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Research Article

ASSESSMENT OF BIOLOGICAL DAMAGE AND TOXIC POTENCY OF COLCHICINE IN GLADIOLUS (*GLADIOLUS GRANDIFLORUS*) PLANTS

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Abstract

Colchicine is one of the important aqueous solutions that has been used to induce mutation or ploidy in many plant species long ago. However, its proper concentrations plays a vital role, increased or inappropriate concentrations may cause mortality, stunted growth, morphological deformation, etc. in plant species. Thus, this study was conducted to evaluate the different colchicine concentrations on the growth and development of gladiolus corms. Gladiolus corms were treated with 0.2%, 0.4%, and 0.6% concentrations for 24 hours. Colchicine toxicity was evaluated during the early growth stage showed a higher concentration of 0.6% significantly reduced corm survival (47%) and caused a 51.9% survival reduction over control. In terms of growth parameters, all concentrations of colchicine reduced plant height with a number of leaves along with inducing different forms of morphological abnormalities and chlorophyll mutants. To optimize the dose for successful ploidy induction, LD30 and LD50 on mortality rate through an analysis were calculated to be 0.31% and 0.57% whereas GR50 based on plant height and the number of leaves reduction was found to be 0.17% and 0.34%. Hence, the results from this study could be used in the future for further breeding programs by reducing the genotoxicity of colchicine on gladiolus.

Keywords: Abnormality, colchicine optimization, growth reduction, mutation, toxicity.
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1. INTRODUCTION

Improvement in plant germplasm requires efficient breeding techniques, among which polyploidy induction is one of the important techniques utilized to introduce diversification in the gene pool (Limera et al., 2016). Polyploidy induction or duplication of chromosomes through antimitotic chemicals is an effective way to obtain polyploids and increase the mutation rate in ornamental plants (Lan et al., 2020). Chromosome doubling, also known as somatic mutation, does not generate new germplasm instead it creates additional copies of existing genes and chromosomes. However, antimitotic chemical treatment induces genomic alteration, epigenetic changes, modulating gene expression and loss of duplicated genes which leads to phenotypic variation in plants (Laere et al., 2011; Ahmadi et al., 2013).

Among different antimitotic chemicals, colchicine has been commonly used to induce polyploidy since the 1930s (Limera et al., 2016) with doubling efficiency up to 70% (Hailu et al., 2020). Colchicine without damaging chromosomes induces a temporary inactivation of spindle formation (Kumar and Dwivedi, 2014). Manipulation of ploidy level through colchicine has been used by breeders to produce improved varieties of economically important plants

(Touchell et al., 2020). Colchicine chemical name is N-[(7S)-5,6,7,9-tetrahydro-1,2,3, 10-tetramethoxy-9-oxobenzo[*a*] heptalen-7-yl-) acetamide] and its molecular formula is $C_{22}H_{25}NO_6$ (Ade and Rai, 2010). It is a tricyclic alkaloid derived from the amino acid tyrosine and phenylalanine of *Colchicine autumnale* (Le et al., 2020) and extracted from meadow saffron (*Colchicine autumnale*) plant and *Gloriosa superba* L. roots. It has a complex mechanism of action and multiple properties (Eng et al., 2021).

It works by inhibiting the spindle fiber formation by combining tubulins during somatic cell division and inducing polyploidy by interfering with microtubule formation and chromosome anadol movement in the middle of the cell division process (Kwon et al., 2014). It also induces polyploidy by preventing chromosome segregation during meiosis (Ade and Rai, 2010). It causes dynamic instability in microtubules during the cell division process (Khan et al., 2017) (Figure 1). Colchicine is lethal to dividing animal cells but plant cells after colchicine treatment survive in a state of mitotic arrest (C-mitosis) for several days and return to normal cell division process, once the colchicine is removed (Bharati et al., 2006). It is considered as a simple and rapid method utilized for breeding as it induces polyploidy in plant cells in a shorter period of time (Seneviratne et al., 2020). In addition to that, colchicine also induces gene mutation by causing a mismatch of bases or binding with DNA (Salwee and Nehvi, 2014; Chen et al., 2020).

Colchicine is used for polyploidy induction because it is not as toxic to plant cells even if applied as a plant extract. Moreover, they can be transported to different plant parts without changing its properties and induce mutation for a very long time. It also does not change the gene structure (Lertsutthichawan et al., 2017) and remain stable at high temperature therefore it can be sterilized in a culture medium without losing its efficiency (Zhang et al., 2007).

However, low doses of colchicine are not successful in inducing polyploidy while extremely high concentration proves to be lethal to plant cells (Gallone et al., 2014). A high concentration of colchicine leads to the doubling of already doubled chromosomes which results in the production of undesirable ploidy levels (Allum et al., 2007). The reason behind this is, high-level polyploid plants can be malformed, stunted and cause abnormal growth which may be due to somatic instability and extreme gene redundancy that leads to chimera tissues production (Abu-Qaoud and Shtaya, 2014). Undesirable effects linked to colchicine treatment are chromosome losses, rearrangement and aberration, gene mutation, abnormal morphology, growth and sterility (Xie et al., 2015; Manawadu et al., 2016). Colchicine has a strong effect on changing allele frequency (Dhingra and Pokhriyal, 2012). Colchicine also causes mitotic abnormalities such as anaphase bridges, vagrant chromosome, c-metaphase, sticky chromosome, and increased micronuclei frequency with reduction in mitotic index (Hosseini et al., 2018). Furthermore, colchicine induced different physiological changes in plants such as point mutation, changes in hormonal balance, that affect somatic characteristics like changes in chloroplast bodies and chromosomal organelles (Eeckhaut, 2003). Overall, it induces many morphological, histological, cytological and genomic changes in plant cells by altering signal pathways (Le et al., 2020; Talei and Fotokian, 2020). Moreover, the effect of colchicine is related to exposure time, short time duration causes disassembly of spindle fibers, interphase microtubule network, prophase band and phragmoplast. While longer time exposure results in the production of tubular array with variable ultrastructural organization (Bharati et al., 2006).

A low concentration of colchicine for a longer time duration will help in reducing its toxic effect (Sajjad et al., 2013). It

effectively induced polyploidy at a concentration range of 0.001% to 1% from a time duration of 6-72 hrs (Hailu et al., 2020). Colchicine concentration varies from 0.00001% in senno (*Lychnis senno*) to an extremely high concentration of 1.5% in Japanese quince (*Chaenomeles japonica*) for successful ploidy induction (Castro et al., 2018). For each species and type of plant material used, an efficient colchicine concentration should be estimated as tolerance to colchicine is specific to the specie and explant (Rodrigues et al., 2011). Although it is toxic to plants, its low concentration for a longer time period will reduce its toxicity and yield successful polyploids (Sajjad et al., 2013). To get the desired results, colchicine concentration, time duration and plant organ type have to be assessed (Hassanzadeh et al., 2020). As, the successful production of stable polyploids depends upon the balance between effective concentration and exposure duration in order to double the genome of each cell effectively without adversely affecting mechanisms of plant growth and development (Harbard et al., 2012). Therefore the present research aimed to assess the toxic effect the colchicine on gladiolus growth and development and to estimate an effective concentration for successful ploidy induction.

2. MATERIAL AND METHODOLOGY

2.1. Research area

The present research was carried out in October 2021 in the field area of Barani Agricultural Research Institute, Chakwal (32°55′ Latitude and 72°43′ Longitude, 522m altitude). The climate of the studied location is arid to semi-arid with annual rainfall ranging from 500-1000 mm and soil is piedmont alluvial (EC: 0.3 dS/m, pH: 7.68) (Aziz et al., 2021).

2.2. Plant material

Gladiolus corms cv Alexandra of tetraploid ploidy level (4x=60) were used for treatment. Dry, healthy and uniform size corms were purchased for Sky seed store, Lahore. Pakistan.

2.3. Colchicine Treatment

For ploidy induction, gladiolus corms were immersed in an aqueous solution of colchicine at concentrations of 0.2%, 0.4%, 0.6% for 24 hr. For the control treatment, corms were dipped in distilled water at the same time duration. After treatment, corms were rinsed under running tap water for 1 h to remove adhering chemical residues and then placed between folds of blotted paper to remove excess moisture. Both treated and control corms were sown in pots placed in a greenhouse having 75% relative humidity, 28 ^{o}C maximum and 20 ^{o}C minimum temperature (Manzoor et al., 2018). The experiment was designed under complete randomized design (CRD) with three replication (15 corms per replication).

2.4. Data Collection

2.4.1. Emergence/sprouting of corms

Emergence %, Emergence speed index (ESI), Mean emergence time (MET) were recorded to evaluate colchicine toxicity.

Emergence percentage were recorded from 7th day till 30th day after sowing (DAS).

Emergence speed index was recorded through a formula proposed by Alvarez Holguin et al., 2019.

$$ESI=\sum ni/t$$

Where, ESI= emergence speed, ni = need of corms sprouted per day, t= number of days for sprouting

Mean emergence time were calculated by following formula (Khan et al., 2017).

$$\sum (d\times n)/S$$

d= days after sowing; n= number of sprouts emerged on a day (d); S= total number of sprouts emerged

2.4.2. Lethality

To study the lethal effect of colchicine, survival parameters and lethal dose (LD_{50}) were estimated to find out suitable concentration and to prevent toxic effects by standardizing dose.

2.4.2.1. Survival percentage:

Survival percentage of emerged corms was calculated after 45 days of sowing by counting a total number of survived sprouts divided by a total number of emerged sprouts. While percent (%) survival over

control was recorded by Treated / Control × 100 and percent reduction over control (lethality over control) was estimated by formula (Control-Treated / Control) × 100 (Roychowdhury et al., 2012; Parthasarathi et al., 2020).

Table 1: Effect of different colchicine concentration on sprouting/emergence parameters

Treatment	Sprouting percentage	Emergence speed index	Mean emergence time
Control	98.00 ± 2.00 a	11.31 ± 0.92 a	9.47 ± 0.90 d
0.2 %	89.67 ± 2.51 b	9.71 ± 0.46 b	14.03 ± 0.64 c
0.4 %	77.67 ± 4.04 c	7.61 ± 0.19 c	16.63 ± 0.77 b
0.6 %	68.33 ± 3.51 d	6.98 ± 0.22 d	19.46 ± 0.62 a

2.4.2.2. LD_{50}

The LD_{50} for colchicine was calculated after 45 days of sowing (DAS) by probit analysis (Finney, 1978) in MS Excel 2016. The probit function is the inverse cumulative distribution function or quartile function linked with standard normal distribution. The procedure for LD_{50} estimation through probit analysis is as follows:

(1)- Conversion of colchicine doses to $\log_{10}$ values. (2)- The mortality percentage of gladiolus sprouts due to colchicine treatment was determined and rounded off to the nearest whole number. (3)- Calculated *mortality* % were converted into empirical probits using Finney table (probit transformation table). (4)- Dose response regression line were drawn by plotting $\log_{10}$ doses on X-axis and empirical probits on Y-axis. (5)- Regression line passing through most of the plotted lines were used to estimate the $\log_{10}$ value with respect to probit 5. (6)- Antilog to determine log10 value corresponding to the probit 5 was calculated to find out LD_{50} value of colchicine (Parathasarathi et al., 2020).

2.4.3. Growth analysis:

The effect of colchicine on inducing plant injury was observed through morphological parameters *viz*; seedling height and number of leaves.

2.4.3.1. Morphological assessment:

Growth parameters (seedling length and number of leaves were recorded after every 15 days interval (15^{th}, 30^{th}, 45^{th} and 60 days after sowing).

2.4.3.2. Growth reduction GR_{30}, GR_{50}:

Growth reduction (GR_{30}, GR_{50}) was estimated through a simple linear regression model by fitting straight line equation y = mx + c, where y represent response variable (height reduction %), x is independent variable (colchicine concentration), m signify slope while c is for constant (Wanga et al., 2020).

2.4.4. Biological damage:

Biological damage in treated plants with colchicine was evaluated in terms of chlorophyll mutants and growth abnormalities.

2.4.4.1. Chlorophyll mutants:

Chlorophyll mutants were observed in survived seedlings after 45 days of sowing. Different chlorophyll mutants in treated plants were identified and characterized according to the classification proposed by Gustafson (1940) and their frequency was calculated through the following formula:

$$F\ (\%) = \frac{\text{Number of chlorophyll mutants}}{\text{Total number of survived plants}}$$

2.4.4.2. Growth Abnormalities:

Abnormalities in survived treated plants were identified in terms of sprout shape, seedling texture and leaf architecture. Moreover, their frequency % were calculated through formula mentioned above.

2.5. Statistical Analysis:

Data for germination parameter, survival %, seedling height and number of leaves were subjected to analysis of variance (ANOVA) using Statistix 8.1 software. Means were compared using Fisher's least significant difference at 5% probability level.

3. RESULTS

3.1. Sprouting:

To identify the most effective colchicine concentration for polyploidy induction, the impact of different colchicine concentration on sprouting parameters were evaluated

after 30 days of sowing (Table 1). According to statistical analysis, all colchicine treatments were significantly affected on sprouting/emergence of corms and it reduced with an increase in concentration. The highest sprouting percentage were observed in the control (98%) while in colchicine treatments it ranged from 89-68% respectively. Colchicine not only affects corm sprouting but also delays the emergence of sprouts from the corm. In control, 9.5 days were taken by sprout to emerge from the soil whereas in 0.6% colchicine treatment, sprouts emerged 19.5 days after sowing. Whereas emergence speed index was also correlated with mean emergence time and maximum value (11.3) was observed in control treatment indicating fast emergence rate in comparison to colchicine treatments where decreased in index value from 9.7-6.9 depicts delayed emergence.

3.2. Survival/Mortality:

Lethal effect of colchicine on treated corms in terms of survival parameters evaluated 45 days after sowing was illustrated in table 2. Survival percentage was 97% in control while it continuously decreased with an increase in colchicine concentration and lowest survival percentage (47%) was observed at highest colchicine concentration (0.6%). While, percent survival over control in treated plants also reduced from 82.2% to 48.2% in different colchicine concentration. However, percent reduction over control (%) depicted an increasing trend from 17.8-51.9% with an increased colchicine dose which described a high lethality rate (low survival percentage) in colchicine treated plants.

Table 2: Effect of different colchicine concentration on survival parameters

Treatment	Survival percentage	Percent survival over control	Percent reduction over control
Control	97.33± 1.15 a	100 ± 0.0 a	-
0.2 %	80.00± 2.00 b	82.20 ± 2.94 b	17.78 ± c
0.4 %	62.67± 4.50 c	64.37 ± 5.25 c	35.57 ± b
0.6 %	47.00 ± 2.00 d	48.23 ± 1.62 d	51.93 ± a

3.3. Lethal dose estimation:

The lethal dose (LD 30, 50) in colchicine treated gladiolus plants were estimated through probit analysis on the basis of mortality percentage after 45 days of sowing. Calculated lethal dose (LD 30, 50) and probit units of mortality percentage are presented in table 3 and 4. Lethal dose LD_{30} and LD_{50} for colchicine concentration were found to be 0.31% and 0.56%. These calculated values indicated that at 0.31% concentration 30% of treated plants died while at 0.56% concentration 50% mortality occurred.

3.4. Growth Reduction:

Colchicine treatment had adverse effect on growth of treated plants as illustrated in Table 5. Growth of control plants were at a faster rate while stunted growth was observed in colchicine treated plants. After 15^{th} days of sowing, control plants achieved a height of 27.4 cm but in treated plants reduction in mean plant height with an increase in colchicine concentration were observed and it decreased from 4.1 cm in 0.2% colchicine treatment to 1.7 cm at highest concentration (0.6%). After 30 days of sowing control plants were at vegetative stage (46.8 cm) but the colchicine treated plants in all concentration were still at emergence stage (2.3-4.4 cm). While after 60 days, control plant had completed their vegetative stage with maximum plant height (66.8 cm) whereas colchicine treated had minimum increase in plant height (3.5-7.4 cm) and had just started vegetative phase. Overall, there was 89.3-94.7% reduction in plant height was examined as compared to control plant. Just like plant height, similar trend were observed for leaves growth and each increase in colchicine concentration significantly inhibited number of leaves in treated plants. After 60 days of treatment, highest number of leaves were produced in control plants (8.3) while at 0.2% concentration 4.6

leaves, 0.4 % concentration 4.0 leaves and at 0.6% concentration only 2.3 leaves were fully developed. Whereas, reduction over control showed that colchicine also reduced number of leaves from 43.5 - 65.8% in treated plants.

In order to study the impact of colchicine on growth of treated plants, mean reduction in growth (GR_{50}) for plant height and number of leaves were calculated through linear regression equation. As colchicine causes a decrease in both growth parameters with an increase in colchicine concentration. Therefore, 50% reduction in plant height was estimated to be at 0.17% (Figure 2) concentration whereas 50% reduction in number of leaves were at 0.34% concentration (Figure 3). Based on the calculated doses, it was studied that colchicine had a significant deleterious effect on plant height while it may less affected on leaves development in treated plants.

Table 3: Assessment of colchicine toxicity through probit analysis for LD_{50}, LD_{30}

Concentration	Log_{10} (Concentration)	Mortality (%)	Probit unit	Lethal dose
0.2 %	-0.70	20 %	4.16	LD_{50}: 0.57%
0.4 %	-0.40	37 %	4.67	LD_{30}: 0.31%
0.6 %	-0.22	53 %	5.08	

Table 4: Linear regression equation parameters for LD_{30}, LD_{50}

Parameters	Coefficients
Intercept	5.47
X variable	1.89
Linear equation: y=1.89x+5.47 X= -0.52, LD_{30}= 0.31% X= -0.25, LD_{50}= 0.57%	

3.5. Chlorophyll mutants:

From the results, it was evident the colchicine proves to be hazardous for causing chlorophyll degradation in treated plants, thus producing chlorophyll mutants. Study of frequency and spectrum on chlorophyll mutant at seedling stage showed that chlorophyll mutants were obtained in all concentration (Table 6 and Figure 4). Mutation frequency of chlorophyll by colchicine ranges from 20.2% in 0.2% concentration to 52.7% in highest concentration 0.6%. While the spectrum of chlorophyll mutant consists of 10 different types of mutants included *albino*, *albino green*, *maculata*, *striata*, *xantha*, *viresence*, *aurea*, *chlorina*, *virids* and *tigirina*. In 0.2% and 0.6% treatment, albino green was predominant with 5.0% and 12.7% frequency whereas in 0.4% colchicine concentration, striata produced maximum mutation (7.9%). Overall mutant specturum frequency observed in all treatments were albino green (22.5%) > chlorina (15.1%) > striata (12.9%) > virids (12.2%) > xantha (11.1%) > aurea (9.0%) > virescence (8.7%) > maculate (8.5%) > albino (5.5%) > tigirina (4.2%).

A brief description of identified mutants were as follow:

Table 5: Effect of different colchicine concentration on plant height reduction in treated plants

Treatment	Plant height (cm)				Reduction over control %
	15 days	**30 days**	**45 days**	**60 days**	
Control	27.40 ±1.90 a	46.86 ± 2.71 a	57.03 ± 2.40 a	66.83 ± 1.34 a	-
0.2 %	4.06 ± 0.60 b	4.40 ± 0.56 b	5.00 ± 0.70 b	7.40 ±1.01 b	89.37 ±b
0.4 %	2.43± 0.91 bc	3.13 ± 0.92 b	3.86 ± 0.98 b	4.40 ±0.96 c	93.43 ±a
0.6 %	1.73± 0.76 c	2.30 ± 0.36 b	2.93 ± 0.23 b	3.53 ±0.25 c	94.70 ±a
Treatment	**Number of leaves**				**Reduction over control %**
	15 days	**30 days**	**45 days**	**60 days**	
Control	3.67 ± 0.57a	5.00 ± 0.01 a	6.33 ± 0.57 a	8.33 ± 0.57 a	-
0.2 %	2.33 ± 0.57 b	3.33 ± 0.57 b	4.00 ±0.01 b	4.67 ± 0.57 b	43.50 ±b
0.4 %	2.00 ± 0.01 b	2.00 ± 0.01 c	3.33 ± 0.57 b	4.00 ± 0.01 b	51.83± b
0.6 %	1.00 ± 0.01 c	1.67 ± 0.57 c	2.00 ± 0.01 c	2.33 ± 0.57 c	65.83 ±a

- **Albino**: Mutants were devoid of chlorophyll, carotenoids or any other pigment and had white leaves.
- **Albino green**: Seedling consist of white and green parts
- **Maculata**: Spots of chlorophyll and/or carotenoids destruction present on different areas of seedling
- **Striata**: Seedlings had longitudinal strips of white color due to longitudinal destruction of chlorophyll
- **Xantha**: Seedling had yellow/pale yellow color leaves and carotenoids were present but chlorophyll was absent
- **Virescence**: Light green color of seedlings
- **Aurea**: Mutants had golden yellow colored leaves
- **Chlorina**: Seedlings of yellowish green leaves
- **Viridis**: Viridine green color of seedlings
- **Tigirina**: Seedlings had yellow and green colored leaves

3.1.Morphological abnormalities

During early growth stage, colchicine treatment not only stunted the plant growth but also induced different morphological malformation in treated plants and their frequency increased with an increased in colchicine concentration (21.3%-55.3%) (Table 7 and Figure 5). Observation revealed that four major morphological abnormalities were found such as abnormal shaped sprouts, curved shaped true leaves, leathery texture of seedlings and deformed seedlings. Among these phenotypic aberrations, higher frequency (17.0%) at maximum concentration were observed for both abnormal shape sprouts and seedlings with leathery texture whereas frequency for deformed seedlings were minimum in all concentrations (8.8%) respectively. Thus percentage for healthy plants (free from any abnormalities) were also lowest at highest concentration (44.7%)

4. DISCUSSION

Sprouting percentage is one the most important parameters to be studied in colchicine treatments as sprouting rate predicts the extent of damage occur to gladiolus corms because of exposure to different concentration of colchicine for 24 hr (Muhammad et al., 2021). As corm sprouting/seed germination is one the critical and first stage of plant development and the agronomic performance of treated corms/seeds in terms of sprouting and seedling establishment has significant effect on determining plant health and growth (Hosseini et al., 2013). According to results, sprouting percentage decrease with an increase in colchicine concentration which may be due to delay in mitosis process, chromosomal damage (aberration) and induction of enzymes activity (lipase and catalase) (Mostafa and Abou-Alhamd, 2016). Furthermore colchicine also reduced sprouting percentage due to its interaction with DNA that causes changes in base pair relation (Roychowdhury and Tah, 2011). However, at lower concentration of colchicine (0.2%) slightly reduction in sprouting percentage occur because of colchicine inability to diffuse or reach to target tissues through the cells, or may be the target tissues have high resistance towards colchicine (Sungkaew et al., 2015). Reduction of seed germination in coriander (*Coriandrum sativum*) upto 13% is due to damage or destruction of cell constituents at molecular level (Kolhe et al., 2020) while in spinach (*Spinacia oleracea*) due to strong toxic effect of colchicine in destroying spindle fibre, germination % reduced to 86.1% (Roughani et al., 2017) whereas by interfering with development of enzymes involved in germination process, colchicine reduced germination to 60% in parsley (*Petroselinum crispum*) (Nasirvand et al., 2018).

The first visible effect of colchicine is delayed sprouting and growth of treated corms as depicted in present research. The reason behind increased in mean sprouting time due to injuries inflicted by colchicine to corms. Thus a time is required by corms to build-up their defence mechanism in

Table 6: Frequency and spectrum of chlorophyll mutants in different colchicine concentration

Treatment	**Number of survived plants**	**Chlorophyll mutants**										
		Albino	**Albino green**	**Maculata**	**Striata**	**Xantha**	**Viresence**	**Aurea**	**Chlorina**	**Virids**	**Tigirina**	**Total (Frequency %)**
Control	97	0	0	0	0	0	0	0	0	0	0	0
0.2% col	80	1 (**1.3**)	4 (**5.0**)	2 (**2.5**)	4 (**5.0**)	0 (**0.0**)	1 (**1.3**)	0 (**0.0**)	1 (**1.3**)	3 (**3.8**)	0 (**0.0**)	16 (**20.2**)
0.4% col	63	0 (**0.0**)	3 (**4.8**)	1 (**1.6**)	5 (**7.9**)	3 (**4.8**)	2 (**3.2**)	3 (**4.8**)	2 (**3.2**)	4 (**6.3**)	0 (**0.0**)	23 (36.6**)**
0.6% col	47	2 (**4.2**)	6 (**12.7**)	2 (**4.2**)	0 (**0.0**)	3 (**6.3**)	2 (**4.2**)	2 (**4.2**)	5 (**10.6**)	1 (**2.1**)	2 (**4.2**)	25 (**52.7%)**
Total		**5.5 %**	**22.5 %**	**8.3 %**	**12.9 %**	**11.1 %**	**8.7 %**	**9.0 %**	**15.1 %**	**12.2 %**	**4.2 %**	

Table 7: Frequency and types of morphological abnormalities in different colchicine concentration

Treatment	**No of survived plants**	**Growth Abnormalities**					
		Abnormal shape sprout	**Curved shaped true leaves**	**Seedling Leathery texture**	**Deformed seedlings**	**Total**	**% Healthy plants**
Control	97	0	0	0	0	0	0
0.2% col	80	4 (**5.0**)	5 (**6.3**)	6 (**7.5**)	2 (**2.5**)	17 (**21.3**)	78.7%
0.4% col	63	6 (**9.5**)	7 (**11.11**)	5 (**7.9**)	4 (**6.3**)	22 (**34.8**)	65.2%
0.6% col	47	8 (**17.0**)	10 (**21.3**)	8 (**17.0**)	0 (**0.0**)	26 (**55.3**)	44.7%
		31.5 %	38.7 %	32.4 %	8.8 %		

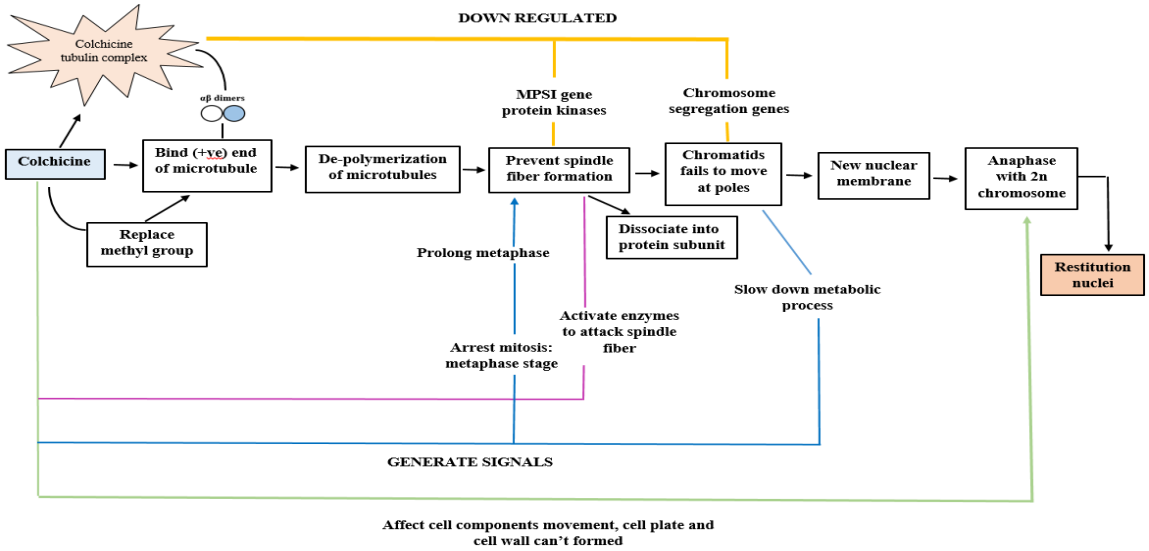

Figure 1: Colchicine induced polyploidy in mitotic cells through direct and indirect ways. Direct action (**Black arrows**) involves binding of colchicine molecules with positive end of tubulin dimers (sub unit of microtubules) by replacing methyl group causes disruption of microtubule polymerization which prevent spindle fiber formation by dissociating them into protein subunits. This leads to failure of chromatids to moves at poles thus results in formation of new nuclear membrane at anaphase stage forming restitution nuclei having 2n chromosomes. Indirect mode of action consists of series of reactions **(i)** Colchicine generate signals that prolong metaphase thus delaying metaphase stage to anaphase stage. Also it slows down metabolic process which delays separation of sister chromatids **(Blue line). (ii)** Affect movement of cell components and cell plate which prevent cell division **(Green line). (iii)** Activate enzymes which attack spindle fiber (**Purple line**). **(iv)** Down regulated MPSI gene expression (that monitor spindle fiber apparatus integrity) causes monopolar mitosis due to failure of duplication of spindle body. Also down regulated gene expression related to segregation of sister chromatids **(Orange line).**

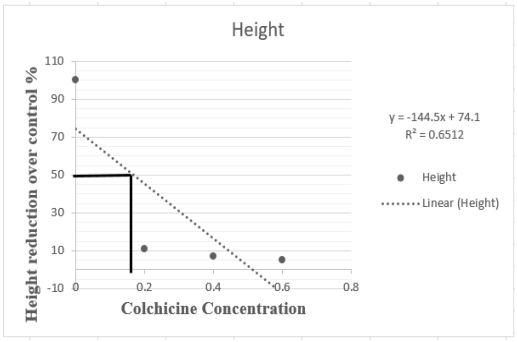

Figure 2: Growth reduction (GR_{50}) on basis of height reduction over control (%)

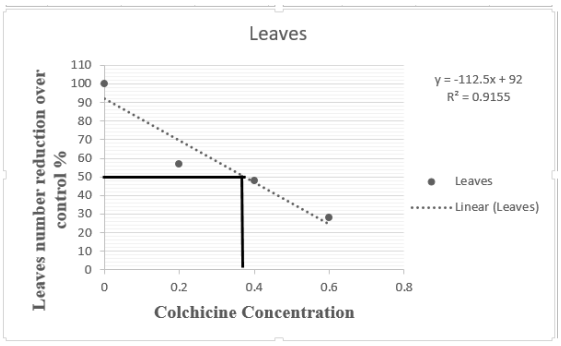

Figure 3: Growth reduction (GR_{50}) on basis of leaves number reduction over control (%)

GR_{50} for height
Where Y = 50
50 = -144.5X + 74.1
X = 0.17, Therefore GR_{50} = 0.17%

GR_{50} for leaves
Where Y = 50
50 = -122.5X + 92
X = 0.34, Therefore GR_{50} = 0.34%

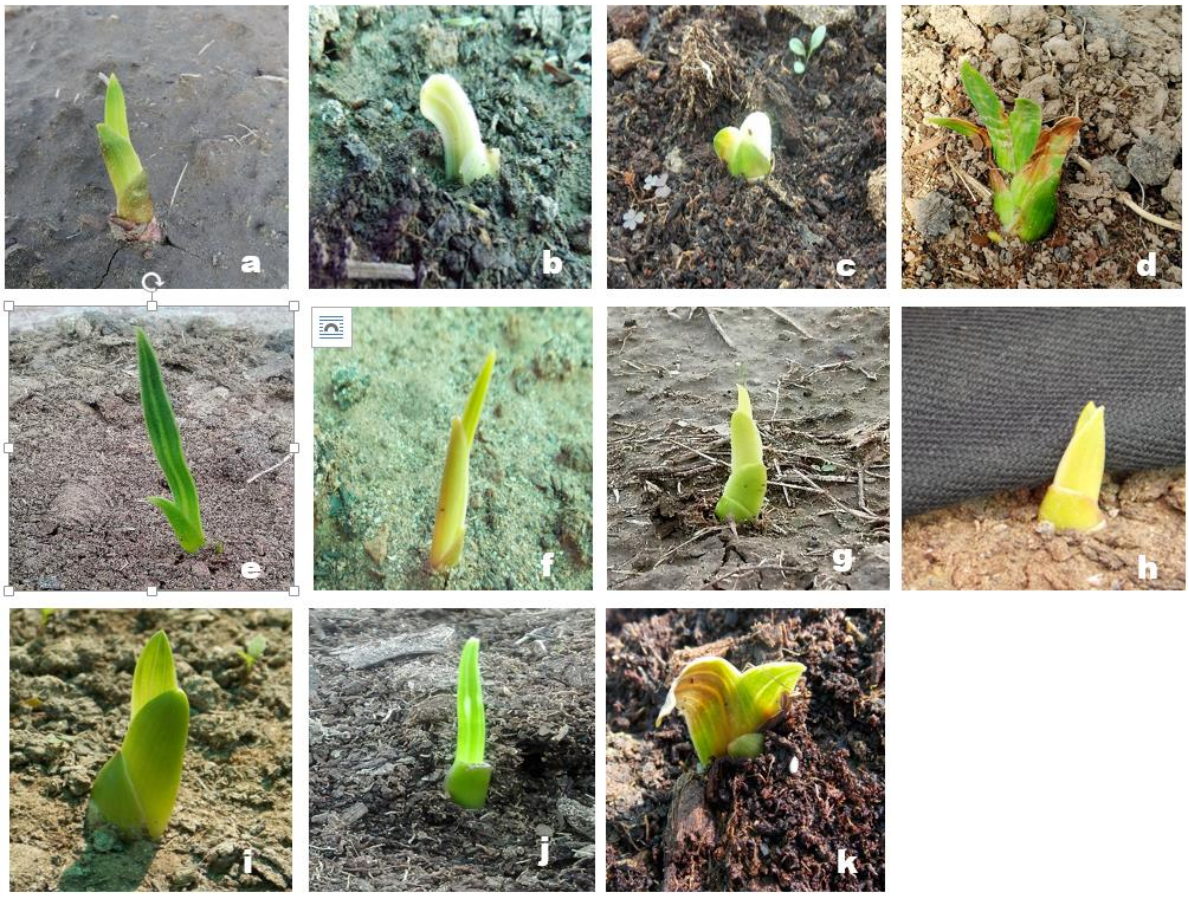

Figure 4: Chlorophyll mutants produced at different colchicine concentration: **(a)** Control **(b)** Albino **(c)** Albino green **(d)** Maculata **(e)** Striata **(f)** Xantha **(g)** Virescence **(h)** Aurea **(i)** Chlorina **(j)** Viridis **(k)** Tigirina

Figure 5: Morphological abnormalities observed in different concentration of colchicine (a) emerging sprout has straight shape while in treated plants (b-d) abnormal shaped sprouts emerged. (e) Control seedlings had smooth texture with normal shape leaves whereas (f) curved shaped true leafs (g) with leathery seedling texture and (h) deformed shape seedlings were noted in treated plants.

order to recover themselves from toxic effect of colchicine before they can germinate (Eng et al., 2021). Increase in colchicine dosage from 0.5% to 1.5 % increases days to germination of pigeon pea (*Cajanus cajan*) from 10 days to 26 days due to disturbance in biochemical pathways (Udensi and Ontui, 2013). Delayed seed germination due to colchicine treatment were also observed in black locust (*Robinia pseudoacacia*) (Suliman and Asander, 2020) and soybean (*Glycine max*) (Mangena, 2020).

The main objective of colchicine treatment is to obtain a large number of surviving plants with highest polyploidy induction rate (Omezzine et al., 2012). However, colchicine treatment is always associated with high mortality rate which is mainly due to its role in causing physiological disturbance which affect biochemical pathways and also due to toxic effect which arise from direct interaction between DNA molecules and colchicine (Brisibie et al., 2011; Mensah et al., 2013). Mortality is also related to poor seedling vigour of treated plants due to their inability to overcome lethal effect of colchicine treatment (Tiwari and Mishra, 2012). Decrease in survival rate of gladiolus corms was attributed to higher colchicine concentration with longer exposure duration as more colchicine were absorbed into cells and were diffused towards different parts of cells interfering with different cellular mechanism which increased mortality rate (Kashtwari et al., 2021). Moreover, colchicine along with reducing cell division also affect cytoplasm viscosity which leads to improper cell functions (Ragasova and Ondrasek, 2016; Boonbongkarn et al., 2013). Furthermore, in colchicine treated plants, gene expressions linked to plant hormone signal transduction, phenylalanine and phenylpropanoid were down regulated whereas gene expression of carcinogensis chemicals were increased which described the reasons behind low survival rate in colchicine treatment as both phenylpropanoid and phenylalanine have vital role in protecting plant cell from biotic and abiotic stresses (Zhou et al., 2017). Also, during stress or harsh conditions, increase overproduction of ROS effected scavenging system which leads to burst of oxidative stress as ROS attacked on important biomolecules such as DNA, which causes disruption in cell metabolism and resulted in phenomenon of own cell death (Azoush et al., 2014). Colchicine significantly reduced gerbera (*Gerbera hybrida*) seedling survival rate from 88.3% to 58.0% (Bhattarai et al., 2021) while in echeveria (*Echeveria elegans*), saffron (*Crocus sativus*) and chrysanthemum (*Chrysanthemum carinatum*) survival rate decreased with an increase in concentration (Cabahug et al., 2021; Kashtwari et al., 2021; Kushwah et al., 2021)

At low concentration, cells at prophase stage shows sensitivity however at high concentration, detrimental effect of colchicine block all cells at metaphase stage leading to apoptosis and cell death (Kashtwari et al., 2021). Lowest survival rate (47%) at highest 0.6% colchicine concentration depicts that change of genetic material in cells after colchicine treatment causes mutated cells to rearrange themselves according to their natural cell biology. Cells that able to find new chromosomal arrangement will survive while that fail to form new balance will be deleted during cell division process in early growth stages (Afifah et al., 2020; Qalby et al., 2020). Hosseini et al. (2018) also observed that inability of plant to preserve their chromosome sets in balance at high colchicine concentration leads to highest mortality in rose periwinkle (*Catharanthus roseus*). Similarly, in effort to overcome colchicine toxic effect, poor seedling vigor leads to death of phlox (*Phlox drummondi*) treated plants (Tiwari and Mishra, 2012).

LD_{50} (Mean lethal dose) is defined as a dose which results in death of 50% plant population in response to applied chemical (Afifah et al., 2020). It depends upon

species, plant material, type and stage at which lethality is measured (Hailu et al., 2020). It is used to find out the lethal toxicity of a specific chemical (Parthasarathi et al., 2020) as the nature of genetic changes/mutation because of chemical treatment can be estimated by calculation the optimal dose/concentration of a chemical (Bonde et al., 2020). Therefore, It is important to study the lethal dose (LD_{50}) of chemicals in order to assess the sensitivity of plant species to specific chemical (Jeloudar et al., 2019).

In artificially induced polyploidy through colchicine treatment, it is considered as a dose at which desirable polyploids are obtained with minimum negative effects. Determination of LD_{50} is also important to reduced colchicine lethality in plants and to obtain beneficial genetic changes (Sao et al., 2020) as it is used to determine the appropriate concentration and soaking time to get polyploid plants (Afifah et al., 2020). Plants at low colchicine concentration had highest survival rate because at this concentration, colchicine does not initiate ethylene synthesis but only depolarized cell enlargement, whereas high mortality at higher concentration was due to inhibition of respiration and solution uptake by colchicine and damage different parts of cells which leads to higher mortality percentage (Jeloudar et al., 2019; Cabahug et al., 2021).

Mutagens usually caused lethality at seedling stage (Roychowdhury et al., 2012). Therefore estimation of LD_{50} at early growth stages in gladiolus corms through mortality rate helps in assessing the toxic effect of colchicine. Khan et al (2017) find out 0.01% colchicine concentration lethal in tinda (*Praecitrullus fistulosus)* because it results in 37.5% death of treated plants. While LD_{50} calculated to be 0.132% in patchouli (*Pogostemon cablin*) (Afifah et al., 2020). LD_{50} in marigold (*Tagetes erecta*) calculated through probit analysis were found to be 0.1% colchicine for 1 hour as it cause 52% mortality (Nandhini et al., 2019) and in garlic (*Allium sativum*) 0.145% for 6 h was estimated after 45 days of planting (Mahajan et al., 2015).

Inhibition of growth after antimitotic chemical is a common phenomenon and reported in many crops (Ajayi et al., 2014). Reduced growth rate of treated plants were due to reduction/disturbance in natural rate of cell division. As chemicals penetrates into apical cell layers and affect normal cell division (Thao et al., 2003). In present research, reduction in plant height and number of leaves in treated gladiolus plants may occur due to loss of cortical microtubules that results in cell expansion instead of elongation (Le et al., 2020). Moreover, presence of residual colchicine harm newly tender bulbs thus slowing growth (Hailu et al., 2020). High concentrations of colchicine (0.4% and 0.6%) proves to be toxic as colchicine block spindle fiber development, modifies differentiation process and leads to formation of C-mitosis that inhibited cell multiplication (Bennici et al., 2006; Luo et al., 2018). Reduced growth at higher colchicine concentration were due to sudden increase in seed metabolic status, physiological disorder, increase in growth promoters, auxin synthesis inhibition, increase in growth inhibitors or decline in assimilate mechanism (Roychowdhury and Tah, 2011). Growth inhibition also occur due to injury to meristematic cells or interaction of spindle poison with cell elongation (Amin et al., 2015).

Reduction in plant height in sesame (*Sesamum indicum*) was due to toxic effect by colchicine on activities of cytokinin which is one of the important hormone in plant developmental process such as cell division, enlargement and morphogenesis (Nura et al., 2013) whereas, increase in ethylene synthesis by colchicine treatment inhibited garlic (*Allium sativum*) growth because ethylene has role in inhibiting auxin and cytokinin activities which explains why treated plants can't had faster growth rate (Hailu et al., 2020). Slow growth rate of cowpea (*Vigna unguiculata*) treated plants occur because of deleterious

effect of colchicine on apical region that leads to disturbance of natural rate of cell cycle (Ajayi et al., 2014). Due to colchicine toxicity, asparagus (*Asparagus officinalis*) and chrysanthemum (*Chrysanthemum carinatum*) seedlings grow at a slower rate as compared to control (Chen et al., 2020; kushwah et al., 2021) while slow physiological and biochemical process, inhibited growth at early stages of seedlings in Benghal dayflower (*Commelina benghalensis* L) (Shaikh et al., 2021). Colchicine treatment also reduced plant height and number of leaves in pineapple (*Ananas comosus*) genotype "Bangka" (Rosmaina et al., 2021), calendula (*Calendula officinalis*) (El-Nashar and Ammar, 2016) and cyrsanthemum (*Crysanthemum morifolium*) due to retardation of cell division by arresting mitotic division (Ghormade et al., 2020).

Usually exposure of plant material etc seeds, seedling, roots to higher dose of colchicine leads to significant reduction in plant growth with severe physiological alteration. Determination of effective colchicine dose for ploidy induction were estimated by evaluating the damage occur to plant through growth parameters (Lv et al., 2021). Mean growth reduction (GR_{50}) is a dose at which 50% reduction in plant occur, thus calculation of GR_{50} helps in studying the genotoxic effect of colchicine (Shaikh et al., 2021). Colchicine inhibited growth due to its role in sucrose degradation which in involved in cell wall synthesis and cell enlargement. Also it inhibit DNA synthesis and cell division (Lv et al., 2021). Mean reduction in growth reduction (GR_{50}) was studied with a fact that low dose causes minimum effect to plant phenotype whereas high dose results in extreme genomic changes which produced abnormal plants of stunted growth (Shrivastva et al., 2021). Colchicine induced physiological changes reduced growth in garden cress (*Lepidium sativum*) during first month of growth (Aqafarini et al., 2019) while in wild ginseng (*Panax ginseng*) initial growth retardation may occur due to c-mitotis or formation of sticky supercoiled chromosome and loss of microtubule as usually microtubule loss causes cell to expands rather than elongate thus producing shorter and thicker plants (Le et al., 2020). After one month of growth, petunia (*Petunia hybrida*) seedlings had significant reduction in plant height with malformed leaves and poor growth (Ning et al., 2009).

In many crop species, high colchicine concentration with longer exposure duration causes chromosome deletion yielding plant tissue abnormalities (Megbo, 2010; Lan et al., 2020). At high colchicine concentration, phytotoxicity, toxic contamination and plant abnormality are main causes of plant death (Hosseini et al., 2018). In gladiolus growing seedlings, phytotoxic effect of colchicine produced different growth abnormalities which was Abnormal growth rate by colchicine is attributed due to imbalance in growth hormones or because of non-heritable physiological changes (Hewawasam et al., 2004). Abnormal growth are usually chimeric in nature and occur due to diplontic selection (Mensah et al., 2007). Exposure of colchicine often leads to morphological abnormalities in seedlings which includes foliar irregularities (Lam et al., 2014). Development of abnormal leaves were attributed by changes in ascorbic acid concentration, imbalance enzyme activity and reduction in auxin level, auxin destruction or chromosome aberration (Obute et al., 2007; Hewawasam et al., 2004). Colchicine treated Seedlings of Persian poppy (*Papaver bracteatum*) deviate from normal growth and turned into dark brown necrotic tissues (Esfahani et al., 2020). High colchicine concentration (0.1%) and longer soaking period (4.5 hr) produced abnormal appearance plants in katokkon chilli (*Capsicum chinense*) (Kasmiati et al., 2020). While in pointed gourd (*Trichosanthes dioica*), colchicine treated seeds produced seedlings having sturdy and brittle leaves with stunted growth (Hassan et al., 2020).

Chlorophyll, a green pigment is one of the most important component throughout the plant life due to its role in photosynthesis (Dudits et al., 2016) and its development is controlled by different genes located on chromosomes adjacent to centromere and proximal segments and by non-chromosomal DNA (Kolar et al., 2015). While chlorophyll loss is linked with environmental stress and variation in carotenoids/chlorophyll content is an ideal indicator for stress study in plant (Azoush et al., 2014). In present research, significant reduction in chlorophyll content along with production of different chlorophyll mutants were observed in treated plants. Reduction in chlorophyll content (green pigment level) in tissue is one of the most common characteristics of colchicine treatment which leads to reduced leaf area and less synthesis of food material (carbohydrates). As treated plants which were already in stress may suffer from food shortage (nutrient deficiency) due to which its generally morphology appears to be weak and distorted (Ahmad et al., 2010). Overall, colchicine leads to plants in stress which alter the chlorophyll content in treated plants (Ajayi et al., 2014).

Chlorophyll mutation is one the reliable indicators used to assess sensitivity of crop towards a specific chemical (Chaudhari et al., 2015). It is also used to evaluate the effectiveness and efficiency of different doses of mutagen in treated plants for viable mutations (Darkwah et al., 2019) and are used as genetic markers in basic and applied research (Kolar et al., 2011). Therefore scoring of chlorophyll mutants proves to be an reliable method to measure the phytotoxic effect of mutagen on plant genotype (Selvan and Raju, 2017). Reduction in chlorophyll content was may be due to breakdown of thylakoid membrane in treated plants (Xu et al., 2010) or might occur due to due to deficiency of chlorophyll and carotenoids or both in plastid genes which leads to variegation in leaves (Nura et al., 2013). Chlorophyll deficiency in sesame (*Sesamum indicum*) can also be due to disturbance in chlorophyll apparatus or because of diplontic selection (Mensah et al., 2013) and colchicine concentration range of 0.1%-2.0% produced chlorophyll deficient mutants in sesame (*Sesamum indicum*) (Nura et al., 2013). While in Chang dang orchids (*Rhynchostylis gigantean*), tetraploid plants had less number of chloroplast in comparison to diploid due to abnormal cell structure (Kerdsuwan and Te-chato, 2012).

5. CONCLUSION

Selection of a specific concentration at its optimal rate is a pre-requisite for success in induction of ploidy and ultimately in crop improvement program. Therefore, for production of maximum viable plants with minimum damage, optimization of mean lethal dose (LD) and mean growth reduction (GR) of colchicine is important to induce successful desirable changes in plant genome by keeping in mind the other biological damages caused by colchicine on gladiolus growth and development. These parameters to study the effect of colchicine on growth and development of gladiolus during early stages of growth can be used in large scale breeding studies for improvement in gladiolus genepool.

6. DECLARATION OF INTEREST

All contributed authors have no conflict of interest

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