РАСТЕНИЕВЪДНИ НАУКИ, ГОД. LI, No. 6
PLANT SCIENCE, VOL. LI, No. 6
София. 2014. Sofia

IN VITRO POLYPLOIDIZATION OF *Lilium martagon*

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Abstract

This study investigated the capacity of the antimitotic agents: colchicine, oryzalin and trifluralin for inducing polyploidization *in vitro* of *Lilium martagon* var. Album (2n = 24). The one part of scales was soaked (4 h) in solution of colchicine or oryzalin. The second part of scales was transferred to the media enriched with oryzalin or trifluralin. Flow cytometry was used to estimate ploidy level of regenerated plants. The highest number of survived and regenerated scales (13), and the highest number of autotetraploids (69) were obtained when colchicine was used at the concentration of 1 g/l[1]. Oryzalin significantly affected the survival rate and appeared to be more phytotoxic and inhibiting for regeneration of bulblets than treatment of colchicine. After the soaking of scales in oryzalin solution the number of recovered autotetraploids was low because oryzalin strongly damaged scales and inhibited regeneration of bulblets. Enrichment of MS multiplication medium with oryzalin or trifluralin completely damaged the scales.

Key words: *Lilium*, tetraploids, antimitotic agents, *in vitro*

The genus *Lilium* belongs to Liliaceae family which comprises of about 80 species. *Lilium martagon* L. (Martagon section) is an endangered and protected species in many European countries, including Poland (List of plants protected as natural rarities, 2012). *Lilium martagon* var. Album is a vigorous bulbous perennial plant to 2 m in height, with beautiful pure white flowers. It is an important species that is cultivated for cut flowers and as pot plant and grown in gardens. This popular lily is a diploid and has a chromosome number 2n = 24 (Richardson, 1936; Inceer et al., 1999; Gomurgen and Altinozlu, 2004).

Many vegetatively propagated crops, including lily, are synthetic polyploids. *In vitro* techniques have great potential for improving the efficiency of polyplidization because this method offers more controlled environment (Dhooghe et al., 2011). Polyploidy is not simply genome duplication, but it results in molecular and physiological modification (Adams and Wendel, 2005). Polyploids often show novel morphology that is not observed in their diploid form. The reasons for using polyploids in lily breeding are larger flowers and the stronger stems of tetraploids than in the diploids (2n = 2x = 24) (Van Tuyl et al., 1992). Additionally, polyploidization can results in a higher resistance to diseases or/and stress and in a prolonged flowering time. In interspecific hybridization of lily, the F1-sterility at the diploid level is restored at tetraploid level (Van Tuyl and Van Holsteijn, 1996). Mitotic polyploidization is based on the chromosome doubling of somatic tissues (Ramsey and Schemske, 1998).

The largest group of antimitotic agents, including colchicine, oryzalin and trifluralin, are metaphase inhibitors (Dhooghe et al., 2011). Colchicine is an alkaloid that is extracted from plants of *Colchicum autumnale*. Oryzalin and trifluralin are antimicrotubule herbicides belonging to dinitroanilines. The most commonly used antimitotic agent is colchicine, which binds poorly to plant tubulins and must be used in relatively high concentrations. Oryzalin inhibits spindle formation at approximately one thousandth of the concentration of colchicine (Allum et al., 2007). In many plant species, colchicine causes side effects such as sterility, abnormal growth, chromosome losses or rearrangements and gene mutation (Luckett, 1989). Also, colchicine is very toxic to humans (Van Tuyl et al., 1992). In contrast, oryzalin and trifluralin specifically bind to plant tubulins and can be used at lower concentrations (Van Tuyl et al., 1992; Dhooghe et al., 2009 a, b). It was reported that *in vitro* treatment of lily bulb scales with oryzalin appeared to be less inhibiting for the regeneration and resulted in a higher number of polyploid plants than treatment with colchicine (Van Tuyl et al., 1992). For other plant crops it has been shown that the optimal antimitotic agent, its concentration and its exposure time for successful polyploidization is species dependent (Dhooghe et al., 2011).

The aim of the study was to compare the capacity of the antimitotic agents colchicine, oryzalin and trifluralin for *in vitro* inducing of tetraploids in *Lilium martagon* var. Album.

MATERIAL AND METHODS

The study was carried out on the bulblets of *L. martagon* var. Album virus-tested by ELISA. Initial culture of this genotype was established using seeds as the

starting explants. Afterwards, the obtained bulblets were propagated on MS (Murashige and Skoog, 1962) medium containing 0.1 mg/l NAA and 30 g/l sucrose. A culture cycle was 10 weeks, after this period the plants were transferred to fresh culture medium or used for polyploidization. In the experiment, the scales of this genotype were dissected from the bulbs grown *in vitro.* The one part of scales was placed in colchicine (0.5 and 1 g/l) or oryzalin (10 and 100 mg/l) solutions for 4 hours. The second part of scales was transferred to media enriched with oryzalin (0.5 and 5 mg/l) or trifluralin (0.5 and 5 mg/l). Colchicine was dissolved in water, oryzalin in NaOH and trifluralin in ethanol. All solutions were filter-sterilized and added to the media after autoclaving or to the soaking solutions. For regeneration of new bulblets, the scales were transferred on MS multiplication medium with 0.1 mg/l NAA. After a period of 8 weeks, the regenerated adventitious bulblets were transferred onto the fresh media without antimitotic agents for an additional 8 weeks prior to flow cytometric analysis. Each treatment consisted of 5 jars with 5 scales. Culture conditions: photoperiod – 16 h of light provided by cool-white fluorescent lamps (Philips TLD 36W/95) at 80 µmol/m^2.sec, temperature 20 °C. The observations and measurements were recorded after 8 weeks of culturing. The number of survived scales and the number of bulblets per scale were determined.

After 16 weeks of lily bulblet culture, the flow cytometry was used to estimate ploidy level of regenerated plantlets (Figure 1). One leaf sample was collected from each plantlet. Approximately 0.5 cm^2 of leaf tissue was chopped with a sharp razor blade in 0.5 ml of staining solution containing 4',6-diamidino-2-phenylindole (DAPI) (CyStain UV Ploidy, Parec Gmbh, Germany). Then, 1.5 ml of this staining solution was added and the samples were incubated at room temperature for 5 minutes, filtered through a 30 µm filter, and analyzed in Partec Flow Cytometer using UV excitation (HBP-Lamp, UV-Laser). Samples from plantlets derived from untreated initial scales were used as standards.

RESULTS AND DISCUSSION

To obtain the tetraploid of *L. martagon* var. Album plant, the scales of bulblets were treated *in vitro* with three different antimitotic agents: colchicine, oryzalin and trifluralin. Bulblet scale survival and rate of tetraploid induction depended on the type and the concentration of antimitotic agent, and on the method of their application. For soaking the scale in the colchicine solutions (0.5 g/l and 1.0 g/l), scale survival was the highest – 52% (13 scales) (Table 1). Scale survival rates for oryzalin treatments were depressed overall compared to colchicine.

Flow cytometric analysis showed that the efficiency of chromosome doubling depended on the type of antimitotic agents and their concentration and method of application (Table 1, Figure 1 A, B) The most efficient conditions for inducing tetraploids seemed to be soaking in colchicine solution (0.5 and 1 g/l). The highest number of survived and regenerated scales (13), and the highest number of autotetraploids (69) of *Lilium martagon* var. Album were obtained when colchicine was used at the concentration of 1 g/l (Table 1, Figure 2 B, C). Oryzalin significantly affected the survival rate and appeared to be more phytotoxic and inhibiting for regeneration of bulblets than treatment of colchicine. After the soaking of lily scales in oryzalin solution (10 and 100 mg/l) the number of recovered autotetraploids was low (6-9) because oryzalin strongly damaged scales and inhibited regeneration of bulblets (Table1, Figure 2 D, E). Similarly, in *Rhododendron* and *Ranunculus*, the survival rate upon colchicine treatment was better compared to oryzalin (Väinölä, 2000; Dhooghe et al., 2009a). In turn oryzalin was found to be more efficient

Table 1. Effect of different antimitotic agents on the survival rate, regeneration ability and chromosome doubling in *Lilium martagon* var. Album *in vitro*

Antimitotic agents	Concentration	Number of cultured scales	Number (%) of survived scales	Number of bulblets per scale	Number of regenerated plantlets	Number (%) of tetraploids obtained
The soaking of scales (4 h)						
Control	0	25	19 (76)	5.0	7	0 (0)
Colchicine	0.5 g/l 1.0 g/l	25 25	13 (52) 13 (52)	5.3 6.7	126 260	11 (8.7) 69 (26.5)
Oryzalin	10 mg/l 100 mg/l	25 25	8 (32) 8 (32)	2.8 2.8	28 16	9 (32.0) 6 (37.5)
The media enriched with antimitotic agents (exposure of scales – 16 weeks)						
Control	0	25	20	5.0	6	0
Oryzalin	0.5 mg/l 5.0 mg/l	25 25	0 0	0 0	0 0	0 0
Trifluralin	0.5 mg/l 5.0 mg/l	25 25	0 0	0 0	0 0	0 0

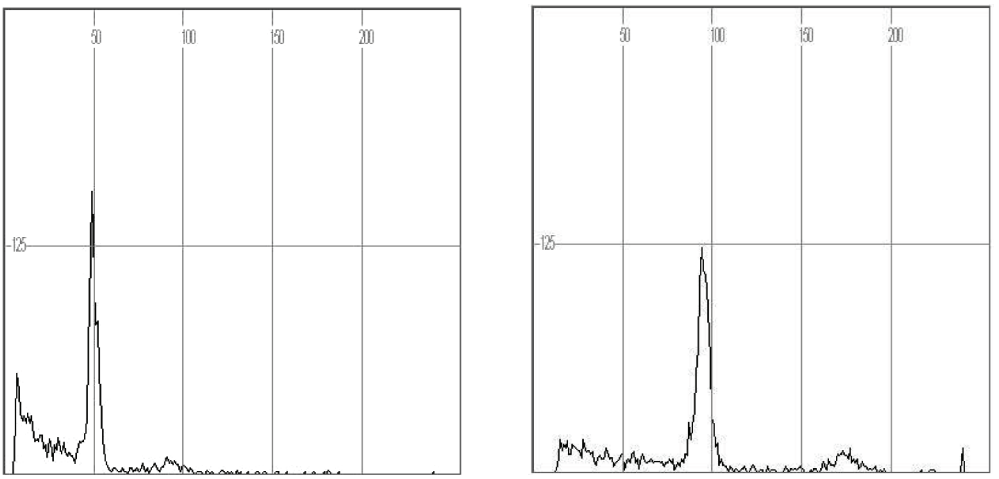

Fig. 1. Histograms of relative nuclear DNA content of nuclei isolated from young leaves of Lilium martagon var. *Album plants regenerated in vitro*
A – diploid plant (control – untreated standard), **B** – autotetraploid plant (treated with colchicine – 1.0 g/l)

Fig. 2. Plants of Lilium martagon var. Album regenerated in vitro
A – diploid plant (control – untreated standard), **B – E** autotetraploid plants obtained after soaking the scale in the solution of antimitotic agents: **B** – colchicine 0.5 g/l, **C** – colchicine 1.0 g/l, **D** – oryzalin 10 mg/l, **E** – oryzalin 100 mg/l.

than colchicine in inducing of chromosome doubling in interspecific hybrids of *Lilium longiflorum* × 'Whilito' and *L. henryi* × *L. candidum* (Van Tuyl et al., 1992). Colchicine was absolutely ineffective in inducing polyploidisation in *Helleborus* species, while oryzalin and trifluralin induced tetraploids (Dhooghe et al., 2009b). In our previous studies with *Hemerocallis,* oryzalin and trifluralin induced polyploidization but the highest number of tetraploids was obtained for the treatments with amiprophos-methyl (APM) (Podwyszyńska et al., 2010). In tulip, both oryzalin and APM induced polyploids with similar efficiency (Podwyszyńska, 2012). All scales of *Lilium martagon* var. Album isolated on the medium enriched with oryzalin (0.5 and 5 mg/l) or trifluralin (0.5 and 5 mg/l) were completely damaged and killed. In contrary, the incubation (12 weeks) of *Helleborus niger* and *H.* x *nigercors* shoots on the media with oryzalin or trifluralin was effective for inducing tetraploids (Dhooghe et al., 2009b).

In conclusion, the best results in chromosome doubling of *Lilium martagon* var. Album were obtained upon soaking the scales in colchicine solution (0.5 and 1 g/l). The autotetraploid plants of *Lilium martagon* var. Album can be used in lily breeding programs to develop new varieties.

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Acknowledgements.

This work was supported by The National Centre for Research and Development, Poland, Project No. N R12 0063 06/2009.