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Production of Tetraploid Plants of *Trollius chinensis* Bunge Induced by Colchicine

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Abstract

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Trollius chinensis Bunge is one of the important ornamental and medicinal plants cultivated in China. In this study, the tetraploid plants of *Trollius chinensis* were successfully induced by colchicine and their morphological, cytological and fertility characteristics were investigated. The results indicated that the tetraploid species of *Trollius chinensis* ($2n = 4x = 32$) was efficiently induced from the diploid species ($2n = 2x = 16$) by colchicine under 0.05% to 0.2% concentrations, with the best induction under treatment combinations of 0.1%, 24h duration and 0.2%, 12h duration. In comparison with the diploid plants, the tetraploid plants exhibited dramatically varied phenotypic features, showing increased leaf length and width, petiole, flower and pollen diameters. In addition, they also exhibited increased stomatal size and chloroplast numbers in guard cells. Tetraploidy reduced the pollen fertility. Nevertheless, the maintenance of female fertility makes the tetraploid a potential germplasm for further polyploid breeding. Our investigation provides a potential design for an effective pathway to generate varieties of this plant species with chromosome doubling.

Keywords: morphological, cytological and fertility characteristics; polyploidy induction; *Trollius chinensis*

Trollius chinensis Bunge is a diploid species belonging to the family Ranunculaceae. It is widely distributed in mountainous areas at elevations from 1000 to 2200 m a.s.l. in North China (Li *et al.* 2012). *Trollius chinensis* plants exhibit interesting phenotypic features of leaves and gold-coloured flowers. These features allow the species to be used as an excellent option for gardens, parks and flower decorations (Zhu 2003). In addition, the flower of *Trollius chinensis* contains various organic substances such as flavonoids, organic acids, and volatile oils, which have potential medical value e.g. in inhibiting pathogenic infection, counteracting inflammation, and resisting the virus invasion (Li *et al.* 2002; Lu *et al.* 2015). Thus, this species has already been widely cultivated as medicinal plant in mountainous areas. It has important commercial value and application prospects. However, the plant growth at a high elevation habitat limits its capacity to thrive in regions of lower elevation, where the high temperatures during the summer season affect the normal plant growth and development.

It was previously reported that the polyploid plants exhibit advantages in tissue and organ establishment, generally exhibiting improved vegetative tissue size, enlarged flower shape, and elevated resistance to environmental stresses in comparison with the diploid species (Liu *et al.*2011; Meng *et al.* 2011). Additionally, doubling of the chromosome of medicinal plants showed increased active ingredients (Gao *et al.* 1996; Chen & Gao 2000; Zhang *et al.* 2002). These findings suggest that it is possibly feasible to extend the planting area of *Trollius chinensis* by induction of polyploid plants to improve tolerance to environmental stresses and enhance its resistance to a warmer climate. Colchicine, as one of the

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efficient chemical agents of chromosome doubling, has been successfully used to modify the chromosome numbers in diverse plant species (Takamura & Miyajina 1996; Kermani *et al.* 2004; Liu *et al.* 2007, 2013; Pansuksan *et al*. 2014).

To date, numerous studies have been performed in *Trollius chinensis*, focusing on the extraction of medical ingredients, the growth and yield investigation under demonstration trials, and establishment of techniques of seed germination, regeneration of plantlets through tissue culture (Li *et al.* 2002; Yan *et al.* 2008; Yang *et al.* 2011). However, only a few studies on polyploidy induction in this species have been reported.

The aim of the study was to establish an efficient system for the production of polyploid *Trollius chinensis* plants through colchicine treatment. Polyploidy induction was performed as follows: 100 seeds were treated with 500 mg/l of gibberellin (GA_3) solution for 24 h. After thorough washing with distilled water, the seeds were germinated for 3 days and then treated with colchicine at respective concentrations of 0.05, 0.10, and 0.2% for 12, 24, 36, and 48 h under darkness. After colchicine treatment, the seeds were immersed in a 10% (v/v) sodium hypochlorite solution for 10 min and rinsed with sterile distilled water 3–5 times. Surface-sterilized seeds were transferred to agar-solidified MS media (Murashige & Skoog 1962) supplemented with 0.5 mg/l 6-benzylaminopurine and 0.1 mg/l 1-naphthaleneacetic acid (pH = 5.8–6.0) in tissue culture vessels of 350 ml volume and cultivated under a 16 h photoperiod of 36 µmol/m^2/s of photosynthetically available radiation (PAR) at 25 ± 2°C. After 6 weeks, the seed germination rate and seedling survival rate in each treatment were determined by conventional approaches. Survived seedlings were transplanted in pots with a 3:1 mixture of peat and perlite and cultured in a greenhouse under relatively consistent environmental conditions (12 h photoperiod of 180 µmol/m^2/s of PAR at 25 ± 2°C). The experiment was repeated three times.

Colchicine-induced plants with significantly modified phenotypic features were subjected to the observation of the chromosome numbers. Diploid plants were used as a control. Root tips from 6-week cultures were pretreated with 2 mmol/l of 8-hydroxyquinoline solution at 20°C for 2 h. Pretreated root tips were fixed in absolute ethanol-glacial and acetic acid (3 : 1) at 4°C for 24 h. Fixed root tips were hydrolyzed in concentrated HCl for 5–6 min at 60°C in water bath. After hydrolysis, root tips were stained with propionic acid-jarosite-chloral hydrate-haematoxylin and observed under an optical microscope (Olympus BH-2, Olympus,Tokyo, Japan) to record the chromosome numbers. For each plant derived from colchicine treatment, 20 cells with metaphase chromosomes in the root tips were investigated.

A subset of morphological traits of tetraploid plants such as leaf length and width, and petiole and flower diameters were measured after 25 weeks of culture by means of conventional methods. Average pollen diameters of tetraploid and diploid plants were counted from 50 pollen grains spread on a microscope slide after being dried in silica gel for 24 h.

To investigate stomata characteristics, the epidermal layers at the lower leaf surface were gently removed using forceps and stained in 1% of I-KI solution for 3 min. The tissue was observed on a slide under an optical microscope (magnified 15 × 40 times) equipped with microscopic linear micrometer. The mean number of chloroplasts in the guard cells was recorded according to Zhang *et al*. (2008) as well.

To analyse the fertility of tetraploids, pollen germination and cross tests were performed. For the *in vitro* pollen germination test, pollens were spread on a solid culture medium (10% sucrose, 0.01% boric acid and l% agar) at 25°C for 3 h. The pollen germination rate was calculated at the stage when the length of the germinated pollen tube was greater than the pollen diameter. Reciprocal crosses between tetraploid and diploid plants were also performed. Flowers of the female parent were emasculated before anthesis. The fruit set rate, average number of seeds per fruit and germination rate of seeds were calculated with the help of conventional methods.

All colchicine treatments had negative effects on seedling growth and survival rates (Table 1). Additionally, the survival rates decreased with increased colchicine concentration and prolonged treatment duration. The 24 h treatment with a 0.1% colchicine concentration resulted in the highest phenotypic variation among seedlings as well as the highest survival rate of seedlings. Hence, this treatment is thought to be optimal to induce the polyploid plants of *Trollius chinensis*.

Compared with the controls ($2n = 2x = 16$) (Figure 1A), most of the plants with varied phenotypic features exhibited doubled chromosome numbers ($2n = 4x = 32$) (Figure 1B). A total of 32 plants with tetraploid genomes from all colchicine treatments were obtained. Additionally, some plants were identified as aneuploids (Figure 1C and D), possibly

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Table 1. The inductive effect of various colchicine treatments after 6 weeks of culture

No.	Colchicine concentration (%)	Duration (h)	Percentage of survival (%)	Percentage of variant plants (%)
1 (c)	0.00	24	49.67 ± 0.89^{a}	0.00^{g}
2	0.05	12	32.33 ± 0.44^{d}	2.33 ± 0.44^{f}
3	0.05	24	26.67 ± 0.89^{e}	3.67 ± 0.89^{ef}
4	0.05	36	10.33 ± 0.44^{j}	3.33 ± 0.44^{ef}
5	0.05	48	8.33 ± 0.44^{k}	4.67 ± 0.44^{de}
6	0.10	12	39.67 ± 0.89^{b}	9.33 ± 1.11^{b}
7	0.10	24	24.67 ± 1.11^{f}	12.67 ± 0.44^{a}
8	0.10	36	18.33 ± 0.44^{h}	7.33 ± 0.89^{c}
9	0.10	48	13.67 ± 0.44^{i}	5.67 ± 0.44^{d}
10	0.20	12	35.33 ± 0.89^{c}	10.33 ± 0.44^{b}
11	0.20	24	22.33 ± 0.44^{g}	9.33 ± 1.11^{b}
12	0.20	36	10.67 ± 1.11^{j}	5.67 ± 0.89^{d}
13	0.20	48	7.33 ± 0.44^{k}	3.67 ± 0.89^{ef}

The means in columns followed by the same letter are not significantly different from each other at $P < 0.05$ of Duncan test

resulting from the uneven chromosome replication and exchange during mitosis.

The phenotypic features of the tetraploid plants were largely altered (Figure 2). Namely, the leaf length, leaf width, petiole diameter, flower diameter and pollen diameter in such plants were increased by 90.07, 80.58, 46.48, 37.50, and 37.76% respectively, compared to control plants (Table 2). These results suggest that doubling the chromosome numbers can significantly modify plant growth features in *Trollius chinensis.*

Significant differences, based on the statistical analysis ($P < 0.05$), were observed between the diploid and tetraploid plants in the stomatal guard cell size and the number of their chloroplasts. In comparison with the diploids, the length and width of guard cells increased by 48.25 and 52.62%, respectively, and the

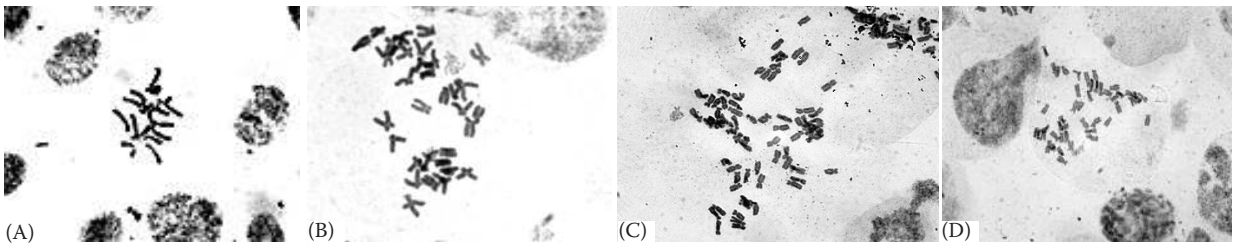

Figure 1. Identification of the chromosome numbers in colchicine-treated *Trollius chinensis* plants: diploid (A), tetraploid (B), aneuploid (C, D)

Figure 2. Phenotypic features of cotyledons, flowers, and leaves of diploid (A) and tetraploid (B) plants

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Table 2. Comparison of morphological and physiological characteristics of diploid and tetraploid *Trollius chinensis* plants after 25 weeks of culture

Ploidy	Diploid	Tetraploid
Leaf length (mm)	30.02 ± 0.29[b]	50.74 ± 0.53[a]
Leaf width (mm)	20.06 ± 0.18[b]	30.72 ± 0.60[a]
Petiole diameter (mm)	0.71 ± 0.02[b]	1.04 ± 0.13[a]
Flower diameter (mm)	40.65 ± 0.42[b]	50.96 ± 0.57[a]
Length of stomata (μm)	49.58 ± 2.00[b]	73.50 ± 3.97[a]
Width of stomata (μm)	38.33 ± 3.76[b]	58.50 ± 4.64[a]
Number of chloroplasts per guard cell	8.50 ± 1.05[b]	18.50 ± 2.07[a]
Pollen diameter (μm)	32.10 ± 1.22[b]	44.22 ± 2.40[a]
Pollen germination rate (%)	88.60 ± 2.67[a]	47.30 ± 3.45[b]

Different small letters in the same row indicate significant differences from each other at $P < 0.05$ of Duncan test

Table 3. Selected characteristics of crosses among tetraploid and diploid *Trollius chinensis* plants

Cross combination	Pollinated flowers count	Fruit count	Fruit set rate (%)	Seed count/fruit	Germination rate of seeds (%)
Diploid × diploid (control)	90	74	82.22	126.00	45.34
Tetraploid × diploid	51	34	66.67	104.70	12.67
Diploid × tetraploid	51	23	45.10	60.80	4.33

chloroplast numbers in the guard cells increased by 117.65% in the tetraploid plants. These findings revealed that the genome modifications of *Trollius chinensis* can largely alter the stomata properties. In previous studies, many researchers (Przywara *et al*. 1988; Cohen & Yao 1996; Pansuksan *et al.* 2014) also examined bigger stomatal cells in polyploids than in diploids. Thus, the size of stomatal guard cells seems to be an important determination marker to distinguish polyploid plants of *Trollius chinensis*. An *in vitro* pollen germination test indicated that the tetraploid pollen germination rate was reduced by 46.61% compared to diploid plants (Table 2). If diploid plants were pollinated with pollens of tetraploid plants, the fruit set rate, seed count and germination ability remarkably decreased compared to reciprocal crosses and crosses between controls (Table 3). Similar results were already reported in other tetraploid plants, such as *Citrullus lanatus* (Jaskani *et al*. 2005) and *Vicia villosa* (Tulay & Unal 2010). Our findings are most likely connected with the ploidy status of regenerated plants. Nevertheless, if tetraploid plants were crossed with diploids used as male components, the fruit set and germination rates were significantly higher than those from reciprocal crosses and provided a sufficient amount of hybrid seeds for further utilization (Table 3). Our results indicated that the maintenance of female fertility makes the tetraploid a potential germplasm for further polyploid breeding. In addition to cross breeding, anther culture of tetraploid plants can be used to regenerate plants for the preparation of new varieties. Our investigation provides an effective approach to improve *Trollius chinensis* germplasm and extend its growing areas via the chromosome doubling pathway.

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