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Research Paper

Polyploid Induction in *Viola acuminata* Ledebour using Colchicine

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Abstract

This study was aimed to obtain polyploid plants of viola (*Viola acuminata* Ledebour) using the antimitotic agent colchicine. Experiments consisted in using colchicine on ungerminated seeds. The seeds were plunged into colchicine solutions in concentrations of 0.01, 0.05, 0.10 and 0.20% for 24 and 48 h. Polyploidy levels (diploid, tetraploid and hexaploid) were firstly detected by chromosome counting (karyotype), and then confirmed by anatomical and morphological parameters. The greatest percentage of polyploids was induced with 0.10% colchicine for 24 h. The chromosome number was 2n = 18 in diploids, 2n = 36 in tetraploid, and 2n = 54 in hexaploid. Differences in some morphological and anatomical parameters were significant between polyploid and control plantlets. The highest number of chloroplasts in guard cells was obtained in plantlets obtained from treated seed with 0.10% colchicine for 24 h. Exposure times of 48 h did not significantly influence polyploidisation. New ornamental varieties with improved characteristics may be produced by polyploid induction.

Keywords: Antimitotic agents, Chromosome doubling, Ornamentals breeding, Tetraploidy, Violaceae.

INTRODUCTION

Viola (*Viola acuminata* Ledebour) is an herbaceous species in the family Violaceae, with self-supporting growth habit. Some species of *Viola* are under International Union for Conservation of Nature and Natural Resources (IUCN) red list of threatened plants. Species of family Violaceae from different genera and sections are known to produce cyclotides, cyclic polypeptides of much interest due to their medicinal properties and chemical structure.

Biotechnology of ornamental plants aims to improve productivity and accelerate breeding programs (Silva *et al*., 2019). One of the most important biotechnology tools in horticulture industry is polyploidy induction. Polyploidy plays a valuable role in the evolution of plant species, genetic diversity, developing improved hybrids, alteration of plant architecture and the formation of new species and varieties with useful traits (Dhooghe *et al*., 2011). Polyploid plants show vigorous growth, better quality and distinct advantages such as having bigger organs, increased floral pieces and quality like color, faster metabolism, and protection from deleterious mutations and better resistance to biotic and abiotic stresses (Huy *et al*., 2019). Polyploidization has the most important role in the hybridization, improvement and production of excellent plants and varieties in some ornamental plants (Miguel and Leonhardt, 2011; Huy *et al*., 2019).

Induction of polyploidy is done by inhibiting the spindle fiber formation during cytokinesis, chromosome gets multiplied but cell divisions do not occur. Polyploidy can be induced by several antimitotic agents that inhibit mitosis, resulting in chromosome doubling. The most common used antimitotic agent in plant chromosome set doubling is colchicine. Colchicine as an anti-mitotic substance stops cell division at the metaphase stage, after the chromosomes have been doubled, leading to polyploidy induction (Pinheiro *et al*., 2000). The commonly used concentration of colchicine is between 0.01–1.00 %. The concentration and exposure time duration of colchicine for polyploidy induction is species-dependent (De Mello Silva *et al*., 2000).

Various plant parts such as apical meristem, flower bud, root tip and particularly seed can be used to induce polyploidy. Success of polyploidy induction depends upon some factors such as the antimitotic agents application method, plant part (explant) used, species and cultivar type, concentration and exposure time duration. There are no any reports on the induction of polyploidy in *Viola*. Therefore, this investigation reports the induction of polyploidy in *Viola acuminata* Ledebour by colchicine, for the first time.

MATERIALS AND METHODS

Plant materials

Seeds of viola (*Viola acuminata* Ledebour) were used as materials in this study. To investigate the best cultivation bed for germination of seeds; humus, cocopeat and combination of humus and cocopeat in ratio of 1:1 were used (data not shown). Finally, we selected last cultivation bed for seeds germination.

Polyploidy induction

Seeds of *V. acuminata* with chromosome number 2n = 18 were used for induction of polyploidy. Uniform sized seeds were purchased from Parks and Greenery Office of Karaj Municipality, Karaj, Iran. The non-dormant seeds were soaked in 0.01%, 0.05%, 0.10% and 0.2% colchicine (Sigma Co., USA) solution for 24 and 48 h at room temperature, while control seeds were soaked in distilled water.

Culture condition

After the germination of the seeds (around 14 days after cultivation), all treated and untreated (control) seedlings were transferred into the plastic pots containing humus and cocopeat (1:1) for further growth.

Chromosome number determination

For chromosome counting, root tips were excised from plantlets. Excised root tips were plunged into the 0.05 g/1000 ml distilled water for 24 h followed by washing with distilled water. Root tips were kept in acetic acid 90% for 30 min. After this time, specimens were rinsed with distilled water twice. Then, they were plunged in 1 N of HCl for 10 min. at 60°C followed by rinsing in distilled water. Root tips were stained using Orcein solution for 15 min. at room temperature. Stained root tips were put on the slide and squashed (Gao *et al*., 1996). Slides were observed and photographed under light microscope in the metaphase phase.

Morphological observations

Morphological parameters were compared between control and colchicine treated plantlets. Morphological measurements were done 60 days after seed germination. Stem length, root length, leaf length, leaf width and leaf number were measured by a ruler. Leaf length was calculated from leaf tip to the base of leaf where it connects to the petiole. Leaf width was calculated from the widest section of each leaf.

Anatomical observations

A thin layer of epidermis tissues was removed from the abaxial surface of the leaves and stained by iodine solution on slides along with a drop of water (Dwivedi *et al*., 1986). The slides were analyzed for guard cells size and number and the number of chloroplasts in guard cells under a light microscope (K200, Nikon, Japan) with magnification of 400 (×400). Three slides were prepared for each specimen and mentioned parameters on each slide were measured.

Experimental design and data analysis

A factorial experiment in a completely randomized block design with four replications was employed. Factors were colchicine concentrations (0.01, 0.05, 0.10 and 0.2%), and exposure time to colchicine solutions (24 and 48 h). The analysis of variance (ANOVA) procedure for a factorial experiment was used to test for significant effect of treatments. The data were analyzed using GenStat v. 12 statistical software and means were compared using Fisher's protected LSD test at 5% level of significance.

RESULTS AND DISCUSSION

Table 1 and Figs. 1 and 2 show the results of polyploid induction in *V. acuminata*. Chromosome counting from the root tip meristems of surviving treated plantlets was performed by karyotype analysis. Chromosome counting (Fig. 1) confirmed the polyploidy induction. Chromosome counts were obtained for diploid, tetraploid and hexaploid plantlets treated with colchicine. Tetraploids had a doubled number of chromosomes (2n = 4x = 36) compared to the diploids (2n = 2x = 18). In hexaploids, chromosomes set contained 2n = 6x = 54.

Generation of polyploids by treatment of plants with mitotic spindle poisons such as colchicine is a common technique used in plant breeding for many years (Dhooghe *et al*., 2011). Polyploid induction plays a significant role for the improvement of plants particularly in ornamentals. Our study revealed the effectiveness of colchicine application on polyploid induction of *V. acuminata*. The effectiveness of colchicine application and polyploid induction depends on some factors especially the plant species, type of explant, the colchicine concentration applied, the penetration of the compound and duration of treatment (Allum *et al*., 2007; Hannweg *et al*., 2013). Colchicine concentration and duration of treatment are two most important factors. Young tissues containing actively dividing cells are preferred to obtain high efficiency in polyploid plant formation through colchicine treatment (Huy *et al*., 2019).

Table 1. Effect of colchicine treatments on different morphological and anatomical parameters of *Viola acuminata* Ledebour.

Colchicine (%)	Exposure time (h)	Plantlet height (cm)	Stem length (cm)	Leaf length (cm)	Leaf width (cm)	Leaf number	Root length (cm)	Stomata diameter (µm)	Stomata number	Chloroplast number
0.00	0	9.40c	4.50c	1.06d	0.84e	5.05a	4.90e	17.66a	10.54a	10.35d
0.01	24	10.90b	4.80c	1.87c	0.90cd	5.25a	6.10c	18.90a	10.46a	11.23d
	48	9.90c	4.75c	1.75c	0.96c	5.20a	5.15d	19.56a	11.13a	12.75c
0.05	24	16.27a	5.87b	2.00ab	1.05b	5.55a	10.40b	17.89a	11.93a	12.85c
	48	9.87c	4.77c	1.95b	1.02b	4.90a	5.10d	17.40a	9.40a	14.45b
0.10	24	17.30a	6.25a	2.26a	1.28a	5.10a	11.05a	20.20a	9.74a	18.84a
	48	9.77c	5.45b	1.90b	0.98c	5.00a	4.32e	18.80a	9.50a	10.10d
0.20	24	11.40b	6.10a	1.72c	0.83e	5.00a	5.30d	18.57a	9.35a	12.12c
	48	7.79d	4.63c	1.08d	0.78e	5.00a	3.16f	17.44a	8.89a	10.67d

In each column, means with similar letter(s) are not significantly different (P<0.05) using the Duncan's multiple range test.

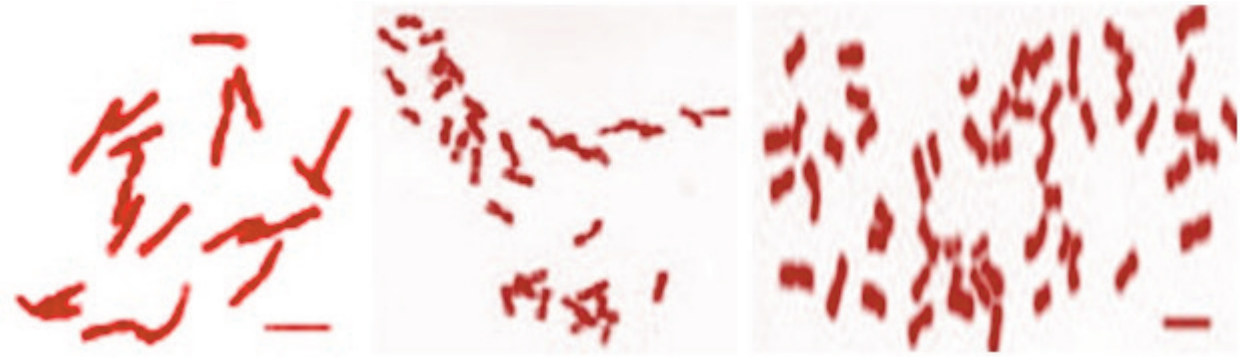

Fig. 1. Micrographs of chromosomes (karyotype) of the root tip cells in *Viola acuminata* Ledebour. Left: Diploid control plantlet (2n = 18), Middle: Induced tetraploid plantlet (2n = 36), and induced hexaploid (2n = 54).

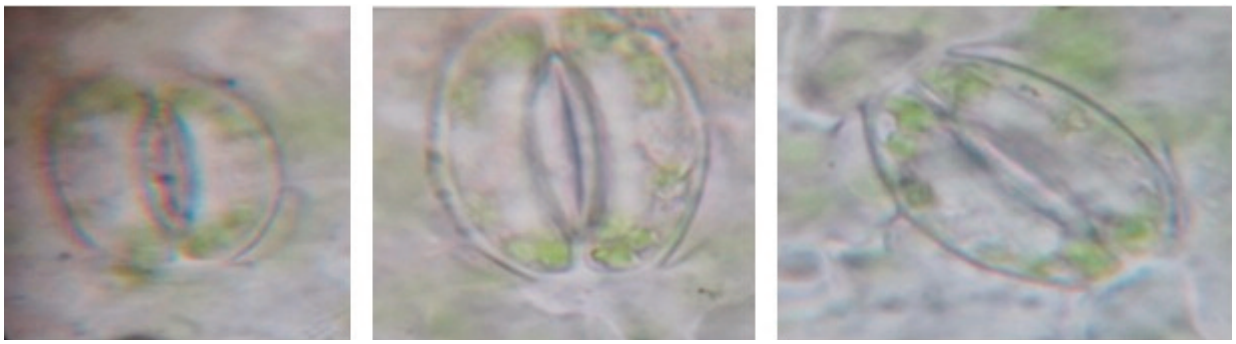

Fig. 2. Micrographs of stomata containing chloroplasts. Left: Diploid control plantlet (2n = 18), Middle: Induced tetraploid plantlet (2n = 36), and induced hexaploid (2n = 54).

In current study, seed was proper explant for colchicine treatment and polyploidy induction. Explants must be exposed to antimitotic agents at levels and times high enough to saturate plant tissues and induce polyploidy (Allum *et al*., 2007). Optimum concentration and duration of colchicine are different for each species and variety, even within the same family of plants (Sarathum *et al*., 2010). Polyploid induction by colchicine was reported in some other ornamental plants (Dhooghe *et al*., 2009; Zhang *et al*., 2010; Cai *et al*., 2015; Lucía *et al*., 2015; Manzoor *et al*., 2018). These researchers showed significant differences in vegetative and generative parameters between control and polyploid plants.

Colchicine has been used at 0.05–0.20% concentrations and 2–9 days for most species (Huy *et al*., 2019). In the present investigation, polyploidy has been induced using 0.10 and 0.20% concentrations of colchicine for 24 h. Polyploidy induction by colchicine was reported by some researchers on orchids (Qiang *et al*., 2009; Atichart, 2013; Bunnag and Hongthongkham, 2015). Mortality caused by colchicine is different for various species, mainly depending upon its concentration. High concentrations of colchicine are associated with plant cell death due to the highly toxic effect of this antimitotic agent (Pintos *et al*. 2007; Blasco *et al*., 2015). In current study, the maximum number of viable plantlets was observed on 0.10 % treatment.

Among all methods for analysis of polyploidy, flow cytometery and karyotype are the most efficient, precise, accurate, quick and reproducible tool for detection of changes in ploidy levels of a large numbers of samples (Doležel, 1997; Blasco *et al*., 2015). In the present study, chromosome counting revealed induction of tetraploidy and hexaploidy in *V. acuminata*. The chromosome number of diploid *V. acuminata* was 2n = 18, tetraploid was 2n = 36, and hexaploid was 2n = 54 (Fig. 1). Morphological and anatomical parameters were measured to confirm chromosome counting. However, ploidy level determination by morphological or anatomical assays alone has some limitations (Chen *et al*., 2009; Dhooghe *et al*., 2011).

Some changes in stem length, leaf length, leaf width, root length and chloroplasts number in guard cells (Table 1), due to polyploidy, were observed between colchicine-treated plantlets and untreated diploids. Significant differences were observed in these morphological and anatomical parameters measured in plantlets treated with various levels of colchicine (Table 1). These observations were utilized to confirm the results obtained by chromosome counting. There were no obvious differences in leaf number, stomata diameter and stomata number between induced polyploid with diploid plantlets (Table 1). The vegetative growth of plantlets treated with colchicine was faster than that of control. The concentration of colchicine had a marked influence on the growth of plantlets. The highest plantlets length (17.30 cm) was obtained in tetraploids. This length is two times the length of control plantlets with 9.40 cm. Leaves characteristics of diploids and tetraploids were significantly different in the items of leaf length and width, and chloroplast number in guard cells (Table 1). Maximum average stomatal guard cell diameter of 20.20 µm was obtained from tetraploid plantlets treated with 0.10% colchicine for 24 h.

Comparison of stomatal guard cells size is a simple, effective, and economical method to select polyploids from diploids (Huy *et al*., 2019). Ploidy assessment by stomatal guard cell measurement allows breeders the opportunity for early screening of potential polyploids without investing in the time and space to growth a large population of plants. This method requires less time which permits simple and rapid analysis of a large number of species and varieties (Chen *et al*., 2009; Dhooghe *et al*., 2011). However, this method should be applied in combination with other modern methods in order to obtain clearer results (Huy *et al*., 2019). Several researchers showed increase in size of guard cells of plants treated with colchicine in comparison with untreated plants (De Mello Silva *et al*., 2000; Choopeng *et al*., 2019). Our study showed that chloroplast number increased at concentration of 0.10 colchicine for 24 h increased chloroplasts causes more chlorophyll and darker green leaves. Similar to our finding, the leaves of orchids tetraploid plantlets

treated with colchicine were longer and dark green than those of the diploids (Sarathum *et al*., 2010; Bunnag and Hongthongkham, 2015).

CONCLUSION

Induction of polyploidy in *Viola acuminata* Ledebour was attained through the treatment of seeds with colchicine. Unlike stomata diameter and number, traits such as stem length, root length, leaf length, leaf width and leaf number were significantly influenced by colchicine. Treatment of seeds with the concentration of 0.10% for 24 h was the best. Polyploid induction is a proper approach for improvement of ornamental plants. The flower characteristics of *V. acuminata* polyploids plants may be investigated in future works.

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