horticulturae

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Nitrous Oxide Treatment after Pollination Induces Ploidy Changes in Statice (*Limonium* sp.)

Juana Cordoba-Sanchez [1,*], Keith Funnell [2], Duncan Hedderley [2], Nick Roskruge [1] and Ed Morgan [2]

[1] School of Agriculture and Environment, Massey University, Palmerston North 4410, New Zealand
[2] New Zealand Institute for Plant and Food Research Limited, Palmerston North 4410, New Zealand; keith.funnell@plantandfood.co.nz (K.F.); duncan.hedderley@plantandfood.co.nz (D.H.); ed.morgan@plantandfood.co.nz (E.M.)
* Correspondence: juana.cordoba@gmail.com

Abstract: The production of statice (*Limonium* sp.) plants with higher ploidy through induction of whole-genome duplication (WGD) via the spindle disrupter nitrous oxide (N_2O) was examined as a strategy to increase the germplasm diversity of the species. Furthermore, the impact of the resulting ploidy changes on the morphological features of the progeny was examined. Intraspecific crosses between diploid plants of *Limonium sinuatum* (L.) Mill and *L. perezii* (Stapf) Hubb. were conducted daily for seven consecutive days, with subsequent exposure to N_2O. Within the resulting progeny, between 16% and 35% of plants were polyploid when N_2O was applied between one and four days after pollination. A comparative analysis between diploid and tetraploid progeny was conducted, using a selection of 10 *L. sinuatum* (5 diploids and 5 tetraploids) and 7 *L. perezii* (4 diploids and 3 tetraploids) genotypes. The results revealed differences between tetraploids and their diploid counterparts for most of the evaluated characteristics. Tetraploid plants of *L. sinuatum* and *L. perezii* exhibited pollen grains 1.5 times larger in plan area; the leaves and main floral stem diameter were 1.2 and 1.5 times thicker for *L. sinuatum* and *L. perezii*, respectively, the guard cell length was 1.4 times greater for both species, while the stomatal density was 0.6 times lower for *L. perezii* and 0.8 for *L. sinuatum*. The leaf area and main floral stem wings were affected by the ploidy increase only for *L. sinuatum*. In this regard, tetraploid plants of *L. sinuatum* displayed leaves 1.8 times bigger and main floral steam wings 2.4 times wider in comparison to diploid plants. In conclusion, the production of tetraploid *Limonium* plants using N_2O shortly after pollination creates new diversity for breeding. These findings underscore the potential for leveraging polyploidy as a strategy to enhance desirable traits in *Limonium* species.

Keywords: autopolyploid; gene dosage effect; mitotic disruption; ornamental plants; polyploidization; whole-genome duplication

Citation: Cordoba-Sanchez, J.; Funnell, K.; Hedderley, D.; Roskruge, N.; Morgan, E. Nitrous Oxide Treatment after Pollination Induces Ploidy Changes in Statice (*Limonium* sp.). *Horticulturae* **2024**, *10*, 816. https://doi.org/10.3390/horticulturae10080816

Academic Editor: Davide Neri

Received: 22 June 2024
Revised: 21 July 2024
Accepted: 27 July 2024
Published: 1 August 2024

1. Introduction

As a cut flower, *Limonium* species and their hybrids are primarily used as a "filler" in flower arrangements and frequently as dried flowers. *Limonium* is the fifteenth most cut flower by volume sold [1], resulting in significant interest in delivering new cultivars. Hybridization offers an opportunity to increase diversity by introducing new characteristics and has been frequently used to generate new cultivars, e.g., "Ocean blue" [2] and the "siNZii" series [3]. Nevertheless, in interspecific hybrids, sterility and infertility are frequent, limiting the further use of the hybrids for breeding purposes [4]. An alternative approach to increase the germplasm diversity demanded by the ornamental industry is the production of seedlings with double the number of chromosomes, i.e., whole-genome duplication (WGD).

When utilizing diploid parental lines, WGD contributes to increasing germplasm diversity by means of producing tetraploid and mixoploid plants [5] with novel morphological features [6–8], although mixoploids are not desirable. In addition, ploidy increases

have been associated with the "gene dosage effect" where the phenotype is suggested to be directly attributed to the number of copies of a particular gene [9]. Therefore, morphological changes such as bigger organs and lower stomatal density are expected to occur in polyploid plants [10–13].

At a cellular level, WGD is a consequence of the disruption of the mitotic spindle during cell reproduction, which can occur naturally or be induced [14]. In nature, four significant WGD events have been identified during the evolution and diversification of angiosperms (see [5,15] for a review), while in *Limonium* natural polyploids have occurred at least twice [10,16]. Possible mechanisms involved in the production of polyploids in *Limonium* are allopolyploidization or hybridization between diploid genotypes with different basic chromosome numbers, and fusion between a reduced gamete with an unreduced gamete or between unreduced gametes [10,16]. Hence, while WGD does occur naturally, its frequency is perhaps less than may be desirable within breeding programs. As a consequence, the generation of synthetic polyploids using mutagens such as nitrous oxide (N_2O), colchicine, or oryzalin has been evaluated [4,11–13,17–19]. Ploidy manipulation in *Limonium* has previously been achieved using colchicine or oryzalin as the inductive agent [4,11,13], and recently the efficacy of N_2O for in vivo induction of unreduced gametes was evaluated [20]. Treatment with N_2O as a gas has induced WGD in other plant species such as wheat (*Triticum aestivum*; [17]), canary grass (*Phalaris canariensis*; [18]), rye (*Secale cereale*; [12]) and lilium (*Lilium* × *formolongi*; [19]). In those studies, for the induction of WGD, different pressures (i.e., from 608 Kpa to 1013 Kpa), durations (i.e., from 4 h to 72 h), and zygote/embryo developmental stages (i.e., between 5 and 13 days after pollination (DAP)) have been evaluated.

Treatment with N_2O disrupts the mitotic/meiotic spindle by depolymerizing microtubules. As a result, chromosomes are retained in the center of the cell before the formation of the cell plate, resulting in one daughter cell having double the number of chromosomes and the other having none [21]. For the present study, N_2O was selected over oryzalin or colchicine as N_2O penetrates intact plant tissues, reaching the internal organs to exert its effect, and N_2O does not remain in the plant, rapidly dissipating from the plant following treatment. Additionally, N_2O is not considered to affect the survival and growth of treated plants, as can occur with both colchicine and oryzalin [21–24].

The developmental stage of the embryo is of great consequence with regard to the induction of WGD. Embryos that are younger are less developed, with a smaller number of cells. Immediately after fertilization, the embryo is single celled and thereafter, during the formation of a multi-celled embryo, cell divisions may not be synchronized [25]. This means that treatment at a given time after pollination may result in only one or few dividing cells within an embryo being affected by the treatment. It is thus considered important to time the treatment to coincide with the first cell division to ensure that all the cells resulting from subsequent cell divisions are tetraploid. Treating cells at the time of the first cell division therefore reduces the probability of the formation of mixoploids. Thus, the aim of N_2O treatment for WGD was to disrupt the mitotic spindle in zygotes/proembryos during the first embryonic division, and to experimentally determine the best timing of treatment to increase the production of tetraploids while minimizing the occurrence of mixoploids.

In the current study, we chose to use flow cytometry to determine the ploidy of the progeny derived after treatment with N_2O for WGD. In comparison to chromosome counting, the determination of ploidy by flow cytometry enhances accuracy in ploidy determination by simultaneously analyzing multiple nuclei to estimate ploidy, with no differences detected between the results of flow cytometry and chromosome counting for other plant species, such as oil-tea (*Camelia oleifera*; [26]) and *Hibiscus* [27]. Moreover, automation of flow cytometry enables the analysis of a large number of plants in a short amount of time. Additionally, flow cytometry has previously been used for determination of the plant ploidy of hybrids or their derived allopolyploids in *Limonium* [4,11,13]. In the research described here, gaseous N_2O was applied so as to disrupt the mitotic spindle, ideally targeting single-celled zygotes [12]. As reported for wheat [17], 24 h N_2O treatment

is the recommended exposure time, and our own investigations with *Limonium* support this (unpublished). While N_2O has been used for the induction of unreduced pollen (2n) of *Limonium* [20], the successful generation of progeny of increased ploidy by means of inducing WGD using N_2O has not previously been reported. Therefore, the aim of this study was to demonstrate that treatment with N_2O of zygotes of *Limonium sinuatum* (L.) Mill and *Limonium perezii* (Stapf) Hubb., at the right DAP, would result in plants with increased ploidy (i.e., tetraploid or mixoploid). The effects of WGD on the phenotypes of the *L. sinuatum* and *L. perezii* progeny were also described.

2. Materials and Methods

2.1. Plant Material and Cultivation

Two diploid *L. sinuatum* (2*n* = 16) genotypes (i.e., sin16, and sin18) and two *L. perezii* genotypes (i.e., per11 and per273) were used as parents in the crosses conducted in the current study. The genotypes were selected based on their contrasting stigma, synchronous flowering times, and greater-than-75% seed set following intraspecific pollination. Emasculation prior to anther dehiscence was not necessary as *L. sinuatum* and *L. perezii* are self-incompatible and present with pollen/stigma dimorphism [28]. Each genotype was available as multiple clones for experimentation.

Individual *L. sinuatum* flowers were pollinated manually during spring, while the crosses in *L. perezii* were completed during summer. Containerized plants were grown in a commercially sourced, bark-based growing medium (Daltons Landscape Supplies Ltd., Matamata, New Zealand Base growing medium, (CAN Fines A Grade 50%; fiber 30%, Matamata, New Zealand), Pacific pumice 7 mm 20%, 0.5 kg/m^3 superphosphate (9.1P–11S–20Ca), 2.0 kg/m^3 each of agricultural lime and dolomite (21Ca–10Mg; Prebble Seeds, Christchurch, New Zealand), and 1 kg/m^3 gypsum), with 4.3 kg/m^3 of 8–9-month Osmocote® 16N–3.5P–10K (Grace-Sierra International, The Netherlands), 0.4 kg/m^3 calcium ammonium nitrate (27N–6Ca–4Mg), 0.5 kg/m^3 potassium sulfate (42K–18S), and 3 kg/m^3 Osmocote® (23N) under greenhouse conditions in Palmerston North, New Zealand (40.9006 °S, 174.8860 °E), with daily air temperatures set at 20 °C for heating and 24 °C for venting, and a natural photoperiod of 11 h in spring and 14.30 h in summer.

2.2. Experimental Design

For the induction of WGD in zygotes of intraspecific crosses of either *L. sinuatum* or *L. perezii*, 10 recently opened individual flowers of each parental genotype were crossed in a reciprocal crossing design on each selected day prior to treatment with N_2O. Following the methodology outlined previously [29], whole plants carrying the pollinated flowers were treated with N_2O within a pressure-tolerant cylinder for 24 h at c. 600 kPa. After application of N_2O, the plants were returned to the greenhouse conditions until ovule/embryo rescue was undertaken at one month after pollination. For example, plants treated on day seven (7 DAP) were pollinated and seven days after pollination were exposed to N_2O.

In the first experiment, *L. perezii* genotypes were used with an experimental design comprising a 2 × 7 arrangement of factors: seed-bearing genotype (i.e., per11 or per273) and seven different treatment times (1 DAP to 7 DAP) when N_2O was applied. In a subsequent experiment, *L. sinuatum* genotypes were used in a 2 × 7 arrangement of factors, i.e., seed-bearing genotype (i.e., sin16 or sin18) and seven treatment times (2 DAP to 8 DAP), for application of N_2O.

In both experiments, a completely randomized design was utilized. Within each species, each genotype served as both pollen-donor and seed-bearing parent. As each pollinated flower can develop only one zygote, the experimental unit therefore corresponded to each pollinated flower, having 10 replicates per treatment.

As control treatments within the experiments, one pair of plants of each cross combination (i.e., sin16 × sin18; and per11 × per273) was used for performing crosses on two separate dates, but without N_2O application.

For the parents and derived seedlings, measured and calculated response variables included proportion of pollinated flowers that successfully produced zygotes (i.e., cross success), ploidy of seedlings derived (and the proportion of tetraploid seedlings or seedlings with tetraploid tissues in the case of mixoploid plants), plan area of pollen grains (i.e., surface area of the image of a pollen grain as viewed under a microscope; refer to Section 2.4.1), guard cell length, stomatal density, leaf thickness and area, main floral stem diameter, and main floral stem wing width [30]. The efficacy of N_2O treatment in inducing tetraploids and mixoploids was evaluated by determining the proportion of tetraploid and mixoploid seedlings relative to the total number of seedlings obtained for each species individually. Any triploid occurrences were omitted from the analysis.

2.3. In Vitro Ovule Culture

One month after the pollinations were undertaken, the zygotes were established in vitro by culturing the ovules enclosing the zygotes, utilizing a base medium comprising Murashige and Skoog macro and micro salts and iron [31], Gamborg B5 vitamins [32], 30 g/L sucrose, 7.5 g/L agar [33], and 0.03 mL/L Kathon® LXE (active ingredient chloromethylisothiazolinone/methylisothiazolinone). Once germinated, seedlings were transferred to a pre-rooting medium comprising the base medium [33] for further growth. Finally, the plants were transferred to a root induction medium comprising the base medium amended with Gamborg B5 vitamins [32] and 12 mg/L indole-3-butyric acid (IBA) for one month.

In all cases, the medium pH was adjusted to 5.7 with either 0.1 N NaOH or 0.1 N HCl prior to autoclaving at 121 °C at 103 kPa for 15 min. Seedlings were cultured in disposable plastic culture vessels (Alto Packaging, Hamilton, New Zealand) containing approximately c. 50 mL of above-mentioned media. The laboratory growing conditions were 25 ± 1 °C, at a photosynthetic photon flux density of 30 ± 5 µmol m^2/s and 16 h photoperiod provided by cool-white fluorescent tubes.

2.4. Morphological Evaluation of L. sinuatum and L. perezii

Rooted plants of progeny were deflasked and grown under greenhouse conditions until flowering using the commercially sourced, bark-based growing medium detailed above. When at least 80% of the calyces were open [30], morphological evaluation was undertaken. A total of ten *L. sinuatum* genotypes were chosen for evaluation, comprising five diploids (sin15, sin16, sin18, sin191, and sin192) and five tetraploids (sin193, sin194, sin202, sin203, and sin204), as confirmed by prior flow cytometry analysis. Similarly, seven *L. perezii* genotypes were selected, consisting of four diploids (per11, per181, per271, and per52) and three tetraploids (per53, per54, and per91).

2.4.1. Floral Characteristics

Using a Vernier caliper (0–150 mm, Protech, Rydalmere, Australia, TD2082), diameter of the main floral stem and width of the main floral stem wings were recorded on three main stems of plants of each genotype.

For determination of plan area of pollen grains, pollen was collected from three flowers taken from three different corymbs of each genotype. The pollen from the three flowers was mixed and stored in an ethanol/glycerol fixation solution containing 85% ethanol and 100% glycerol in a 3:1 proportion [34,35] until measured. For each genotype, samples were prepared for microscopy using 30 µL of the solution containing this pooled pollen and 10 µL of modified Alexander's stain [36] and observed using a Zeiss microscope Imager.Z2 (Axio Imager Z2, Carl Zeiss Microscopy GmbH, Gottingen, Germany). Pollen grains were photographed at 50x magnification using a digital camera (Axiocam 305 color, Carl Zeiss Microscopy GmbH, Gottingen, Germany) and the software ZEN2.6 (blue edition version 2.6.76.00000) [37]. The plan area of each of 100 grains was measured and recorded using the software Fiji version 1.54f [38]. Pollen grains that stained purple were recorded as viable, and those that stained brown or with doubtful coloration were recorded as aborted [36].

2.4.2. Vegetative Characteristics

For each genotype, three recently fully expanded leaves were selected for the measurement of leaf area and thickness. For leaf thickness, a Vernier caliper (0–150 mm, Protech, Rydalmere, Australia, TD2082) was used, and one measurement taken. In contrast, for the leaf area, photographic images (Nikon D5100with VR lens; 18–55 mm 1:3.5–5.6 G, Nikon, Melville, NY, USA) were obtained and processed using the software Fiji version 1.54f [38].

Guard cell length and stomatal density were measured from similar regions of two recently fully expanded leaves, each corresponding to replicates. Transparent impressions of the abaxial surface of leaves were created using a polyvinyl acetate wood glue (PVA) technique [39]. Guard cells were observed at 100× magnification (Zeiss imager.Z2, Axio Imager Z2, Carl Zeiss Microscopy GmbH, Gottingen, Germany), with the length of 100 guard cells measured per leaf, each guard cell corresponding to a subsample.

Quantification of stomatal density was based on transparent impressions of the abaxial surface of leaves photographed at 100× magnification using a digital camera (Axiocam 305 color, Carl Zeiss Microscopy GmbH, Gottingen, Germany) and the software ZEN2.6 (blue edition version 2.6.76.00000) [37]. Photographs were processed using the software Fiji version 1.54f [38], with the stomatal density calculated as the number of stomata in 1 mm^2. Two leaves were taken from each genotype, representing the replicates, while five areas of each leaf were used for the density estimation and corresponded to subsamples.

2.5. Determination of Ploidy Level

For the determination of the ploidy level, leaves from two-month-old seedlings (parental genotypes as well as progeny) were sampled and analyzed using flow cytometry, utilizing the methodology of [40]. Leaf tissue was finely chopped, together with that of *Trifolium repens* or *Bellis* sp. as an internal standard, in 400 µL buffer extraction containing citric acid 0.5% and Tween 0.5%. This mix was filtered through CellTrics 30 µm filter (Sysmex, Görlitz, Germany). Next, 1.6 mL of 4′,6-diamidino-2-phenylindole (DAPI) staining solution (final concentration 2.5 µg/mL) was added, and the samples measured using a Partec PAII Flow Cytometer (SYSMEX CyFlow Space, Sysmex, Lincolnshire, (IL), USA). The DAPI relative fluorescence was compared with that of a known diploid *L. sinuatum* plant. One thousand events were counted for each sample peak. The software Flowmax flow cytometry version 2.9 (2014) (CyPAD Windows^{TM}-based software, Sysmex, Lincolnshire, IL, USA) was used for data acquisition and analysis.

2.6. Statistical Analysis

Data analysis was conducted using the software R version 4.2.2 (The R Foundation for Statistical Computing, Vienna, Austria) [41] with ggplot 2 version 3.5.1 [42] utilized for graphic outputs.

The response variable cross success followed a binary distribution while stomatal density followed a Poisson distribution. In both cases, a generalized linear model (glm function from the stats package version 4.4.1 [41]) with the relevant distribution was used to model the effects of seed-bearing genotype and DAP. The other response variables were analyzed with linear models (lm function from the stats package version 4.4.1 [41]).

Pairwise differences between means were tested using LSD (Least Significant Difference) test, adjusting the *p*-values by the Bonferroni method [43]. For the generalized linear models (glm), pairwise likelihood ratio tests were used to compare between the levels of factors (similar to LRPAIR in [44]). For all analyses, the *p*-value used was 0.05.

To visualize the effect of higher ploidies on plant morphology, a Principal Component Analysis (PCA) was completed of the variables: ploidy and each of the seven morphological characteristics were evaluated. The PCA utilized the software R version 4.2.2 (The R Foundation for Statistical Computing, Vienna, Austria) [41] using the packages FactoMineR version 2.11 [45] and factoextra version 1.0.7 [46]. The classical means of data summarizing, dimensionality reduction, and visualization for a multi-component analysis were used.

3. Results

3.1. Seedlings Obtained

For plants treated with N_2O, the number of pollinated flowers that successfully produced zygotes varied from 7 to 53 (Online Supplementary Table S1), representing between 10% and 75% cross success. A lower number of zygotes occurred for *L. perezii* in comparison with *L. sinuatum*. In *L. perezii*, between 9% and 20% of zygotes were obtained in treated plants and control plants, respectively, whereas in *L. sinuatum* 67% of the zygotes were produced in treated plants and 70% in control plants (Online Supplementary Table S1). In tissue culture, embryo mortality was between 15% for *L. sinuatum* and 85% for *L. perezii* genotypes.

3.2. Effect of N_2O Treatment on Ploidy

Flow cytometry results (Online Supplementary Data S2) revealed that the treatment of *L. perezii* with N_2O for 24 h after pollination resulted in the production of five tetraploid progeny when it was applied between 1 and 3 DAP, with only diploids (five plants) and mixoploids (three plants) resulting when applied on other days after pollination (Figure 1; Online Supplementary Table S3). In *L. perezii*, the efficacy of the treatment with N_2O in inducing tetraploids was 38%, whereas in mixoploids it was 23%, for a total of 61%. The progeny with N_2O-induced doubling (i.e., tetraploids) was obtained between 1 DAP and 3 DAP.

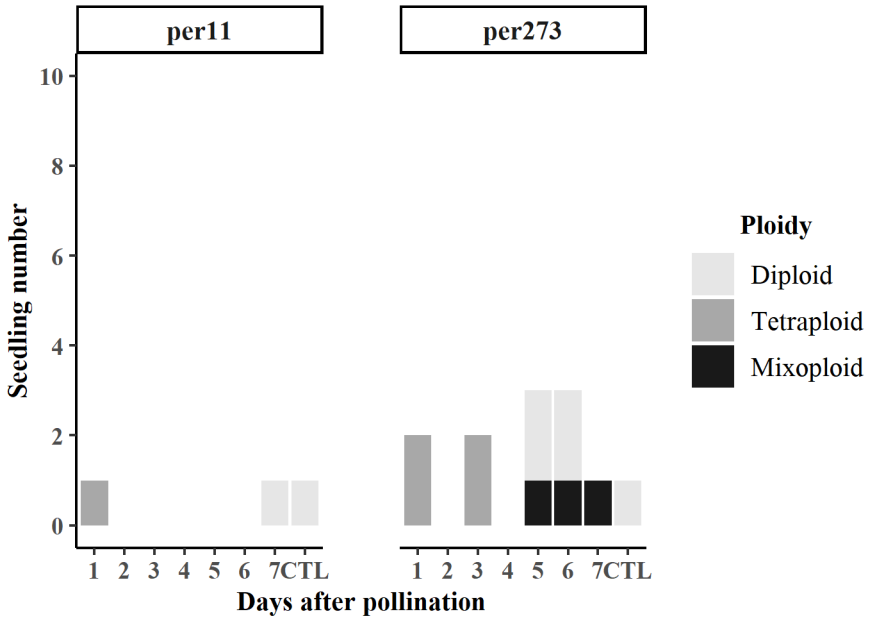

Figure 1. Number of diploid, tetraploid, and mixoploid progeny obtained in two seed-bearing genotypes of *Limonium perezii* on each day after pollination (DAP) when N_2O was applied. Gaps in the data mean zero seedlings survived after tissue culture.

In the case of *L. sinuatum*, the flow cytometry results (Online Supplementary Data S4) showed that N_2O treatment for 24 h resulted in the production of one tetraploid progeny when the treatment was applied 2 DAP, and three mixoploid progeny when applied 4 and 5 DAP (Figure 2; Online Supplementary Table S5). Pollinations in the control plants resulted in no tetraploid progeny, though one triploid progeny was produced, presumably as the result of fertilization of an unreduced gamete (data not presented and excluded from the calculations of the efficacy of N_2O to induce polyploidization). In this species, the efficacy of N_2O treatment to induce tetraploidy was 1%, whereas for mixoploidy it was 3%, for a total of 4%.

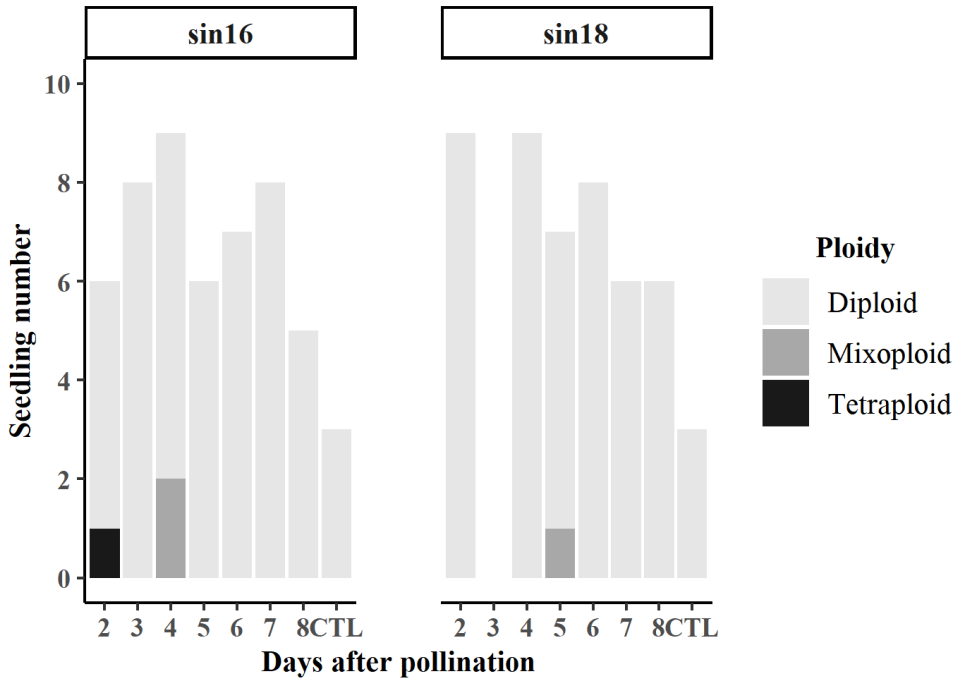

Figure 2. Number of diploid, tetraploid, and mixoploid progeny obtained in two seed-bearing genotypes of *Limonium sinuatum* on each day after pollination (DAP) when N_2O was applied. Gaps in the data mean zero seedlings survived after tissue culture.

The success of zygotes in being regenerated as plants (mean 0.72 ± 0.13 per flower) was not affected by the seed-bearing genotype (sin16 or sin18; $p > 0.05$) nor by the N_2O treatment ($p > 0.05$).

3.3. Morphological Evaluation

3.3.1. Floral Characteristics

The plan area of individual, purple-stained (modified Alexander's stain) i.e., viable, pollen grains ranged between 1195 μm^2 and 4500 μm^2 across all *L. sinuatum* and *L. perezii* genotypes examined, which included diploids and tetraploids. The differences observed were associated with the ploidy and species ($p < 0.05$; Table 1). For example, the mean of the plan area of the pollen collected from *L. sinuatum* (2000.4 μm^2 ± 658 μm^2) was 9% larger compared to that of *L. perezii* (1829.1 μm^2 ± 728.3 μm^2). With regard to the effect of ploidy, the pollen grain area in *L. sinuatum* varied from 1595.5 ± 405.9 μm^2 for diploids to 2406.2 ± 611 μm^2 for tetraploid genotypes. In *L. perezii*, the pollen grain area for diploids was 1519.6 ± 395.4 μm^2 and for tetraploids it was 2240.3 ± 960.5 μm^2. In summary, the pollen plan grain area of the tetraploid progeny of *L. sinuatum* and *L. perezii* was 1.5 times larger in comparison with their diploid counterparts (Online Supplementary Figure S6C).

The floral characteristics of the main floral stem diameter and main floral stem wing width increased in the tetraploid genotypes of *L. sinuatum* in comparison with their diploid counterparts ($p < 0.05$; Table 1). The main floral stem diameter was 1.5 times thicker in tetraploid plants, and the main floral stem wings were 2.4 times wider (Online Supplementary Figure S7). In contrast, as floral stem wings are absent in *L. perezii*, only the main floral stem diameter increased significantly in tetraploid plants, being 1.2 times thicker ($p < 0.05$; Table 1).

Table 1. Plan area (μm^2) by ploidy of purple-stained pollen collected from *Limonium sinuatum* genotypes used as parents or corresponding to their progeny treated as young zygotes with nitrous oxide (N_2O) for 24 h.

Genotype	Ploidy	Pollen Plan Area (μm^2) [z]
sin15 (parent)	Diploid	2200 ± 212 [b]
sin16 (parent)	Diploid	1811 ± 274 [c]
sin191 (progeny)	Diploid	1946 ± 180 [c]
sin192 (progeny)	Diploid	1854 ± 363 [c]
sin193 (progeny)	Tetraploid	2551 ± 407 [a]
sin194 (progeny)	Tetraploid	2597 ± 353 [a]
sin201 (progeny)	Tetraploid	2670 ± 329 [a]
sin202 (progeny)	Tetraploid	2439 ± 336 [a]
sin203 (progeny)	Tetraploid	2437 ± 276 [a]
sin204 (progeny)	Tetraploid	2543 ± 528 [a]

[z] LSD test was performed for analysis of the pairwise differences between means. Different letters represent different group means (p-value = 0.05).

3.3.2. Vegetative Characteristics

The length of guard cells varied across all genotypes from 15 to 66 µm, and the differences observed were associated with both ploidy and species ($p < 0.05$; Table 2). The guard cells from the tetraploid genotypes were 1.4 times larger in comparison with the diploids for both *L. sinuatum* and *L. perezii*, displaying a mean value of 28.43 ± 4.40 for diploids versus 39.94 ± 7.65 for tetraploids (Online Supplementary Figure S6B).

Table 2. Guard cell length and stomatal density by ploidy of *Limonium sinuatum* genotypes used as parents or corresponding to their progeny treated as young zygotes with nitrous oxide (N_2O) for 24 h.

Genotype	Ploidy	Guard Cell Length (µm) [z]	Stomatal Density [x y]
sin15 (parent)	Diploid	23.8 ± 4.1 [e]	2.2 ± 1.4 [a]
sin16 (parent)	Diploid	24.0 ± 4.9 [de]	2.3 ± 1.3 [a]
sin191 (progeny)	Diploid	26.3 ± 3.2 [e]	1.8 ± 1.0 [b]
sin192 (progeny)	Diploid	30.3 ± 3.7 [d]	1.3 ± 0.4 [c]
sin193 (progeny)	Tetraploid	31.2 ± 4.6 [c]	1.7 ± 1.6 [c]
sin194 (progeny)	Tetraploid	33.7 ± 3.9 [c]	1.4 ± 0.6 [e]
sin201 (progeny)	Tetraploid	42.8 ± 5.8 [b]	1.0 ± 0.6 [f]
sin202 (progeny)	Tetraploid	38.2 ± 5.3 [c]	1.3 ± 1.1 [g]
sin203 (progeny)	Tetraploid	47.5 ± 5.6 [a]	1.1 ± 1.0 [h]
sin204 (progeny)	Tetraploid	45.1 ± 4.8 [b]	1.0 ± 0.9 [g]

[z] LSD test was performed for analysis of the pairwise differences between means. Different letters represent different group means (p-value = 0.05); [x] Number of stomata per mm^2; [y] LRPAIR test grouping was performed for the stomatal density mean. Different letters represent different group means (p-value = 0.05).

Across genotypes, the stomatal density varied between 31.21 and 65.33 stomata per mm^2, and the differences observed were associated with both ploidy and species ($p < 0.05$; Table 2). The average density of the stomata was between 0.6 times and 0.8 times lower in tetraploid *L. perezii* and *L. sinuatum* plants in comparison with their corresponding diploids (Online Supplementary Figure S6).

The leaf thickness increased in the tetraploid genotypes of *L. perezii* and *L. sinuatum* in comparison with their diploid counterparts ($p < 0.05$; Table 2). As a result, in the tetraploids, the leaves were 1.2 times thicker in *L. perezii* and 1.5 times thicker in *L. sinuatum*.

In the case of the leaf area, the increment in ploidy only produced a significant increase in area of the leaves of *L. sinuatum*, represented by leaves that were 1.8 times bigger in the tetraploid genotypes (Table 2).

An analysis of the morphological characteristics by PCA using diploid and tetraploid plants of *L. sinuatum* and *L. perezii* revealed two components that explained 78% of the

observed variance in morphological measures (Figure 3). In the primary component (PC1), 54.11% of the variability was explained by factors such as the pollen grain plan area, main floral stem diameter, leaf thickness, and guard cell length, all of which exhibited a concurrent increase with ploidy; and by stomatal density, which showed a decrease with increasing ploidy. The variance within the second component (PC2; 25.24%) was linked to species differentiation between *L. perezii* and *L. sinuatum*. Notably, *L. perezii* had smaller main floral stem wings, a larger leaf area, a greater guard cell length, and higher stomatal density in comparison to *L. sinuatum*.

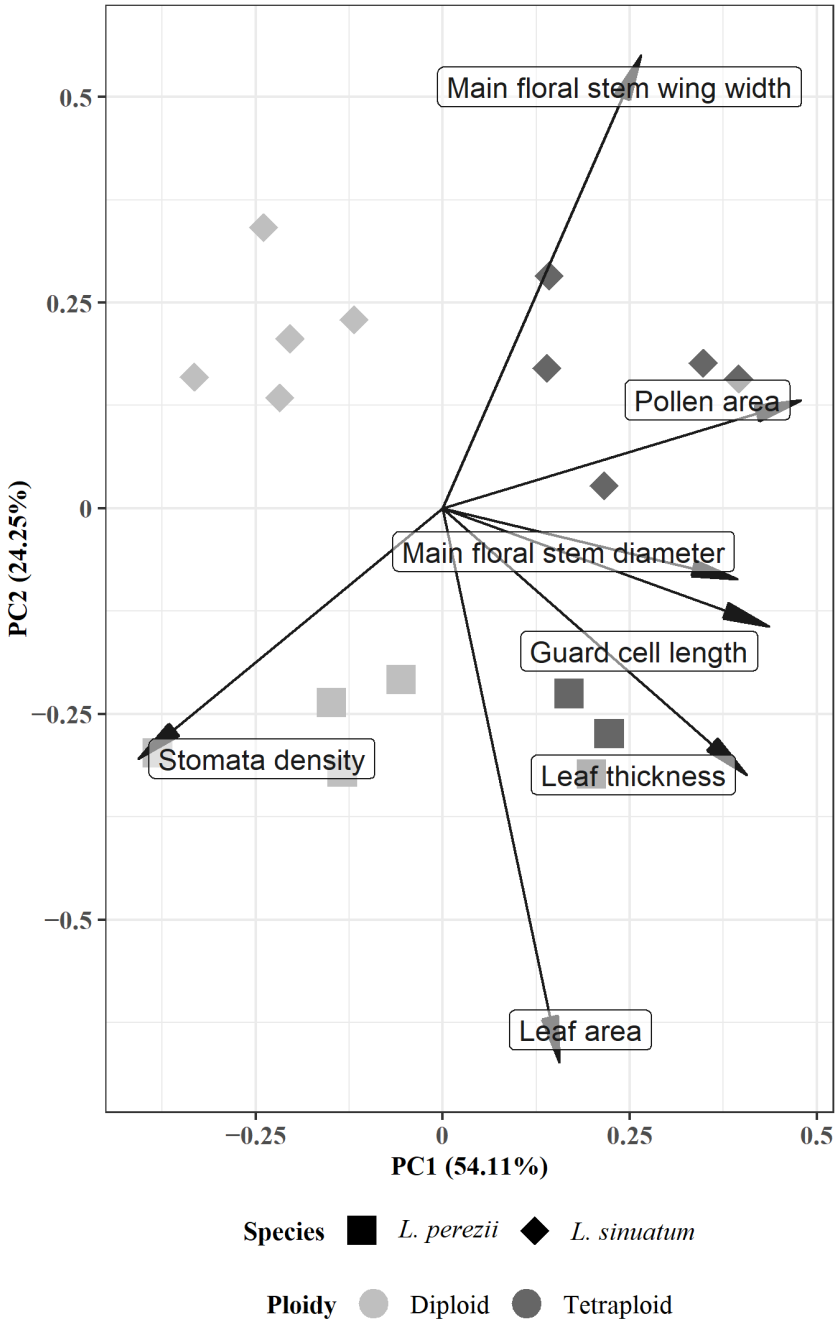

Figure 3. Principal Component Analysis (PCA) of the morphological response variables evaluated (pollen area, guard cell length, stomatal density, leaf thickness and area, main floral stem diameter, and main floral stem wing width) in diploid and tetraploid *Limonium sinuatum* genotypes. The arrows represent the eigenvectors and point in the direction of increasing magnitude of the measurement. The dimensions are described as PC1 (Dimension 1) and PC2 (Dimension 2).

4. Discussion

N_2O treatments were shown to induce WGD in two *Limonium* species when applied between 1 and 3 DAP. If applied later, however, e.g., at 4-6 DAP, the progeny could be mixoploid or diploid (Figures 1 and 2). These effects of timing agreed with those reported for rye (*Secale cereale*; [12]). Thus, when N_2O affects the mitotic spindle later in zygote development, only mixoploid or diploid progeny are obtained (Figures 1 and 2). We interpret this as potentially being due to cell divisions in multi-celled zygotes not being synchronous (reviewed by [47]). As only dividing cells can be affected by the treatment, the result is a mix of diploid and tetraploid cells within one zygote.

While a genotype-specific effect of the induction of WGD has been reported in rye [12] and maize [14], in the current experiment no apparent genotypic specificity was evident, although the number of parent plants were small (two genotypes of each species). Both *L. sinuatum* and *L. perezii* produced between one and five tetraploids when their zygotes were treated with N_2O between 1 DAP and 3 DAP (Figures 1 and 2).

In the current study, the total proportion of tetraploid progeny observed in the *L. perezii* using N_2O (38%) was similar to that reported for wheat (>30%; [17]); *L. sinuatum* with only one tetraploid progeny was excluded from this comparison (1% tetraploidization). Nevertheless, when compared to recent studies utilizing oryzalin to induce tetraploidization in *L. sinuatum*, our study revealed a doubling of the tetraploidization rate for *L. sinuatum* and a 37% increase for *L. perezii*, as compared to the tetraploidization of *L. sinuatum* using oryzalin [13]. Additionally, unlike previous findings where physiological abnormalities were observed in tetraploid *L. sinuatum* plants induced by oryzalin treatment [13], or in plantlets from unpublished studies conducted by our team, no such issues were noted in the tetraploid plants induced with N_2O. While improving the frequency of polyploidization remains to be achieved, especially with the genotypes of *L. sinuatum*, these results highlight the effectiveness of N_2O treatment for inducing whole-genome duplication (WGD) in *Limonium* when applied between 1 DAP and 4 DAP.

In the present study, the induction of WGD increased the germplasm diversity, as suggested in [5], through the production of plants with different ploidies, i.e., tetraploid *L. sinuatum* and *L. perezii* plants. In addition, new morphological features represented by the organ size increased in *L. sinuatum* and *L. perezii* between 1.2 and 1.6 times in the tetraploid plants in comparison with their diploid counterparts (Figure 3). The increased organ size noted in this research corroborates the "gene dosage effect" [9] and findings reported in other *Limonium* species, such as *L. bellidifolium* [11] and *L. sinuatum* [13], and in other crops, such as *Phlox amabilis* [6], *Gerbera hybrida* [8], and *Brassica* [7]. In the case of pollen size, the increase in size with increased ploidy seen here in *L. sinuatum* agrees with previous reports on *L. sinuatum* [20] but contrasts with *L. ovalifolium* and *L. multiflorum*, which did not display the direct ploidy–pollen size relation observed in our study [10].

The consistency of the morphological changes in leaf thickness, main floral stem diameter, guard cell length, and stomatal density observed in polyploid plants of both *L. sinuatum* and *L. perezii* provides a simple screening protocol to minimize the number of plants that require testing using flow cytometry to confirm ploidy status. Although pollen size, leaf thickness, main floral stem diameter, guard cell length, and stomatal density showed a direct association with ploidy in both *L. sinuatum* and *L. perezii*, because of the ease of measurement, measuring pollen size and guard cell length has the potential to be used for pre-selection of polyploid plants in *L. sinuatum* and *L. perezii*, hence reducing the resource costs within a breeding program.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae10080816/s1, Supplementary Table S1: Number of crosses done, embryos/ovules rescued and seedlings obtain in *Limonium sinuatum* or *Limonium perezii* plants treated with nitrous oxide (N_2O) or untreated (control).; Supplementary Data S2: Flow cytometry results for *Limonium perezii*; Supplementary Table S3: Percentage of diploid, tetraploid and mixoploid seedlings obtained in two seed-bearing genotypes of *Limonium perezii* treated with

nitrous oxide (N_2O) at different day after pollination (DAP) or untreated (control). n = 10 crosses; Supplementary Data S4: Flow cytometry results for *Limonium sinuatum*; Supplementary Table S5: Percentage of diploid, tetraploid and mixoploid seedlings obtained in two seed-bearing genotypes of *Limonium sinuatum* treated with nitrous oxide (N_2O) at different day after pollination (DAP) or untreated (control). n = 10 crosses; Supplementary Figure S6: Morphological comparison between diploid and tetraploid plants of *Limonium perezii*. (A) Whole plants, scale bar = 10 cm (B) Guard cells, scale bar = 100 µm (C) pollen grains, scale bar = 200 µm; Supplementary Figure S7: Morphological comparison between diploid and tetraploid plants of *Limonium sinuatum*. (A) Whole plants, scale bar = 10 cm (B) Main floral stems, scale bar = 10 cm (C) Detailed view main floral stem wings from a diploid plant, scale bar = 2 cm (D) Detailed view main floral stem wings from a tetraploid plant, scale bar = 2 cm.

Author Contributions: J.C.-S. conceived, designed, and implemented the experiments with review input from K.F., E.M. and N.R. In coordination with D.H. and J.C.-S. analyzed and interpreted the data. J.C.-S. wrote the manuscript with review input from K.F., N.R. and E.M. All authors have read and agreed to the published version of the manuscript.

Funding: This manuscript forms a portion of a thesis submitted by J.C.-S. to fulfil a Ph.D. requirement. J.C.-S.'s doctoral studies were supported by a New Zealand Development Scholarship from the New Zealand Ministry of Foreign Affairs and Trade (PLC-0000003678). This work was supported by The New Zealand Institute for Plant and Food Research Limited SSIF funding 1972—"Breeding Technology Development".

Data Availability Statement: The data presented in this study are available on request from the corresponding author due to legal restrictions.

Acknowledgments: This work forms a portion of a thesis submitted by J.C.-S. to fulfil a PhD requirement. We thank Andrew Mullan, Belinda Diepenheim, and Steven Ray for technical assistance, Sylvia Erasmuson for the flow cytometry analysis, and Philippa Barrell for reviewing the manuscript.

Conflicts of Interest: Authors Keith Funnell, Duncan Hedderley and Ed Morgan were employed by the New Zealand Institute for Plant and Food Research Limited. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflicts of interest.

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