Eur. J. Hortic. Sci. *81*(4), 219–226 | ISSN 1611-4426 print, 1611-4434 online | http://dx.doi.org/10.17660/eJHS.2016/81.4.5 | © ISHS 2016

Original article

Polyploidy induced by colchicine in *Dendranthema indicum* var. *aromaticum*, a scented chrysanthemum

Miao He[1], Wenjie Gao[1], Yaohui Gao[2], Yingzhu Liu[1], Xue Yang[1], Hongbin Jiao[1] and Yunwei Zhou[1]

[1] Department of Landscape Architecture of Northeast Forestry University, Harbin, Heilongjiang, China
[2] Beijing Forestry University

Summary

***Dendranthema indicum* var. *aromaticum* from the genus *Chrysanthemum* is a rare species with a rich flavor. The aim of this study was to find a suitable treatment combination that would effectively induce polyploidy of diploid *Dendranthema indicum* var. *aromaticum* and to lay the foundation for the late cultivation of aromatic *Chrysanthemum*. In this study materials, colchicine concentration and treatment duration were examined for improving the induction of polyploidy. The combinations of three materials (shoot tips, pre-germinated seeds and grin), five colchicine concentrations (100, 200, 500, 1,000 and 2,000 mg L^{-1}) and three treatment durations (12, 24 and 48 h) were tested in *Dendranthema indicum* var. *aromaticum*. A total of 7 tetraploids and 301 chimeras determined by chromosome number analysis were obtained. The treatment of grin seeds with 1,000 mg L^{-1} colchicine for 24 h (14.5%) and shoot tips with 1,000 mg L^{-1} colchicine for 7 d (40%) were suitable for induction of chromosome doubling. The tetraploid plants displayed much larger stomata with lower density, as well as a higher chloroplast count than the diploid paints. Moreover, tetraploid plants developed larger, thicker leaves, greater flower diameter, more epidermis hairs and shorter plant height than the diploid plants.**

Keywords
Colchicine, *Dendranthema indicum* var. *aromaticum*, induction, identification, polyploid

Significance of this study

What is already known on this subject?

- *Dendranthema indicum* var. *aromaticum* is characterized by a special scent, which is an important source of aroma in genus *Dendranthema*. Hybridization is the good way to produce new scented chrysanthemum. However, it is difficult to cross breed if one species is a diploid while the other is a higher ploidy level. Therefore, we decided to double the somatic chromosomes of *D. indicum* var. *aromaticum*, in order to increase the success of crosses between the two species.

What are the new findings?

- In this study, the tetraploid plants displayed much larger stomata with lower density, as well as a higher chloroplast count, than the diploid plants. Moreover, tetraploid plants developed larger, thicker leaves, greater flower diameter, more epidermis hairs and shorter plant height than the diploid plants.

What is the expected impact on horticulture?

- We had got the tetraploids of *Dendranthema indicum* var. *aromaticum*, which were important parents for scented *Chrysanthemum.* Later, we will cross between the tetraploid and cultivated *Chrysanthemum* and get the scented *Chrysanthemum*, which has important significance for improving the ornamental traits of horticultural plants.

Introduction

Chrysanthemum (*Dendranthema* × *grandiflora*), one of the famous traditional flowers, is widely cultivated in the world. The flowers of Chrysanthemum are usually bright in color, but they do not possess fragrance or other slight scents. Hence, introducing aroma to the flowers of Chrysanthemum is an important topic in Chrysanthemum's breeding.

Dendranthema indicum var. *aromaticum* (*D. indicum* var. *aromaticum*) is a herbaceous perennial plant that is commonly found in Shennongjia, Hu Bei province, P.R. China (Liu and Zhang, 1983). The plants are characterized by a special scent, which is an important source of aroma in genus *Dendranthema*. Previous studies have shown that *D. indicum* var. *aromaticum* is diploid (2*n*=2*x*=18) (Du et al., 1989) and *Dendranthema* × *grandiflora* is polyploid (2*n*=6*x*=54).

Hybridization can be used to produce new scented Chrysanthemum. However, it is difficult to cross breed if one species is a diploid while the other has a higher ploidy level (Li et al., 2008). Studies have found that it is possible for Chrysanthemum to cross as tetraploid in *Dendranthema* × *grandiflora*. Therefore, we decided to double the somatic chromosomes of *D. indicum* var. *aromaticum*, in order to improve the likelihood of successful cross between the two species.

Materials and methods

Plant materials

The plant material used in this study include the seeds and shoot tips of the diploid *D. indicum* var. *aromaticum* (2*n*=2*x*=18). The budding branches of *D. indicum* var. *aromaticum* (12–15 cm) were collected and stored in a water box with sterile Hogland culture fluid at 23 ± 2°C, 5,000 lux which was replaced every week. At the time of complete flower

opening, a brush was used for self-pollination 3 times. The wilting flowers were dried naturally and the seeds were collected. Robust foot buds (3–5 cm) of *D. indicum* var. *aromaticum* were cut into the sand, and placed in the greenhouse for conventional management (23 ± 2°C) until two pieces of true leaves could be processed.

D. indicum var. *aromaticum* were obtained from Shen-nongjia in the Hu Bei province, P.R. China, in 2008. The samples were maintained in the garden nursery at the College of Landscape Architecture and Horticulture, Northeast Forestry University, Harbin, P.R. China.

Polyploidy induction

Dry seeds were incubated in warm water for 5 h. The seeds that became visibly swollen were selected and surface sterilized by immersion in 2% sodium hypochlorite for 10 min and rinsed with sterile distilled water 6–8 times. The seeds were placed in a constant temperature incubator. The pre-germinated seeds and grin were used to induce polyploidy. Colchicine was applied to seeds at concentrations of 100, 200, 500, 1,000, 2,000 mg L^{-1} and at treatment durations of 12, 24, 48 h. The seeds that were soaked in distilled water were used as a control. For each treatment, 200 seeds were treated, with 3 replications. The seeds were washed with distilled water for 3 times and then seeded in a plug for regular management.

The bunched cotton balls placed on shoot tips were treated with colchicine solution by using a micropipette to place 200 ul → 200 ul onto the cotton balls between 9:00–10:00 am. A range of various concentrations of colchicine was applied at 100, 200, 500, 1,000, 2,000 mg L^{-1} for 7 days. Distilled water was used as a control solution. The processing of the shoot tips was always carried out in the dark environment. For each treatment, 30 shoot tips were treated, with three replications. Finally, the bunched cotton balls were removed, after which they were washed with distilled water and transferred to greenhouse conditions.

Collection of root tips

After the colchicine treatment, the treated explants (7–10 cm) were transferred to the water box and cultured for 20 days. The explants were incubated at regular intervals at 25 ± 2°C with a 16 h photoperiod and illumination of 2,000 lux. When the roots reached 1–2 cm, their tips were used to observe the number of chromosomes.

Observation of chromosomes and determination of ploidy

Root tips that were approximately 2–3 mm in length were taken from the plantlets (external morphological change) and pretreated with 2 mM 8-hydroxyquinoline for 7 h at 18°C. After washing with distilled water, the root tips were transferred to fresh Carnoy's solution (acetic acid: alcohol, 3:1) for fixing at 4°C for at least 24 h. After being washed with distilled water, the fixed root tips were hydrolyzed in 1M HCL at 60°C for 8 min. After washing with distilled water 3–5 times, the hydrolyzed root tips were stained with improved Carbol Fuchsin for 30 min. The chromosome numbers were observed under a fluorescence microscope (DM250) with × 1,000 magnification. Thirty meristematic cells of root were observed to determine the number of chromosomes in each plant.

Morphological observation

In order to compare diploid and tetraploid plants, morphological characteristics of seven diploids and seven tetraploid plants were observed in different growth stages. Several characters such as plant height, leaf length and width were measured in 120 days of seedling age and flower diameter was measured during the full extended of the flower with a vernier caliper.

Stomata observation

Leaf samples were obtained from plants when they reached the 5–6 true-leaf stage. For stomatal measurements, an area about 0.5 cm^2 on the under epidermis of the leaves was coated with nail polish. After it dried, the nail polish impression was removed using a strip of scotch tape. The tape was then stuck on to a microscope slide and observed under a light microscope. The number of chloroplasts was observed with 1% w/v I_2-KI solution for 10 minutes in the under epidermis of the leaves. Thirty stomata were measured for each leaf. To determine the stomatal density, stomata in 10 microscopic fields were counted for each plant. Two leaves were chosen from the same part of each of the seven diploid control plants and each of the seven tetraploid plants.

Statistical analysis

All the data were analyzed using Microsoft Office Excel 2007 software with one-way ANOVA. Data were evaluated by the analysis of variance and significant by Duncan's multiple range test (DB, 1955) with the statistical program SPSS Software Version 19.5. A difference was considered statistically significant when $p < 0.05$ and significant differences were denoted in the tables.

Results

Effect of colchicine treatment on the seeds

The effects of colchicine on polyploidy survival and seeds induction were examined 120 days after the treatment. The results were affected by colchicine concentration, duration and seed germination (Tables 1 and 2). All of the seeds treated with colchicine demonstrated lower survival rates than those of the control. In general, the rate of seeds survival was decreased when the concentration of colchicine and duration was increased (Figure 1). The significant analysis showed that the survival rate of the treated group was significantly lower than that of the control group after the treatment concentration was higher than 1,000 mg L^{-1} ($p < 0.05$). Especially for the 48 h duration (1,000, 2,000 mg L^{-1}), the survival rates of pre-germinated seeds were 36.17%, 31.17%, and grin seeds were 29.83%, 23.67%. Polyploidy induction was increased when the concentration of colchicine and duration were increased. For pre-germinated seeds, the highest percentage of polyploidy occurred after a 48 h treatment using 2,000 mg L^{-1} colchicine, the induction rate was 11%, significantly higher than other treatment groups ($p < 0.05$). Followed by 500 mg L^{-1} for 48 h (8%), 1,000 mg L^{-1} for 48 h (7.67%) and 2,000 mg L^{-1} for 24 h (7.33%), they had similar induction effects. For grin seeds, the highest percentage of polyploids occurred after a 24 h treatment using 1,000 mg L^{-1} colchicine, the induction rate was 14.5%. Then 2,000 mg L^{-1} for 48 h (12.5%), significantly higher than other treatment groups ($p < 0.05$). Among them, 1,000 mg L^{-1} for 48 h (9.83%) and 2,000 mg L^{-1} for 24 h (7.33%) also have similar induction effects. This suggests that high concentrations of colchicine low duration and high duration low concentration can achieve the same desired effect induced.

Table 1. Induction effects of different treatment time and concentrations of colchicine on pre-germinated seeds of *D. indicum* var. *aromaticum*.

Treatment		The survival after 30 days		Number of polyploids obtained		Induction rate (%)
Concentration (mg L^{-1})	Time (h)	Number of surviving	Survival rate (%)	Tetraploids	Chimeras	
0	12	196.00±2.00a	98.00±1.00a	0.00±0.00b	0.00±0.00h	0.00±0.00g
0	24	195.12±0.58a	97.56±0.29a	0.00±0.00b	0.00±0.00h	0.00±0.00g
0	48	194.86±1.24a	97.43±0.62a	0.00±0.00b	0.00±0.00h	0.00±0.00g
100	12	190.33±0.58ab	95.17±0.29ab	0.00±0.00b	0.00±0.00h	0.00±0.00g
100	24	165.33±1.53ef	82.67±0.76ef	0.00±0.00b	0.00±0.00h	0.00±0.00g
100	48	163.33±0.58fg	81.67±0.29fg	0.00±0.00b	0.00±0.00h	0.00±0.00g
200	12	191.67±0.58ab	95.83±0.29ab	0.00±0.00b	1.00±1.00gh	0.50±0.00fg
200	24	173.67±3.51d	86.83±1.76d	0.00±0.00b	2.33±0.58g	1.17±0.29f
200	48	158.67±3.51g	79.33±1.76g	0.00±0.00b	0.00±0.00h	0.00±0.00g
500	12	186.33±7.37bc	93.17±3.69bc	0.00±0.00b	10.33±0.58de	5.17±0.29cd
500	24	170.33±5.03de	85.17±2.52de	0.00±0.00b	9.00±0.00ef	4.50±0.00de
500	48	111.67±5.51k	55.83±2.75k	0.00±0.00b	16.00±2.00b	8.00±1.00b
1,000	12	181.33±3.51c	90.67±1.76c	0.00±0.00b	8.00±0.00f	4.00±0.00e
1,000	24	134.00±1.00i	67.00±0.50i	0.00±0.00b	10.33±1.53de	5.17±0.76cd
1,000	48	72.33±2.52l	36.17±1.26l	1.33±1.53a	14.00±0.00c	7.67±0.76b
2,000	12	146.33±0.58h	73.17±0.29h	0.00±0.00b	11.00±2.00d	5.50±1.00c
2,000	24	117.00±2.00j	58.50±1.00j	0.00±0.00b	14.67±2.52bc	7.33±1.26b
2,000	48	62.33±2.52m	31.17±1.26m	1.00±1.00a	21.00±0.00a	11.00±0.50a

Data represents mean ± SD of three replicates.
Means (in the same column) followed by the same letter did not have significant difference by Duncan's multiple range test $p < 0.05$.

Table 2. Induction effects of different treatment time and concentrations of colchicine on Grin seeds of *D. indicum* var. *aromaticum.*

Treatment		The survival after 30 days		Number of polyploids obtained		Induction rate (%)
Concentration (mg L^{-1})	Time (h)	Number of surviving	Survival rate (%)	Tetraploids	Chimeras	
0	12	194.00±1.00a	97.00±0.50a	0.00±0.00a	0.00±0.00i	0.00±0.00i
0	24	195.12±0.58a	97.56±0.29a	0.00±0.00a	0.00±0.00i	0.00±0.00i
0	48	194.86±1.24a	97.43±0.62a	0.00±0.00a	0.00±0.00i	0.00±0.00i
100	12	181.67±1.53bc	90.83±0.76bc	0.00±0.00a	0.00±0.00i	0.00±0.00i
100	24	171.33±2.08d	85.67±1.04d	0.00±0.00a	1.00±1.00hi	0.50±0.50hi
100	48	152.67±2.52e	76.33±1.26e	0.00±0.00a	0.00±0.00i	0.00±0.00i
200	12	185.00±2.65b	92.50±1.32b	0.00±0.00a	2.33±0.58hi	1.17±0.29hi
200	24	175.33±5.03d	87.67±2.52d	0.00±0.00a	4.00±4.00gh	2.00±2.00gh
200	48	142.00±1.00f	71.00±0.50f	0.00±0.00a	7.00±1.00fg	3.50±0.50fg
500	12	176.67±1.53cd	88.33±0.76cd	0.00±0.00a	8.00±4.00f	4.00±2.00f
500	24	156.00±8.00e	78.00±4.00e	0.00±0.00a	12.67±1.53e	6.33±0.76e
500	48	83.33±5.03i	41.67±2.52i	0.00±0.00a	6.33±0.58fg	3.17±0.29fg
1,000	12	172.00±6.24d	86.00±3.12d	0.00±0.00a	16.00±1.00d	8.00±0.50d
1,000	24	114.33±4.04g	57.17±2.02g	2.00±0.00a	27.00±0.00a	14.50±0.00a
1,000	48	59.67±1.53j	29.83±0.76j	0.00±0.00a	19.67±3.51c	9.83±1.76c
2,000	12	139.33±1.53f	69.67±0.76f	0.00±0.00a	13.00±2.00e	6.50±1.00e
2,000	24	101.33±2.52h	50.67±1.26h	0.00±0.00a	17.33±0.58cd	8.67±0.29cd
2,000	48	47.33±1.53k	23.67±0.76k	1.00±0.00a	24.00±1.00b	12.50±0.50b

Data represents mean ± SD of three replicates.
Means (in the same column) followed by the same letter did not have significant difference by Duncan's multiple range test $p < 0.05$.

Table 3. Induction effects of different treatment concentrations of colchicine on shoot tips of *D. indicum* var. *aromaticum*.

Treatment	The survival after 30 days		Number of polyploids obtained		Induction rate (%)
Concentration (mg L^{-1})	Number of surviving	Survival rate (%)	Tetraploids	Chimeras	
0	30.00±0.00a	100.00±00.00a	0.00±0.00b	0.00±0.00e	0.00±0.00e
100	28.67±0.58a	95.56±1.92a	0.00±0.00b	2.00±1.00d	6.67±3.33d
200	25.00±1.00b	83.33±3.33b	0.00±0.00b	4.33±0.58c	14.44±1.92c
500	18.67±2.52c	62.22±8.39c	0.00±0.00b	7.00±0.00b	23.33±0.00b
1,000	14.00±2.00d	46.67±6.67d	1.00±1.00a	11.00±1.00a	40.00±6.67a
2,000	7.00±1.00e	23.33±3.33e	1.00±0.00a	5.00±0.00c	20.00±0.00bc

Data represents mean ± SD of three replicates.
Means (in the same column) followed by the same letter did not have significant difference by Duncan's multiple range test $p < 0.05$.

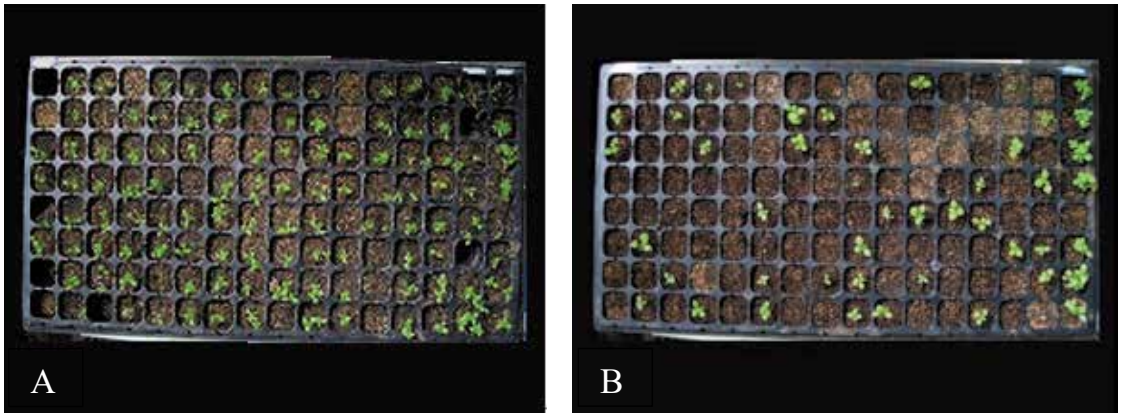

Figure 1. Treatment *D. indicum* var. *aromaticum* grin seeds with 500 mg L^{-1} colchicine (A) 24 h, (B) 48 h.

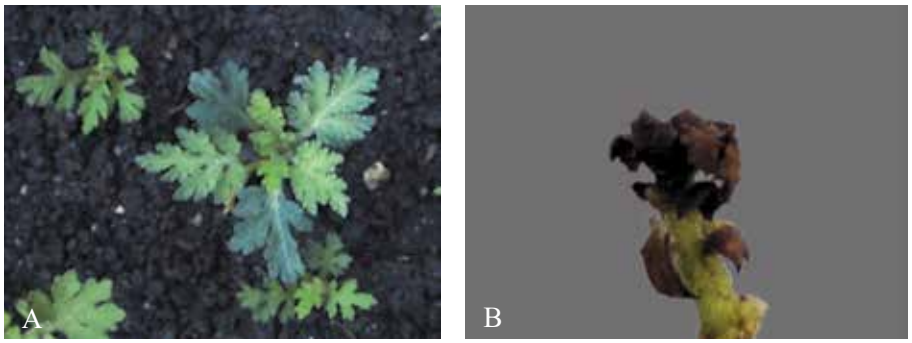

Figure 2. Treatment *D. indicum* var. *aromaticum* shoot tips with colchicines and shoot tips of damage. (A) Normal shoot tips, (B) Victims of shoot tips.

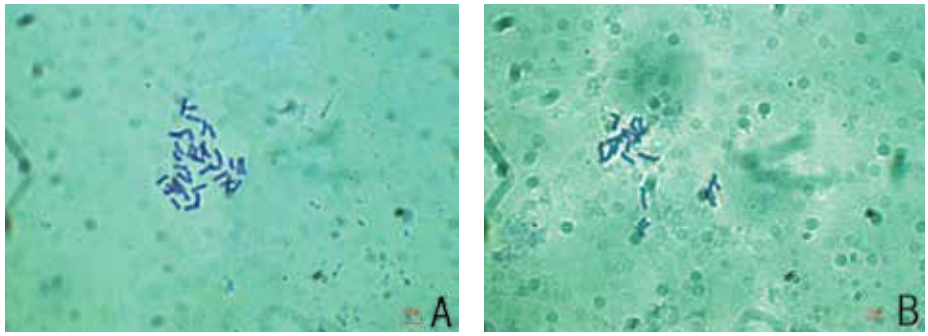

Figure 3. Chromosome number of root tip between tetraploid (A) and diploid (B) plants (×1,000).

Table 4. Flowers and leafs comparison of diploid and tetraploid.

Ploidy level	Plant height (cm)	Length of leaf (cm)	Width of leaf (cm)	Flower size (cm)
Diploid	47.85±3.73a	6.02±0.56b	4.63±0.30b	1.53±0.26a
Tetraploid	38.64±1.81b	9.17±0.29a	7.26±0.37a	2.12±0.54a
Rangeability (%)	-19.12±2.53	52.91±9.45	56.90±2.18	37.19±12.15

Data represents mean ± SD of three replicates.
Means (in the same column) followed by the same letter did not have significant difference by Duncan's multiple range test $p < 0.05$.

Effect of colchicine treatment on shoot tips

The effects of colchicine on polyploidy survival and the induction of shoot tips were examined 60 days after the treatment. The results showed that there were significant effects of colchicine concentration (Table 3). In general, higher concentrations of colchicine resulted in lower survival of the shoot tips and higher induction. The survival rates of the shoot tips were 46.67% with 1,000 mg L^{-1} colchicine treatment and 23.33% with 2,000 mg L^{-1} colchicine treatment, significantly different from the control group (100%) ($p < 0.05$). The highest induction rates of the shoot tips were 40% with 1,000 mg L^{-1} colchicine treatment. The second was 23.33% with 500 mg L^{-1} and 20% with 2,000 mg L^{-1} colchicine treatment. However, when the treatment concentration was 2,000 mg L^{-1}, most shoot tips could not grow normally and nearly half of the badly damaged shoot tips were brown or dark brown (Figure 2). Therefore, the most suitable concentration is 1,000 mg L^{-1} to induce polyploidy plants with colchicine.

Chromosome numbers

The chromosome numbers of the root tips were observed under a microscope to detect the ploidy level of the treated plants. It showed that the chromosome number of diploid plants of *D. indicum* var. *aromaticum* was $2n = 2x = 18$ and that of tetraploid plants was $2n = 4x = 36$ (Figure 3). In total, 301 chimeras and 7 tetraploid plants were obtained.

Morphological comparison of diploid and tetraploid plants

The variants in the morphological characteristics of leaf, stem and flower were observed between 2*x* and 4*x* plants under the same growing condition. Tetraploid plants had larger, thicker leaves, greater flower diameter, more epidermis hairs and the total plant height was shorter than that of diploid control plants (Figure 4). Leaf length was increased from 6.02 ± 0.56 (diploid) to 9.17 ± 0.29 cm (tetraploid), and leaf width was increased from 4.63 ± 0.3 (diploid) to 7.26 ± 0.37 cm (tetraploid). Flower diameters of diploid and tetraploid plants were 1.53 ± 0.26 and 2.12 ± 0.54 cm, respectively, and the difference was statistically significant (Table 4).

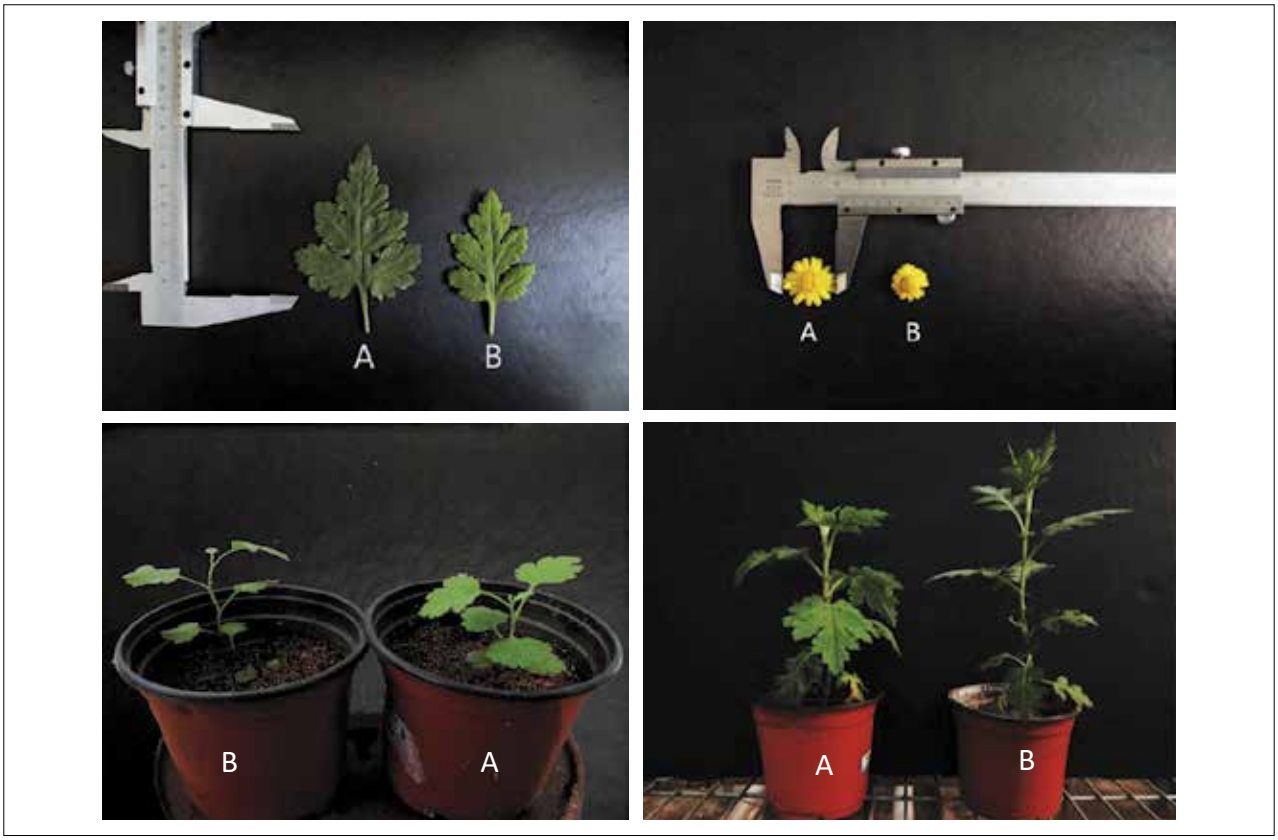

Figure 4. Comparison of tetraploid (A) and diploid (B) plants, leaves and flowers.

Stomatal comparison of diploid and tetraploid plants

In diploid and tetraploid plants, the stomata density and size were found to be significantly different (Figure 5). The stomata of 2*x* plants averaged 24.63 ± 1.22 × 19 ± 1.37 um (length × width) and the tetraploid stomata averaged 37.54 ± 1.83 × 23.26 ± 1.76 um. Diploid plants had more stomata (22 ± 2.74/0.1 mm^2) than the tetraploids (9 ± 1.75/0.1 mm^{-2}). The chloroplast number of guard cells differed significantly between the 2*x* and 4*x* plants, and the average number was 12 ± 1.58 in diploids and 18 ± 2.43 in tetraploids (Table 5).

Discussion

Polyploidy has played an important role in plant breeding as a valuable method for inducing variation and producing superior plants (Majdi et al., 2010; Stanys et al., 2006). Artificially induced polyploid technology has been used to improve flower morphology, fruit size, yields, break self-incompatible system, restore fertility, and improve resistance to diseases and pests (Kim et al., 2004; Schepper et al., 2001; Tel-Zur et al., 2011; Ye et al., 2010). In our study, tetraploids of *D. indicum* var. *aromaticum* had shown superior morphological characteristics than the diploids, such as larger, thicker leaves, greater flower diameter, and more epidermis hairs. These characteristics that were reported in this study are also observed in other plants as well (Gantait et al., 2011; Shao et al., 2003; Urwin et al., 2007).

For polyploidy induction, this study has found that colchicine concentration and treatment duration were the two key factors. The previous study has found that high concentrations of colchicine combined with short durations of treatment or, conversely, low concentrations of colchicine combined with long durations of treatment are preferred (Ye et al., 2010). Various colchicine concentrations (from 100 to

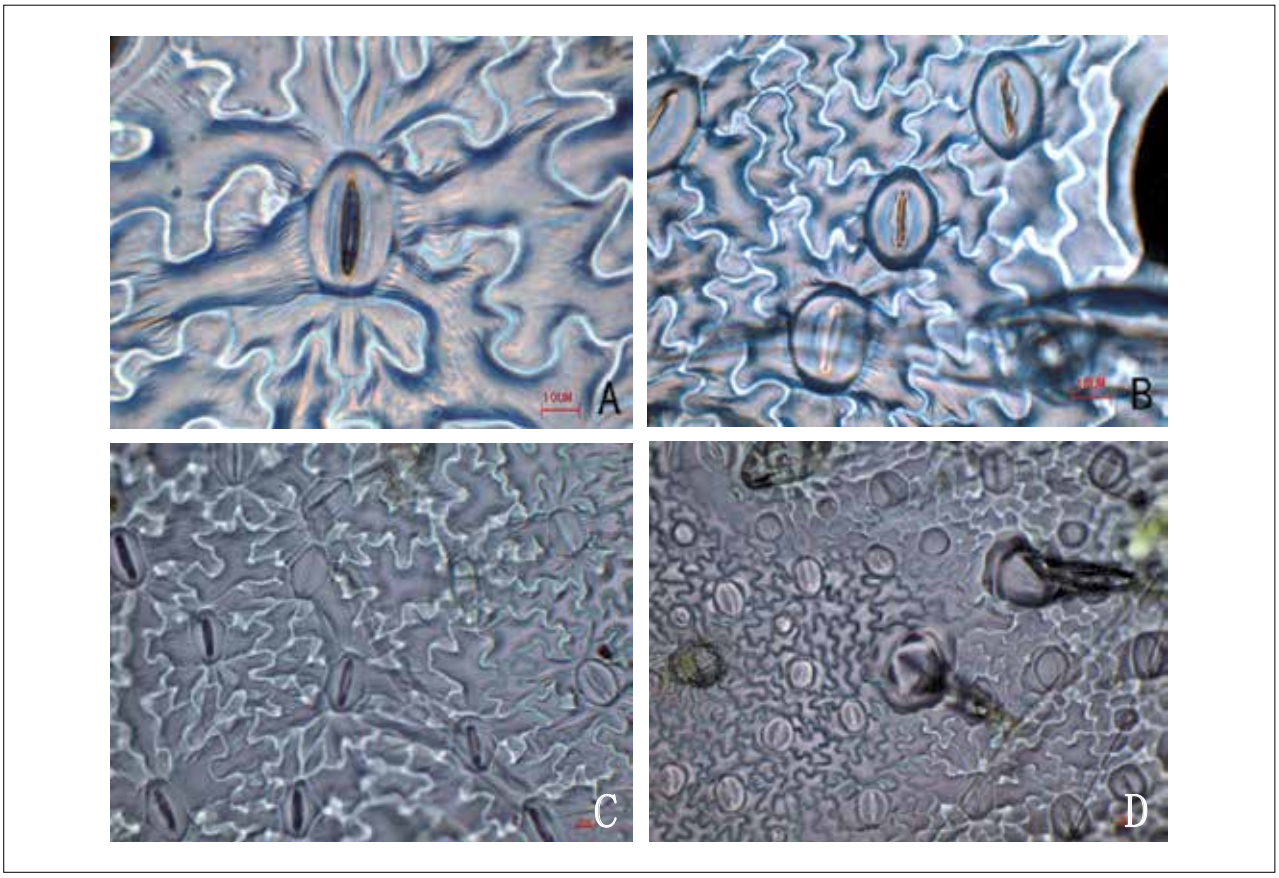

Figure 5. Stomata of leaves between tetraploid (A, C) and diploid (B, D) plants. (A, B×1,000; C, D×400).

Table 5. Comparison of chloroplasts number, guard cell size of leaf and stomata density between diploid and tetraploid.

Ploidy level	Length of stomata (um)	Width of stomata (um)	Width of guard cell (um)	Number of chloroplasts	Stomata density (No./0.1 mm^2)
Diploid	24.63±1.22b	19.00±1.37b	8.88±1.42a	12.00±1.58b	22.00±2.74a
Tetraploid	37.54±1.83a	23.26±1.76a	11.32±1.58a	18.00±2.43a	9.00±1.75b
Rangeability (%)	52.42±0.12	22.40±0.44	27.76±2.67	49.95±0.51	-59.33±2.91

Data represents mean ± SD of three replicates.
Means (in the same column) followed by the same letter did not have significant difference by Duncan's multiple range test $p < 0.05$.

5,000 mg L^{-1}) combined with treatment duration (6 h to 7 days) were carried out following the methods of in vitro chromosome doubling (Roy et al., 2001; Song et al., 1997; Thao et al., 2003), which were found to be effective in this study. Our results also showed that the treatment of grin seeds with 1,000 mg L^{-1} colchicine for 24 h (14.5%) and shoot tips with 1,000 mg L^{-1} colchicine for 7 d (40%) were suitable for chromosome doubling in *D. indicum* var. *aromaticum*. In contrast, previous studies found that the suitable treatment of seeds was 500 mg L^{-1} concentration for 48 h (83.1%) in *Dendranthema nankingense* (Chen et al., 2002) and 500 mg L^{-1} concentration for 12 h (23%) in *Chysanthemum lavandulifolium* (Mo et al., 2010). This suggested that plants in the same genus can have varied sensitivity to colchicine treatment.

Currently, there are various methods available for the identification of polyploid plants (Li et al., 1991; Tao et al., 2009; Yang et al., 2006). Earlier studies of artificial and spontaneous tetraploid plants have often found that the size and number of stomata and the number of chloroplasts within the guard cells change significantly in the case of chromosome doubling, compared with the diploid state (Beck et al., 2003; Urwin et al., 2007). In our study, traits such as stomatal density and size, and the chloroplast number of guard cells were evaluated to determine whether they could be used to identify or provide supporting evidence for putative polyploids in a preliminary scanning. Tetraploid *D. indicum* var. *aromaticum* had significantly greater values for stomatal density and size than the controls. Many researchers have reported that tetraploid plants generally have bigger stomata than diploid plants (Miguel and Leonhardt, 2011; Oliveira et al., 2004). Therefore, stomatal sizes could be used to analyze the level of ploidy in future studies. At the same time, some studies indicate that it is not reliable to select putative tetraploids simply based on stomatal sizes if chimeras exist in the population for the selection (Chen et al., 2006). Flow cytometry and chromosome counts are both practical and accurate methods of screening the ploidy level of plants (Praça et al., 2009). Flow cytometry is more effective and convenient, but can be expensive when compared to chromosome counts (Nimura et al., 2006). So we confirm the polyploid status by chromosome counts.

A higher frequency of chimera occurrence is usually associated with colchicine (Ackerman and Dermen, 1972; Schifino and Fernandes, 1987). Because the colchicine works effectively only during dividing cells, thereby the polyploidization generally does not equally occur in all explant cells, leading to the occurrence of mixoploids and chimeras (Wan et al., 1989). The data presented in this paper supports previous findings that the high production rates of chimeras are observed in other plants treated with colchicine, such as *Miscanthus sinensis* (Petersen et al., 2003) and *Echinacea purpurea* L. (Nilanthi et al., 2009). However, another study found that chimeras were not detected in any of the plants examined. He pointed out that both experimental conditions and the genotype seem to influence the induction of chimeras. Additionally, in the case of ploidy level, the developmental stage of the examined material may have also affected the results (Głowacka et al., 2010). In addition to colchicine, oryzalin is also a kind of suitable for plant chromosome doubling. In recent years, some researchers have applied oryzalin to the chromosome doubling of apple, kiwi, pear, lily and other plants (Bartish et al., 1996; Chalak and Legave, 1996; Bouvier et al., 2002; Rhee et al., 2005). Compared with colchicine, oryzalin has the characteristics of low toxicity, low cost, and high efficiency of induction of chromosome doubling (Allum et al., 2007). Induction of tetraploids in ornamental *Alocasia* through colchicine and oryzalin treatments, oryzalin applied at 0.01% for 24 h showed the best result with four tetraploids out of 26 explants examined (15.4%), at 0.05% or higher, colchicine did induce mostly chimeras (Thao et al., 2003). Further experiments are necessary to produce tetraploids with oryzalin in *D. indicum* var. *aromaticum*.

The confirmed tetraploid plants, which have been transferred to pots, could develop normal looking inflorescences. Related studies have used these tetraploid plants to cross with hexaploid *Dendranthema* × *grandiflora* for breeding new varieties. As described above, *D. indicum* var. *aromaticum* has rich fragrance and this trait is very useful for flower aroma breeding.

Acknowledgments

This research was supported by the Fundamental Research Funds for the Central Universities (2572015EY03) and National Natural Science Foundation of China (31400590).

References

Ackerman, W., and Dermen, H. (1972). A fertile colchiploid from a sterile interspecific camellia hybrid. Journal of Heredity *63*, 55–59.

Allum, J.F., Bringloe, D.H., and Roberts, A.V. (2007). Chromosome doubling in a *Rosa rugosa* Thunb. hybrid by exposure of in vitro nodes to oryzalin: the effects of node length, oryzalin concentration and exposure time. Plant Cell Reports *26*, 1977–1984. http://dx.doi.org/10.1007/s00299-007-0411-y.

Bartish, I.V., Korkhovoy, V.I., Fomina, Y.L., and Lim, Y.K. (1996). A new approach to obtain polyploid forms of apple. In Eucarpia Symposium on Fruit Breeding and Genetics *484*, pp. 561–564.

Beck, S.L., Dunlop, R.W., and Fossey, A. (2003). Stomatal length and frequency as a measure of ploidy level in black wattle, *Acacia mearnsii* (de Wild). Botanical Journal of the Linnean Society *141*, 177–181. http://dx.doi.org/10.1046/j.1095-8339.2003.00132.x.

Bouvier, L., Guerif, P.H., Djulbic, M., Durel, C.E., Chevreau, E., and Lespinasse, Y. (2002). Chromosome doubling of pear haploid plants and homozygosity assessment using isozyme and microsatellite markers. Euphytica *123*, 255–262. http://dx.doi.org/10.1023/A:1014998019674.

Chalak, L., and Legave, J.M. (1996). Oryzalin combined with adventitious regeneration for an efficient chromosome doubling of trihaploid kiwifruit. Plant Cell Reports *16*, 97–100. http://dx.doi.org/10.1007/BF01275459.

Chen, F., Jiang, J., and Fang, W. (2002). Study on induction of polyploid *Dendranthema nankingense* with colchicine. Acta Agriculturae *18*, 46–50.

Chen, L.-p., Wang, Y.-j., and Zhao, M. (2006). In vitro induction and characterization of tetraploid Lychnis senno Siebold et Zucc. HortScience *41*, 759–761.

Duncan, D.B. (1955). Multiple range and multiple F test. Biometrics *11*, 1–42.

http://dx.doi.org/10.2307/3001478.

Du, B., Liu, Q., Zhu, C., and Ke, S. (1989). Karyotype studies of two species on *Dendranthema*. Journal of Wuhan Botanical Research *7*, 293–296.

Gantait, S., Mandal, N., Bhattacharyya, S., and Das, P.K. (2011). Induction and identification of tetraploids using in vitro colchicine treatment of *Gerbera jamesonii* Bolus cv. *Sciella*. Plant Cell, Tissue and Organ Culture (PCTOC) *106*, 485–493. http://dx.doi.org/10.1007/s11240-011-9947-1.

Głowacka, K., Jeżowski, S., and Kaczmarek, Z. (2010). In vitro induction of polyploidy by colchicine treatment of shoots and preliminary characterisation of induced polyploids in two *Miscanthus* species. Industrial Crops and Products *32*, 88–96. http://dx.doi.org/10.1016/j.indcrop.2010.03.009.

Kim, Y.-S., Hahn, E.-J., Murthy, H.N., and Paek, K.-Y. (2004). Effect of polyploidy induction on biomass and ginsenoside accumulations in adventitious roots of ginseng. Journal of Plant Biology *47*, 356–360. http://dx.doi.org/10.1007/BF03030551.

Li, M., Zhang, X., and Johnson, D.E. (1991). Plant Chromosome Technology. Northeast Forestry University Publisher, pp. 48–118.

Li, X., Chen, F., and Zhao, H. (2008). Compatibility of interspecific cross in *Dendranthema* genus. Acta Horticulturae Sinica *35*, 257–262.

Liu, Q., and Zhang, S. (1983). A new variety of *Dendranthema gaertn* from Shennongjia of Hubei. Journal of Wuhan Botanical Research *2*, 237–238.

Majdi, M., Karimzadeh, G., Malboobi, M.A., Omidbaigi, R., and Mirzaghaderi, G. (2010). Induction of tetraploidy to feverfew (*Tanacetum parthenium* Schulz-Bip.): Morphological, physiological, cytological, and phytochemical changes. HortScience *45*, 16–21.

Miguel, T.P., and Leonhardt, K.W. (2011). In vitro polyploid induction of orchids using oryzalin. Scientia Horticulturae *130*, 314–319. http://dx.doi.org/10.1016/j.scienta.2011.07.002.

Mo, G.-z., Sun, M., Pan, H.-t., Zhang, Z, and Q.-x. (2010). Polyploid of *Dendranthema lavandulifolium* induced by colchicines. Journal of Nuclear Agricultural Sciences *24*, 527–531.

Nimura, M., Kato, J., Horaguchi, H., Mii, M., Sakai, K., and Katoh, T. (2006). Induction of fertile amphidiploids by artificial chromosome-doubling in interspecific hybrid between *Dianthus caryophyllus* L. and *D. japonicus* Thunb. Breeding Science *56*, 303–310. http://dx.doi.org/10.1270/jsbbs.56.303.

Nilanthi, D., Chen, X.-L., Zhao, F.-C., Yang, Y.-S., and Wu, H. (2009). Induction of tetraploids from petiole explants through colchicine treatments in *Echinacea purpurea* L. BioMed Research International 2009, 1–7.

Oliveira, V.M. d., Forni-Martins, E.R., Magalhães, P.M., and Alves, M.N. (2004). Chromosomal and morphological studies of diploid and polyploid cytotypes of *Stevia rebaudiana* (Bertoni) Bertoni (*Eupatorieae*, *Asteraceae*). Genetics and Molecular Biology *27*, 215–222. http://dx.doi.org/10.1590/S1415-47572004000200015.

Petersen, K.K., Hagberg, P., and Kristiansen, K. (2003). Colchicine and oryzalin mediated chromosome doubling in different genotypes of *Miscanthus sinensis*. Plant Cell, Tissue and Organ Culture *73,* 137–146. http://dx.doi.org/10.1023/A:1022854303371.

Praça, M.M., Carvalho, C.R., and Clarindo, W.R. (2009). A practical and reliable procedure for in vitro induction of tetraploid tomato. Scientia Horticulturae *122*, 501–505. http://dx.doi.org/10.1016/j.scienta.2009.05.032.

Rhee, H.K., Cho, H.R., Kim, K.J., and Kim, K.S. (2004). Comparison of pollen morphology in interspecific hybrid lilies after in vitro chromosome doubling. In IX International Symposium on Flower Bulbs *673*, 639–643.

Roy, A., Leggett, G., and Koutoulis, A. (2001). In vitro tetraploid induction and generation of tetraploids from mixoploids in hop (*Humulus lupulus* L.). Plant Cell Reports *20*, 489–495. http://dx.doi.org/10.1007/s002990100364.

Schepper, S. d., Leus, L., Mertens, M., Debergh, P., Bockstaele, E. v., and Loose, M. d. (2001). Somatic polyploidy and its consequences for flower coloration and flower morphology in azalea. Plant Cell Reports *20*, 583–590. http://dx.doi.org/10.1007/s002990100372.

Schifino, M.T., and Fernandes, M.I.M. (1987). Induction of polyploidy and cytological characterization of autotetraploids of *Trifolium riograndense* Burkart (*Leguminosae*). Euphytica *36*, 863–872. http://dx.doi.org/10.1007/BF00051871.

Shao, J., Chen, C., and Deng, X. (2003). In vitro induction of tetraploid in pomegranate (*Punica granatum*). Plant Cell, Tissue and Organ Culture *75*, 241–246. http://dx.doi.org/10.1023/A:1025871810813.

Song, P., Kang, W., and Peffley, E.B. (1997). Chromosome doubling of *Allium fistulosum* × *A. cepa* interspecific F_1 hybrids through colchicine treatment of regenerating callus. Euphytica *93*, 257–262. http://dx.doi.org/10.1023/A:1002957800957.

Stanys, V., Weckman, A., Staniene, G., and Duchovskis, P. (2006). In vitro induction of polyploidy in Japanese quince (*Chaenomeles japonica*). Plant Cell, Tissue and Organ Culture *84*, 263–268. http://dx.doi.org/10.1007/s11240-005-9029-3.

Tao, D., Li, X., Wang, L., Zhou, J., Chen, Y., and Huo, W. (2009). Progresses on determination of cell chromosome ploidy level of plants. Life Science Research *13*, 453–458.

Tel-Zur, N., Dudai, M., Raveh, E., and Mizrahi, Y. (2011). In situ induction of chromosome doubling in vine cacti (*Cactaceae*). Scientia Horticulturae *129*, 570–576. http://dx.doi.org/10.1016/j.scienta.2011.04.027.

Thao, N.T.P., Ureshino, K., Miyajima, I., Ozaki, Y., and Okubo, H. (2003). Induction of tetraploids in ornamental *Alocaias* through colchicine and oryzalin treatments. Plant Cell, Tissue and Organ Culture *72*, 19–25. http://dx.doi.org/10.1023/A:1021292928295.

Urwin, N.A., Horsnell, J., and Moon, T. (2007). Generation and characterisation of colchicine-induced autotetraploid *Lavandula angustifolia*. Euphytica *156*, 257–266. http://dx.doi.org/10.1007/s10681-007-9373-y.

Wan, Y., Petolino, J., and Widholm, J. (1989). Efficient production of doubled haploid plants through colchicine treatment of anther-derived maize callus. Theoretical and applied genetics *77*, 889–892. http://dx.doi.org/10.1007/BF00268344.

Yang, X., Cao, Z., An, L., Wang, Y., and Fang, X. (2006). In vitro tetraploid induction via colchicine treatment from diploid somatic embryos in grapevine (*Vitis vinifera* L.). Euphytica *152*, 217–224. http://dx.doi.org/10.1007/s10681-006-9203-7.

Ye, Y., Tong, J., Shi, X., Yuan, W., and Li, G. (2010). Morphological and cytological studies of diploid and colchicine-induced tetraploid lines of crape myrtle (*Lagerstroemia indica* L.). Scientia Horticulturae *124*, 95–101. http://dx.doi.org/10.1016/j.scienta.2009.12.016.

Received: Mar. 22, 2016
Accepted: Jul. 1, 2016

Addresses of authors:
Miao He [1], Wenjie Gao[1], Yaohui Gao [2], Yingzhu Liu[1], Xue Yang[1], Hongbin Jiao[1] and Yunwei Zhou[1,*]
[1] Department of Landscape Architecture of Northeast Forestry University, 26 Hexing Road, Harbin, Heilongjiang 150040, China
[2] Beijing Forestry University
* Corresponding author;
E-mail: zhouyunwei1970@163.com

This research was supported by the Fundamental Research Funds for the Central Universities (2572015EY03) and National Natural Science Foundation of China (31400590).