Induction and Identification of Polyploidy by Colchicine Treatment in *Lilium regale*

Nouraddin Izadi Jeloudar[1], Esmaeil Chamani[1]*,
Ali-akbar Shokouhian[1] and Rasoul Asghari Zakaria[2]

[1] Horticulture and Landscaping Dep., Agriculture and Natural Resources Fac. University of Mohaghegh Ardabili, Ardabil, Iran
[2] Agronomy and Plant Breeding Dep., Agriculture and Natural Resources Fac. University of Mohaghegh Ardabili, Ardabil, Iran

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Summary In order to produce an autotetraploid population of *Lilium regale*, different concentration of colchicine was used as chemical mutagen treatment to induce *in vitro* polyploidy. Bulb scales were soaked in various filter sterilized colchicine solutions (0.01, 0.05 and 0.1%) for 6, 12 and 24 h separately, then bulb scales were shifted to colchicine free MS medium for regeneration and normal growth. The ploidy level of the regenerated plantlets from treated bulb scales was estimated by chromosome counting in root tips. However, the length of stomatal guard cell, density and chloroplast number guard cell used as cytological parameters to compare between diploid and polyploid plantlets. The results of the experiment showed that high tetraploid plantlets (27.3%) were produced by 0.01%+ 24 h colchicine treatment. However, the highest survival rate (72%) was observed in 0.01%+ 6 h colchicine treatment. The stomatal number was reduced significantly ($p \leq 0.01$) in tetraploid compared to diploid. The result of the evaluation revealed that an increase in the length of stomatal and chloroplast number guard cell was found in polyploid plantlets as well as a reduction in stomatal number per unit area. It is found that colchicine at rate 0.01% for 24 h was the most effective treatment in inducing polyploidy in *L. regale* plantlets.

Key words *Lilium regale*, Colchicine, Polyploidy, Chromosome, Stoma.

The genus *Lilium*, the Liliaceae family includes 100 species, subspecies, and varieties which wide spreads mostly throughout the cold and temperate regions of East Asia (Wu and Raven 2000). *Lilium regale* is a rare wildflower germplasm resource in some counties of China and was used as an important parent in the breeding process as well as a source of higher values in medicine, ornamentation and food industry. It is known as king lily, half-cliff flower, trumpet flower or evening primrose (Zhang and Niu 2002).

Alternative usage, option for the addition of value is one of the most convenient and efficient ways to achieve this purpose. (Ollitrault *et al.* 2008, Wu *et al.* 2012, Marasek-Ciolakowska *et al.* 2014). Breeding through changing ploidy level is an effective method with a short duration and easy operation in comparison with the traditional cross-breeding and mutation breeding methods, which can help to increase breeding germplasm resources (Kashkush *et al.* 2002, Thao *et al.* 2003, Madon *et al.* 2005). Among ornamental plants, many polyploid cultivars have been produced and developed because they have many horticultural desirable characteristics such as the extension of flowering longevity, deep flower color, larger flowers (Okazaki and Hane 2005) and greater adaptability than diploid plants (Gao *et al.* 1996). Polyploid lilies have many advantages such as thicker leaves, stronger stems (especially important for forcing during the winter period) and restoration of F_1 sterility at the tetraploid level in interspecific hybridization (Van Tuyl *et al.* 1990, Van Holsteijn 1994). Production of polyploid plants is one of the current issues in lily breeding (Van Tuyl *et al.* 1990). It is reported that polyploid plants could be obtained through using artificial cell chromosome doubling by treatment of vegetative tissue with spindle inhibitors such as colchicine (Blakeslee and Avery 1937, Emsweller 1988).

To make a genetic variation, chromosome doubling has been widely used in plant breeding programs. It has been also used for the production of new cultivars (Griesbach 1990) and for the restoration of fertility in interspecific or intergeneric hybrids (Anderson *et al.* 1991). It's found that some species of lily has 24 chromosomes in their cells ($2n=2x=24$) (McRae 1998). To induce polyploids, colchicine as a chemical mutagen was used in somatic cells. Colchicine is very harmful to humans and in some cases shows undesirable mutagenic activity on plants (Van Tuyl *et al.* 1990). A high frequency of chimera formation is normally associated with chromosome doubling treatments under *in vivo* condition (Schifino and Fernandes 1987). It has been proved that chromosome doubling under *in vitro* condition is

* Corresponding author, e-mail: echamani@uma.ac.ir
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an excellent technique because of the high efficiency of polyploidy production with negligible chimera formation (Chen and Goeden-Kallemeyn 1979). Hence, there is a good scope for using *in vitro* techniques in polyploidy studies in lily and other bulbous ornamentals. To achieve further success in global competitiveness in the floriculture trademark, the production and development of new and popular cultivars by polyploidization, hybridization and mutation are seeming essential.

The objective of this study was to induce polyploidy in *L. regale* and aimed to determine an effective concentration and duration of colchicine treatment as an important step to obtain new varieties of *L. regale*. This study was an attempt to make an indirect method for the induction of polyploidy in *L. regale*.

Materials and methods

Plant material

Bulbs of *Lilium regale* were obtained from the Institute of Vegetables and Flowers of the Chinese Academy of Agricultural Science. Bulb scales were separated and surface sterilized for 30 s in ethanol 70% and 10 min in 2% NaOCl (w/v) and finally used as an explant. Regenerated explants were placed on solid MS medium according to described by Murashige and Skoog (1962) and was adjusted to pH 5.7 then 30 mL of the MS medium was poured into a 200 mL glassy drum and autoclaved for 15 min at 121°C under a pressure of 1.2 kg cm^{-2}. All the cultures kept at 25°C, 16 h photoperiod per day and under continuous illumination (50 μmol m^{-2} s^{-1}) with white fluorescent lamps.

After three months, when *in vitro* plantlets regenerated, scales with 1.5 to 2.0 g were collected and used for the present study.

Colchicine treatment

The effect of colchicine concentrations on plantlets was separately evaluated for any duration of treatments. The outer and healthy scales were selected and ten days cultured on bud initiation medium to mitotic activates and then soaked in various colchicine aqueous solutions (0.01, 0.05 and 0.1%) for 6, 12 and 24 h at room temperature. Afterward, the treated bulblet scales were rinsed thoroughly with distilled water and cultured carefully on solid MS medium and were incubated at 25°C with a 16 h photoperiod per day at a light intensity of 60 μmol photons m^{-2} s^{-1} from fluorescent lamps. Survival rate and regeneration were observed after 30 days of colchicine treatment. The growing and brown scales were considered as dead and removed from the culture. Healthy explant cultured in new MS medium and allowed to regeneration and normal growth.

Ploidy level determination

To determine the ploidy level for each plantlet, chromosome number was counted separately. The procedure of chromosome counting approximately was similar to described by Park *et al.* (1999). The newly grown root tips derived from plantlets were collected, pretreated for 24 h in iced water (0°C), and fixed in the mixture solution of 1% chromic acid and 10% formalin (1 : 1, v/v) at 4°C for 24 h, subsequently rinsed three times. The samples were hydrolyzed with 1 M NaOH for 10 min at 60°C, rinsed with distilled water. Root tips were slightly dried on filter paper and for staining transferred to hematoxylin for 15 h at room temperature. After washing with distilled water three times, the root tips were treated with sitase enzyme for 1–2 h and then cut to about 1–2 mm. Samples placed on a slide glass and mashed with a steel needle afterward added a drop of acetic acid 45% on the sample and placed lamella and finally squashed with the several slow hits. Chromosome numbers were counted directly from these slides under a light microscope and photographed. To proliferate the specified polyploid plantlet, the explants were cultured in three times on the new medium at intervals of 2–3 months.

Stomatal observations

The cytological features of diploid and putative polyploid plantlets were evaluated and compared with each other. Stomata guard cell length and stomatal density measurement used for identification of polyploidy plantlets. For this purpose, leaf samples of diploid and putative polyploid plantlets were used by nail varnish technique. In this study 10 plantlets of diploid (control) and 10 plantlets of putative polyploids randomly were selected. Measurement and counting stomata guard cell length and stomata density were performed for the well expanded leaf of each plantlet. Ten leaves were taken from diploid and putative polyploid plantlets and then the lower epidermal segments (about 1–2 mm^2) were selected from the central portion of each leaf. This part of the leaves was covered with a thin layer of clear nail polish and allowed to dry (Hamill *et al.* 1992). After drying the polish, it was removed carefully with glass glue then placed on a glass slide and observed through the light microscope and photographed. After taking photographs, the stomata guard cell length and stomata density were recorded. For study of the chloroplast number in the stomatal guard cells, ten leaves from diploid and putative tetraploid plantlets leaves randomly selected and samples of lower epidermal layer from each ploidy levels were obtained and this epidermal layer then placed on a glass slide, observed by light microscope and counted separately chloroplast number per guard cells (Guimaraes and Stotz 2004). Obtained data in putative polyploid and diploid plantlets were analyzed using ANOVA and Microsoft Excel 2016 statistically.

Table 1. Influence of various concentrations and soaking times on induction of polyploid plantlets from the *in vitro* bulb scales of *L. regale*.

Colchicine		No. of treated scales	Survival rate (%)	No. of plants tested	Tetraploidy production plantlets	Polyploidy production efficiency (%)
Time (h)	Conc. (%)					
6	0.01	25	72	18	1	5.6
6	0.05	25	40	10	0	0
6	0.1	25	24	6	0	0
12	0.01	25	40	10	2	20.0
12	0.05	25	28	7	0	0
12	0.1	25	24	6	0	0
24	0.01	25	44	11	3	27.3
24	0.05	25	16	4	0	0
24	0.1	25	16	4	0	0

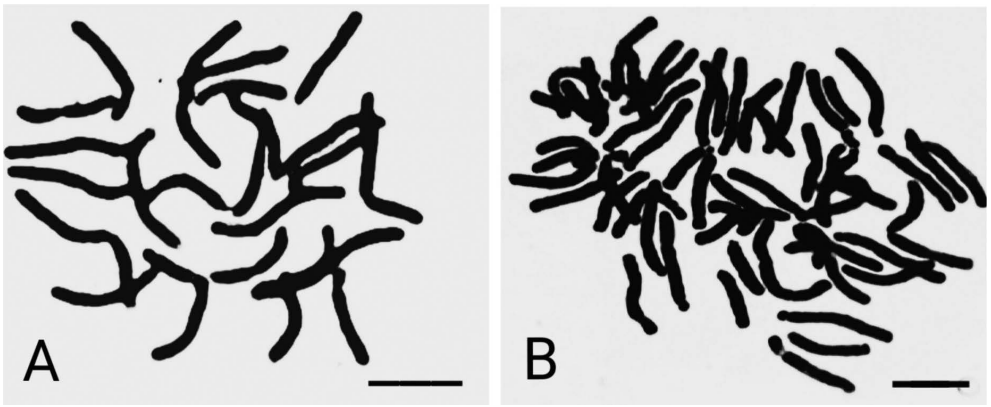

Fig. 1. Metaphase chromosomes in root tip cells of *L. regale* from colchicine-treated bulblets. A. Diploid ($2n=2x=24$). B. Tetraploid ($2n=4x=48$). Scale bar=10 μm.

Results

Survival rate

Treated explants showed stress symptoms due to colchicine, but symptoms rate was varied from 16 to 72% depending on the concentration of the colchicine solution and the soaking time. The survival rate in this study was specified approximately 3–4 weeks after culture. However, non-growing brown scales were considered as dead. The results of the experiment showed that there were significant differences ($p\leq0.01$) between survival rate and colchicine concentrations. A higher survival rate was observed with lower (0.01%) colchicine concentration. In addition, increasing of soaking period significantly ($p\leq0.05$) decreased the bulb scale survival rate (Table 1). In general, among the treated bulb scales, the highest percentage survival rate (72%) was observed in colchicine treatment at rate of 0.01% for 6 h treatment duration.

Polyploidization

All the regenerated plantlets from the colchicine treatments were evaluated after two months for their ploidy level determination. The chromosome counting results showed that colchicine successfully induced six tetraploid plantlets from bulb scale (Table 1). Studies of cell division in newly grown root tips clearly indicated the tetraploidy of the samples which due to chromosome doubling of diploid plantlets (Fig. 1).

The results showed that there were significant differences ($p\leq0.01$) between polyploidy and colchicine concentrations. All the tetraploid plantlets were obtained from the lowest concentration of colchicine (0.01%). No polyploid plantlet was found in other concentrations. The results also revealed that there are significant differences ($p\leq0.05$) between polyploidy and treatment duration which resulted that the highest treatment duration (24 h) produced the maximum tetraploid plantlets (Table 1).

Morphological analysis

The identification of polyploids can be based on stomatal morphology (Hamill *et al.* 1992). Stomata density is not influenced by external factors such as temperature and water content of the plant tissue and the stomata counting is a suitable, easy and reliable method for many plantlets can be estimated (Silva *et al.* 2000). The length of stomata guard cell and stomatal density significantly ($p\leq0.01$) differed between polyploid and diploid plantlets (Table 2). The result of our experiment showed that stomata length was 11.57 μm and 8.11 μm in tetraploid and diploid plantlets respectively.

The results of stomatal density showed that tetraploid plantlets significantly ($p\leq0.01$) had lower stomatal density than diploid plantlet. There was a clear difference between the chloroplast number in guard cells between

diploid and tetraploid plantlets (Fig. 2). The results of our experiment revealed that 66.7 chloroplasts were found in guard cells of diploid plants compared to 96.8 chloroplasts in tetraploids (Table 2).

Discussion

The survival rate is a very important factor when evaluating the efficiency of polyploid induction after soaking bulb scales in colchicine solution. The results of survival rate agree with the work of Heo *et al.* (2016) who reported the negative effect of high colchicine concentration on survivability in *L. leichtlinii* plantlets. However, Singh and Roy (1988) also reported high colchicine concentration can cause the death of plantlets by damaging to several parts of cells. Many other researchers demonstrated colchicine had a negative toxic effect on explants and decreased the survival and regeneration rate of cultures (Jähne and Lörz 1995, Cohen and Yao 1996, Van Duren *et al.* 1996, Song *et al.* 1997).

The results of polyploidization were similar to work of Heo *et al.* (2016) who concluded that the longest soaking treatment with low colchicine concentrations induced the highest percentage of polyploidy production efficiency among the other treatments in *L. leichtlinii*. Many other researchers have demonstrated that colchicine treatment is an effective method for chromosome doubling in plants, but their experiment resulted that the response to colchicine differs depending upon the plant species (Zhang *et al.* 2008, Sarathum *et al.* 2010, Blasco *et al.* 2015).

Based on the total bulb scale numbers and focusing on the data of the survival rate and regenerated tetraploid plantlets, we concluded the most suitable treatment to induce tetraploidy was 0.01% colchicine treatment for 24 h due to losses low number of bulb scales as well as

Table 2. Comparison of morphological traits of stomata in diploid and tetraploid plantlets *in L. regale*.

Ploidy level	Diploid (2*x*)	Tetraploid (4*x*)
	Mean	Mean
Stomata length (μm)	8.11b	11.57a
Stomata density (1000 μm^2)	6.11a	3.37b
Chloroplast/guard cell	66.7b	96.8a

Mean separation within columns by Duncan's multiple range test ($p \leq 0.01$).

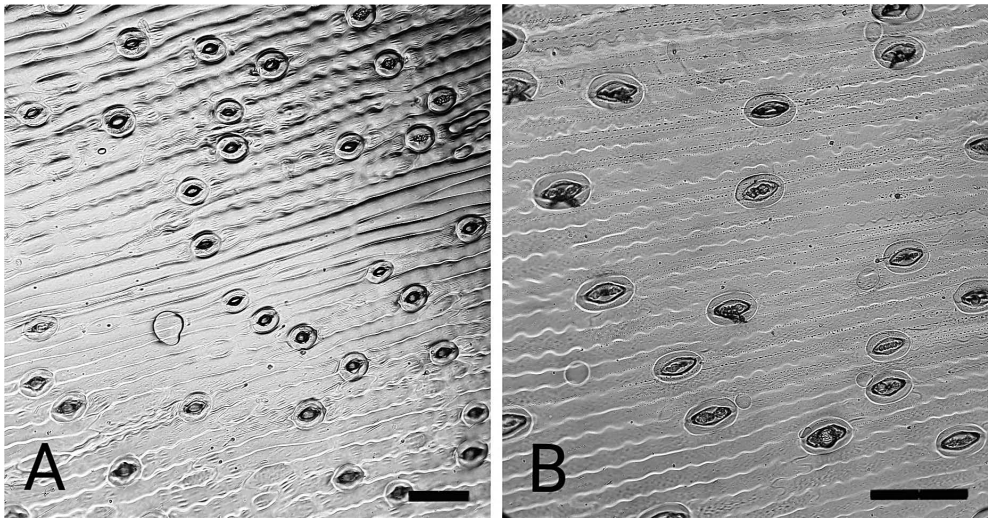

Fig. 2. Difference in stomata size and density between diploid and tetraploid in *L. regale*. A. Diploid ($2n=2x=24$). B. Tetraploid ($2n=4x=48$). Scale bar=20 μm.

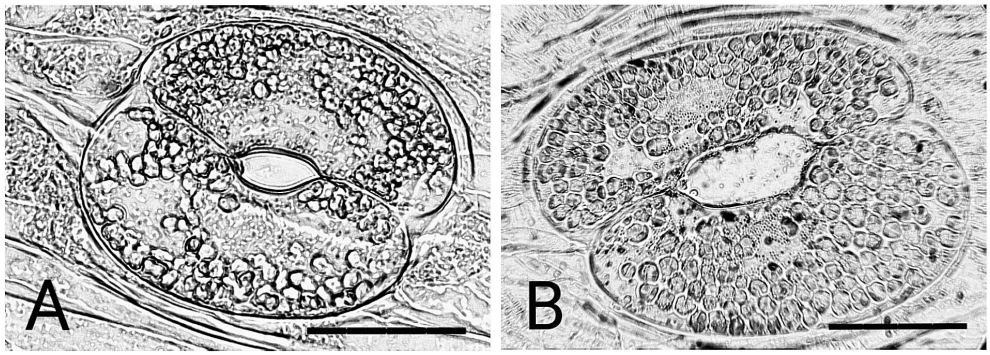

Fig. 3. Morphology of stomatal guard cells with chloroplasts in *L. regale*. A. Diploid ($2n=2x=24$). B. Tetraploid ($2n=4x=48$). Scale bar=0.25 μm.

inducing the high percentage of polyploidy production efficiency (27.3%) rather than other treatments.

Base on our findings of Morphological analysis we conclude that the relationship between the stomata density and ploidy level was negative, where the lower density values were obtained in tetraploid plants. Hence, this strong negative correlation between stomatal density and ploidy level can be used as an indicator of ploidy level determinator in *L. regale*.

The size of stomata has been used to differentiate diploid and polyploid regenerants of many plants such as orchids (Silva *et al.* 2000), *Stevia rebaudiana* (Zhang *et al.* 2018). The differences in stomatal number have also been used to identify polyploids (Vandehout *et al.* 1995). Our findings in case of significant differences ($p \leq 0.01$) of chloroplast number in guard cells of diploid and tetraploid plantlets (Table 2 and Fig. 3) was in agreement with results of Yetisir and Sari (2003). Omidbaigi *et al.* (2010) also reported the chloroplast number in guard cells of polyploid basil (*Ocimum basilicum* L.) significantly increased and compared to the diploid plant.

Our results indicated that stomata density, stomata length, and chloroplast number were useful tools for rapid prescreening of plantlet polyploidy to reduce the plantlets number to be maintained for the next studies. The choice of method for ploidy identification will depend on time and budget.

The tetraploid plantlets obtained from this study were subcultured in several time and were prepared for adaptation in soil and evaluation of their value as an ornamental source. We believe that the polyploid plantlets obtained from this study will be able to be used for the development of new *L. regale* cultivars with attractive ornamental characteristics.

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