number of florets. Colchicine significantly reduces flowering upto 6.3-12.5% at different concentrations because it not only slows down the cell division but also interferes with the cellular mechanism that affects the cytoplasm viscosity and causes the cells to function abnormally (Boonbongkarn et al., 2013). Flowering was decreased in physic nut (*Jatropha curcas*) plants after colchicine treatment because these tetraploid plants do not manufacture sufficient photosynthetic product which ultimately retard growth and development and thus reduced flowering percentage (Niu et al., 2016). Another novel floral trait developed through colchicine treatment was production of bright colored gladiolus flowers which may be due to polyploidy induction. In tetraploid plants of rose (*Rosa centifolia*), doubling of chromosomes increased the gene number that caused a change in enzyme activity and isozyme diversity. These changes altered the biosynthesis pathway of pigments (anthocyanin) which led to the modification in flower color (Osburn et al., 2003).

Change in pollen grain diameter occurred due to chromosome doubling. Pollen grains had larger diameter at 0.3% colchicine concentration as compared to control ones. Size of pollen grain can be used as primary indicator to identify plants of different ploidy levels. In autopolyploid plants of night blooming cactus (*Hylocereus undatus*) increase in pollen grain diameter was correlated with chromosome doubling. Change in chromosome number altered the cellular structure (increase size) in order to support the growth and development of doubled genome (Cohen et al., 2013). Pollen grain fertility percentage significantly reduced from 82.9% in control to 71.8% at 0.3% colchicine concentration. Lower pollen grain fertility is one of

the universal effects of induced polyploidy and it occurs due to production of unreduced gametes. In polyploid plants of grain amaranthus (*Amaranthus cruentus*), Milan (2008) studied that reduced pollen grain fertility may have been resulted from different meiotic abnormalities like lagging of chromosome and formation of multivalents and univalents that led to the development of unbalanced gametes which were non-viable. In present study, increase in chromosome numbers increased the dimensions of stomata size but decreased the stomatal density of colchicine treated plants. Changes in stomatal size are usually associated with the development of polyploids and it is simple and non-destructive step to identify the different levels of induced polyploidy. In colchicine treated phlox (*Phlox drummondii*) plants, increase in size of polyploidy cells maximizes the stomatal dimensions. Cells with larger genomic material grow bigger to retain the constant ratio between the cytoplasmic and nuclear volume and enhance the expression of proteins due to the increase in number of genes. This increase in cell size may result in the development of larger plants (Tiwari and Mishra, 2012).

CONCLUSIONS

Colchicine at 0.1% and 0.3% concentration has effectively enhanced the gladiolus ornamental value by improving its floral value such as vase life and floret diameter. This plant material is a valuable germplasm which will be used in future to increase the genetic diversity in gladiolus in the form of new allelic combinations and to provide more opportunities in gladiolus breeding.

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AUTHOR CONTRIBUTIONS

A.M. – designed and executed research work and wrote the research paper; T.A. – helped in writing manuscript along with designing and supervising the research work; M.A.B. – helped in analyzing statistical data, writing and revisions of the manuscript; A.A.Q. – helped in performing research work; I.A.H., M.M.Q.B. and M.K.N.S. – helped in writing and improving the manuscript.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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