Production and Identification of a Tetraploid Germplasm of Edible *Lilium davidii* var. *unicolor* Salisb via Chromosome Doubling

Yayan Feng and Leifeng Xu

Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing 100081, China

Panpan Yang

College of Landscape Architecture, Nanjing Forestry University, Nanjing 210037, China; and Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing 100081, China

Hua Xu, Yuwei Cao, Yuchao Tang, Suxia Yuan, and Jun Ming¹

Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing 100081, China

Additional index words. edible lily, chromosome doubling, colchicine, oryzalin, tetraploid

Abstract. *Lilium davidii* var. *unicolor* Salisb is a cultivar of *Lilium* (Liliaceae) with important edible and ornamental characteristic. The application and production of *Lilium davidii* var. *unicolor* Salisb were still facing large problems because of its several disadvantages such as narrow range of adaptability, small annual growth increment, and low fertility. To achieve broader environmental adaptability and obtain a more nutritious germplasm, we used colchicine and oryzalin to induce chromosome doubling via the soaking method. Tissue culture bulbs were treated with colchicine at 0.03%, 0.05%, or 0.08% for 32, 40, or 48 hours or with oryzalin at 0.002%, 0.005%, 0.008%, or 0.01% for 3, 6, 9, 12, or 24 hours before being transferred to a differentiating medium. The results showed that colchicine treatment resulted in the highest induction rate when applied at 0.05% for 48 hours, whereas oryzalin treatment produced fewer tetraploid plants. The chromosome number of induced plants with small stoma density and longer guard cells is twice than that of the diploid. The plants were identified as tetraploid. In this study, a new germplasm of *Lilium davidii* var. *unicolor* Salisb was innovative and showed novel genetic characteristic.

Lilium davidii var. *unicolor* Salisb, known as the Lanzhou lily in Chinese, has been cultivated in China for nearly 500 years as a useful, but expensive vegetable. The main production area of Lanzhou lily is Lanzhou, Gansu Province; large-scale cultivation has also been undertaken in Pingliang and Yongjing. In traditional Chinese medicine, Lanzhou lily has antitussive and moistening properties that benefit the lungs. Polysaccharides are the key physiologically active components of Lanzhou

lily (Chen et al., 2008; de Souza et al., 2007; Ma et al., 2012; Yin et al., 2010), in addition to colchicine, which also have important physiological functions. However, the poor adaptation ability of Lanzhou lily has limited its growth in other areas, and its low fertility has limited its application for breeding. Chromosome doubling could improve the genetic adaptability, fertility, and nutritive value of this species (Chen and Kirkbride Jr., 2000; Dhooghe et al., 2011; Gomes et al., 2014; Lu and Bridgen, 1997; Zonneveld, 2009). Therefore, we used chromosome doubling to obtain a new germplasm with stronger adaptability and a higher nutrient content, laying a foundation for new varieties with improved breeding and food value.

Polyploidy, defined as the presence of three or more genomes in somatic cells, is one form of speciation (Adams and Wendel, 2005; Blanc and Wolfe, 2004; Bogart, 1980; Schultz, 1980). Polyploid plants produced through genome amplification exhibit many genetic differences from their diploid counterparts (Lokker et al., 2004), including morphological and cytological changes, reflected in parameters such as the leaf

shape index, stomatal density, and guard cell size, which can directly affect metabolism (Beck et al., 2003; Dhooghe et al., 2011; Masterson, 1994; Rhoades and Dempsey, 1966). These phenomena are widespread in many species, including *Zantedeschia* (Cohen and Yao, 1996), *Triticum aestivum* L. (Hansen and Andersen, 1998), and *Miscanthus sinensis* (Petersen et al., 2002). Genetic adaptability and tolerance to environmental changes can also be increased by polyploidy (Beers et al., 2005; Dhooghe et al., 2011; Xiong et al., 2006). Moreover, duplicated genetic material can restore fertility, but not for all species (Dunn and Lindstrom, 2007); the reason for such differences between species remains unclear. For crops with medical and economic value in particular, genome amplification is the best way to increase the content of active compounds (Berkov and Philipov, 2002; Gomes et al., 2014). There have been many studies on chromosome doubling in *Lilium* (Lim et al., 2001; Siljak-Yakovlev et al., 2003; van Tuyl et al., 1992). Such research has indicated that tetraploid plants exhibit fully restored fertility for breeding, although the edibility and genetic characteristics of the plants related to health care use have not been noted as differences among the selected materials.

The first application of colchicine for somatic tissue chromosome doubling was reported in the 1930s (Blakeslee and Avery, 1937). Since then, colchicine has become the most frequently used inducer. However, this high rate of induction is accompanied by high toxicity to humans and undesirable mutagenic effects on plants (Borisy and Taylor, 1967; Cohen and Yao, 1996; Liu et al., 2007). Oryzalin is considered a preferable alternative to colchicine because of its lower toxicity and lower required dosage (Bouvier et al., 1994; Kermani et al., 2003; Ramulu et al., 1991; van Tuyl et al., 1992). The effective concentration of oryzalin appears to vary from 0.001% to 0.1%.

Polysaccharides are considered the main functional components of lily bulbs, exhibiting antioxidant activity and immunoregulatory and hypoglycemic properties (Chen et al., 2008; Ma et al., 2012; Yin et al., 2010). Polysaccharides isolated and purified from many plant species have become a topic of great interest in natural medicine research (Chen et al., 2008; de Souza et al., 2007; Ma et al., 2012). Few studies have measured the polysaccharide content of artificial tetraploid *Lilium*. Phenol-vitriolic colorimetry is considered the most convenient and accurate method for polysaccharide content determination (Yang et al., 2004).

Here, for the first time, *Lilium davidii* var. *unicolor* Salisb was used as the material subjected to in vitro chromosome doubling. The induction rate was confirmed both indirectly and directly, through karyotype analysis, determination of genome size, and the measurement of stomata and polysaccharide contents, with the aim of obtaining a new germplasm with stronger adaptability and a high nutrient content and laying a foundation

Received for publication 5 Apr. 2017. Accepted for publication 8 June 2017.

This study was supported by Key Techniques of the Flower Seed Industry with Chinese Characteristics (2012BAD01B07), the National Natural Science Foundation of China (31272205), the National Special Public Welfare Project (2005DIB3J022), the Science and Technology Innovation Program of the Chinese Academy of Agricultural Sciences, and the Key Flower Projects of Beijing (YLHH2006001, YLHH201300101). This research was conducted at the Key Laboratory of Biology and Genetic Improvement of Horticultural Crops, Ministry of Agriculture, China.

¹Corresponding author. E-mail: mingjun@caas.cn.

for new varieties with high nutritional value and breeding utility.

Materials and Methods

Plant materials. The experiments were conducted in the cultivated species *Lilium davidii* var. *unicolor* Salisb, which is diploid (Fig. 1). For scale differentiation culture, modified Murashige and Skoog (MS) medium containing sucrose (3% w/v), 1-naphthaleneacetic acid (NAA) ($0.1 \text{ mg}\cdot\text{L}^{-1}$), and 6-benzylaminopurine (6-BA) ($1.0 \text{ mg}\cdot\text{L}^{-1}$) was used. The pH was adjusted to 5.8 with 1 M NaOH. An adequate number of aseptic bulbs were obtained through primary culturing. The materials were subcultured in an MS-based medium containing sucrose (9% w/v). All plant materials were cultured in a seed chamber at a constant temperature of 23°C with a 16 h light/8 h dark photoperiod.

In vitro chromosome doubling. In these experiments, we used two inducers: colchicine and oryzalin (Sigma, St. Louis, MO). Both inducers were applied to explants via the soaking method. The mother liquors of the above mentioned inducers were prepared by dissolving 200 mg of the inducer in 20 mL of dimethyl sulfoxide [DMSO (2%)], with 400 mg of NaOH. These solutions were then vacuum filter-sterilized (0.22 μm pore size) and aseptically added to 2% sterilized DMSO to a final volume of 50 mL. For colchicine treatment, three levels (0.03%, 0.05%, and 0.08%) and four treatment durations (24, 32, 40, and 48 h) were tested in all combinations. For oryzalin treatment, using concentrations from a previous study (van Tuyl et al., 1992), we tested four concentrations (0.002%, 0.005%, 0.008%, and 0.01%) and five processing times (3, 6, 9, 12, and 24 h). The treated scales (1 cm $\times$ 1 cm) were hermetically soaked and placed on a shaking table with a rotation speed of 120 r/min at 25°C . The explants were washed three times with sterile water and transferred to the differentiating medium with $0.1 \text{ mg}\cdot\text{L}^{-1}$ NAA and $1.0 \text{ mg}\cdot\text{L}^{-1}$ 6-BA for the regeneration of bulblets. Each treatment included three replicates, and 40 explants were cultured in each replicate. All cultures were cultivated under the same conditions described previously. The control was treated with the same

quantity of medium in the same environment, but without the inducers.

Flow cytometry ploidy identification. For the screening of initial polyploidy, young leaves were employed as the experimental materials. About 200 mg of leaf tissue was soaked with 2 mL of cold cell lysate in a 5 cm-diameter petri dish. We tested five types of cell lysis solutions (Doležel et al., 2007). First, the leaves were quickly chopped with a razor blade on ice. The obtained nuclear suspension was passed through a $30 \mu\text{m}$ -mesh nylon filter into a 2 mL centrifuge tube, which was then centrifuged off-center for 3 min (2000 r/min). The precipitated nuclei from the underlayer were stained with $500 \mu\text{L}$ of $50 \mu\text{g}/\text{mL}$ propidium iodide (PI; Sigma) for 10–15 min in the dark. Polyploidy was detected through BD FACSCalibur flow cytometry.

Chromosome counting and karyotyping. For cytological identification and to produce idiograms, 50 cells were detected. We used the root tips of plants grown in a rooting medium containing 9% sucrose without any growth regulator as the sample material. The root tips were pretreated with $0.7 \text{ mmol}\cdot\text{L}^{-1}$ cycloheximide for 8–9 h at room temperature and then fixed in Carnoy solution (absolute alcohol:glacial acetic acid = 3:1) for at least 24 h in the refrigerator. Next, the chromosomes were dissociated in 1 N HCl at 60°C for 10 min, and the materials were stained with carbol fuchsin for 5 min, after three rinses with distilled water. Chromosomes were counted in a minimum of 30 well-spread cells, and at least three different roots were examined. Karyotype analysis was performed using chromosome length and the relative arm ratios as standards (Levan et al., 1964).

Estimation of nuclear genome size. The nuclear genome size was estimated via flow cytometry using the protocol described previously. The young leaves of tissue culture seedlings were selected as experimental materials. The diploid and tetraploid genome sizes were determined using wheat as reference standard with a $2C$ value of 16 G. The pretreatment procedure was the same as for flow cytometry detection. Five samples from each species were measured. The variance of the data were checked through analysis of variance (ANOVA).

Determination of stomatal density and guard cell size. Stomatal density and guard cell measurements were performed on leaf under the epidermis via light microscopy at $\times 10$ and $\times 40$ magnifications. Diploid and tetraploid tissue culture seedlings at the same developmental stage were selected as the experimental materials. The obtained leaves were soaked in Carnoy solution until they were thoroughly faded. The fixed leaves were rinsed with distilled water for 2–5 min before staining with 1% iodine (potassium iodide) for 30 s. About 50 fields (10×40) were evaluated to determine the number of guard cells and their lengths. Statistical significance was evaluated through univariate ANOVA.

Analysis of polysaccharide contents. The polysaccharide content was determined using the phenol-vitriolic colorimetric method (Yang et al., 2004). Glucose was chosen to generate the standard curve. A linear regression equation was used to calculate the concentration from the absorbance values obtained through ultraviolet spectroscopy. Next, the extract liquid was examined. A 10 g lily bulb sample was subjected to continuous drying at 60°C and degreased with 95% alcohol for 3, 2, and 2 h before filtration and dialysis. The polysaccharide content was calculated according to the following formula: polysaccharide content (%) = $C.D.f \times 100/W$.

Results

Polyploidy induction. To determine ploidy levels, flow cytometry detection was used to screen for polyploidy. The reliability of this method was assessed through root-tip chromosome counting. The diploid plant was selected as the standard species. The DNA content of a sample was calculated using the G_1 peak means. The G_1 peak of the control was set to 200; the mean G_1 peak of the tetraploid was located at 400 and those of the chimera were set to 200 and 400. The DNA ploidy of unknown samples was determined as follows:

$$\begin{aligned} \text{Sample ploidy (integer)} \\ &= (\text{sample mean } G_1 \text{ peak} / \text{standard mean } G_1 \text{ peak}) \\ &\quad \times \text{standard ploidy} \end{aligned}$$

In diploid metaphases, a chromosome number of $2n = 24$ was detected, whereas that of the induced tetraploids was $2n = 48$. After this two-step detection, the induction effect was analyzed using schematics (Table 1). The two inducers had different effects on polyploidy induction. In the colchicine treatments, a total of 1560 scales were cultured in the regeneration medium. The differentiation rate ranged from 29.4% to 59.9%, whereas that of the contrast combination was 96.0%. The optimal treatment was achieved using a 0.05% solution for 48 h. Treatment with oryzalin did not efficiently produce tetraploid plants, but the ratio of the obtained mixoploids was higher. The preexisting scales were initially greatly inhibited,

Fig. 1. Characteristics of *Lilium davidii* var. *unicolor* Salisb flowers (A) and bulbs (B).

and some growth abnormalities resulted from colchicine toxicity. Sixty-six tetraploid plants were obtained via induction with colchicine, whereas only one tetraploid plant was obtained via induction with oryzalin (data not shown). The tetraploid bulbs were cultured in a subculture medium for propagation.

Karyotype analysis. According to the standard (Levan et al., 1964), natural diploids showed $2n = 24$ chromosomes in mitotic metaphase, whereas tetraploids showed $2n = 48$ chromosomes (Fig. 2). The karyotype and related parameters are summarized in Table 2. There was one median region (m) pair with an arm ratio between 1.0 and 1.7; two submedian region (sm) pairs with an arm ratio between 1.7 and 3.0; three subterminal region (st) pairs with an arm ratio between 3.0 and 7.0; and seven terminal region (t) pairs with an arm ratio greater than seven. The karyotype formula for the diploid was $2n = 24 = 2m + 2sm + 6st + 14t$. The tetraploids exhibited an expected karyotype of $4n = 48 = 4m + 4sm + 12st + 28t$ (Fig. 3). Karyotype analysis showed that chromosome pairing was normal in colchicine-induced tetraploid plants as no aneuploidy was observed. Karyotype analysis is the most accurate method for confirming the success of tetraploid induction.

Genome size. To assess the genome sizes of the diploids and tetraploids, flow cytometry was used to calculate the 2C value, and ‘Chinese Spring’ was selected as the reference standard, in which the 2C value is 16 G (Chapman et al., 2015). The peak for ‘Chinese Spring’ was set to 200 as the standard peak. The 2C value was calculated according to the following formula: (mean of the sample peak/mean of the standard peak) $\times$ standard 2C value. The diploid plants exhibited an average 2C value of 35.3 ± 0.9 G, whereas the tetraploid plants exhibited an average 2C value of 70.2 ± 0.6 G (Fig. 4).

Stomatal size and density. Guard cell length and stomatal density were measured via light microscopy on the hypodermis at $\times 10$ and $\times 40$ magnifications. In general, there was a positive correlation between stomatal size and ploidy, whereas stomatal density and ploidy were negatively correlated. Fifty fields were observed, and each stoma was measured. The diploid hybrids exhibited a higher stomatal density (average of 18–20) in every field (10×40), and the guard cell length was 23–25 μm . The tetraploid presented a lower stomatal density of 9–11 per field and a guard cell length of 32–33 μm (Table 3). These data should have considerable value for the selection of tetraploids. The differences between the tetraploid and diploid regarding stomatal density and guard cell length were significant (Fig. 5). Thus, stomatal measurements represent an indirect method for ploidy screening.

Polysaccharide quantification. To assess the medicinal value of the plants, their polysaccharide content was detected using the

Table 1. Number of mixploid and tetraploid regenerated bulbs under the treatment with colchicines.

					Percentage of regenerated bulbs (Number of regenerated bulbs)	
Inducer	DMSO	Concn (%)	Soak time (h)	Differentiation number	Mixploid	Tetraploid
Control				39.3 ± 1.0	— ^z	—
Colchicine	2%	0.03	24	27.3 ± 0.6	3.7 (1) ^y	0 (0)
			32	24.3 ± 1.5	4.1 (1)	4.1 (1)
			40	20.6 ± 2.5	9.7 (2)	4.9 (1)
			48	19.7 ± 2.1	11.2 (2)	10.2 (2)
		0.05	24	21.7 ± 3.2	4.6 (1)	9.2 (2)
			32	19.3 ± 2.1	10.4 (2)	10.4 (2)
			40	18.7 ± 1.5	16.0 (3)	16.0 (3)
			48	15.0 ± 1.0	33.3 (3)	33.3 (5)
		0.08	24	21.3 ± 1.5	4.7 (1)	9.4 (2)
			32	15.7 ± 1.5	12.7 (2)	12.7 (2)
			40	15.3 ± 1.5	19.6 (3)	19.6 (3)
			48	13.3 ± 1.5	30.1 (4)	22.6 (3)
Total number				25	26	

^zAll regenerated plants were diploid; $\pm$ sd.

^yThe numbers within parentheses mean regenerated bulbs with each ploidy in each treatment. The number of diploids was not included in the table.

Fig. 2. Root tips used for chromosome counting. (A) Chromosomes of diploid plants and (B) chromosomes of tetraploid plants.

Table 2. Chromosome parameters of *Lilium davidii* var. *unicolor* Salisb.

Chromosomes no.	Relative length %			Arm ratio	Type
	Long arm	Short arm	Full length		
1	2.8	1.9	4.7	1.47	M
2	2.8	1.0	3.8	2.80	Sm
3	3.1	0.4	3.5	7.75	T
4	2.9	0.4	3.3	7.25	T
5	2.4	0.5	2.9	4.80	St
6	2.5	0.3	2.8	8.33	T
7	2.4	0.3	2.7	8.00	T
8	2.1	0.5	2.6	4.20	St
9	2.2	0.4	2.6	5.50	St
10	2.3	0.3	2.6	7.66	T
11	2.2	0.3	2.5	7.33	T
12	1.9	0.2	2.1	8.50	T
Total	29.6	6.5	36.1		

Fig. 3. Ideogram of the diploid (A) and tetraploid (B) chromosomes.

Fig. 4. G₂ peaks of the sample and standard materials. (A) The sample peaks were set at the 200 and (B) standard peaks for the sample set.

Table 3. T-test of stomata density and guard cell length.

Variable	The unit view		T-value
	Diploid	Tetraploid	
Stomata density	18–20	9–11	–10.55**
Length of guard cell (um)	23–25	32–33	–24.19**

**Showed very significant difference.

phenol-vitriolic colorimetric method. The polysaccharide contents of the diploid and tetraploid scales were $14.6 \pm 0.8\%$ and $23.1 \pm 1.0\%$, respectively, indicating that the polysaccharide content increases with ploidy, showing a 1.5-fold increase with chromosome doubling. These results show that the edible value and medicinal value had been improved.

Discussion

Genome amplification is a critical step for improving the genetic characteristics of plants (Beers et al., 2005; Dean et al., 2002; Pinard et al., 2006). The key step for successful induction depended on the concentration of the inducer and the processing time in the induction stage. The confirmation step included direct and indirect methods for measuring chromosome doubling. In previous studies, colchicines have been considered the most effective inducers for achieving high induction rates, but they are also highly toxic to humans and plants (Cohen and Yao, 1996; Gao et al., 1996; Hansen and Andersen, 1998). Oryzalin is an alternative to colchicines that shows lower toxicity and a high efficiency of chromosome doubling (Dunn and Lindstrom, 2007; Morejohn et al., 1987; Petersen et al., 2002; Thao et al., 2003).

In this study, colchicine was found to be the best inducer for chromosome doubling, whereas oryzalin induced only diploid and mixoploid plants. This result agrees with many previous reports from other species (Abdoli et al., 2013; Gomes et al., 2014; Majdi et al., 2010; Thao et al., 2003). However, in previous studies, oryzalin was considered a preferable alternative to colchicines for chromosome doubling because of its high efficiency and low toxicity (Dunn and Lindstrom, 2007; Kermani et al., 2003; Morejohn et al., 1987; van Tuyl et al., 1992). The poorer results

we observed for oryzalin may be because of the use of an inappropriate concentration range or the different genotype of the experimental plant materials (Dhooghe et al., 2011; Kermani et al., 2003). We speculate that a higher concentration and longer processing time may be required.

There are many ways to detect tetraploid plants, including genetic, cytological, morphological, and chemical detection (Aina et al., 2012; Dhooghe et al., 2011; Yu et al., 2009). As previously reported, flow cytometry is a precise and rapid tool for large-scale polyploidy detection, but it is difficult to confirm aneuploidy (Doležel and Bartos, 2005; Doležel et al., 1998, 2007; Zonneveld et al., 2005). Cytological detection, which relies on chromosome counting, is considered the most accurate method for identifying tetraploid plants. In the present study, we chose flow cytometry as a time-saving initial screen for polyploidy. In this method, the leaf condition and the cutting method can significantly affect flow imaging. At the detection stage, a high degree of accuracy was crucial to evaluate the standard genome size (Leeton and Smyth, 1993; Zonneveld, 2009). As expected, the DNA content of the tetraploid was twice as high as that of the diploid. The genome size of the wild *Lilium* family ranges from 30–40 G; thus, the detected genome size of 35.3 ± 0.9 G was highly credible (Zonneveld et al., 2005). Root-tip chromosome counts were used to confirm the detection of tetraploids (Coe, 1959; Taylor et al., 1976). Genome size, stomatal measurements, and polysaccharide quantification further analyzed the characteristics of tetraploid germplasm. In total, 67 tetraploid plants were obtained using the above mentioned methods for selection and propagation.

Our karyotype analysis revealed that the mitotic metaphases of diploids and tetraploids contained 24 and 48 chromosomes,

respectively. This study also showed various dissimilarities compared with previous work, which may have been caused by differences in the geographical and ecological environment (Levan et al., 1964; Siljak-Yakovlev et al., 2003).

There are significant differences between stomatal characteristics and plant ploidy (Vandenhout et al., 1995). The direct relationship between genome size and ploidy level is a universal phenomenon in most angiosperms (Beaulieu et al., 2008; Masterson, 1994). Considering the consistency of the genetic and environmental background, we confirmed that the differences in stomatal parameters were caused by chromosome reduplication. The larger size and lower density of stomata may play an important role in plant physiological and ecological activities (Beck et al., 2003; Borrino and Powell, 1988; Hetherington and Woodward, 2003; Liu et al., 2007; Sari et al., 1999).

Tetraploid plants may show stronger photosynthesis. The deep color may result from an increased chlorophyll content to photosynthesis, which can increase adaptability and tolerance to environmental stresses (Beaulieu et al., 2008; Hetherington and Woodward, 2003; Masterson, 1994; Vandenhout et al., 1995). The obtained materials grew well in tissue culture vessels, where the inner temperature and pH differed greatly from those of the originating environment. We speculate that the materials were highly adaptable (Dhooghe et al., 2011). Many studies have shown that polysaccharides, as one of the three key biopolymers, have many effects that benefit the health of human beings (Chen et al., 2008; Ma et al., 2012; Yang et al., 2006). The edibility of *L. davidii* has been attributed to its high polysaccharide content. Chromosome doubling can restore fertility in many species (Dhooghe et al., 2011; van Tuyl et al., 1992). The fertility of tetraploid plants will be verified by tests that follow plants until the flowering stage. These tetraploid plants can be widely used in health care and breeding activities (de Souza et al., 2007; Yang et al., 2006; Yin et al., 2010).

In summary, we have demonstrated the application of in vitro treatment with colchicine and oryzalin to obtain tetraploid plants of the important edible plant *Lilium* for the

Fig. 5. General characteristics and comparison of diploid and tetraploid plants. (A) Bulbs of the diploid (left) and tetraploid (right); (B) leaves of the diploid (left) and tetraploid (right); (C) stoma measurements of the diploid; and (D) stoma measurements of the tetraploid.

first time, with the aim of obtaining well-adapted, fertile materials with high nutritional value, thereby unlocking new potential for the broader application of this species in production and breeding. In the following stage of our work plan, we will transplant the tissue culture seedlings in the field and employ the gas chromatography mass spectrometry method for metabolic profiling and the determination of CO₂ uptake to assess photosynthetic efficiency.

Literature Cited

- Abdoli, M., A. Moieni, and H. Naghdi Badi. 2013. Morphological, physiological, cytological and phytochemical studies in diploid and colchicine-induced tetraploid plants of *Echinacea purpurea* (L.). *Acta Physiol. Plant.* 35:2075–2083.
- Adams, K.L. and J.F. Wendel. 2005. Polyploidy and genome evolution in plants. *Curr. Opin. Plant Biol.* 8:135–141.
- Aina, O., K. Quesenberry, and M. Gallo. 2012. In vitro induction of tetraploids in arachis paraguariensis. *Plant Cell Tissue Organ Cult.* 111:231–238.
- Beaulieu, J.M., I.J. Leitch, S. Patel, A. Pendharkar, and C.A. Knight. 2008. Genome size is a strong predictor of cell size and stomatal density in angiosperms. *New Phytol.* 179:975–986.
- Beck, S.L., R.W. Dunlop, and A. Fossey. 2003. Stomatal length and frequency as a measure of ploidy level in black wattle, *Acacia mearnsii* (de Wild). *Bot. J. Linn. Soc.* 141:177–181.
- Beers, C.M., R. Barba-Gonzalez, A.A. van Silfhout, M.S. Ramanna, and J.M. van Tuyl. 2005. Mitotic and meiotic polyploidization in lily hybrids for transferring botrytis resistance. *Acta Hort.* 673:449–452.
- Berkov, S. and S. Philipov. 2002. Alkaloid production in diploid and autotetraploid plants of *datura stramonium*. *Pharm. Biol.* 40:617–621.
- Blakeslee, A.F. and A.G. Avery. 1937. Methods of inducing doubling of chromosomes in plants. *J. Hered.* 28:393–411.
- Blanc, G. and K.H. Wolfe. 2004. Functional divergence of duplicated genes formed by polyploidy during Arabidopsis evolution. *Plant Cell* 16:1679–1691.
- Bogart, J.P. 1980. Evolutionary implications of polyploidy in amphibians and reptiles, p. 341–378. In: W.H. Lewis (ed.). *Polyploidy: Biological relevance*. Plenum, New York, NY.
- Borisy, G.G. and E.W. Taylor. 1967. The mechanism of action of colchicine. Binding of colchicine-3H to cellular protein. *J. Cell Biol.* 34:525–533.
- Borrino, E.M. and W. Powell. 1988. Stomatal guard cell length as an indicator of ploidy in microspore-derived plants of barley. *Genome* 30:158–160.
- Bouvier, L., F.R. Fillon, and Y. Lespinasse. 1994. Oryzalin as an efficient agent for chromosome doubling of haploid apple shoots in vitro. *Plant Breed.* 113:343–346.
- Chapman, J.A., M. Mascher, A. Buluç, K. Barry, E. Georganas, A. Session, V. Strnadova, J. Jenkins, S. Sehgal, L. Oliker, J. Schmutz, K.A. Yelick, U. Scholz, R. Waugh, J.A. Poland, G.J. Muehlbauer, N. Stein, and D.S. Rokhsar. 2015. A whole-genome shotgun approach for assembling and anchoring the hexaploid bread wheat genome. *Genome Biol.* 16:16–26.
- Chen, H., M. Zhang, Z. Qu, and B. Xie. 2008. Antioxidant activities of different fractions of polysaccharide conjugates from green tea (*Camellia sinensis*). *Food Chem.* 106:559–563.
- Chen, J.-F. and J.H.K. Kirkbride, Jr. 2000. A new synthetic species of cucumis (*Cucurbitaceae*) from interspecific hybridization and chromosome doubling. *Brittonia* 52:315–319.
- Coe, E.H., Jr. 1959. A line of maize with high haploid frequency. *Amer. Nat.* 93:381–382.
- Cohen, D. and J.-L. Yao. 1996. In vitro chromosome doubling of nine *Zantedeschia* cultivars. *Plant Cell Tissue Organ Cult.* 47:43–49.
- Dean, F.B., S. Hosono, L. Fang, X. Wu, A.F. Faruqi, P. Bray-Ward, Z. Sun, Q. Zong, Y. Du, J. Du, M. Driscoll, W. Song, S.F. Kingsmore, M. Egholm, and R.S. Lasken. 2002. Comprehensive human genome amplification using multiple displacement amplification. *Proc. Natl. Acad. Sci. USA* 99:5261–5266.
- de Souza, M.C.R., C.T. Marques, C.M.G. Dore, F.R.F. da Silva, H.A.O. Rocha, and E.L. Leite. 2007. Antioxidant activities of sulfated polysaccharides from brown and red seaweeds. *J. Appl. Phycol.* 19:153–160.
- Dhooghe, E., K. Van Laere, T. Eeckhaut, L. Leus, and J. Van Huylbroeck. 2011. Mitotic chromosome doubling of plant tissues in vitro. *Plant Cell Tissue Organ Cult.* 104:359–373.
- Doležel, J. and J. Bartos. 2005. Plant DNA flow cytometry and estimation of nuclear genome size. *Ann. Bot.* 95:99–110.
- Doležel, J., J. Greilhuber, S. Lucret, A. Meister, M.A. Lysak, L. Nard, and R. Obermayer. 1998. Plant genome size estimation by flow cytometry inter-laboratory comparison. *Ann. Bot.* 82:17–26.
- Doležel, J., J. Greilhuber, and J. Suda. 2007. Estimation of nuclear DNA content in plants using flow cytometry. *Nat. Protoc.* 2:2233–2244.
- Dunn, B.L. and J.T. Lindstrom. 2007. Oryzalin-induced chromosome doubling in buddleja to facilitate interspecific hybridization. *HortScience* 42:1326–1328.
- Gao, S.L., D.N. Zhu, Z.H. Cai, and D.R. Xu. 1996. Autotetraploid plants from colchicine-treated bud culture of *Salvia miltiorrhiza* Bge. *Plant Cell Tissue Organ Cult.* 47:73–77.
- Gomes, S.S.L., C.W. Saldanha, C.S. Neves, M. Trevizani, N.R.B. Raposo, M.M. Notini, M. de Oliveira Santos, J.M.S. Campos, M.S. Campos, and L.F. Viccini. 2014. Karyotype, genome size, and in vitro chromosome doubling of *Pfaffia glomerata* (Spreng.) Pedersen. *Plant Cell Tissue Organ Cult.* 118:45–56.
- Hansen, N.J.P. and S.B. Andersen. 1998. In vitro chromosome doubling with colchicine during microspore culture in wheat (*Triticum aestivum* L.). *Euphytica* 102:101–108.
- Hetherington, A.M. and F.I. Woodward. 2003. The role of stomata in sensing and driving environmental change. *Nature* 424:901–908.
- Kermani, M.J., V. Sarasan, A.V. Roberts, K. Yokoya, J. Wentworth, and V.K. Sieber. 2003.

- Oryzalin-induced chromosome doubling in Rosa and its effect on plant morphology and pollen viability. *Theor. Appl. Genet.* 107:1195–1200.
- Leeton, P.R.J. and D.R. Smyth. 1993. An abundant line-like element amplified in the genome of *Lilium speciosum*. *Mol. Gen. Genet.* 237:97–104.
- Levan, A., K. Fredga, and A.A. Sandberg. 1964. Nomenclature for centromeric position on chromosomes. *Hereditas* 52:201–220.
- Lim, K.B., J. Wennekes, J.H. de Jong, E. Jacobsen, and J.M. van Tuyl. 2001. Karyotype analysis of *Lilium longiflorum* and *Lilium rubellum* by chromosome banding and fluorescence in situ hybridisation. *Genome* 44:911–918.
- Liu, G., Z. Li, and M. Bao. 2007. Colchicine-induced chromosome doubling in *Platanus acerifolia* and its effect on plant Morphology. *Euphytica* 157:145–154.
- Lokker, A.C., R. Barba-Gonzalez, K.-B. Lim, M.S. Ramanna, and J.M. van Tuyl. 2004. Genotypic and environmental variation in production of 2n-gametes of oriental x Asiatic lily hybrids. *Acta Hort.* 673:453–456.
- Lu, C. and M.P. Bridgen. 1997. Chromosome doubling and fertility study of *Alstroemeria aurea caryophyllaea*. *Euphytica* 94:75–81.
- Ma, L., H. Chen, Y. Zhang, N. Zhang, and L. Fu. 2012. Chemical modification and antioxidant activities of polysaccharide from mushroom *Inonotus obliquus*. *Carbohydr. Polym.* 89:371–378.
- Majdi, M., G. Karimzadeh, M.A. Malboobi, R. Omidbaigi, and G. Mirzaghaderi. 2010. Induction of tetraploidy to feverfew (*Tanacetum parthenium* Schulz-Bip.): Morphological, physiological, cytological, and phytochemical changes. *Hort. Sci.* 45:16–21.
- Masterson, J. 1994. Stomatal size in fossil plants: Evidence for polyploidy in majority of angiosperms. *Science* 264:421–424.
- Morejohn, L.C., T.E. Bureau, J. Molè-Bajer, A.S. Bajer, and D.E. Fosket. 1987. Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization in vitro. *Planta* 172:252–264.
- Petersen, K.K., P. Hagberg, and K. Kristiansen. 2002. In vitro chromosome doubling of *Miscanthus sinensis*. *Plant Breed.* 121:445–450.
- Pinard, R., A. de Winter, G.J. Sarkis, M.B. Gerstein, K.R. Tartaro, R.N. Plant, M. Egholm, J.M. Rothberg, and J.H. Leamon. 2006. Assessment of whole genome amplification-induced bias through high-throughput, massively parallel whole genome sequencing. *BMC Genomics* 7:216.
- Ramulu, K.S., H.A. Verhoeven, and P. Dijkhuis. 1991. Mitotic blocking, micronucleation, and chromosome doubling by oryzalin, amiprofos-methyl, and colchicine in potato. *Protoplasma* 160:65–71.
- Rhoades, M.M. and E. Dempsey. 1966. Induction of chromosome doubling at meiosis by the elongate gene in maize. *Genetics* 54:505–522.
- Sari, N., K. Abak, and M. Pitrat. 1999. Comparison of ploidy level screening methods in watermelon: *Citrullus lanatus* (Thunb.). *Matsum. and Nakai. Sci. Hort.* 82(3):265–277.
- Schultz, R.J. 1980. Role of polyploidy in the evolution of fishes, p. 313–340. In: W.H. Lewis (ed.). *Polyploidy: Biological relevance*. Plenum, New York, NY.
- Siljak-Yakovlev, S., S. Peccenini, E. Muratovic, V. Zoldos, O. Robin, and J. Valles. 2003. Chromosomal differentiation and genome size in three European mountain *Lilium* species. *Plant Sys. Evol.* 236:165–173.
- Taylor, N.L., M.K. Anderson, K.H. Quesenberry, and L. Watson. 1976. Doubling the chromosome number of trifolium species using nitrous oxide. *Crop Sci.* 16:516–518.
- Thao, N.T.P., K. Ureshino, I. Miyajima, Y. Ozaki, and H. Okubo. 2003. Induction of tetraploids in ornamental *Alocasia* through colchicine and oryzalin treatments. *Plant Cell Tissue Organ Cult.* 72:19–25.
- Vandenhout, H., R. Ortiz, D. Vuylsteke, R. Swennen, and K.V. Bai. 1995. Effect of ploidy on stomatal and other quantitative traits in plantain and banana hybrids. *Euphytica* 83:117–122.
- van Tuyl, J.M., B. Meijer, and M.P. van Diën. 1992. The use of oryzalin as an alternative for colchicine in in-vitro chromosome doubling of *Lilium* and *Nerine*. *Acta Hort.* 325:625–630.
- Xiong, Y.C., F.M. Li, and T. Zhang. 2006. Performance of wheat crops with different chromosome ploidy: Root-sourced signals, drought tolerance, and yield performance. *Planta* 224: 710–718.
- Yang, B., J. Wang, M. Zhao, Y. Liu, W. Wang, and Y. Jiang. 2006. Identification of polysaccharides from pericarp tissues of litchi (*Litchi chinensis* Sonn.) fruit in relation to their antioxidant activities. *Carbohydr. Res.* 341:634–638.
- Yang, L.-S., Y.-X. Li, M.-L. Li, and J. Liu. 2004. Determination for the content of polysaccharides in the *Lilium brownii* by phenol-vitriolic colorimetry. *Chinese J. Info TCM* 8:027.
- Yin, J.Y., S.P. Nie, C. Zhou, Y. Wan, and M.Y. Xie. 2010. Chemical characteristics and antioxidant activities of polysaccharide purified from the seeds of *Plantago asiatica* L. *J. Sci. Food Agr.* 90:210–217.
- Yu, Z., K. Haage, V.E. Streit, A. Gierl, and R.A.T. Ruiz. 2009. A large number of tetraploid *Arabidopsis thaliana* lines, generated by a rapid strategy, reveal high stability of neo-tetraploids during consecutive generations. *Theor. Appl. Genet.* 118:1107–1119.
- Zonneveld, B.J.M. 2009. The systematic value of nuclear genome size for “all” species of *Tulipa* L. (*Liliaceae*). *Plant Sys. Evol.* 281:217–245.
- Zonneveld, B.J.M., I.J. Leitch, and M.D. Bennett. 2005. First nuclear DNA amounts in more than 300 angiosperms. *Ann. Bot.* 96:229–244.