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ORIGINAL ARTICLE

An efficient method for inducing multiple genotypes of tetraploids *Lilium rosthornii* Diels

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Abstract

Polyploids generally show strong environmental adaptation and diverse morphological variations. Therefore, polyploid induction is an important protocol for plant breeding. *Lilium rosthornii* is a wild lily species with high horticultural value and excellent disease resistance. In this study, seeds of *L. rosthornii* were subjected to polyploidy induction treatments to obtain multiple genotypes of tetraploids. Germinated seeds were immersed in two antimitotic agents at different concentrations for various times. In total, 199 tetraploid genotypes were obtained. The most efficient treatments of each agent were immersed in 0.05% colchicine for 36 h and in 0.01% oryzalin for 24 h; the induction rate of the former (27.78%) was significantly higher than that of the latter (22.22%). The swollen hypocotyl phenotype after colchicine and oryzalin treatments was strongly correlated with tetraploidy (0.989** and 0.975**, respectively), suggesting that this phenotype could serve as an early ploidy selection trait. The correlations were weaker between stomata length/density and tetraploidy (0.773** and 0.695**, respectively), implying that stomatal characters are affected by both the ploidy level and genotype. After several rounds subculture in vitro, the morphology and growth traits were not significantly different between diploids and tetraploids, but there were wider variations in these parameters in tetraploids than in diploids. After transplanting, the bulblet germination rate was higher in tetraploids than in diploids. The leaf phenotype did not differ between tetraploids and diploids initially, but the leaves of tetraploids became larger than those of diploids over time. Together, these suggest that tetraploids, may contribute diverse characteristics to lily breeding.

Key message

Multiple genotypes of tetraploid *Lilium rosthornii* were induced by colchicine and oryzalin. Swollen hypocotyl was associated with polyploidy. Compared with diploids, tetraploids showed wider variations in morphological parameters.

Keywords *Lilium rosthornii* · Polyploidy induction · Multiple genotypes · Swollen hypocotyl · Morphological variation

Abbreviations

ANOVA	One-way analysis of variance
BA	Benzylaminopurine
FCM	Flow cytometry
MS	Murashige–Skoog
NAA	Naphthylacetic

Introduction

Polyploid induction is an important protocol for plant breeding. According to the chromosome origin, polyploids can be classified as allopolyploids and autopolyploids. Compared with diploids, most allopolyploids show superior growth and stress resistance because of heterosis and heterozygosity, whereas autopolyploids may show either enhanced or

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reduced these traits due to differences in heterozygosity among different genotypes (Podwyszyńska et al. 2014, 2018; Pozo and Elena 2015; Zhang et al. 2017). Wild plants have many genes and diverse phenotypes that may have been lost from cultivated varieties, so they are important germplasm resources (Kasteele and Stoop 1974). To acclimatize wild plants, seeds with multiple genotypes may provide wider range of morphological variations and stress resistance (Acosta-Gallegos et al. 2007; Tian et al. 2013; Mei et al. 2009). Chromosome duplication using seeds as the starting material can yield multi-genotypic autopolyploids with diverse heterozygosity (Bingham et al. 1994; Eliášová et al. 2014; Pozo and Ramirez-Parra 2014).

The efficiency of polyploidy induction depends on many variables, including explant type, the type and concentration of the antimitotic agent, and the exposure time. The most commonly used antimitotic agent is colchicine, which has a high affinity for microtubules in animal cells but a much lower affinity for plant tubulins (Dhooghe et al. 2010; Morejohn et al. 1984). Alternative antimitotic agents, such as oryzalin, trifluralin, surflan, and amiprohpos methyl (APM), have been used in previous studies (Khosravi et al. 2008; Nimura et al. 2006). Their efficiency depends on their concentration, as well as exposure time and solvent (Petersen et al. 2003, 2010; Zhang et al. 2017; Zhou et al. 2016). Leaves, stem apex, and seeds are the most widely used plant materials in induction treatments (Tu et al. 2018; Yu et al. 2015). The pre-culture period of plant materials also affect the polyploid induction efficiency (Kafawin and Chen 1991). For the induction of polyploids in *Lilium*, scales and shoots are the most widely used materials to date (Emsweller and Brierley 1940; Zhang et al. 2017). These studies for polyploidy induction using scales or shoots have used only a few individuals, so that the characteristics of polyploidization may have reflected only a few genotypes. Seeds, produced by fertile lily, may have multiple genotypes (Lim et al. 2008). However, seeds have rarely been used for polyploid induction in lily. Frist, hybrid seeds may lose the target characteristics, because character segregation may occur during seed formation (Lim et al. 2008). Second, many important variates are interspecific hybridization, such as OA hybrids, which are infertility and cannot produce seeds (Barba-Gonzalez et al. 2006; Tuyl and Arens 2010). Unlike varieties, seeds are an important propagative organ for majority of wild *Lilium* species (Liang and Tamura 2000).

Lilium rosthornii (2n = 2x) is a wild lily species with high horticultural value and excellent disease resistance (Du 2014). To make full use of this excellent material, we devised a method to induce polyploids of *L. rosthornii*. To establish an efficient method for inducing multiple genotypes of tetraploids, seeds were treated with two antimitotic agents at various concentrations for a range of exposure times. Moreover, to select abundant variations for acclimation and further breeding, we compared the morphological characters and growth rate between the genotypes of diploids and tetraploids in tissue culture and after transplanting.

Materials and methods

Seed pre-culture

Mature seeds of *L. rosthornii* were collected from Hunan Province. After 2 months of stratification in cold sand, the disinfected seeds were cultured on germination medium (Murashige–Skoog (MS) medium containing 0.02 mg L^{-1} naphthylacetic acid (NAA), 30 g L^{-1} sugars, and 6 g L^{-1} agar, pH 5.8). The plant materials were incubated at 23 ± 2 °C, under a 16/8 h (light/dark) photoperiod with light (40 μm m^{-2} s^{-1}) supplied by cool-white fluorescent lamps.

Polyploidy induction and morphology investigation during seeds germination

Germinated seeds with hypocotyl 0.2–0.5 cm in length, were selected as the materials for polyploidy induction. Oryzalin was dissolved in small amount of 1 M NaOH then diluted with distilled water to final concentrations of 0.005%, 0.01%, and 0.02% (w/v). Colchicine was directly dissolved in distilled water and diluted to final concentrations of 0.025%, 0.05%, and 0.1% (w/v). The solutions were sterilized by autoclaving and then used to treat the germinated seeds for different periods (12 h, 24 h, and 36 h). The control groups were treated with sterile distilled water. Each treatment contained 30 seeds and had three repeats. After induction, seeds were cultured on germination medium and the hypocotyl length, euphylla number, and other phenotypic traits were observed every 7 days. The survival rate of seeds was determined in the 3rd and 6th month after treatments. Each surviving seed and seeding propagated from it was marked as a clone. Newly generated shoots were transplanted onto proliferation medium (MS medium containing 0.1 mg L^{-1} NAA, 2 mg L^{-1} 6-Benzylaminopurine (6-BA), 30 g L^{-1} sugars, and 6 g L^{-1} agar, pH 5.8) and subcultured every 60 days. After three to four rounds of subculturing, some shoots were transplanted onto rooting medium (MS medium containing 0.02 mg L^{-1} NAA, 60 g L^{-1} sucrose, and 6 g L^{-1} agar, pH 5.8) to promote bulblet growth and obtain roots for chromosomal counting, and some shoots were further cultured on proliferation medium to obtain more microshoots.

Polyploidy level detection

Flow cytometry (FCM) identification

Young leaf tissue (0.5 × 0.5 cm) was selected for analysis by flow cytometry (FCM). The FCM identification was

conducted according to a previously reported protocol, with minor modifications (Zhang et al. 2017). Histograms were generated after analyzing ≥ 5000 nuclei using FSC 3.0. Flow-Cytometry Express software.

Chromosomal counting

After FCM identification, tetraploids were randomly reconfirmed by chromosomal counting using a method of Zhou (2007) with a minor modifications.

Stomatal characteristics and comparison of diploid and tetraploid growth in tissue culture

After 60 days of cultivation on MS medium (containing 0.5 g L^{-1} active carbon), uniformly sized leaves were selected from 30 diploid and 30 tetraploid genotypes to investigate the stomatal characteristics of each genotype (with three replications). The nail polish impression method was used to analyze stomatal characteristics (Ozturk et al. 2014). The stomata size and density were observed under 10 × lens of a light microscope (Lecia DM500, Heerhrugg, Switzerland). Stomata density was observed under three different horizons. The length and width of 10 stomata were measured for each genotype. The stoma index was calculated by dividing stoma length by stoma width. To calculate the scale differentiation rate for each ploidy level, 90 outer-layer scales were selected from similarly sized bulblets of 15 genotypes and then cultured on proliferation medium. The degree of differentiation was observed every 5 days and the number of newly generated microshoots was recorded after 60 days. We selected 32 diploid and 32 tetraploid shoots with a uniform size from eight genotypes of each ploidy level and cultured them on MS medium (containing 0.5 g L^{-1} activated carbon) to compare shoot growth characteristics. Roots length was recorded every 7 days to calculate the root generation rate. After 3 months of culture, phenotype parameters including the increase of shoot weight, root number and length, and leaf number and size were determined.

Comparison of growth of diploids and tetraploids in the greenhouse

Tissue-cultured bulblets (diameter, 0.8–1.0 cm) of 30 diploid genotypes (N = 2863) and 30 tetraploid genotypes (N = 2818) were transplanted into pots (5 × 5 cm, 2:1 mixture of grass peat and sands) after 2 months of dormancy breaking. After 3 months of growth, the seedlings were transplanted into large pots. The germination rate of bulblets was investigated after 1 month. For each genotype, the number of leaves, and the length and width of new fully extended leaf blades were recorded as morphological characters. The plants were cultivated in the greenhouse from 12 November 2018 to 31 June 2019 (13 to 27 °C in winter and 20 to 33 °C in spring during day, 5 to 15 °C in winter and 10–20 °C in spring during night).

Statistical analyses

Data were processed using Microsoft Excel 2010 software (Microsoft Corp, Richmond, CA, USA) and IBM SPSS Statistics 22 (IBM, Chicago, IL, USA). The significance of differences among treatments was evaluated using independent-samples T test or one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. The significance of differences in each parameter was evaluated using multivariate analysis of variance (in a general linear model) followed by Duncan's multiple range tests. The linear relationship between two variables was evaluated by Pearson's correlation analysis. Differences at $P < 0.05$ were considered significant. Graphs and figures were generated using GraphPad Prism 7.00 and Adobe Photoshop CS3 software.

Results

Changes in seeds morphology and growth after polyploidy induction

As illustrated in Fig. 1, the survival rate, morphology, and development rate of seeds differed significantly between those treated with antimitotic agents and the control groups. In the control groups, 100% of seeds survived from 0 to 36 h after water treatment. The hypocotyl rapidly extended to 4–5 cm in length over 2 weeks, then stopped elongating. The hypocotyl was thin, yellowish green, and had a smooth surface with abundant root hairs (Fig. 1b). Seeds in the control groups generated 1–2 euphylla in 2–4 weeks (Fig. 1d). In the treated groups, most generating seeds showed abnormal hypocotyl phenotype, apart from a few that were not significantly different from the control groups. As shown in Fig. 1c, the abnormal hypocotyl was swollen, had a rough surface, a yellowish-green color, and few root hairs. The swollen hypocotyl extended to 0.5–2 cm in length in 2 weeks, and then stopped elongating. No euphylla were generated in 2 months. After 2–3 months of cultivation, the color of the swollen hypocotyl became brown. One part of the hypocotyl gradually produced new shoots (Fig. 1e). Other parts of the hypocotyl did not generate new shoots in 3 months and died, but one seed generated callus in 6 months (Fig. 1f). Finally, 137 oryzalin-treated and 239 colchicine-treated seeds were survival, of which 87 oryzalin-treated and 125 colchicine-treated seeds formed abnormal hypocotyl (Table 1).

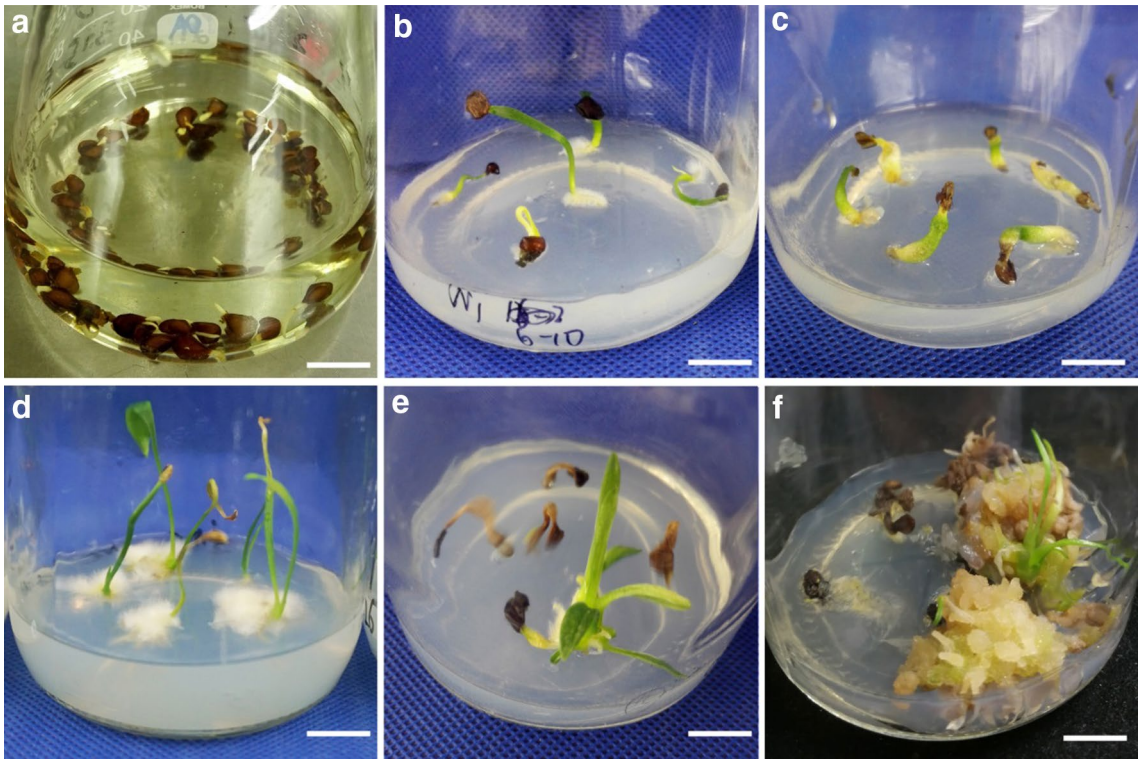

Fig. 1 Phenotype of *L. rosthornii* seeds after treatment with anti-mitosis agents. Germinated seeds immersed in anti-mitosis agent (**a**); phenotype of seeds in control group after 14 days (**b**); variant hypocotyl phenotype of seeds at 14 days after anti-mitosis treatments (**c**); phenotype of seeds in control group at 30 days (**d**); shoots directly variant generated from swollen hypocotyl (**e**); shoots indirectly generated from callus (**f**) in 6th month of culture; bars = 1 cm

Ploidy level after induction by oryzalin and colchicine

The ploidy levels of seeds in each treatment were identified by FCM (Table 1). There were 81 tetraploids in the oryzalin-treated group, 118 in the colchicine-treated group, and none in the control group. As shown in Fig. 2, selected tetraploids were randomly checked by chromosomal counting, and all re-identified plants had 48 chromosomes, double the number of chromosomes in diploids (2n = 2x = 24). The induction efficiency of treatments was compared by ANOVA. We compared 18 combinations of the two antimitotic agents, and found that the most efficient induction treatment was 0.05% colchicine for 36 h (27.78%), followed by 0.1% colchicine for 24 h (23.33%), 0.01% oryzalin for 24 h (22.22%) and 0.05% colchicine for 24 h (21.11%). The former treatment was significantly more efficient than the other treatments, and the latter three treatments were not significantly different from each other in terms of efficiency.

To determine the influence of each parameter, between-subjects effects were tested in multivariate analyses (Tables 2 and 3). The survival rate, frequency of swollen hypocotyl, and induction frequency of tetraploids were significantly affected by the concentration of each antimitotic agent, treatment time, and the interaction between concentration and treatment time ($P < 0.01$). An ANOVA multiple comparison analysis was conducted to analyze the effects of agent concentration and treatment time (Table 4). The numbers of swollen hypocotyl and tetraploids were markedly lower in the 0.02% oryzalin treatment than in the 0.005% and 0.01% oryzalin treatments, and not significantly different between the latter two treatments. The numbers of swollen hypocotyl and tetraploids were significantly higher after the 24-h oryzalin induction treatment than after the 12-h and 36-h oryzalin induction treatments. The numbers of swollen hypocotyl and tetraploids were not significantly different between the 0.05% and 0.1% colchicine treatments, but were significantly higher in those treatments than in the 0.025% colchicine treatment. The numbers of swollen hypocotyl and tetraploids were almost same after the 24-h and 36-h colchicine induction treatments, and were significantly higher after those treatments than after 12-h colchicine induction treatment. In conclusion, the highest relative efficiency of tetraploid induction was achieved with 0.005–0.01% oryzalin treatments for 24 h, and 0.05–0.1% colchicine treatments for 24 to 36 h.

Table 1 Effects of colchicine and oryzalin treatments on *L. rosthornii* seeds

Mitosis inhibitor	Concentration (%)	Treated time (h)	No. of treated seeds	No. of survival seeds	No. of swollen-hypocotyl	No. of swollen-hypocotyl at each ploidy level		
						Tetraploid	Mixploid	Diploid
Oryzalin	0.005	12	90	30	7	3ij (3.33%)	1	3
	0.005	24	90	25	18	16c (17.78%)	0	2
	0.005	36	90	16	13	13d (14.44%)	0	0
	0.010	12	90	23	6	6gh (6.67%)	0	0
	0.010	24	90	20	20	20b (22.22%)	0	0
	0.010	36	90	11	11	11de (12.22%)	0	0
	0.020	12	90	7	7	7 fg (7.78%)	0	0
	0.020	24	90	4	4	4hi (4.44%)	0	0
	0.020	36	90	1	1	1j (1.11%)	0	0
Total			810	137	87	81	1	5
Colchicine	0.025	12	90	40	5	1j (1.11%)	2	2
	0.025	24	90	32	10	8 fg (5.56%)	1	1
	0.025	36	90	27	13	13d (14.44%)	0	0
	0.050	12	90	29	6	6gh (6.67%)	0	0
	0.050	24	90	27	19	19b (21.11%)	0	0
	0.050	36	90	25	25	25a (27.78%)	0	0
	0.10	12	90	25	13	12ef (14.44%)	1	0
	0.10	24	90	21	21	21b (23.33%)	0	0
	0.10	36	90	13	13	13d (14.44%)	0	0
Total			810	239	125	118	4	3
Water	0.00	0	90	90	90	0	0	0
	0.00	12	90	90	90	0	0	0
	0.00	24	90	90	90	0	0	0
	0.00	36	90	90	90	0	0	0
Total			360	360	360			

Each treatment contained 30 seeds and had three repeats; Different lowercase letters within a column indicate significant difference at $P < 0.05$ level (one-way analysis of variance; Duncan's multiple range tests)

Correlation between swollen hypocotyl phenotype and ploidy level

The results of FCM analyses showed that the plantlets developing from hypocotyl with a normal phenotype were diploids, while the majority of shoots developing from swollen hypocotyl had altered ploidy levels. As shown in Table 1, the 87 plants that developed from swollen hypocotyl after oryzalin treatment consisted of 81 tetraploids, one mixploid and five diploids; and the 125 plants that developed from swollen hypocotyl after colchicine treatment consisted of 118 tetraploids, four mixploids, and three diploids. In correlation tests between the swollen hypocotyl phenotype and the frequency of tetraploids, the Pearson's coefficient values were 0.975** and 0.989** ($P < 0.01$) for the oryzalin and colchicine treatments, respectively (Table 5).

Stomatal characteristics of tissue cultured plantlets in diploid and tetraploid populations

As shown in Table 6, the stomatal length, width, and stomatal index of tetraploids was 61.17%, 22.33%, and 32.47% higher than those of tetraploids, respectively, while stomata density was 78.33% lower in tetraploids than in diploids. Except for stomata density, all other stomatal parameters showed larger coefficients of variation in tetraploids than in diploids. The results of correlation analyses between stomata characteristics and ploidy levels for tissue-cultured plantlets are summarized in Table 7. The Pearson's coefficient for stomatal traits of tetraploids (length, width, and density) were 0.773**, 0.504**, and 0.695** ($P < 0.01$), respectively.

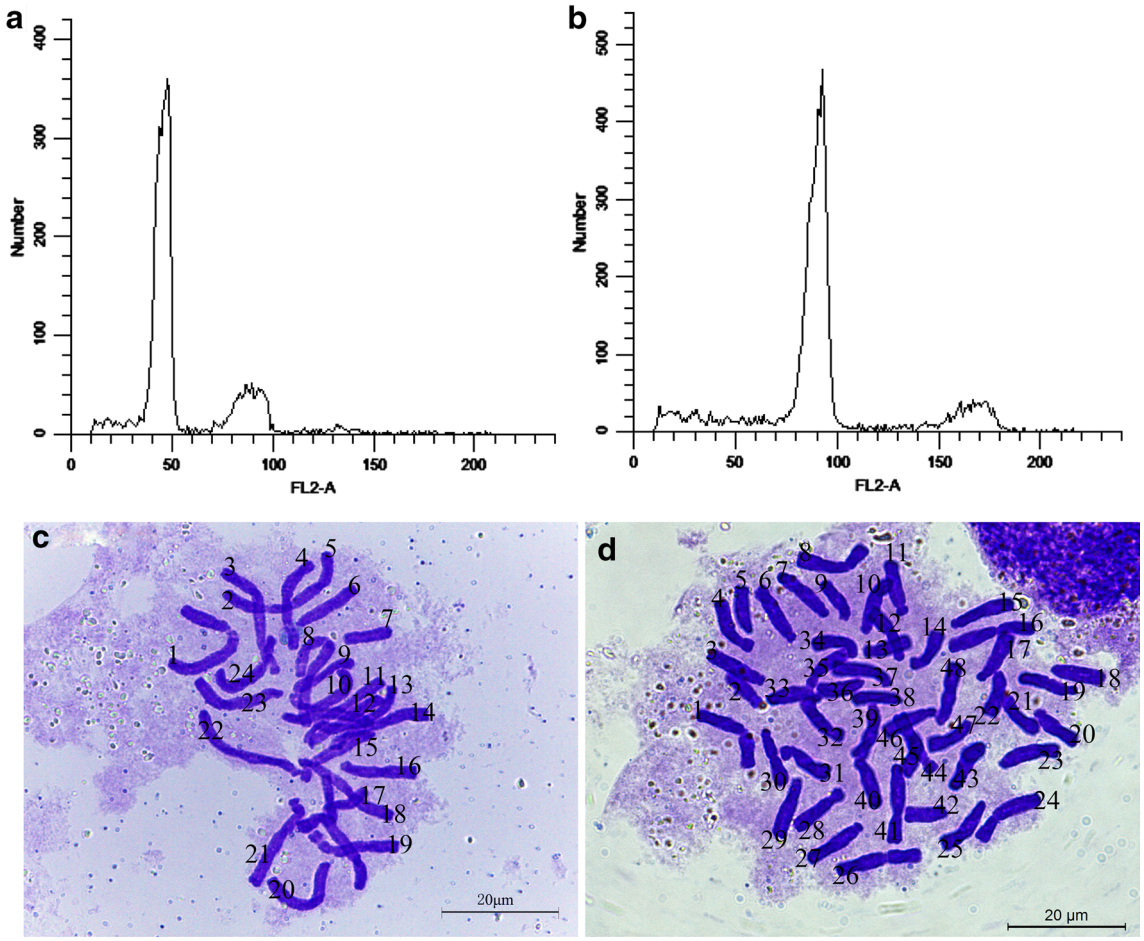

Fig. 2 Ploidy confirmation of diploid and tetraploid plantlets by flow cytometry and chromosomal counting. Flow cytometry histograms for control group (**a**) and tetraploids (**b**); chromosomes in root tip cell from diploid 2n = 2x = 24 (**c**) and tetraploid 2n = 4x = 48 (**d**); bars = 20 μm

Table 2 Tests of between-subject effects in oryzalin treatments

Source	Dependent variable	Type III sum of squares	df	Mean square	F value	Sig
Concentration	Survival number	204.963	2	102.481	345.875	0.000
	Swollen hypocotyl	48.222	2	24.111	65.100	0.000
	Tetraploid number	38.889	2	19.444	32.813	0.000
Treatment time	Survival number	58.741	2	29.370	99.125	0.000
	Swollen hypocotyl	29.556	2	14.778	39.900	0.000
	Tetraploid number	32.667	2	16.333	27.563	0.000
Concentration × treatment time	Survival number	6.815	4	1.704	5.750	0.004
	Swollen hypocotyl	30.222	4	7.556	20.400	0.000
	Tetraploid number	37.778	4	9.444	15.938	0.000
Error	Survival number	5.333	18	0.296		
	Swollen hypocotyl	6.667	18	0.370		
	Tetraploid number	10.667	18	0.593		
Total	Survival number	971.000	27			
	Swollen hypocotyl	395.000	27			
	Tetraploid number	363.000	27			

Concentration × treatment time: interaction between concentration and treatment time

Table 3 Tests of between-subject effects of colchicine treatments

Source	Dependent variable	Type III sum of squares	df	Mean square	F value	Sig
Concentration	Survival number	59.556	2	29.778	40.200	.000
	Swollen hypocotyl	31.630	2	15.815	22.474	.000
	Tetraploid number	50.963	2	25.481	38.222	.000
Treatment time	Survival number	50.889	2	25.444	34.350	.000
	Swollen hypocotyl	52.074	2	26.037	37.000	.000
	Tetraploid number	69.407	2	34.704	52.056	.000
Concentration × treatment time	Survival number	24.889	4	6.222	8.400	.001
	Swollen hypocotyl	35.926	4	8.981	12.763	.000
	Tetraploid number	33.926	4	8.481	12.722	.000
Error	Survival number	13.333	18	.741		
	Swollen hypocotyl	12.667	18	.704		
	Tetraploid number	12.000	18	.667		
Total	Survival number	2125.000	27			
	Swollen hypocotyl	711.000	27			
	Tetraploid number	682.000	27			

Concentration × treatment time: interaction between concentration and treatment time

Table 4 Multiple comparison analysis of concentration and treatment time in ANOVA

Antimitotic agent	Item	Gradient of parameter	No. of survival seeds	No. of swollen hypocotyl	No. of tetraploid
Oryzalin	Concentration (%)	0.005	7.89 ± 0.30a	4.22 ± 0.37a	3.56 ± 0.59a
		0.01	6.00 ± 0.30b	4.11 ± 0.37a	4.11 ± 0.59a
		0.02	1.33 ± 0.30c	1.33 ± 0.37b	1.33 ± 0.59b
	Treatment time (h)	12	6.67 ± 0.30a	2.22 ± 0.37b	1.78 ± 1.07c
		24	5.44 ± 0.30b	4.67 ± 0.37a	4.44 ± 1.07a
		36	3.11 ± 0.30c	2.78 ± 0.37b	2.78 ± 1.07b
Colchicine	Concentration (%)	0.025	10.11 ± 0.74a	3.11 ± 0.70b	2.44 ± 0.67b
		0.05	9.00 ± 0.74b	5.56 ± 0.70a	5.56 ± 0.67a
		0.1	6.56 ± 0.74c	5.22 ± 0.70a	5.11 ± 0.67a
	Treatment time (h)	12	10.44 ± 0.74a	2.67 ± 0.70b	2.11 ± 0.67b
		24	8.00 ± 0.74b	5.56 ± 0.70a	5.33 ± 0.67a
		36	7.22 ± 0.74b	5.67 ± 0.70a	5.67 ± 0.67a

Values are mean ± standard deviation; different lowercase letters within a column indicate significant difference at $P < 0.05$; Significance of differences in each parameter was evaluated using multivariate analysis of variance (general linear model), followed by Duncan's multiple range tests

Table 5 Correlation analysis between the swollen hypocotyl phenotype and tetraploidy

			No. of swollen hypocotyl plants	No. of tetraploid plants
Oryzalin	No. of swollen hypocotyl	Pearson correlation	1	0.975**
		Sig. (2-tailed)		0.000
		N	9	9
Colchicine	No. of swollen hypocotyl	Pearson Correlation	1	0.989**
		Sig. (2-tailed)		0.000
		N	9	9

Scale differentiation and adventitious shoot growth of plantlets with different ploidy levels in tissue culture

Scale differentiation and adventitious shoot growth were monitored in plantlets (Table 8). Scale differentiation did not differ significantly between diploids and tetraploids. The scales were swelled by 10 days of culture and generated new microshoots in 25 days. After 60 days of culture, diploids and tetraplolids had generated an average of 2.40 and 2.61 adventitious shoots, respectively. The rooting percentage from diploid and tetraploid adventitious shoots was 39.59% and 40.12%, respectively, at 14 days and 100% at 30 days (on MS medium containing 0.5 g L^{-1} activated carbon). Finally, diploids and tetraploids formed, on average, 9.38 and 10.88 roots, respectively, with an average length of 2.48 cm and 2.08 cm, respectively. The average increased weight of diploid and tetraploid shoots was 0.47 g and 0.48 g, respectively. The values of four leaf parameters (leaf number, length, width, and area) were all slightly higher for tetraploids than for diploids, but the differences were not significant. There were no significant differences between the two ploidy levels in terms of differentiation, growth, and morphology, but for most parameters, the coefficient of variations was higher for tetraploids than for diploids.

Germination rate of bulblte s and leaf growth rates of plantlets after transplanting

Average bulblet germination rates of diploids and tetraploids after transplanting were 55.08% and 65.31%, respectively (Table 9). The germination rate of diploid genotypes ranged

Table 6 Stomatal characters of tissue-cultured diploid and tetraploid plantlets

Ploidy level	Stomata length (μm)	Stomata width (μm)	Stomata density	Stomata index
2x	46.49 ± 5.94b	30.36 ± 3.52b	92.18 ± 27.56a	1.54 ± 0.16b
4x	74.93 ± 15.67a	37.14 ± 7.86a	51.69 ± 12.24b	2.04 ± 0.27a
Difference (%)	61.17	22.33	− 78.33	32.47
CV-2x (%)	12.78	11.59	29.90	10.39
CV-4x (%)	20.91	21.16	23.68	13.23

Stomata index: stoma length/width. Significance of differences in ploidy level was evaluated using independent-samples T test followed by Duncan's multiple range tests. Coefficient of variation (CV) was calculated by dividing standard deviation (SD) by mean value

Table 7 Correlations between stomatal characteristics of tissue-cultured and ploidy level

	Ploidy level	Stomata length	Stomata width	Stomata density
Ploidy level				
Pearson correlation	1	0.773**	0.504**	0.695**
Sig. (2-tailed)		0.000	0.000	0.000
N	60	60	60	60

Table 8 Scale differentiation and adventitious shoot growth in tissue culture

Ploidy level	Average No. of differentiation	Increased shoots weight (g)	No. of roots	Length of roots (cm)
2x	2.40 ± 1.12a	0.47 ± 0.22a	9.38 ± 3.70a	2.48 ± 1.08a
4x	2.61 ± 1.34a	0.48 ± 0.24a	10.88 ± 5.77a	2.08 ± 1.16a
CV-2x (%)	46.66	48.76	39.48	43.50
CV-4x (%)	51.25	51.69	53.04	55.86
Ploidy level	Leaves number	Leaves length (cm)	Leave width (cm)	Leave area (cm^2)
2x	3.75 ± 0.71a	3.16 ± 0.99a	0.48 ± 0.10a	1.21 ± 0.54a
4x	3.81 ± 0.74a	3.18 ± 0.87a	0.62 ± 0.15a	1.62 ± 0.79a
CV-2x (%)	18.86	31.35	21.79	44.69
CV-4x (%)	19.35	27.46	24.42	48.90

Average number of differentiated scales was counted after 60 days of cultural on prolification medium; other characters were observed after 90 days of culture on MS medium (contain 0.5 g^{-1} active carbon)

Table 9 Germination rates of diploid and tetraploid bulblets after transplanting

Ploidy level	Mean (%)	SD	Minimum (%)	Maximum (%)	CV (%)
2x	55.08	0.09	40.58	70.43	16.63
4x	65.31	0.08	48.39	80.30	12.87

SD standard deviation, *CV* coefficient of variation

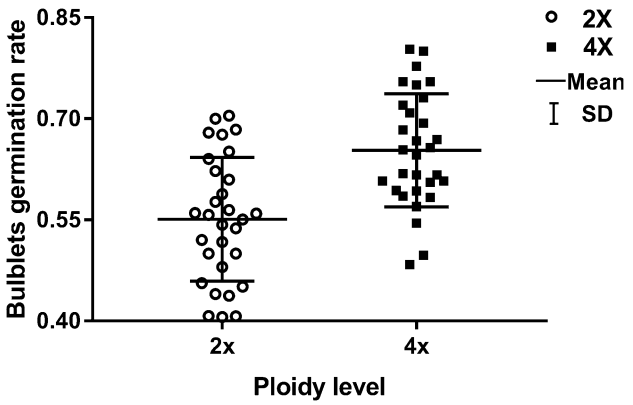

Fig. 3 Bulblet germination rates of diploid and tetraploid genotypes after transplanting

from 40.58 to 70.43% (coefficient of variation, 16.63%), while that of tetraploid genotypes ranged from 48.39 to 80.30% (coefficient of variation, 12.87%). The bulblet germination rate of diploid and tetraploid genotype is shown in Fig. 3. The germination rate of seven tetraploid genotypes was greater than the maximum germination rate of diploids.

Table 10 summarizes morphological characteristics after transplanting. Leaf number did not differ significantly between diploids and tetraploids at each time point. Leaf length was similar between diploids and tetraploids initially, but leaves of tetraploids became significantly larger than those of diploids over time. For example, after 7 months of cultivation, the average leaf length was significantly greater in tetraploids (14.11 cm) than in diploids (11.35 cm). The leaf width of tetraploids was greater than that of diploids at all time points, except in the 3rd month of culture. Leaf area did not differ significantly between the two ploidy levels initially, but was greater for tetraploids than for diploids in the 5th to the 7th month of culture. After 7 months of culture, the coefficient of variation in leaf area was greater in tetraploids (23.06%) than in diploids (14.86%).

Discussion

An efficient method for tetraploid induction is crucial for breeding. To eliminate the effects of poor seed germination, we selected germinated seeds as the materials for induction in this study. The use of germinated seeds to improve the induction rate has been reported in many studies (Ascough and Staden 2008; Dhamayanthi and Gotmare 2010; Feng et al. 2016; Schnell 2015). However, the appropriate period of pre-culture was different among different species (Aqafarini et al. 2019; Feng et al. 2016; Omidbaigi et al. 2010). In this study, we selected seeds with hypocotyl about 0.2–0.5 cm in length because longer hypocotyl was easily detached from the endosperm, and was prone to rotting during subsequent steps. Among the induction treatments, the most efficient one was 36-h immersion in 0.05% colchicine (induction rate, 27.78%). Our results indicated that colchicine is more efficient than oryzalin, as it resulted in a higher tetraploid induction rate (Tables 1 and 4). However, the results of other studies suggested that polyploidy induction is more efficient with oryzalin treatment than with colchicine treatment (Bouvier et al. 2010; Lehrer et al. 2008; Sakhanokho et al. 2009; Tuyl et al. 1990). Oryzalin, a kind of herbicide, is characterized by more affinity to microtubules of plant cells than animal cells and it always resulted in a higher mortality rate compared with colchicine (Bajer and Mole-Bajer 1986; Morejohn et al. 1987; Morejohn and Fosket 1991). Moreover,

Table 10 Growth characteristics of diploid and tetraploid populations of *L. rosthornii* at various times

Transplanted times	Ploidy level	Leaf number	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)
1 month	2x	1.60 ± 0.56a	2.82 ± 0.65a	0.64 ± 0.18b	1.44 ± 0.58a
	4x	1.60 ± 0.67a	2.91 ± 0.61a	0.78 ± 0.24a	1.79 ± 0.66a
3 months	2x	4.73 ± 0.69a	6.18 ± 1.34a	1.31 ± 0.39a	6.64 ± 2.90a
	4x	4.90 ± 0.80a	5.94 ± 1.41a	1.39 ± 0.34a	6.67 ± 2.73a
5 months	2x	5.57 ± 0.82a	8.28 ± 1.36a	1.50 ± 0.28b	9.77 ± 2.47b
	4x	5.87 ± 0.94a	8.85 ± 1.38a	1.69 ± 0.25a	11.70 ± 2.42a
7 months	2x	6.47 ± 0.90a	11.35 ± 1.25b	1.96 ± 0.20b	18.17 ± 2.70b
	4x	6.90 ± 0.88a	14.11 ± 1.65a	2.16 ± 0.24a	24.20 ± 5.58a
	CV-2x (%)	13.91	11.01	10.20	14.86
	CV-4x (%)	12.75	11.69	11.11	23.06

oryzalin cannot directly dissolve in water, so solvents may affect the survival and induction rates as reported in previous studies (Petersen et al. 2003). Inclusion, the higher fatality rate of oryzalin than that of colchicine may directly responsible for less efficiency of polyploidy induction. Similar results have been reported in previous studies (Chung et al. 2014; Petersen et al. 2003, 2010).

The phenomenon that morphological variations in the hypocotyl after the induction treatments was widely reported in previous studies, but the correlation between the altered hypocotyl phenotype and the ploidy level was rarely reported (Ascough and Staden 2008; Dhamayanthi and Gotmare 2010; Schnell 2015). For both of the antimitotic agents, high Pearson's coefficient values were obtained for the relationship between hypocotyl changes and tetraploidy. These findings suggested that morphological variations in the hypocotyl may serve as an early ploidy selection character. Stomatal characters are also widely used as ploidy selection traits. For the majority of plants, the stomata size was positively correlated with ploidy levels, but the stomata density was negative correlated with ploidy levels (Kaensaksiri et al. 2011; Podwyszyńska et al. 2014). In addition, stomatal characters remain stable during cultivation and through generations (Husband et al. 2016; Pavlíková et al. 2017). In this study, stomata length and density were correlated with ploidy levels, as indicated by the Pearson's coefficient values (Table 7). However, some tetraploids had similar stomata length and density to those of diploids (Table 6 and Fig. 3). Similar stomatal characters between diploids and tetraploids have also been reported for some genotypes of apple (Hias et al. 2017), potato (Aversano et al. 2013), *Mentha canadensis* (Yu et al. 2015), and *Rhynchostylis gigantean* var. *rubrum* (Kerdsuwan and Te-chato 2012). These findings suggested that stomatal characters are affected by both the ploidy level and genotype.

Previous studies have been reported that the size of the nucleus and cells increases with whole chromosome duplication, and this affects mitosis and the cell cycle (Pozo and Elena 2015). Slow growth of tetraploids is a typical characteristic of neopolyploids (Allario et al. 2011; Podwyszyńska et al. 2018). In this study, delayed development of seeds and slow differentiation of bulblets only occurred initially. After several rounds of subcultural, there were no differences in scale differentiation, shoot growth, and leaf morphology between tetraploids and diploids (Table 8). There are several possible explanations for the transient growth retardation observed in this study. Firstly, the toxic effect of the antimitotic agent may be decreased or disappeared during long-term cultivation (Drunen and Husband 2018; Munzbergova 2017). Secondly, DNA elimination and/or chromosome rearrangements may be have occurred during genome stabilization (Dar et al. 2013). Thirdly, there may have been changes in DNA methylation patterns (Podwyszyńska et al. 2014; Xu et al. 2017).

Most losses occur when tissue-cultured plants are transplanted. In this study, the average germination rate was higher for tetraploids than diploids, suggesting that tetraploids had better environmental fitness than diploids in these conditions. Several diploid and tetraploid genotypes shown similar germination rates, suggesting that environmental fitness is not only affected by ploidy level but also by genotype. As previous studies reported that the degree of genomic changes after polyploidization is highly variable within a species (Spoelhof et al. 2017) and tetraploids may not vigorous than diploids for some genotypes (Podwyszyńska et al. 2018). Growth characters were also compared after transplanting. Initially, the leaf size was not significantly different between diploids and tetraploids, but the leaf area of tetraploid plants was significantly greater than that of diploid plants after 5 months of cultivation. Previous studies suggested that photosynthetic capability of tetraploid always significantly higher than that of diploid (Cao et al. 2018; Zhou et al. 2016). However, tetraploid primary characteristics by increased in nuclear and cell volume which need speed longer time and greater bulk of materials in building up larger cells (Levan 1943; Knight and Beaulieu 2008). We hypothesis that, the photosynthesis area was increase with increased leaf number and size which may attributed more accumulation of photosynthetic products for tetraploid to building up larger leaves compared with diploid in later stage of growth. This assumption needs further comparison the photosynthesis capability between the two ploidy levels.

Most studies on polyploids have concentrated on one or a few genotypes, and therefore, their results may only reflect characteristics of given genotypes rather than the actual characteristics of polyploidization. Multiple genotypes may provide more opportunities for morphological variation in other characters (Chen et al. 2016; Emsweller and Brielery 1949). In this study, many characters had larger coefficients of variation in tetraploids than in diploids, both in tissue culture and after transplanting. Therefore, compared with diploids, tetraploids may provide a wider range of variation for lily breeding.

Conclusion

In the present study, we have developed an effective protocol for polyploidy induction with germinated seeds treated by oryzalin and colchicine. Both mitotic inhibitors were efficient in polyploidy induction but the treatment with colchicine presented a higher survival rate and higher induction rate than those treatment with oryzalin. After treated with both mitotic inhibitors, the swollen hypocotyl phenotype was prevalence observed and it was strongly

correlated with polyploidy. After transplanting, tetraploids were more vigorous than diploids for higher germination rate of bulblets and larger size of leaves.

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Author contributions G-XJ and L-JW conceived and designed the experiments; L-JW, QZ and XG performed the experiments; L-JW analyzed the data and wrote the manuscript; G-XJ, L-JW and Q-ZC revised the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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