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Chromosome Doubling in *Limonium bellidifolium* (Gouan) Dumort. by Colchicine Treatment of Seeds

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Limonium bellidifolium **(2n = 2x = 18), a perennial statice belonging to the family Plumbaginaceae, is cultivated as a garden plant or for cut flowers and is an important breeding material for the production of hybrid cultivars in the genus. In this study, chromosome doubling in *L. bellidifolium* was attempted to increase the variability in horticultural traits. Seeds of this species were treated with an antimitotic agent, colchicine, at different concentrations and exposure periods. The treated seeds were sown in soil in a cell tray and the seedlings were grown in a greenhouse. More than 50% of the seedlings treated with colchicine for 24 or 48 h, irrespective of the concentration, survived for 4 months after treatment. Most of the seedlings treated for 72 h at any concentration showed very poor growth and abnormal thickening of the hypocotyl, and ultimately died. The surviving seedlings were grown in 9-cm pots. Flow cytometry analysis of leaf tissues showed that 2.5%–5% of plants that received 0.05% colchicine for 72 h, 0.25% colchicine for 24 h, 0.25% colchicine for 48 h, or 0.5% colchicine for 48 h were tetraploid (4x) or mixoploid (2x + 4x). The highest frequencies of tetraploids and mixoploids occurred following treatment with 0.05% colchicine for 72 h. These results showed that colchicine treatment of seeds is effective for chromosome doubling in *L. bellidifolium*. After 3 years of cultivation, the morphological characteristics of diploid and tetraploid *L. bellidifolium* plants at the flowering stage were investigated. The stomatal density was lower in all investigated tetraploid and mixoploid plants than in the control diploid plant. The stomatal length was 1.1- to 1.5-fold higher in all tetraploid and mixoploid plants than in the control. Tetraploid plants tended to have wider and thicker leaves than the control and also produced larger flowers.**

Key Words: antimitotic agent, flow cytometry, perennial statice, polyploidization, tetraploid.

Introduction

The genus *Limonium*, whose members are commonly called statices, in the family Plumbaginaceae comprises over 300 species and has a ubiquitous distribution (Burchi et al., 2006). The perennial statice *L. bellidifolium* (2n = 2x = 18) is native to the Caucasus and Siberia. It has blue florets and its growth is adversely affected by high temperature. This species is cultivated as a garden plant or for cut flowers, and is an important breeding material in the production of hybrid cultivars (Harada, 1992; Tsurushima, 1993).

Chromosome doubling is a valuable tool for improving crops (Thao et al., 2003). In various ornamental crops, polyploidization has produced attractive traits such as thicker stems, greener leaves, increased width-to-length ratio of leaves, and larger flowers (Chen and Goeden-Kallemeyn, 1979; Ishizaka and Uematsu, 1994; Lindsay et al., 1994; Nakano et al., 2006; Nimura et al., 2006; Nonaka et al., 2011; Ogasawara et al., 2014; Takamura and Miyajima, 1996). Chromosome doubling in ornamental plants has been observed to occur spontaneously among plants regenerated through tissue culture (Aida and Shibata, 2002; Lindsay et al., 1994; Nakano et al., 2003, 2006; Oh et al., 1995; Winkelmann and Grunewaldt, 1995), and has been induced by treatment with spindle toxins (Chen and Goeden-Kallemeyn, 1979; Cohen and Yao, 1996; Ishizaka and Uematsu, 1994; Nimura et al., 2006; Nonaka et al., 2011; Ogasawara et al., 2014; Takamura and Miyajima, 1996; Thao et al., 2003). Spindle toxins, such as colchicine, oryzalin, amiprophos-methyl, and trifluralin, have

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been successfully applied to chromosome doubling in plants (Hansen and Andersen, 1996; Nonaka et al., 2011; Sree Ramulu et al., 1991; Thao et al., 2003; Tosca et al., 1995); of these, colchicine is the most commonly used (Hancock, 1997).

In *Limonium* breeding programs, novel cultivars have generally been produced by intra- or inter-specific hybridization. Morgan et al. (2001) reported that the fertility of sterile interspecific hybrids, created by crossing *L. perezii* and *L. sinuatum* using embryo rescue techniques, was restored through oryzalin-induced somatic chromosome doubling. To the best of our knowledge, this is the only polyploidization study in *Limonium*, and thus far, no study has determined the optimum concentrations and/or exposure time for spindle toxin-induced chromosome doubling or provided a precise description of morphological characteristics.

In the present study, chromosome doubling in *L. bellidifolium* was achieved by treating seeds with colchicine to increase horticultural trait variability. The effects of different concentrations and exposure periods of colchicine on plant survival and chromosome doubling were examined. Furthermore, the morphological characters of the polyploids at the flowering stage were investigated.

Materials and Methods

Plant materials and colchicine treatments

Seeds of *L. bellidifolium* were obtained from FUKUKAEN NURSERY & BULB CO., LTD., Nagoya, Japan. The seeds were treated with 0.05%, 0.25%, or 0.5% colchicine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 24, 48, or 72 h. After treatment, they were washed with tap water and sown in soil in a cell tray. The cell trays were cultivated in a greenhouse, where the temperature at night was 18°C. After 3 months, the plants that had survived were potted into 9-cm plastic pots and cultivated in the same greenhouse.

Flow cytometry analysis and chromosome count

The ploidy level of colchicine-treated plants was estimated by flow cytometry (FCM) analysis using a CyFlow flow cytometer (Partec, Münster, Germany). Young leaf tissue samples of approximately 1 cm^2 were cut into small pieces with a razor blade in 400 µL of solution A (Partec High Resolution Staining Kit for Plant DNA Analysis; Partec), and 1.6 mL of DAPI-staining solution (Partec High Resolution Staining Kit for Plant DNA Analysis; Partec) was added. The mixture was filtered through a 40-µm mesh filter to remove debris and analyzed with the flow cytometer.

To verify the ploidy level, the chromosomes in root tip cells of selected plants were observed. Root tips, approximately 5–10 mm in length, were excised from potted plants and soaked in water for at least 8 h at 0°C. They were then fixed in Farmer's fixative (3:1 ratio of absolute alcohol:glacial acetic acid) for at least 24 h and stained with acetocarmine for at least 20 min. The stained root tips were placed on a glass slide with one or two drops of 45% acetic acid, crushed, and observed under a microscope at 400× magnification.

Leaf stomata observations

The mean stomatal density and stomatal size of five randomly selected leaves of a plant for which ploidy level was determined by flow cytometry were measured using the easy replica-method according to Usui and Win (1997). Clear nail polish was applied to the abaxial surface of each leaf and allowed to dry for at least 3 min. Cellophane tape was attached to the abaxial leaf surface to remove the hardened nail polish (a replica of the leaf surface), which was then placed on a glass slide. The prepared slides were observed under a light microscope.

Morphological characterization of chromosome-doubled plants

Morphological characterization of diploid, tetraploid, and mixoploid plants was carried out after 3 years of cultivation during the flowering season. The mean diameter of the three longest shoots, the mean leaf width of five randomly selected expanded leaves, the mean length and width of 10 randomly selected flowers, the number of shoots per plant, and plant height were recorded for chromosome-doubled plants at the flowering stage. The mean soil plant analysis development values of five randomly selected expanded leaves were measured using a chlorophyll meter (SPAD-502 plus; KONICA MINOLTA, INC., Tokyo, Japan). To examine pollen fertility, mature pollen grains were collected from florets immediately after anthesis, stained with acetocarmine, and observed under a light microscope. The mean leaf thickness of randomly selected leaves was measured as follows. Leaves were harvested from potted plants, cut into small pieces, and fixed in FAA (formalin:acetic acid:70% ethanol = 5:5:90, v/v/v). The samples were dehydrated in a graded series of n-butyl alcohol and embedded in paraffin. Serial longitudinal sections (15-µm wide) were cut with a rotary microtome and stained with safranin and fast green. The sections were observed under a light microscope.

Results

Colchicine treatment

The effects of three colchicine concentrations and exposure periods were investigated (Table 1). More than 50% of seedlings treated with colchicine for 24 or 48 h, regardless of the concentration, survived for 4 months following treatment. Most of the seedlings treated for 72 h showed very poor growth, abnormal thickening of the hypocotyl, and ultimately died. The frequencies of surviving seedlings produced from the seeds treated for 72 h were significantly lower than the control.

Table 1. Effect of treating seeds with colchicine on the survival and ploidy level of *Limonium bellidifolium* seedlings.

Concentration (%)	Period (h)	Total no. of seeds treated	Total no. of surviving seedlings[z]	% of surviving seedlings[y]	No. of seedlings at each ploidy level[x]			
					Diploid (2x)	Tetraploid (4x)	Mixoploid (2x+4x)	% of tetraploid+mixoploid seedlings
Control[w]		80	48	60.0±13.2 a	48	0	0	0
0.05	24	120	90	75.0±6.9 a	89	0	1	0.8
0.05	48	120	83	69.2±4.8 a	82	0	1	0.8
0.05	72	120	19	15.8±7.1 b	13	2	4	5.0
0.25	24	120	91	75.8±6.9 a	88	2	1	2.5
0.25	48	120	89	74.2±3.7 a	86	2	1	2.5
0.25	72	120	2	1.7±1.7 b	1	1	0	0.8
0.5	24	120	95	79.2±8.3 a	95	0	0	0
0.5	48	120	65	54.2±8.1 a	62	1	2	2.5
0.5	72	120	4	3.3±1.4 b	2	0	2	1.7

[z] Data were recorded four months after colchicine treatment.
[y] Values represent the means±SE of four independent experiments, each consisting of 30 seeds except the control. In the control treatment, 20 seeds were used in each experiment. Values within the same column followed by different letters are significantly different at the 0.05 level using Tukey's test.
[x] Ploidy level was determined by flow cytometry analysis using a leaf from each plantlet 6 months after colchicine treatment.
[w] Seeds were sowed in soil without colchicine treatment.

An FCM histogram of the control treatment had a single peak corresponding to 2C DNA content (data not shown). The peaks of all 48 plants derived from control seeds appeared at approximately the same position, indicating that they were diploid (2x). However, a single peak, corresponding to 4C DNA content, appeared in the histograms of plants derived from seeds treated with colchicine, indicating that these plants were tetraploid (4x) (data not shown). In some colchicine treatment-derived plants, the histograms showed two peaks at different positions, corresponding to 2C and 4C DNA, indicating that they were mixoploids (2x + 4x) (data not shown).

FCM analysis of leaf tissues showed that 2.5%–5.0% of plants that received 0.05% colchicine for 72 h, 0.25% colchicine for 24 h or 48 h, or 0.5% colchicine for 48 h were tetraploid (4x) or mixoploid (2x + 4x) (Table 1). The highest percentage of tetraploid and mixoploid plants (5.0%) occurred with the 0.05% colchicine for 72 h treatment.

To confirm the ploidy levels, chromosome numbers were counted in root tip cells (Fig. 1). *Limonium bellidifolium* plants with a 4C peak were confirmed to be tetraploid with 2n = 36 chromosomes.

Observation of leaves of chromosome-doubled plants

The morphological characteristics of *L. bellidifolium* leaves were investigated in plants with different ploidy levels (Table 2; Fig. 2). The stomatal densities of all investigated tetraploid and mixoploid plants, except plant no. 3, were significantly lower than those of control diploid plants. The stomatal lengths of all tetraploid and mixoploid plants were 1.1- to 1.5-fold greater than in the control. Tetraploid plants tended to have wider and thicker leaves than diploid plants. The leaf width and thickness of tetraploid plant no. 9 was significantly increased, by 45.9% and 37.9%, respectively.

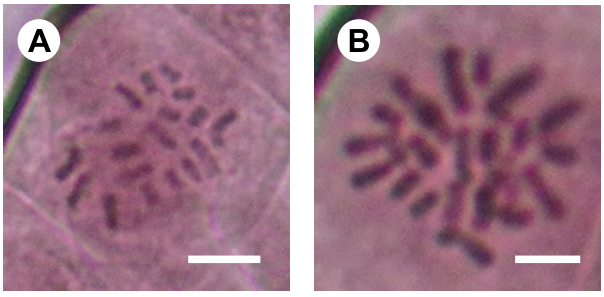

Fig. 1. Somatic chromosomes in root tip cells of *Limonium bellidifolium* plants derived from colchicine-treated seeds. (A) Diploid (2x) chromosomes derived from control seeds not treated with colchicine, and (B) tetraploid (4x) chromosomes derived from seeds treated with 0.25% colchicine for 24 h. Scale bar = 4 μm.

Growth and morphology of chromosome-doubled plants

The morphological characteristics of diploid and tetraploid *L. bellidifolium* plants at the flowering stage are shown in Table 3 and Figure 2. Tetraploid plants showed compact and vigorous growth, and tended to have larger flowers than the diploid controls. Flower length and width were significantly increased by 1.2- and 1.3-fold, respectively, in tetraploid plants compared with the controls. There was no clear cut difference in pollen fertility between diploids and tetraploids.

Discussion

We successfully produced tetraploid *L. bellidifolium*

Table 2. Comparison of morphological characteristics of *Limonium bellidifolium* leaves from plants with different ploidy levels.[z]

Plant strain	Ploidy level	Stomatal density (no.·mm^{-2})	Stomatal size (μm)		Leaf width (mm)	Leaf thickness (μm)	Leaf SPAD value[y]
			Length	Width			
Control[x]	2x	69.4 a [w]	23.4 d	23.4 a	17.2 c	272 b	44.5 b
No.3	4x	59.2 ab	25.2 cd	22.8 a	20.1 bc	284 b	42.7 bc
No.9	4x	49.0 b	34.5 a	22.9 a	25.1 b	375 a	56.4 a
No.13	4x	43.9 b	28.8 b	25.0 a	24.4 b	271 b	37.9 cd
Mixoploid	2x+4x	46.9 b	28.0 bc	23.4 a	32.5 a	263 b	35.5 d

[z] Five randomly selected leaves were measured from each plant.
[y] SPAD value means soil plant analysis development value.
[x] The control was a plant derived from a seed without colchicine treatment.
[w] Values within the same column followed by different letters are significantly different at the 0.05 level using Tukey's test.

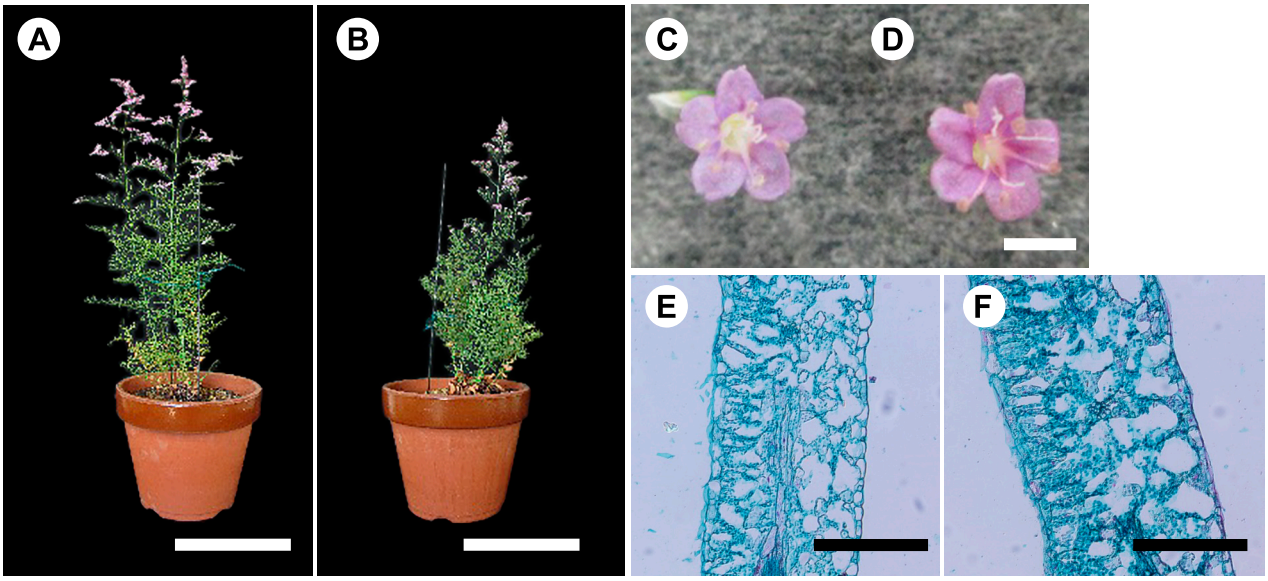

Fig. 2. Flowering plants (A and B), flowers (C and D) and leaf cross sections (E and F) of *Limonium bellidifolium* plants derived from colchicine-treated seeds. (A, C, and E) Diploid (2x) plants derived from control seeds not treated with colchicine, and (B, D, and F) tetraploid plants (4x). Scale bar = 20 cm for A and B, 2 mm for C and D, and 200 μm for E and F.

Table 3. Comparison of morphological characteristics of diploid and tetraploid *Limonium bellidifolium* plants at the flowering stage.

Plant strain	Ploidy level	No. of shoots per plant	Plant height (cm)	Stem diameter (mm)[z]	Flower length (mm)[y]	Flower width (mm)[y]	Pollen fertility (%)[x]
Control[w]	2x	9	60	1.7 a[v]	2.9 b	2.9 b	59 b
No.3	4x	6	50	1.7 a	3.6 a	3.7 a	40 c
No.9	4x	10	77	2.1 a	3.6 a	3.7 a	95 a
No.13	4x	4	65	1.9 a	3.6 a	3.7 a	24 d
Mixoploid	2x+4x	11	57	1.5 a	3.3 a	3.3 ab	89 a

[z] The three longest shoots were investigated from each plant.
[y] Ten randomly selected flowers were investigated from each plant.
[x] One hundred randomly selected pollen grains were investigated from each plant.
[w] The control was a plant derived from a seed without colchicine treatment.
[v] Values within the same column followed by different letters are significantly different at the 0.05 level using Tukey's test.

plants by treating seeds with colchicine *in vivo*. Our results indicate that colchicine treatment is effective for chromosome doubling in this species. However, the highest percentage of tetraploid and mixoploid plants was only 5.0% (Table 1); therefore, future studies are needed to establish more efficient methods for chromosome doubling in this genus. Cohen and Yao (1996) stated that *in vitro* colchicine treatment is more efficient than *in vivo* treatment for the production of polyploids. Culture technology can regenerate plants effectively,

and *in vitro* colchicine treatments show a low frequency of chimeras (Cohen and Yao, 1996). Chromosome doubling by *in vitro* colchicine treatment has been achieved in ornamental plants using a variety of tissues and organs, such as apical meristems (Ogasawara et al., 2014), callus (Chen and Goeden-Kallemeyn, 1979), multiple shoots (Cohen and Yao, 1996), nodal segments (Nimura et al., 2006; Nonaka et al., 2011), and shoot tips (Thao et al., 2003). It will be necessary to conduct *in vitro* colchicine treatments using regenerable organs in *L. bellidifolium*. Oryzalin is an alternative spindle toxin to colchicine, and has been used to polyploidize ornamental plants, such as *Alocasia* sp. (Thao et al., 2003), *Gerbera jamesonii* (Tosca et al., 1995), *Lychnis senno* (Nonaka et al., 2011), *Rhododendron* spp. (Väinölä, 2000), and *Rosa* spp. (Allum et al., 2007; Kermani et al., 2003). Some studies have reported that oryzalin is more effective than colchicine for chromosome doubling (Nonaka et al., 2011; Tosca et al., 1995; Väinölä, 2000). In future studies, we will examine the application of oryzalin in polyploid breeding programs for *L. bellidifolium*.

The abaxial leaf surface of *L. bellidifolium* showed reduced stomatal density and increased stomatal size in tetraploid plants (Table 2). It has been reported that polyploidization reduces stomatal density (Chen et al., 2011; Gantait et al., 2011; Gu et al., 2005), but there are cases where a decrease was not observed, such as *L. senno* (Nonaka et al., 2011). Increased stomatal size after chromosome doubling has been reported in some ornamentals, such as *Anthurium andraeanum* (Chen et al., 2011), *Gerbera jamesonii* (Gantait et al., 2011), and *Tricyrtis* sp. (Otani et al., 2014). In the present study, tetraploid *L. bellidifolium* had larger flowers than a control diploid plant (Table 3). Such morphological alterations have been reported in chromosome-doubled plants, including *Cyclamen* spp. (Ishizaka and Uematsu, 1994; Takamura and Miyajima, 1996), *Dianthus caryophyllus* (Nimura et al., 2006), *Eustoma grandiflorum* (Lindsay et al., 1994), *Hemerocallis flava* (Chen and Goeden-Kallemeyn, 1979), and *Tricyrtis hirta* (Nakano et al., 2006). Tetraploid *L. bellidifolium* with large flowers may be valuable, especially as a garden plant.

The chromosome number often varies in species of the same genus. It is important to match parental species with identical chromosome numbers in interspecific hybridization breeding programs to avoid the ploidy level crossing barrier along with other genomic barriers (Suzuki et al., 2005; Tsuda et al., 2013). For example, Tsuda et al. (2013) reported that the hybridization barrier in the cross between diploid wild blueberry, *Vaccinium bracteatum*, and tetraploid highbush blueberry, *V. corymbosum* 'Spartan', was overcome using wild colchicine-induced tetraploid blueberry plants. In the present study, we obtained tetraploid *L. bellidifolium* with $2n = 36$ chromosomes. There are wild tetraploid *Limonium* plants with 36 chromosomes, such as *L. rariflora* (Choudhuri, 1942). It is expected that novel interspecific hybrids could be produced using tetraploid *Limonium* plants. Tetraploidy is also valuable in the production of triploids, which generally produce abnormal pollen grains and no or less fruit because of fertilization failure. Triploids offer a promising way to extend the flowering period of garden plants because flower senescence is inhibited and enervation of plants is prevented (Onozaki, 2014). We hope to produce useful triploids using the tetraploids obtained in the present study.

Chromosome doubling has been widely used as a breeding tool in ornamentals (Chen and Goeden-Kallemeyn, 1979; Lindsay et al., 1994; Nakano et al., 2006; Nonaka et al., 2011; Takamura and Miyajima, 1996). In this study, we demonstrated that treatment of seeds with 0.05% colchicine for 72 h was the best method for polyploidization of *L. bellidifolium*, and that tetraploids were horticulturally attractive. In the future, these tetraploids will be useful for interspecific hybridization and triploid breeding programs to produce novel cultivars.

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