The Journal of Animal & Plant Sciences, 26(4): 2016, Page: 1111-1116
ISSN: 1018-7081

POLYPLOID PRODUCTION IN *LILIUM LEICHTLINII* VAR. *MAXIMOWICZII* USING COLCHICINE

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ABSTRACT

This study was carried out to assemble the basic data on the breeding of new *L. leichtlinii* var. *maximowiczii* cultivars. Various concentrations and soaking times were investigated to determine the most effective approach to induce polyploid plants via colchicine treatment. In determining the survival rate and root formation degree of bulblets by colchicine treatment, it was found that the higher the colchicine concentration and the longer the soaking time were, the more the survival rate and root formation degree decreased. Chromosome analysis by flow cytometry and chromosome counting revealed that colchicine treatment produced a total of 83 polyploid plants. Although the soaking treatment in 0.2% colchicine for 9 hours induced the highest percentage of polyploid plants among the survived plants, a high percentage of the plants died. Interestingly, soaking treatment in 0.05% colchicine for 9 hours induced 15 polyploid plants, which was about 50% of the total number of bulblets tested in this study, hence this was considered the most suitable treatment to produce polyploid in *L. leichtlinii* var. *maximowiczii*.

Keywords: *L. leichtlinii* var. *maximowiczii*; polyploidy; flow cytometry; chromosome counting; colchicines.

INTRODUCTION

The genus *Lilium* is native to Asia, Europe, and North America in the northern hemisphere and includes approximately 100 species (McRae, 1998). Among these, the bulbs and young leaves of *Lilium leichtlinii* var. *maximowiczii* plants have been used as food and medicine in China and Japan (Kim *et al*., 2015). The *Lilium leichtlinii* var. *maximowiczii* plants continue to hold great value as a functional food in the East Asian floral market with its high profile of consumers, and it is expected that the production of edible lily in Japan and China will gradually increase. Korea shows great promise for the development of a floriculture industry with exports to East Asian countries because of its central location in the region. Hence, by promoting exports, the cultivation and distribution of *L. leichtlinii* var. *maximowiczii* could contribute significantly to the increased income of Korean lily farmers. *L. leichtlinii* var. *maximowiczii* is distributed widely throughout all of Korea (Baranova, 1969), but an evaluation of its genetic resources and breeding potential has not been done. For this reason, bulbs of *L. leichtlinii* var. *maximowiczii* need to be imported from foreign countries for cultivation in Korea. However, the bulbs of *L. leichtlinii* var. *maximowiczii* are extremely expensive, and its extensive cultivation as a food source has not been attempted.

To ensure global competitiveness in the floriculture industry, the development of new varieties of plants with high value beyond the most popular cultivars is essential. The addition of value through alternative usage is one of the most convenient and efficient ways to achieve this. Ploidy manipulation is a proven, promising tool for the genetic improvement of several plant species (Ollitrault *et al*., 2008; Wu *et al*., 2012; Marasek-Ciolakowska *et al*., 2014). Generally, polyploid flowering plants have larger flowers and greater adaptability than diploid plants (Lewis, 1980; Gao *et al*., 1996). Creating polyploid plants can be accomplished by the application of mitotic inhibitors. Colchicine solutions have been used successfully for the polyploidization of flowering plants (Chen and Gao, 2007; Sajjad *et al*., 2013; Yang *et al*., 2015). While polyploid plants have been created in the genus *Lilium* (Liu *et al*., 2011), there are no published reports for inducing polyploidy in *L. leichtlinii* var. *maximowiczii*. *L. leichtlinii* var. *maximowiczii* cv. Hakugin is one of the most-consumed edible lilies in Japan and is resistant to disease. In addition, it stably produces large and uniform bulbs, which are then used as a food source. In this study, we compared the efficacy of inducing polyploidy in bublets of *L. leichtlinii* var. *maximowiczii* cv. Hakugin treated with colchicine as the important step toward the breeding of new varieties of *L. leichtlinii* var. *maximowiczii* in Korea.

MATERIALS AND METHODS

Plant material and colchicine treatment: Bulbs of three year old *L. leichtlinii* var. *maximowiczii* cv. Hakugin were purchased from a Matsunaga seed online shop in Japan. Scales were surface sterilized for 10 min in 1% (w/v) NaOCl. Explants were placed with the abaxial side on 15 ml medium composing of MS macro- and microelements, 30g l^{-1} sucrose, 0.4mg l^{-1} thiamin, 100

mg l^{-1}, 7g l^{-1} bacto-agar (Difco, USA) and 0.05mg l^{-1} NAA and maintained at 20°C with a 12h photoperiod per day at quantum flux density of 60µmol $m^{-2}s^{-1}$ from fluorescent lamps. After 3 months, bulblets with fresh weights between 1.7 and 2.0g were collected and used for the present study. Thirty bublets were soaked in various colchicine aqueous solutions (0.05, 0.1 and 0.2%) for 1, 6 or 9 hours at room temperature on a shaker at 100 rpm. Afterward the treated bulblets were rinsed thoroughly with distilled water and cultured carefully on one-half MS medium containing 30g l^{-1} sucrose and 8g l^{-1} agar. All cultures were incubated at 20°C with a 12 hour photoperiod per day at quantum flux density of 60 µmol $m^{-2}s^{-1}$ from fluorescent lamps. Survival rate and root formation were observed after 60 days of colchicine treatment. Non growing and brown bublets were considered as dead. Root formation was investigated to count number of roots. Afterwards bublets were transferred to the charcoal medium to promote growth of plants.

Investigation of putative polyploidy: When the colchicine treated plants had grown for 90 days on the medium, putative polyploids of plants were investigated by estimating the nuclear DNA contents. The nuclear DNA contents of leaves derived from in the cultures were measured with Ploidy Analyser equipment (Partec GmbH, Germany) using the protocol described by Mishiba *et al*. (2000). Nuclear suspensions of leaf cells were obtained from 1^{-cm2} pieces of fresh leaves lacerated with a razor blade in a buffer solution using the CyStain UV Precise P kit (Partec GmbH, Germany), following the manufacturer's protocol. This kit uses DAPI (4, 6-diamidino-2-phenylindole, dilactate), a blue fluorescent stain that preferentially binds double-stranded DNA with A-T nucleotides. Leaves of onion (2n= 2x = 24) were used as an internal standard. Each sample was first analyzed alone to identify its fluorescence intensity profile and then analyzed with an internal standard to measure their ratio of mean cell fluorescence intensities.

Confirmation of polyploidy: In order to confirm the polyploidy level selected from putative polyploid plants, chromosome number was counted. The procedure of chromosome observation was the same as described by Park *et al*. (1999). The root tips derived from bublets were collected, pre-treated with 2mM 8-hydroxyquinoline at room temperature for 2 hours and fixed in the mixture solution of acetic acid and ethyl alcohol (1:3, v/v) at 4 for 4 hours. Samples were washed with distilled water and macerated in a solution of enzyme mixture for 30 to 60 minutes at 38 . The enzyme mixture consisted of 40% cellulase RS (Yakult), 1% pectolyase Y23 (Seishin Pharmaceutical Co., Korea), 70 mM KCl, and 7.5 mM Na2EDTA, and the pH was adjusted to 4.0 by 1M HCl. After washing with distilled water three times, the macerated root tips were placed on a slide glass with a few drops of acetic alcohol (1:3, v/v) solution. Samples were laid by tapping with fine tweezers and air-dried under room temperature. The air-dried specimens were stained with 4% Giemsa solution diluted with 1/15 M phosphate buffer (pH 6.8) for 5 to 10 minutes and air-dried again. They were mounted in paramount and observed under a microscope of 1000 magnifications.

RESULTS AND DISCUSSION

Mortality is a very important indicator when evaluating the efficiency of the induction of polyploid plants after soaking bulblets in colchicine. Mortality in this study varied from 0 to 33.3% depending on the concentration of the colchicine solution and the soaking time (Table 1). A higher mortality was observed with increasing colchicine treatment concentrations and time. The highest mortality was found in 0.2% colchicine treatment for 9 hours. This result agree with the work of Sajjad *et al*., (2013) who reported the negative effect of high colchicine concentration on survivability in plants. Singh and Roy (1988) also reported that high colchicine concentration can cause the death of plants by inducing the damage to several parts of cells making up trophosome.

The effect of colchicine on root formation was also investigated because root formation can be used to observe stress symptoms in plants. Average number of roots ranged from 3.43 to 1.81 on 0.05% for 1 hour and 0.2% for 9 hour colchicine treatment, respectively, showing high root formation in low concentrations (Table 1). High colchicine concentrations had a negative effect on root formation. They placed considerable stress on the plant cells, resulting in the inhibition of root formation or the death of the bulblets. With the inhibition of root formation, a retarded growth phase was observed. This result strongly suggested that high-concentration colchicine treatment may cause a reduced cell division rate. Similar results were found by Jang *et al*. (2000). They reported colchicine treatment in rice seedlings first leads to the inhibition of root development with stress symptoms in which the apical meristem is uplifted, and finally results in growth retardation. Hess (1989) stated that this kind of symptom can be associated with secondary biochemical effects causing growth inhibition.

The flow cytometric results indicated that colchicine successfully induced polyploid plants from bulblets of *L. leichtlinii* var. *maximowiczii* cv. Hakugin. Figs. 1a and 1b show one dominant peak (except for internal standard peak) corresponding to cells in the G1 phase, clearly indicating the polyploidy of the sample. In the putative diploid and triploid plants, the nuclei appeared at a relative nuclear DNA content of 130 and 190 (channel number), respectively. In the putative

tetraploid and hexaploid plants, one main and one minor peak were evident, as shown in Figs. 1c and 1d. Counting of the mitotic chromosomes demonstrated that the colchicine treatment in this study generated 6 triploid plants, 76 tetraploid plants, and 1 hexaploid plant, respectively (Table 2). The plants derived from the 1 hour-treated bulblets were predominantly diploid, with only a few triploid and tetraploid plants observed, and no difference in the polyploidy rate was found between the colchicine concentrations of 0.05%, 0.01%, and 0.2% applied for a very short soaking of only 1 hour. Other colchicine treatments over 6 hours were effective in obtaining polyploid plants, but their percentage differed based on the concentration and duration of the treatment. In general, the occurrence frequency of polyploid plants was increased by the colchicine concentration and soaking time at each concentration. With a survival rate of 72%, the highest percentage of polyploid plants was obtained using 0.2% colchicine for a duration of 9 hours, followed by 54% and 52%, which resulted from concentrationsof 0.2% over 6 hours and 0.1% over 9 hours, respectively.

Tambong *et al*. (1998) reported colchicine does not induce polyploidy in Cocoyam. In contrast, many other researchers have demonstrated that colchicine is an effective method for producing polyploid plants, but they also showed that responses to colchicine differ depending on the plant species (Zhang *et al*., 2008; Sarathum *et al*., 2010; Blasco *et al*., 2015). In this study, we also found that the effect of colchicine on polyploid induction in *L. leichtlinii* var. *maximowiczii* cv. Hakugin differs according to the concentration and soaking time. Although a combination of the highest concentration and longest duration induced the highest percentage of polyploid plants when only focusing on the data of the survived plants, it led to a high percentage of dead plants and was not the most efficient condition according to the data of the total bulblet numbers used for this study. Based on the total bulblet numbers, the most suitable treatment to induce polyploidy was the 0.05% colchicine treatment for 9 hours because it did not cause the death of the plants whereas it induced the highest rate (50%) of highly valuable polyploid plants.

The polyploid plants obtained from this study were grown at the Kangwon National University farm in Korea and were prepared for an evaluation of their value as a food source. We believe that the polyploid plants obtained from this study will be able to be used for the development of new *L. leichtlinii* var. *maximowiczii* cultivars with attractive food characteristics.

Table 1. Influence of colchicine on mortality, shoot regeneration and root formation.

	Mortality(%)	Shoot regeneration(%)	Root formation(No. of roots)
Colchicine 0.05% 1hr	0	100	3.43
Colchicine 0.05% 6hr	3.3	96.7	3
Colchicine 0.05% 9hr	6.7	93.3	2.5
Colchicine 0.1% 1hr	0	100	2.76
Colchicine 0.1% 6hr	13.3	86.7	2.48
Colchicine 0.1% 9hr	23.3	76.7	2.29
Colchicine 0.2% 1hr	0	100	2.75
Colchicine 0.2% 6hr	20	80	2.06
Colchicine 0.2% 9hr	33.3	66.7	1.81

Table 2. Effect of various concentrations and soaking times on induction of polyploid plants from the in vitro bulblets of *L. leichtlinii* var. *maximowiczii* cv. *Hakugin*

condition	No. of bublets treated	Survival plant						Polyploid production efficiency(%)
		Total	Diploid	Triploid	Tetraploid	Hexaploid	polyploidy/ survival plants(%)	
Colchicine 0.05% 1hr	30	30	29	0	1	0	3.3	3.3
Colchicine 0.05% 6hr	30	29	18	1	10	0	37.9	36.7
Colchicine 0.05% 9hr	30	28	13	1	14	0	53.5	50
Colchicine 0.1% 1hr	30	30	29	1	0	0	3.3	3.3
Colchicine 0.1% 6hr	30	26	14	0	11	1	46.5	40
Colchicine 0.1% 9hr	30	23	10	0	13	0	56.5	43.3
Colchicine 0.2% 1hr	30	30	26	2	2	0	13.3	13.3
Colchicine 0.2% 6hr	30	24	11	0	13	0	54.2	43.3
Colchicine 0.2% 9hr	30	20	9	1	12	0	60	43.3

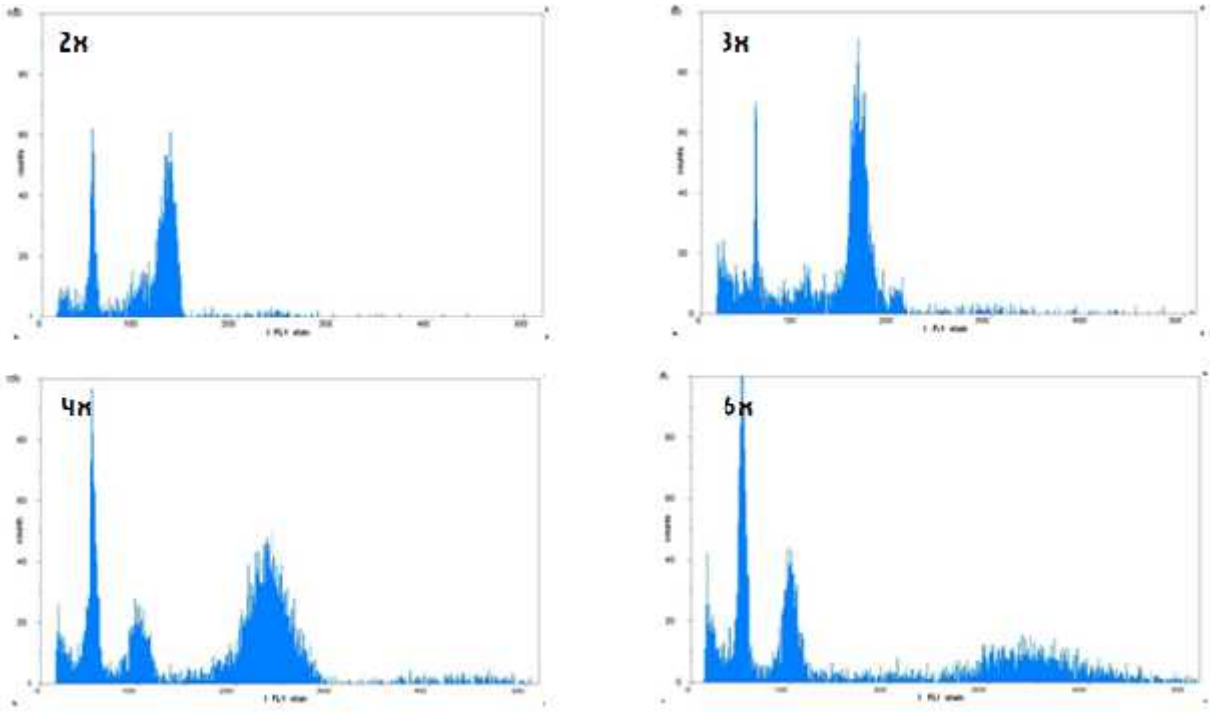

Fig. 1 Flow cytometric histograms of diploid, triploid tetraploid and tetraploid *L. leichtlinii* var. *maximowiczii*.

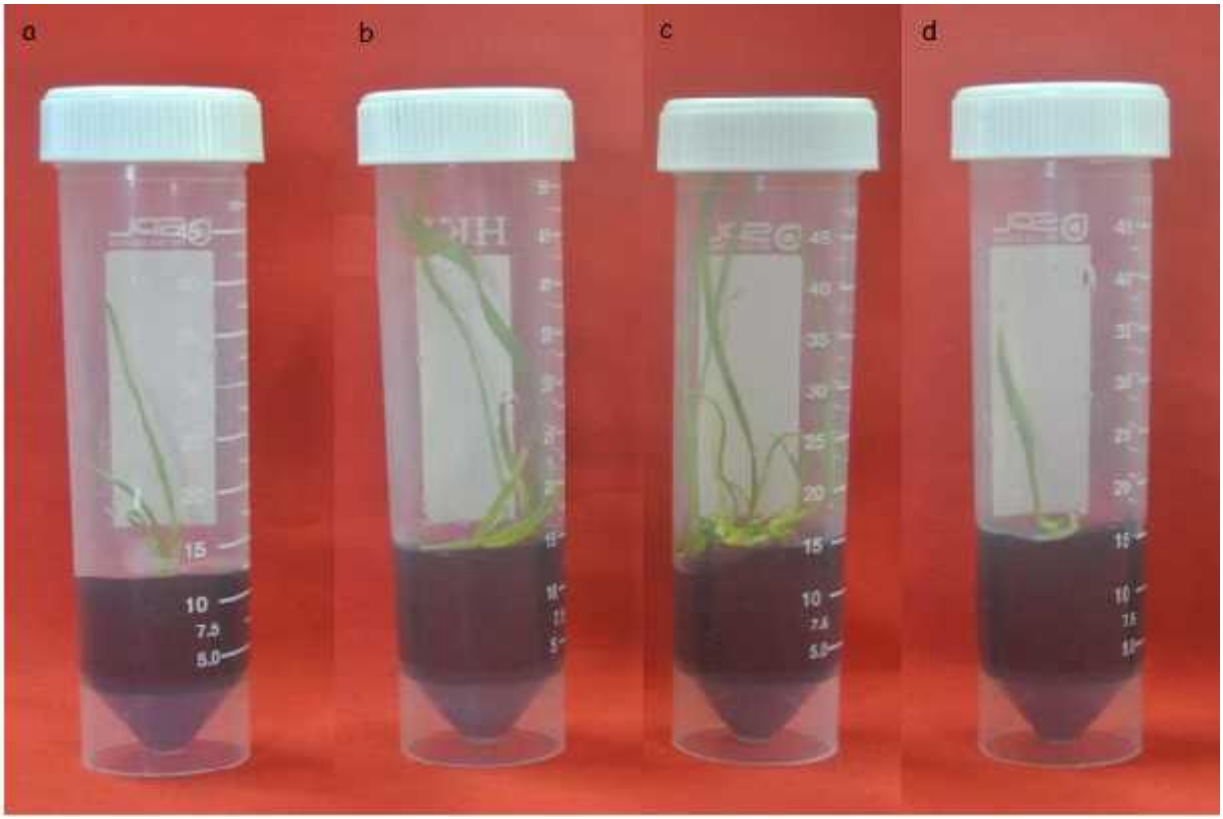

Fig. 2. Living plants after 60 days of colchicine treatment. a) diploid, b) triploid, c) tetraploid and d) hexaploid plant.

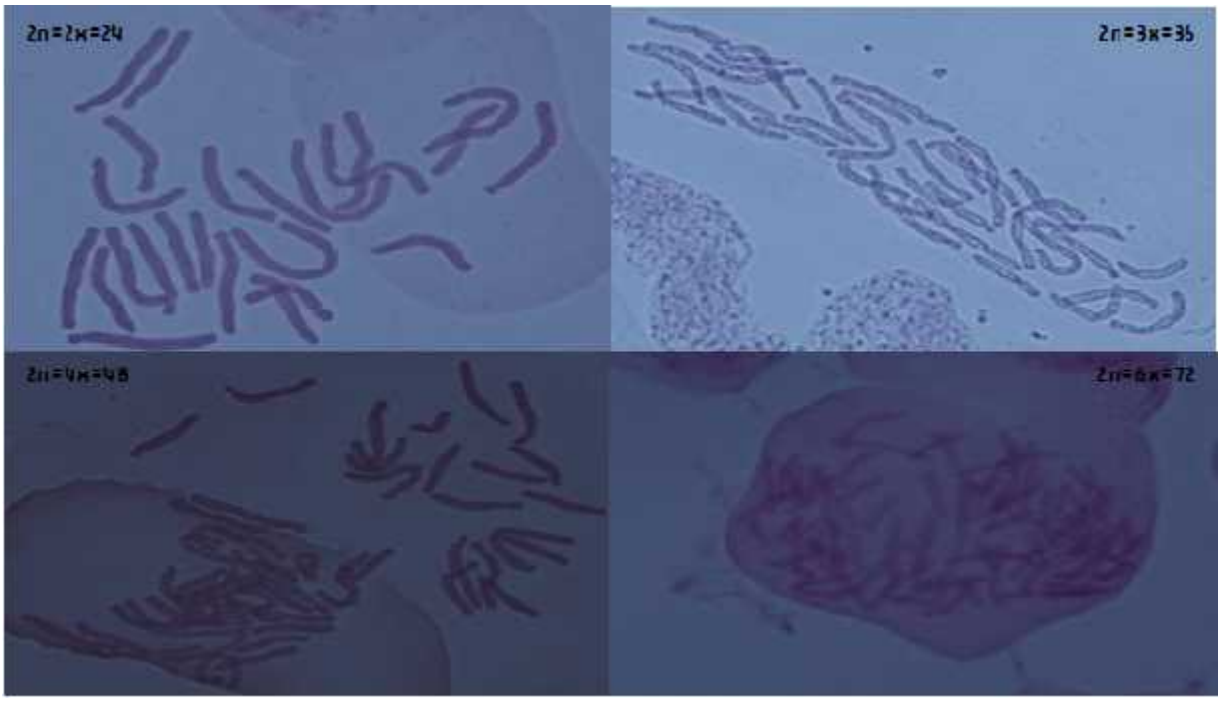

Fig. 3. Metaphase figures in root tip cells of plants from colchicine treated bublets.

Acknowledgments: This work was carried out with the support of the GSP Project (no. 213003-04-1-CGP00), the Ministry of Agriculture, Food and Rural Affairs, Republic of Korea and Kangwon National University (no. C1010819-01-01).

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