



Article

Morphological Characterization of Tetraploids of *Limonium sinuatum* (L.) Mill. Produced by Oryzalin Treatment of Seeds

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Abstract: *Limonium sinuatum* (L.) Mill. ($2n = 2x = 16$) is a popular ornamental plant with dimorphism of pollen grains (type A and type B) and stigmas (papilla and *cob*-like). We applied polyploidy breeding to this species in order to introduce desirable traits. Tetraploid and mixoploid *L. sinuatum* plants were successfully obtained with oryzalin treatment of *L. sinuatum* 'Early Blue' seeds. All three tetraploids had increased leaf width, stomatal size, flower length, and pollen width compared to those of the diploid, and tetraploids had four germinal pores of pollen grains, whereas the diploid had three. All tetraploids had type A pollen grains and *cob*-like stigmas. Furthermore, the growth of cultivated tetraploid plants was slow, with later bolting and flowering times. Mixoploids Mixo-1 and Mixo-3 were estimated to be polyploidy periclinal chimeric plants consisting of a tetraploid L1 layer and diploid L2 layer, and Mixo-2 was estimated to be a polyploidy periclinal chimeric plant consisting of the diploid L1 layer and tetraploid L2 layer. Mixo-4 had tetraploid L1 and L2 layers. Mixoploids, except Mixo-4, had type A pollen grains and *cob*-like stigmas, whereas Mixo-4 had type B pollen grains and papilla stigmas. These polyploids will be useful as polyploidy breeding materials.

Keywords: bolting; cut flower; germinal pore; ornamental plant; polyploidy periclinal chimera; Plumbaginaceae; polyploidy breeding



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1. Introduction

Limonium sinuatum (L.) Mill., commonly known as statice, which belongs to the family Plumbaginaceae native to the Mediterranean area, is a popular ornamental plant because of its wide range of flower colors and long vase life. This species is diploid with $2n = 16$ [1]. In the genus *Limonium*, pollen- and stigma-dimorphism can be observed and is related to the self-incompatibility system. *L. sinuatum* produces type A and type B pollen as well as papilla stigmas and *cob*-like stigmas. The combination of type A pollen and *cob*-like stigma and the combination of type B pollen and papilla stigma do not lead to fertilization [2–5].

Generally, polyploids grow vigorously and their organs are larger than those of diploids. Polyploidization is commonly carried out to introduce novel attractive features to ornamental plants such as plant size, flower enlargement, and intense color of leaves and flowers [6,7]. Phenotypic changes due to chromosome doubling are thought to be caused by increased cell size, allele diversification, gene silencing, and gene dosage effects [6]. Chromosome doubling of plants can be achieved by treatment with polyploidizing agents including colchicine, oryzalin, amiprofos-methyl, and trifluralin. Among them, colchicine is the most commonly used agent [8], whereas oryzalin is recognized as an alternative

because of its high chromosome doubling efficiency and low toxicity [9–13]. To date, polyploids of ornamental plants including *Agastache foeniculum* [14], *Alocasia* sp. [10], *Gerbera jamesonii* [15], *Lychnis senno* [9], *Rhododendron* spp. [16,17], and *Rosa* spp. [12,13] have been obtained by oryzalin treatment.

L. sinuatum is difficult to crossbreed with different species except for some, and polyploid breeding is expected as a method to drastically change the morphology of this species. In the genus *Limonium*, there are a few reports regarding chromosome doubling using polyploidizing agents [18]. Morgan et al. [19] produced allotetraploids of an interspecific hybrid between *L. perezii* and *L. sinuatum* by oryzalin treatment of in vitro shoots. Mori et al. [20] treated the seeds of *L. bellidifolium* with colchicine and obtained autotetraploids, which tended to produce wider, thicker leaves and larger flowers than diploid plants. To the best of our knowledge in the related literature, there are no reports on the production of autotetraploids or detailed morphological characterization of tetraploid plants in *L. sinuatum*.

Enhancing the desired traits in *L. sinuatum* polyploids is a way to create new cultivars with novel attractive traits. Thus, in the present study, we examined the concentration and treatment time of oryzalin required for chromosome doubling in the seeds of *L. sinuatum* in order to achieve polyploidy breeding in *L. sinuatum*. We also investigated the morphology of *L. sinuatum* polyploids.

2. Materials and Methods

2.1. Plant Materials and Oryzalin Treatments

We used the seeds of *L. sinuatum* ‘Early Blue’ (Fukukaen Nursery & Bulb Co. Ltd., Nagoya, Japan). The treatment of seeds with oryzalin was carried out in February 2017. The seeds were surface-disinfected with 70% ethanol for 30 s, immersed in a 1% sodium hypochlorite (NaClO) solution for 10 min, and then rinsed with distilled water. They were treated with 0, 0.0005, 0.001, or 0.005% oryzalin (Wako Pure Chemical Industries Ltd., Osaka, Japan), which was dissolved in dimethyl sulfoxide (DMSO) for 24, 48, or 72 h at 25 °C in the dark on a device (Triple shaker NR-80; Taitec Corporation, Koshigaya, Japan) for shaking culture (80 rpm). Forty seeds were used in one treatment, and five independent experiments were performed. The treated seeds washed with tap water were sown in soil in the cell trays. The cell trays were placed in a greenhouse heated to 20 °C. The grown plants were potted into 7.5-cm plastic pots two months after cultivation, and their survival rate was recorded.

2.2. Flow Cytometry Analysis and Chromosome Count

A flow cytometer (CyFlow PA; Partec GmbH, Görlitz, Germany) was used in flow cytometry (FCM) analysis to estimate the ploidy level of the plants according to the method described by Mori et al. [20] with some modifications. For the analysis, a leaf disc of approximately 1 cm was cut out from a young leaf of plants potted in 7.5-cm pots. Extraction of nuclear DNA and DAPI staining were carried out using a commercial kit (CyStain UV Precise P; Sysmex Corporation, Kobe, Japan). The sample solution filtered using a 40-µm mesh filter was analyzed using a flow cytometer.

To confirm the ploidy level, the chromosomes in the root tip cells of diploid and putative tetraploid plants were observed by using previously reported methods [20]. The prepared samples were examined under a light microscope (CX41; Olympus Corporation, Tokyo, Japan).

2.3. Morphological Characterization

The polyploids potted in 7.5-cm pots were sequentially transferred to 24-cm clay pots from the summer until the autumn of 2017, and then replanted into 45-cm large plastic pots in the summer of 2018.

Morphological characterization of the leaves was performed in October 2017. Five leaves were randomly selected from each plant, and their leaf length, leaf width, leaf

soil plant analysis development (SPAD) value, stomatal size, and stomatal density were examined. A chlorophyll meter (SPAD-502 plus; Konica Minolta, Inc., Tokyo, Japan) was used to measure leaf SPAD values. Guard cells that make up the stomata were observed under a scanning electron microscope (Miniscope[®] TM3030Plus; Hitachi High-Tech Corporation, Tokyo, Japan). The growth of tetraploids at the flowering stage was examined in March 2018. The plant height, number of shoots per plant, stem wing width, flower length, calyx length, pollen size, pollen shape, stigma shape, and its pollen fertility were examined. Pollens and stigmas were observed under a scanning electron microscope (Miniscope[®] TM3030Plus). Carmine acetate staining was used for pollen fertility testing.

In June 2019, morphological characterization of mixoploids cultivated for two years in a greenhouse was carried out by examining its stomatal size, stomatal density, pollen size, pollen shape, and stigma shape. The methods of examination were described as above. In addition, the leaf ploidy level was investigated again using a flow cytometer.

2.4. Spike Culture and Growth Characteristics of Regenerated Tetraploid Plants

Spikes were cultured to reproduce the tetraploids of *L. sinuatum* according to a previous report [21]. Since the spikes are larger than the axillary buds, they are easy to handle and have high reproductive efficiency. The younger the spike, the higher the differentiation rate. Spikes with uncolored calyxes, approximately 1 cm in length, were excised from the bolted flower stalk and surface-disinfected with 70% ethanol for 60 s and immersed in a 2% (*w/v*) NaClO solution containing 0.1% (*v/v*) Tween 20 for 20 min. After three rinses with sterile distilled water, the spikes were cut to approximately 2 mm in length and placed on a shoot regeneration medium, which consisted of the MS medium [22], 1 mg L⁻¹ 6-benzyladenine, 30 g L⁻¹ sucrose, and 8 g L⁻¹ agar, in a test tube. They were cultured in a growth chamber at 20 °C under a 16 h day length (photosynthetic photon flux density 35 μmol m⁻² s⁻¹) with light-emitting diode lights. Multiple regenerated shoots were subcultured every four weeks with the shoot regeneration medium under the conditions described above. Each shoot excised from multiple shoots was placed on the MS medium without plant growth regulators for four weeks and was then placed on the root regeneration medium, which consisted of the MS medium, 1 mg L⁻¹ α-naphthaleneacetic acid, 30 g L⁻¹ sucrose, and 8 g L⁻¹ agar, in a test tube. Diploid or tetraploid plantlets were cultured for four or six weeks, respectively, under the same culture conditions as those for shoot regeneration.

The regenerated plants were acclimated in a growth chamber at 15 °C under 12 h day length (photosynthetic photon flux density 35 μmol m⁻² s⁻¹) with fluorescent lights for three weeks. The cultured plants were potted in 24-cm pots on 15 June 2020, and cultivated in a greenhouse at a ventilation temperature of 10 °C. The number of flower stalks and leaves were recorded every two weeks, and the bolting and flowering days were recorded. Three plants from each strain were investigated.

3. Results

3.1. Oryzalin Treatment

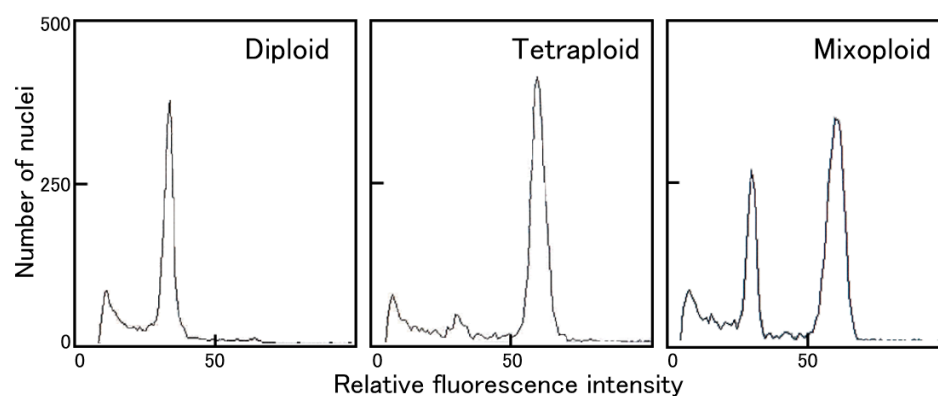
The survival rate of seedlings treated with oryzalin tended to decrease with the increase in treatment time regardless of the treatment concentration (Table 1). The DNA levels of all surviving plants were analyzed using a flow cytometer. Figure 1 presents the histograms from the FCM analysis of the control diploid, tetraploid, and mixoploid plants. A peak of tetraploid was the position with twice that in diploid. Mixoploid plants showed double peaks.

The root tip cells of tetraploid plants had 32 chromosomes, which was twice as high than that of diploid plants. Our analysis showed that three tetraploids were obtained by the seed treatments of oryzalin at 0.001% for 24 h, 0.001% for 72 h, and 0.005% for 48 h. In addition, seven mixoploids were obtained (Table 1).

Table 1. Effect of oryzalin seed treatment on the survival and ploidy level of *Limonium sinuatum* seedlings¹.

Concentration (%)	Period (h)	No. of Seeds Treated	% of Surviving Seedlings	No. of Seedlings at Each Ploidy Level ²		
				Diploid	Tetraploid	Mixoploids
				(2x)	(4x)	(2x + 4x)
0 (control)	0	200	66.0 a ³	123	0	0
0.0005	24	200	43.5 ab	94	0	0
0.0005	48	200	26.5 bc	50	0	1
0.0005	72	200	22.5 bc	31	0	2
0.001	24	200	38.5 bc	74	1	0
0.001	48	200	33.0 bc	62	0	1
0.001	72	200	16.0 cc	16	1	1
0.005	24	200	39.0 bc	74	0	0
0.005	48	200	28.5 bc	49	1	2
0.005	72	200	18.5 bc	25	0	0

¹ Data were recorded two months after the oryzalin treatment. ² Ploidy level was determined by flow cytometry using one leaf from each plant. ³ Values represent the means of five independent experiments, each consisting of 40 seeds. Values within the same column followed by different letters were significantly different at the level of 0.05 according to the Tukey–Kramer test.

**Figure 1.** Histograms from the flow cytometry (FCM) analysis of nuclear DNA content in diploid, tetraploid, and mixoploid *Limonium sinuatum* plants.

3.2. Morphological Characteristics of Tetraploid Leaves

A comparison of the morphological characteristics of leaves in tetraploids, which were named Tetra-1, 2, and 3, is shown in Table 2. The leaf width of all three tetraploids was significantly greater than that of the control plant, Cont-A, and the leaves of tetraploids were rounder (Figure 2A). The leaf SPAD values of tetraploids Tetra-2 and Tetra-3 were significantly higher than those of the diploid. The stomatal size of three tetraploids was significantly greater than that of diploids, and the stomatal density of tetraploids was significantly lower than that of the control plant (Figure 2B).

Table 2. Comparison of leaf morphological characteristics of tetraploid *Limonium sinuatum*¹.

Plant Strain	Ploidy Level	Leaf Length (mm)	Leaf Width (mm)	Leaf Index ²	Leaf SPAD Value ³	Stomatal Size (μm)		Stomatal Density (no. mm ⁻²)
						Length	Width	
Cont-A	2x	98.2 b ⁴	26.2 b	0.26 b	39.6 b	33.4 c	23.4 b	67.0 a
Tetra-1	4x	119.0 ab	51.8 a	0.44 a	39.3 b	46.0 b	30.5 a	51.7 b
Tetra-2	4x	125.0 a	49.4 a	0.40 a	53.0 a	47.2 a	30.4 a	39.0 c
Tetra-3	4x	133.8 a	52.6 a	0.39 a	52.4 a	47.4 a	30.3 a	26.9 d

¹ Five randomly selected leaves were measured from each plant. ² Leaf index represents leaf width/leaf length. ³ SPAD = Soil Plant Analysis Development. ⁴ Values within the same column followed by different letters were significantly different at the 0.05 level according to the Tukey–Kramer test.

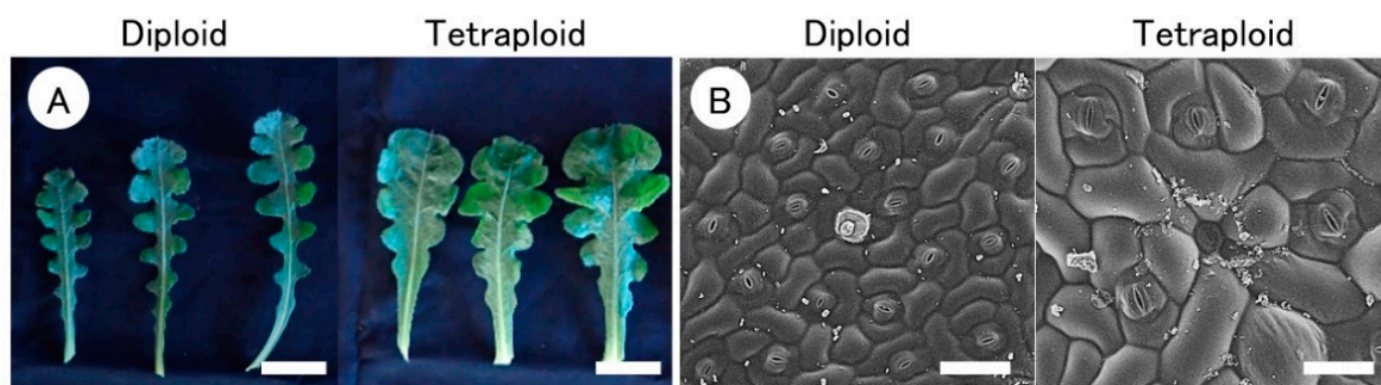


Figure 2. Morphological characteristics of leaves (A), bars = 5 cm and guard cells (B), bars = 100 μ m in diploid and tetraploid *Limonium sinuatum* plants.

3.3. Morphological Characteristics of Tetraploids at the Flowering Stage

A comparison of the morphological characteristics of tetraploids at the flowering stage is shown in Table 3. The stem wings of tetraploids Tetra-1 and Tetra-2 were more than three times wider than those of the control plant. Although the flower length of all tetraploids was significantly longer than that of the diploid plant, there was no difference in calyx length, which is the main part for ornamental use, between the diploids and tetraploids (Figure 3A,B).

Table 3. Comparison of the morphological characteristics of tetraploid *Limonium sinuatum* plants at the flowering stage ¹.

Plant Strain	Ploidy Level	Plant Height (cm)	No. of Shoot per Plant	Stem Wing (mm) ²	Flower Length (mm)	Calyx Length (mm)	Pollen Size (μ m)		No. of Germinal Pores ³	Type of Pollen Grain	Type of Stigma in Pistils
							Length	Width			
Cont-A	2x	52	14	2.2 b	14.2 b ⁴	12.6 a	54.4 a	39.9 b	3	A	cob-like
Tetra-1	4x	59	12	7.9 a	16.5 a	11.7 a	53.9 a	48.5 a	4	A	cob-like
Tetra-2	4x	55	10	8.2 a	17.2 a	13.4 a	56.4 a	52.8 a	4	A	cob-like
Tetra-3	4x	45	12	4.5 b	17.3 a	12.3 a	56.7 a	51.5 a	4	A	cob-like

¹ Five randomly selected shoots, flowers, and pollen grains were investigated from each plant. ² The stem wing width was measured from the center of the stem to the end of the wing. ³ Number of germinal pores of pollen grains was observed. ⁴ Values within the same column followed by different letters were significantly different at the 0.05 level according to the Tukey–Kramer test.

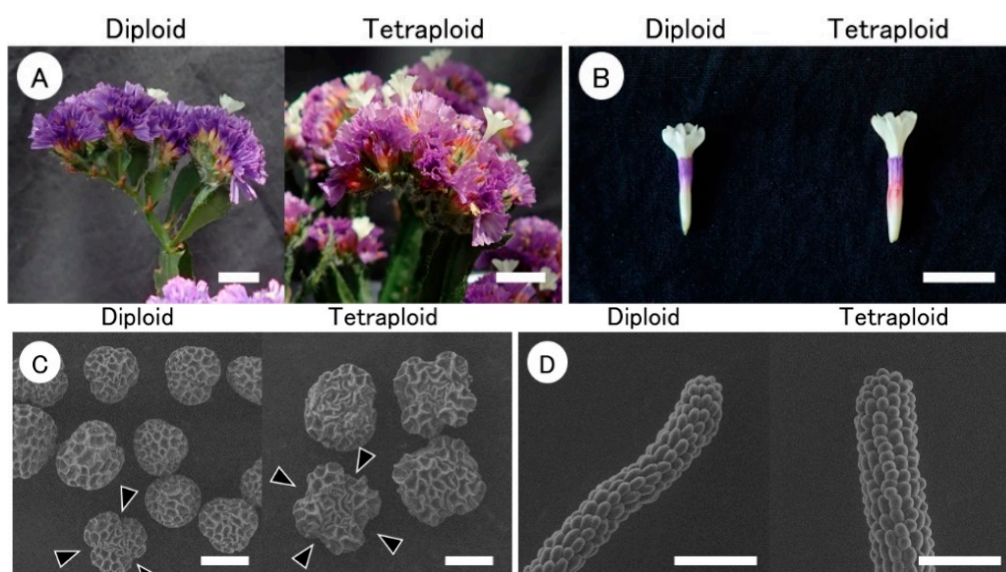


Figure 3. Morphological characteristics of flower clusters (A), bars = 10 mm, flowers (B), bar = 10 mm, pollen grains (C), bars = 30 μ m, and stigmas (D), bars = 100 μ m in diploid and tetraploid *Limonium sinuatum* plants. Arrows indicate the germinal pore.

Pollen and stigma types are involved in cross-compatibility. This information is important when using the obtained polyploids for future breeding. There are two pollen types in *L. sinuatum*: type A with coarse pollen surface, and type B with fine pollen surface. All pollen grains of tetraploids investigated in the present study coincidentally belonged to type A. The pollen width of tetraploids was significantly higher than that of the control, and the tetraploid pollen grains was close to a spherical shape. The pollen grains of diploids were tricolpate with three germinal pores, whereas some observed pollen grains of tetraploids were stephanocolporate with four germinal pores (Figure 3C). There are two types of stigmas in this species: *cob*-like stigmas and papilla stigmas. Both control diploids and tetraploid stigmas investigated in the present study were *cob*-like stigmas (Figure 3D). The pollen fertility of Cont-A, Tetra-1, Tetra-2, and Tetra-3 was 86, 66, 82, and 87%, respectively, and the pollen fertility of Tetra-1 was significantly lower than that of the others (data not shown).

3.4. Morphological Characteristics of Mixoploids

The morphological characteristics of survived mixoploids, which were named Mixo-1, 2, 3, 4, 5, and 6, at the flowering stage in the second year are shown in Table 4. Ploidy levels in the leaves were analyzed by FCM again at the flowering stage in the second year; Mixo-1, Mixo-2, Mixo-3, Mixo-5, and Mixo-6 were polyploid chimeras consisting of diploid and tetraploid cells, and Mixo-4 was detected only in tetraploid cells. Mixoploids investigated in this study, except for Mixo-4, had type A pollen grains and *cob*-like stigmas. However, Mixo-4 contained type B pollen grains and papilla stigmas (Figure 4A,B). The stomatal sizes of Mixo-1, Mixo-3, and Mixo-4 were significantly greater than those of the control diploid, and the stomatal density of Mixo-1 and Mixo-3 was significantly lower than that of the diploid. Mixo-2 had a wide range of pollen grains with four germinal pores. Mixo-4 had large pollen grains with four germinal pores.

Table 4. Comparison of the morphological characteristics of mixoploid *Limonium sinuatum* plants at the flowering stage in the second year ¹.

Plant Strain	Stomatal Size (μm)		Stomatal Density (no. mm^{-2})	Pollen Size (μm)		No. of Germinal Pores ²	Type of Pollen Grains	Type of Stigma of Pistils	Ploidy Level of Leaf ³	L1-L2 Putative Ploidy Level
	Length	Width		Length	Width					
Cont-A	35.1 d ⁴	21.1 de	77.2 bc	52.5 bc	42.5 bc	3	A	<i>cob</i> -like	2x	-
Mixo-1	45.8 b	28.9 b	42.7 d	56.5 ab	46.7 ab	3	A	<i>cob</i> -like	2x + 4x	4x – 2x
Mixo-2	33.1 d	21.9 cd	80.4 bc	57.4 ab	48.9 a	4	A	<i>cob</i> -like	2x + 4x	2x – 4x
Mixo-3	55.8 a	31.4 a	26.9 d	52.0 bc	42.3 bc	3	A	<i>cob</i> -like	2x + 4x	4x – 2x
Mixo-4	40.6 c	24.1 c	58.9 cd	60.8 a	44.9 ab	4	B	papilla	4x	4x – 4x
Mixo-5	27.8 e	19.2 e	130.3 a	49.6 c	39.6 c	3	A	<i>cob</i> -like	2x + 4x	2x – 2x
Mixo-6	28.6 e	20.3 de	100.8 ab	52.6 bc	42.7 bc	3	A	<i>cob</i> -like	2x + 4x	2x – 2x

¹ Three randomly selected leaves and 12 randomly selected pollen grains were investigated from each plant. ² Number of germinal pores of pollen grains was observed. ³ Ploidy levels of leaves were analyzed by flow cytometry at the flowering stage in the second year. ⁴ Values within the same column followed by different letters were significantly different at the 0.05 level according to the Tukey–Kramer test.

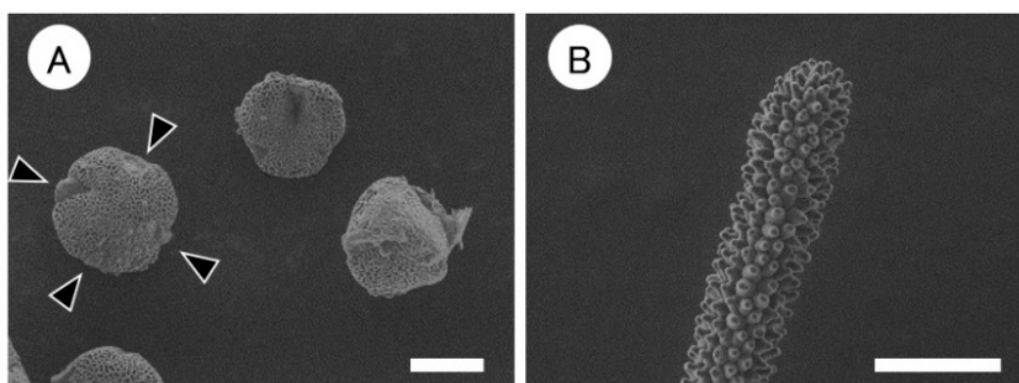


Figure 4. Pollen grains (A), bar = 30 μm and a stigma (B), bar = 100 μm in mixoploid Mixo-4 of *Limonium sinuatum*. Pollens are type B, and stigma is papilla. Arrows indicate the germinal pore.

3.5. Growth of Cultivated Tetraploid Plants

Diploid- and tetraploid-derived cultures except Tetra-2, which were propagated by spike culture, were successfully acclimatized and planted in pots. Tetra-2 could not be successfully cultivated due to complications. In the Cont-A-derived strain (i.e., in the control diploid plants), the leaves were vigorously differentiated, and the plants developed early and produced a large number of flower stalks. The number of leaves and flower stalks of tetraploid Tetra-1- and Tetra-3-derived strains were significantly lower than that of diploids after the middle stage of growth. The number of days from planting to flowering in the Tetra-3-derived strain was high. In the Tetra-1-derived strain, there were few flower stalks, and none reached flowering during the experimental period because of physiological disorders (Figures 5 and 6).



Figure 5. Plants cultivated from diploid Cont-A (A), tetraploid Tetra-1 (B1,B2), and Tetra-3 (C) of *Limonium sinuatum*. The photos were taken three months after planting. Scale bars: all except B1 = 10 cm, B2 = 5 cm.

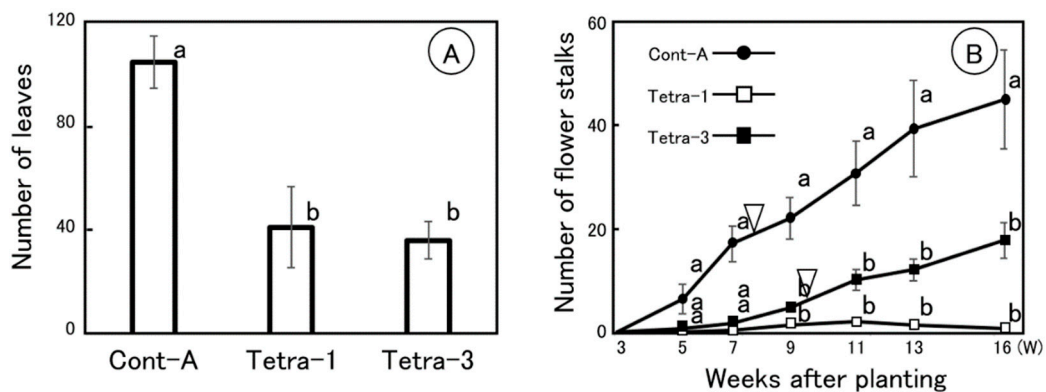


Figure 6. Number of leaves seven weeks after planting (A) and changes in the number of flower stalks (B) in diploid Cont-A-derived strain, tetraploid Tetra-1-derived strain, and Tetra-3-derived strain of *Limonium sinuatum*. Arrows in figure (B) indicate the flowering day. Bars indicate standard errors ($n = 3$). Values followed by different letters were significantly different at the 0.05 level according to the Tukey–Kramer test.

4. Discussion

Our *in vivo* seed treatments with oryzalin successfully produced polyploids of *L. sinuatum* (Table 1). Morgan et al. [19,23] produced interspecific hybrids of *L. perezii* and *L. sinuatum* and obtained allotetraploids after an oryzalin treatment of their embryos. These findings indicate that oryzalin treatment is effective for producing polyploid plants in *Limonium* spp. However, Mori et al. [20] produced tetraploids using *in vivo* colchicine treatment in *L. bellidifolium*, and the production rate of tetraploids was higher than that in the present study using *in vivo* oryzalin treatment in *L. sinuatum*. In the future, it is

necessary to consider the use of colchicine in chromosome doubling of *L. sinuatum* for its further improvement. In addition, in vitro treatments of spindle toxins may increase the efficiency of chromosome doubling in this species because this treatment has been successfully used to produce tetraploids in various ornamental plant species [9,10,24,25].

Tetraploids of *L. sinuatum* have wide leaves, large stomata, low stomata density, and large flowers, and these morphological characteristics were consistent with those of polyploid *L. bellidifolium* [20]. In addition, leaf ovalization [10], stomatal enlargement [26–28], and flower enlargement [29–34] as a consequence of chromosome doubling has been reported in other ornamental plants. Thick stem wings and inflorescence clogging in the tetraploids obtained in this study are not desirable from an ornamental point of view. The ornamental value of the tetraploids is low, and we consider that these need to be improved by breeding. On the other hand, chromosomal doubling is known to bring resistance to environmental stresses such as drought, salt stress, cold, and heat, in addition to morphological changes [35]. Drought tolerance of tetraploids will be associated with low stomatal density [25]. Environmental stress tolerance is also a desirable trait in ornamental plants and future research is expected to explore this aspect deeper.

Generally, pollen grains of the genus *Limonium* have three germinal pores. In this study, the pollen grains of diploids had three germinal pores, whereas those of tetraploids had four germinal pores. An increase in the germinal pore number by chromosome doubling has been widely reported in plants [36,37]. In *L. sinuatum*, the number of germinal pores as well as pollen size in pollen grains is useful as an index to determine the ploidy of pollen.

The cultured *Limonium* tetraploid strains grew slowly and had a later bolting and flowering times. Polyploid plants have lower growth rates and tend to flower later than the related diploids [38]. Pei et al. [39] reported that tetraploid radishes have later bolting and flowering times than those of diploid radishes, and the levels of endogenous phytohormones gibberellin (GA) 1 and GA4, which are presumed to promote flowering, were higher in diploids than in tetraploids, whereas the amount of abscisic acid, which is considered as a floral repressor, was higher in tetraploids than in diploids. Such physiological changes may also occur in tetraploid *Limonium* species.

The shoot apical meristem of many higher plants consists of three cell layers: the outermost epidermal layer (L1), the subepidermal layer (L2), and the inner corpus region (L3) [40–42]. Cells of the L1 layer form the epidermis, those of the L2 layer form the subepidermal mesophyll and germ cells, and those of the L3 layer form the internal and vascular tissues [40,41,43]. In the present study, we estimated the ploidy of the L1 and L2 layers in *Limonium* mixoploids based on morphological observations. Mixo-1 and Mixo-3 were estimated to be polyploidy periclinal chimeric plants consisting of tetraploid L1 tissue and diploid L2 tissue (layer constitution: L1-L2 = $4x - 2x$), because they had larger stomata, and their pollen grains had three germinal pores and were about the same size as those of diploids. Mixo-2 was estimated to be a polyploidy periclinal chimeric plant consisting of diploid L1 tissue and tetraploid L2 tissue (L1-L2 = $2x - 4x$) because the stomatal sizes were approximately the same as those of the diploids, and the pollen grains had four germinal pores and were larger than those of diploids. The second FCM analysis and observation indicated that Mixo-4 had tetraploid leaves and flower stalks. This plant was determined to be a mixoploid according to the first FCM analysis in the early growth period, but it was estimated that tetraploid tissue grew more vigorously than diploid tissue during the two-year cultivation period. In Mixo-5 and Mixo-6, no tetraploid tissues were found in our morphological observations, and detailed investigation is required in the future. The mixoploids obtained in the present study will be useful as breeding materials in *Limonium* polyploidy breeding programs.

In conclusion, tetraploid and mixoploid *L. sinuatum* plants were successfully obtained by oryzalin treatment of the seeds. However, the chromosomal doubling in *L. sinuatum* did not provide a sufficient improvement in ornamental value. In the future, we would like to cross a tetraploid with a diploid to create a triploid with desirable traits such as voluminous inflorescence. On the other hand, it is known that chromosomal doubling may

affect resistance to stress [38]. We want to investigate the physiological characteristics of polyploidy.

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