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PLANT GERMPLASM DIVERSIFICATION STRATEGIES-A CASE STUDY USING *LIMONIUM*

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Plant Science

at Massey University, Palmerston North, New Zealand.

Juana Córdoba-Sánchez

2024

Abstract

Limonium Mill. was chosen for this research as it has an established market for ornamental use and faces significant challenges in breeding including access to germplasm with traits of interest, self-incompatibility in most species, and pollen/stigma dimorphism which limits the types of cross combinations that can be performed. In interspecific crosses, challenges can be the low hybridisation rate (<1%), and the sterility/low fertility of interspecific hybrids. To address these challenges, increase the germplasm diversity available, and speed the development of cultivars, two breeding strategies were evaluated in this research: ploidy manipulation and physically induced mutations.

The main outcomes were:

1. Efficacy of those strategies in:

- Increasing the ploidy levels of the selected genotypes and therefore the germplasm diversity for The New Zealand Institute for Plant & Food Research Ltd (PFR)'s germplasm bank.
- Increasing the hybridisation rate in interspecific crosses.
- Fertility restoration in interspecific hybrids

2. Development of plants with new characteristics or different ploidy levels (i.e., triploids, tetraploids, or even higher ploidy levels).

3. Breeding protocols for *Limonium* using N_2O which could be further used in other plant breeding programmes.

The use of *Limonium* for the development of a new breeding strategies for increasing genetic diversity has a direct impact on other breeding programmes i.e., food crops. In addition, the *Limonium* industry could have access to continued novelty, ensuring industry access to new cultivars would benefit New Zealand's economy mainly through royalties.

Acknowledgements

"Cultivate the habit of being grateful for every good thing that comes to you, and to give thanks continuously. And because all things have contributed to your advancement, you should include all things in your gratitude."

-Ralph Waldo Emerson

I have only words of gratitude to my supervisors Dr Keith Funnell, Ed Morgan, and Prof. Nick Roskruge for giving me the opportunity to do this PhD and for guiding me all the way through it. Keith, thank you for all your patience and friendship; the endless hours of reading drafts of manuscripts and teaching me to improve my writing and my speaking, to organise my thinking and to be a more critical scientist. Thank you, Ed, for giving me the freedom to conduct and lead my research, and for always being there when I needed you. Our different perspectives broaden my thinking and challenge me to look beyond for answers and explanations. Thanks for teaching me to be in a helicopter when first approaching a research problem in a global context before finding a place to land. To Nick, thank you for your guidance and advice, not only in the academia, but also in everyday life in New Zealand, and for connecting my family and me to the Latino community.

To Steven Ray for maintaining the plants and operating the nitrous oxide chamber, without your help this research would not have been possible.

To Duncan Hedderley for your help and guidance with the statistical analysis. I really enjoy our conversations and the process of data analysis as I learn applied statistical analysis.

To Andrew Mullan and Belinda Diepenheim for their help in the plant tissue culture carried out in this research.

To Cara Norling, Andrew Wotton, and Ryohei Kaji for their technical support and assistance.

To Sylvia Erasmuson and Beatrice Fulton for the flow cytometry analysis carried out in this research and for their advice on data interpretation.

To Jan Grant for her patiently teaching me the art of chromosome observation and counting.

To Ross Bicknell and Philippa Barrell for the advice on some of the experiments conducted in this research and feedback on the published work.

To John Monro and Mishra for all their support.

Financial support from a New Zealand Development Scholarship from the New Zealand Ministry of Foreign Affairs and Trade and The New Zealand Institute for Plant and Food Research Limited SSIF funding: 1972 – "Breeding Technology Development".

To all my friends in New Zealand and Plant and Food Research who have made this journey more enjoyable and the burdens of being far from home lighter. Thank you for your friendship!

To my husband Fredy and my children Ana and Juan who embarked with me on the adventure of living in a country "down under" as Aotearoa with a different culture, language, and opportunities. After almost 5 years in New Zealand, we have not only obtained two PhDs (my husband's and mine), but also an important and rewarding life experience full of friends, traditions, and a different way of life.

Thanks to my parents Margarita and Jorge and my brother Jorge for their love and support in our family's decision to live abroad for a while.

Disclosure

JCS conceptualized, designed, and executed the experiments with guidance from Keith Funnell[1], Nick Roskruge[2], and Ed Morgan[1]. Data analysis was conducted by JCS with assistance of Duncan Hedderley[1]. Flow cytometry analyses were carried out by Sylvia Erasmuson[3] and Beatrice Fulton[3], with data interpretation performed by JCS. Alice Boyd[1,2], under JCS's direction, developed a method for germinating pollen from *Limonium perezii*, *Limonium sinuatum*, and their interspecific hybrids. Andrew Mullan[1] and Belinda Diepenheim[1] prepared the plant tissue culture media, while Steven Ray[1] maintained the plants in the greenhouse.

[1] The New Zealand Institute for Plant and Food Research Limited, Palmerston North, New Zealand.

[2] Massey University, School of Agriculture and Environment, Palmerston North, New Zealand.

[3] The New Zealand Institute for Plant and Food Research Limited, Lincoln, New Zealand.

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Publications and presentations

Published publications

Boyd, A., **Cordoba Sanchez, J.**, Funnell, K., & Morgan, E. (2022). Development of a method to germinate pollen of *Limonium sinuatum*, *L. perezii* and their interspecific hybrids. Internal Report. The New Zealand Institute for Plant & Food Research Ltd.

Submitted publications

Córdoba J., Roskruge N., Funnell K, Hedderley D, Morgan E. Nitrous oxide treatment after pollination induces ploidy changes in statice (*Limonium* sp.). 2023. Rev. Bras. Bot. (Under review).

Córdoba J., Roskruge N., Funnell K, Hedderley D, Morgan E. Nitrous oxide treatment increases the proportion of viable pollen and pollen size in *Limonium perezii*. 2023. Acta Hortic. (Accepted).

Córdoba J., Roskruge N., Funnell K, Hedderley D, Morgan E. Induction of unreduced gametes in *Limonium* using nitrous oxide (N_2O) and production of sexual polyploids. 2023 (under internal review by Plant and Food Research colleagues).

Oral presentations

Córdoba J., Roskruge N., Funnell K, Hedderley D, Morgan E. Uso del óxido nitroso (N_2O) para la inducción de cambios en la ploidía de un cultivo modelo. XIII Simposio Internacional de Recursos Genéticos para las Américas y el Caribe. Colombia. 30th November-3rd December 2021.

Córdoba J., Roskruge N., Funnell K, Hedderley D, Morgan E. Nitrous oxide (N_2O) and ploidy changes in a model crop. Plant Science Central Conference. Palmerston North, New Zealand. 6th – 8th July 2021.

Córdoba J., Roskruge N., Funnell K, Hedderley D, Morgan E. Plant Germplasm Diversification Strategies- a case study using *Limonium*. Plant & Food Research: Lincoln Seminar Series. Lincoln, New Zealand. 14th April 2021.

Córdoba J., Roskruge N., Funnell K, Hedderley D, Morgan E. Nitrous oxide treatment increases the proportion of viable pollen and pollen size in *Limonium perezii*. XXVII International EUCARPIA Symposium Section Ornamentals-From Nature to Culture: Breeding Ornamentals for Sustainability. Genoa-Italy. 2nd-5th July 2023.

Abbreviations

2,4-D 2,4-dichlorophenoxyacetic acid

BAP Benzyl Amino Purine

C.A.N. Calcium Ammonium Nitrate

COB Cob stigma

DAP Days After Pollination

DBP Days Before Pollination

DMS Dimethylsulfate

EDA Exploratory Data Analysis

EMS Ethyl methane sulfonate

GA_3 Gibberellic acid

GLM Generalized Linear Model

Gy gray (refer to Section Definitions)

IAA Indole-3-Acetic Acid

IBA Indole-3-Butyric Acid

NAA 1- Naphthaleneacetic acid

N_2O Nitrous oxide

MMC Megaspore Mother Cells

PAP Papillate stigma

PGR Plant Growth Regulator

PMC Pollen Mother Cell

PMI Pollen Mitosis I

PMII Pollen Mitosis II

PFR The New Zealand Institute for Plant & Food Research Ltd

PGR Plant Growth Regulator

PPFD Photosynthetic Photon Flux Density

PTC Plant Tissue Culture

ROC curve Receiver Operating Characteristic Curve

TDZ Thidiazuron

UPOV Union for the Protection of New Varieties

Definitions

Apomictic seed: seed developed without male fertilization. Germination of the seed results in a maternal clone.

Apomixis: asexual seed formation.

Centroid: The centroid of a group of points is equivalent to the average position of all the points, calculated by adding up the coordinates of all points and dividing the result by the total number of points.

Complete penetrance: all the individuals (e.g., plants) which have a mutation manifest it.

Corymbose: It is a type of inflorescence characterised by a flattened top.

Cross success: Number of seedlings obtained divided by number of crosses completed.

Cultivar: refers to a cultivated plant variety produced by selective breeding.

E-Brida: E-Brida is a software designed to centralise breeding information and processes, making them easily accessible.

Embryo sac: It is an egg-producing structure also known as female gametophyte. It contains four types of cells: synergids, antipodals, egg cell, and central cell.

Endosperm: It is a triploid tissue situated within the seed, enveloping the embryo of angiosperms. This tissue emerges following the fertilization of the central cell.

Endosperm balance number: it is a theory which states that for embryo development, the maternal to paternal genome ratio needs to be 2 :1 (Ortiz & Ehlenfeldt, 1992).

Fertility restoration rate: Number of fertile polyploids divided by the number of polyploids produced.

Germination rate: number of zygotes germinated *in vitro* divided by number of zygotes obtained (rescued).

Gray (Gy): It refers to the amount of energy absorbed per unit mass of tissue and is the unit of radiation dose in the new international system.

Heteroploidy: *Hetero* means different, and ploidy is the number of sets of chromosomes in a cell. Heteroploidy then refers to an organism that has a different number of sets of chromosomes to another organism to which it is compared.

Heterosis: phenomena observe in progeny which display phenotypic traits that exceed those observed in their parent e.g., higher vigour, greater biomass, etc (Birchler et al., 2010)**.**

Heterozygosity: refer to having different alleles for the same locus (National Human Genome Research Institute, 2023).

Homeologous: duplicated loci after a polyploidization event (Bomblies & Weigel, 2007). Chromosome copies which display partial homology (Bomblies, 2022)

Homologous: chromosome copies with complete homology (Bomblies, 2022).

Homologous chromosome: *Homo* means "same". Chromosomes derived from the same species which are "identical" and during meiosis pair promptly (Glover et al., 2016).

Homeologous chromosome: *Homeo* means "similar". Chromosomes derived from the different species which were probably homologous in the past, but now present differences in shape and genes location mainly (Glover et al., 2016). In the present research, in interspecific hybrids the chromosomes from *L. sinuatum* are homeologous to the genes from *L. perezii.*

Homoploidy: *Homo* means equal, and ploidy is the number of sets of chromosomes in a cell. Homoploidy then refers to an organism whose number of sets of chromosomes is equal to another organism to which it is compared.

Hybridisation rate: number of hybrids obtained by number of crosses completed.

Micro-pollen: pollen grains with plan area 0.5 times the average size for the species.

Mixoploid: It refers to plants with tissues with different ploidy levels.

Oblanceolate: It's a shape that's much longer than it is wide, with the wider part towards the tip**.**

Pollen viable: pollen grains stained purple with modified Alexander's stain (Peterson et al., 2010).

Pollen aborted: pollen grains stained blue-green or unstained (i.e., brown coloured) with modified Alexander's stain but presenting the exine pattern characteristics of the species.

Polyad: in biological terms, polyad is a group of closely related elements. In the present research it refers to the cells which will become either microspores or megaspores (refer to Section 1.3.1.2). The type of polyad is defined by the number of constituent cells (e.g., dyad (2), tetrad (4)).

Polyploidization rate: Total number of polyploid seedlings obtained in the experiment divided by the total number of pollinations performed.

Proportion of polyploids: number of polyploid plants obtained divided by number of seedlings obtained (excluding mixoploids) or adventitious shoots for plants obtained *in vitro*.

Proportion of mixoploids: number of mixoploids obtained divided by number of seedlings obtained or adventitious shoots for plants obtained *in vitro*.

Seedling: Plant grown from a true seed. Typically, these seeds would result from a sexual event (i.e., pollination followed by fertilization). Seeds may also form without involving a sexual event (i.e., apomictic seeds).

Sensitivity: It refers to the probability that a positive test result will be observed in a positive organism.

Specificity: It refers to the probability that a negative test result will be observed in a negative organism.

Triploid block: It is the reproductive barrier that prevents the development of an embryo due to an abnormal development of the endosperm.

Unreduced pollen: pollen grains with plan area at least 1.2 times the average size for the species and twice the nuclear DNA content of normal pollen.

Zygote: It is a cell formed when a sperm cell fertilises an egg cell.

Zygote success: number of zygotes obtained between 15 and 30 days after pollination divided by the number of crosses completed.

CHAPTER 1. General introduction and literature review

1.1. Introduction

Limonium Mill., commonly known as “sea lavender” or statice, includes herbaceous perennial plants with economic value as ornamentals being recognized for their small, colourful, and elegant flowers with papery appearance when dry. The flowers commercialized worldwide as *Limonium* represent different species e.g., *L. sinuatum*, *L. perezii*, *L. sinensis, L. altaica* (BallSB, 2022, 2023; 2019) and are mostly used as fillers.

Limonium hybrids also have value in the international market for their exceptional characteristic combination (BallSB, 2019, 2021; Burchi et al., 2006; Morgan et al., 1998). Interspecific hybridisation is an important creator of germplasm diversity and production of new cultivars (Van Tuyl & Lim, 2003), but is limited by the genetic distance between species and cross-compatibility (Kuligowska et al., 2016) with consequences on hybrids such as inviability, sterility or weakness (Chen et al., 2016; Kuligowska et al., 2016).

Nevertheless, the ornamentals market is always in need of new cultivars with outstanding features focusing mainly on external appearance e.g., flower colour, shape and size; and plant architecture, but also on agronomic performance e.g., tolerance to biotic and abiotic factors (Kuligowska et al., 2016; Teixeira da Silva et al., 2013). Therefore, breeding tools alternative or complementary to hybridisation are being used for creating diversity and novelty in plants and overcoming the hybridisation challenges (Barba-Gonzalez et al., 2005; Datta, 2012; Jansky, 2006).

1.2. *Limonium* sp.

1.2.1. Distribution

The genus *Limonium* Mill. is classified in the Plumbaginaceae family and the tribe Staticeae (Baker, 1948b). It is distributed across five continents and its centre of origin is in the Mediterranean region with at least 70% of the species being endemic (Koutroumpa et al., 2018). New species have been reported in the recent years (Doğan et al., 2020; Mucina & Hammer, 2019) raising the total number of named species from 150-300

(Baker, 1953b; Flora of North America Editorial Committee, 2005) to ca. 600 (Koutroumpa et al., 2018).

1.2.2. Botanical description

Limonium presents mainly two life forms dwarf shrubs or perennial herbs (Doğan et al., 2020; Flora of North America Editorial Committee, 2005) which are predominantly associated to saline habitats of marine coasts or salt lakes (Doğan et al., 2020; Rois et al., 2016).

L. sinuatum and *L. perezii* are two species forming the basis of the research undertaken in this thesis and, therefore, are the focus of this botanical description. *Limonium* plants develop from a woody rhizome which produce roots and shoots from each node (Flora of North America Editorial Committee, 2005). The leaves are located at the base of the plant and distributed as rosettes, being commonly sessile in *L. sinuatum* and petiolate in *L. perezii* (Flora of North America Editorial Committee (2005); Figure 1-1). The leaf blade as well as the base can display a range of forms from elliptic to obovate, with margins entire or toothed (Flora of North America Editorial Committee (2005); Figure 1-1).

Figure 1-1 A. *Limonium sinuatum* plant showing more than 20 inflorescences, attached to winged stems and petiolate leaves with oblanceolate blade and lobate margin. **B.** *Limonium perezii* plant showing 1 inflorescence, on a wingless stem and leaves with petiole longer than 5 cm, ovate blade, and lobate margin. Photos taken by Cordoba-Sanchez, J.

The corymbose inflorescence arises from the plant's basal rosette. The inflorescence stem may be winged, hispid and branched (Flora of North America Editorial Committee (2005); Figure 1-1). Each individual plant can produce varying number of inflorescence stems depending on the species (UPOV, 2019); for example, in the germplasm collection available at PFR in Palmerston North-New Zealand it was observed that *L. sinuatum* produces more than seven and *L. perezii* less than five inflorescences (Figure 1-1). Each inflorescence is supported by a main peduncle or floral stem and is composed of racemes which comprise spikes that contain spikelets (Figure 1-3). The spikelets comprise a varying number of florets 4 to 10 mm long characterized by the papery appearance of the calyces (Figure 1-3; Figure 1-2). The florets are composed of five petals (corolla), five sepals (calyx), five stamens and five segregated styles attached to a unilocular ovary containing a single ovule (Figure 1-2).

Figure 1-3 **A.** *Limonium sinuatum* inflorescences showing the main constituent parts. **B.** Detailed raceme. Photos taken by Cordoba-Sanchez, J.

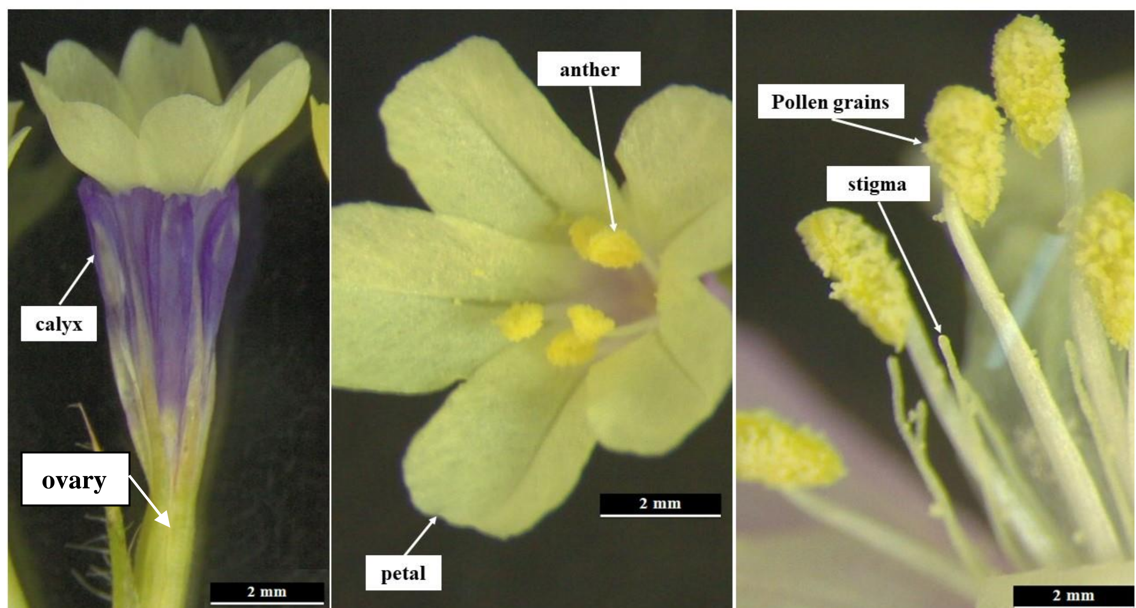

Figure 1-2 Mature recently open *Limonium sinuatum* florets with dehiscent anthers showing some of their constituent parts. Photos taken by Cordoba-Sanchez, J.

1.2.3. Reproductive biology

Eighty five percent of the species from this genus present pollen/stigma dimorphism, displaying two flower types (Baker, 1948b); the first flower type presents a cob stigma (COB) (Figure 1-4C) and pollen type A (A) (Figure 1-4A) while the other displays a papillate stigma (PAP) (Figure 1-4D) and pollen type B (B) (Figure 1-4B) (Baker, 1948b). Dimorphic species are self-incompatible, but cross-compatible, meaning that flowers that are PAP can only be pollinated by pollen A, arising from a flower that has a COB stigma (Zhang, 1995). Morphological distinctions between COB and PAP stigmas primarily arise from differences in the wall structure at the apical region of the papilla (Dulberger, 1975). Specifically, in PAP stigmas, the papillae exhibit minute protuberances, whereas COB stigma displays a smooth surface (Dulberger, 1975; Erdtman & Dunbar, 1966). Additionally, Pollen B features a coarsely reticulate pattern, while Pollen A displays a less coarse reticulation (Erdtman & Dunbar, 1966). For a more in-depth understanding of both pollen and stigma types, refer to Erdtman and Dunbar (1966).

Both *Limonium* species used in this research (*L. sinuatum* and *L. perezii*) present pollen/stigma dimorphism and are self-incompatible.

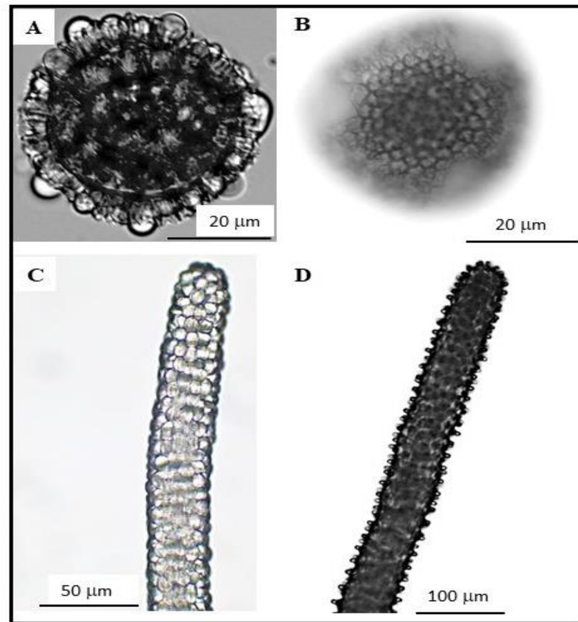

Figure 1-4 Pollen types and stigmata found in the genus *Limonium*. **A**. Pollen Type A exhibits a finer reticulation, distinguishing it from Pollen Type B **B**. Pollen Type B features a coarsely reticulate pattern. C**.** COB stigma displays a smooth surface. **D.** Papillate stigma displays small protruding papillae. Photos taken by Cordoba-Sanchez, J.

1.2.4. Limonium in the ornamental market

Limonium is one of the 20 most cultivated genera in the flower industry worldwide, represented by ca. 29 million Euros at the wholesale level in 2022 (International Association of Horticultural Producers, 2022). Novelty is a key part of the ornamental industry and the production of plants with new commercial phenotypic traits such as colour palette changes, stronger and longer stems, etc is always demanded.

Interspecific hybrids obtained by The New Zealand Institute for Plant & Food Research Ltd (PFR), and marketed as the 'siNZii™' series by BallSB (BallSB, 2021) combine the phenotypic traits of *L. sinuatum* and *L. perezii*, displaying the sharp and strong colours of *L. sinuatum* and the unwinged or conspicuously winged stems of *L. perezii* (BallSB, 2021). Another interspecific hybrid previously produced and commercialized by PFR is "Chorus Magenta" which is tolerant to pests and diseases (Astra Fund, 2023; Morgan et al., 1995).

Despite the advantages of hybrids, they have not replaced the traditional species *L. sinuatum*, *L. perezii*, *L. sinensis, L. altaica* in the ornamental market. There is still a demand for novel phenotypic traits such as different colours and sizes (BallSB, 2023; Plazoleta, 2023).

1.2.5. Breeding in Limonium

As explored in the following Section, breeding strategies that have been applied to *Limonium* include: interspecific hybridisation, ploidy manipulation and chromosomal mutation. These strategies are reviewed so as to identify areas requiring additional investigation which could be undertaken in the current research.

1.2.5.1 Interspecific hybridisation

Within *Limonium*, the breeding strategy of interspecific hybridisation has been successful in obtaining new genetic material for the *Limonium* breeding programme at The New Zealand Institute for Plant and Food Research Limited. For instance, interspecific crosses between *L. perigrinum* and *L. purpuratum* gave rise to the cultivar "Chorus Magenta" (Morgan et al., 1995), while hybridisation between *L. perezii* and *L. sinuatum* gave rise to hybrids that form the basis of a series of cultivars known commercially as 'siNZii™' (BallSB, 2021; Morgan et al., 1998).

Interspecific hybridisation of *Limonium* in New Zealand is a sustainable breeding strategy however, it is limited by some difficulties:

> First, the number of new species that can be imported into New Zealand is restricted to those listed on the New Zealand Plant Biosecurity Index (~ 23; New Zealand Ministry of Primary Industries (2019)). Therefore, this strategy, if conducted in New Zealand, is simply limited by the species available in New Zealand.
>
> Second, the further use of available plant material in interspecific crosses is limited due to pre- and post-zygotic barriers which prevent hybrid formation or development (Chen et al., 2016). The most common pre-zygotic barriers are: geographic isolation, self-incompatibility (Godoy et al., 2018), different flowering times (Baker, 1966), and failure of the pollen-pistil interaction (Jansky,

2006; Zhang, 1995). In contrast, the main post-zygotic mechanisms to prevent the zygote´s development are embryo abortion, infertility, sterility, or albinism (Morgan et al., 2011; Tonosaki et al., 2016; Tuyl & Lim, 2003).

Limonium breeders have implemented strategies to overcome the above listed limitations. For instance, controlled crosses have been complemented with ovule/embryo culture to allow the hybridisation between different species e.g., between *L. perigrinum* × *L. purpuratum* (Seelye et al., 2000) and between *L. perezii* × *L. sinuatum* (Morgan et al., 1998). However, even if the resulting interspecific hybrids develop to become flowering plants, they are sterile or present low fertility. For example, *L. sinuatum* crossed with *L. perezii* resulted in 12% of pollinated flowers containing fertilized ovules and, out of them, less than 5% developed into sterile hybrid plants (Morgan et al., 1998). To restore a hybrid plant's fertility, chromosome duplication methods like treatment with colchicine or oryzalin of the whole plant, shoot, seed or gamete have been successfully used (Jansky, 2006; Morgan et al., 2001; Morgan et al., 1998; Mori et al., 2016; Younis et al., 2014).

As noted above, ovule/embryo culture is useful for obtaining some interspecific hybrids that would otherwise be nonviable, while ploidy manipulation with oryzalin potentially restores the fertility of the hybrid by doubling the number of chromosome sets (Morgan et al. (2001); refer Section 1.2.5.2). However, additional problems exist with interspecific crosses, such as a low interspecific hybridisation rate (<1%; Morgan et al. (1998)), limiting the amount of diversification that can be created from available germplasm. Identifying techniques to overcome this problem is a goal of this research. Therefore, to achieve the required diversification of germplasm, breeding strategies to increase the interspecific hybridisation rate and develop fertile interspecific hybrids requires further exploration and evaluation in *Limonium*. This is discussed in the following sections (refer Sections 1.2.5.2 and 1.2.5.2.2) regarding ploidy manipulation and physically induced mutations.

1.2.5.2 Ploidy manipulation

Ploidy is defined as the basic number of chromosome sets of a living organism, most frequently being diploid (Dewitte et al., 2012). Mutation is defined as any change in the genetic material of an organism, independent of recombination or segregation processes, which can be potentially inherited by the progeny (Harten, 1998). In this regard, the

change in the number of chromosome sets of an organism e.g., to produce plants with ploidy levels higher than diploid (i.e., induction of polyploidy) is considered as a genome mutation (Bado et al., 2015).

Mutation induction therefore refers to the group of techniques used to produce genetic changes, simulating the spontaneous mutagenic process (Bado et al., 2015; Harten, 1998; IAEA, 2017; Pathirana, 2011). In the specific case of induction of polyploidy in plants, chemical treatment (mutagens) with colchicine, oryzalin or N_2O (Ari et al., 2015; Mori et al., 2016; Okazaki et al., 2005) could be used, or polyploid seedlings may arise from crossing plants with different ploidy levels (Harst-Langenbucher, 1990; Morgan et al., 2020).

Polyploid plants present advantages in terms of speciation and adaptation by means of genome "buffering", as represented by the loss or masking of deleterious genes, heterosis, heterozygosity, changes in the reproduction mode, and phenotypic and genotypic diversity (Dewitte et al., 2012; Wang et al., 2016). In addition, polyploid plants show other distinctive characteristics in comparison to diploid plants. For example, polyploids may have larger organs and slower growth rates, higher yield, altered fertility, resistance to diseases or abiotic factors (Dewitte et al., 2012; Mori et al., 2016; Róis et al., 2012). In the case of polyploids of ornamental plants, they may have larger flowers, a longer vase life as cut stems (Eason et al., 2007), and stronger colours (Manzoor et al., 2018; Zeng et al., 2020). These characteristics highlight the potential value of the ploidy manipulation strategy in *Limonium*. The possibility exist to restore hybrid fertility, increase the rate of interspecific hybridisation (refer Section 1.2.5.1) and provide new options for increasing genetic diversity, in addition to modifying morphological characteristics of commercial importance.

As reviewed in this Section, ploidy manipulation in *Limonium* has previously been explored using the application of oryzalin (Morgan et al., 2001), colchicine (Mori et al., 2016) and, more recently, N_2O (Siregar, 2021). Some results include production of tetraploids with restored fertility (i.e., 82% pollen viability compared with 1%) after *in vitro* treatment of shoots of a *L. perezii* × *L. sinuatum* hybrid with oryzalin, facilitating its use in backcrosses (Morgan et al., 2001). In addition, colchicine has been evaluated with seeds of *L. bellidifolium*, where less than 5% of the plants regenerated were

tetraploids (Mori et al., 2016). In the case of N_2O, initial evaluation of its use in *L. sinuatum* established estimates of the best time for it to be applied before pollination to induce the production of unreduced gametes (Siregar, 2021). With regards to the traits displayed by the polyploid *Limonium* plants, a larger genome has not always meant bigger pollen grains (Róis et al., 2012), but it does generally result in thicker and darker-green leaves, bigger florets and stomata, and reduced stomatal density associated with the "gene dosage effect[4]" (Mori et al., 2016).

To date therefore, ploidy manipulation in *Limonium* has illustrated the potential of inducing polyploids for increasing germplasm diversity and restoring hybrid fertility. However, in *Limonium* only an initial exploration has been conducted regarding ploidy manipulation before pollination (i.e., induction of unreduced gametes), and whether this strategy has the potential to increase the interspecific hybridisation rate and develop fertile interspecific hybrids is still pending. Ploidy manipulation before pollination that results in unreduced gametes when used in sexual hybridisation, may increase the interspecific hybridisation rate, and facilitate the development of fertile F1 interspecific hybrids when used in sexual hybridisation (Barba-Gonzalez et al., 2004). In the case of ploidy manipulation after pollination (i.e., at the first zygote cell divisions), it also restores hybrid fertility, as observed in maize (Kato & Geiger, 2002). Ploidy manipulation before or after the pollination could create opportunities to more rapidly increase the genetic diversity represented by seedlings with new phenotypic traits or different ploidies.

1.2.5.2.1 Polyploid induction using nitrous oxide (N_2O)

Ploidy manipulation in *Limonium* has most frequently been reported as achieved using colchicine or oryzalin as the inductive agent (Morgan et al., 2001; Mori et al., 2016). Studies using plant species such as wheat (*Triticum aestivum*; Hansen et al. (1988), canary grass (*Phalaris canariensis*; Ostergren (1957), rye (*Secale cereale*; Gordej et al. (2019) and lilium (*Lilium × formolongi*; Sato et al. (2010) have reported treatment with N_2O to induce WGD at different pressures (i.e., from 608 Kpa to 1013 Kpa), durations (i.e., from

[4] "Gene dosage effect" is a hypothesis that states that the phenotype is directly related with the number of copies of a particular gene Birchler, J., & Veitia, R. (2012). Gene balance hypothesis: Connecting issues of dosage sensitivity across biological disciplines. *Proc. Natl. Acad. Sci. U.S.A., 109*(37), 14746-14753. https://www.pnas.org/content/pnas/109/37/14746.full.pdf

4 h to 72 h) and zygote/embryo developmental stages (i.e., between 5 to 13 DAP). Recently, the efficacy of N_2O as an alternative to oryzalin and colchicine for ploidy manipulation *in vivo* through the induction of unreduced gametes in *Limonium* has been evaluated (Siregar, 2021). Nevertheless, complementary studies are still required on the use of N_2O for whole genome duplication (WGD) and production of sexual polyploids by inducing unreduced gametes.

N_2O is a gas that disrupts the mitotic/meiotic spindle by depolymerising microtubules. As a result, chromosomes are retained in the centre of the cell before the formation of the cell plate and one daughter cell will have the double of chromosomes and the other will be null (Kitamura et al., 2009). This effect of N_2O on cells allows it to be used to produce tetraploid plants (Ostergren, 1957) and to restore fertility (Dewitte et al., 2010; Nukui et al., 2011). The main advantage of N_2O in comparison with other mutagens (e.g., oryzalin or colchicine) is that N_2O is capable of penetrating the plant through the epidermis and reaching the internal organs for effect; it does not remain in the plant, rapidly dissipating after the plant is taken out of the treatment chamber. As an additional advantage, N_2O does not affect the survival and growth of treated plants, as can occur with both colchicine and oryzalin (Dewitte et al., 2012; Kato & Geiger, 2002; Kitamura et al., 2009; Nukui et al., 2011). Additionally, N_2O treatment applied to intact plants can give rise to multiple genetically distinct progeny directly from the cross via seed, without the need for time and equipment for subsequent WGD.

In conclusion therefore, given that the research programme reported here has the primary goals of increasing both diversity and hybridisation rate, and develop fertile interspecific hybrids the research opportunities in *Limonium* are to assess the potential of N_2O for:

- ploidy manipulation before pollination for inducing unreduced gametes and developing fertile polyploid seedlings.
- ploidy manipulation after pollination for induction of WGD.

1.2.5.2.2 Ploidy manipulation during mitosis

Disrupting the mitotic spindle during division of somatic cells induces WGD. The disruption of the mitotic spindle could take place in prometaphase, metaphase, or anaphase causing chromosome missegregation (Figure 1-5). As consequence, the sister

chromatids will not separate between the daughter cells and the two new cells will contain either double the number of expected chromosomes (e.g., tetraploid) or no chromosomes (i.e., nullisomic cell; Walczak et al. (2010)). All chromosome sets of the cell with the doubled number of chromosomes are genetically the same which contrasts with polyploid induction during meiosis as explained below (refer to Section 1.2.5.2.3). Hence, mitotic production of polyploids is useful when pure lines obtained from haploids need to be induced as in maize (Kato & Geiger, 2002); to restore or increase fertility (Kato & Geiger, 2002; Morgan et al., 2001); and to facilitate the crossing between plants with different ploidy levels (Carputo & Barone, 2005; Dewitte et al., 2012; Kato & Geiger, 2002).

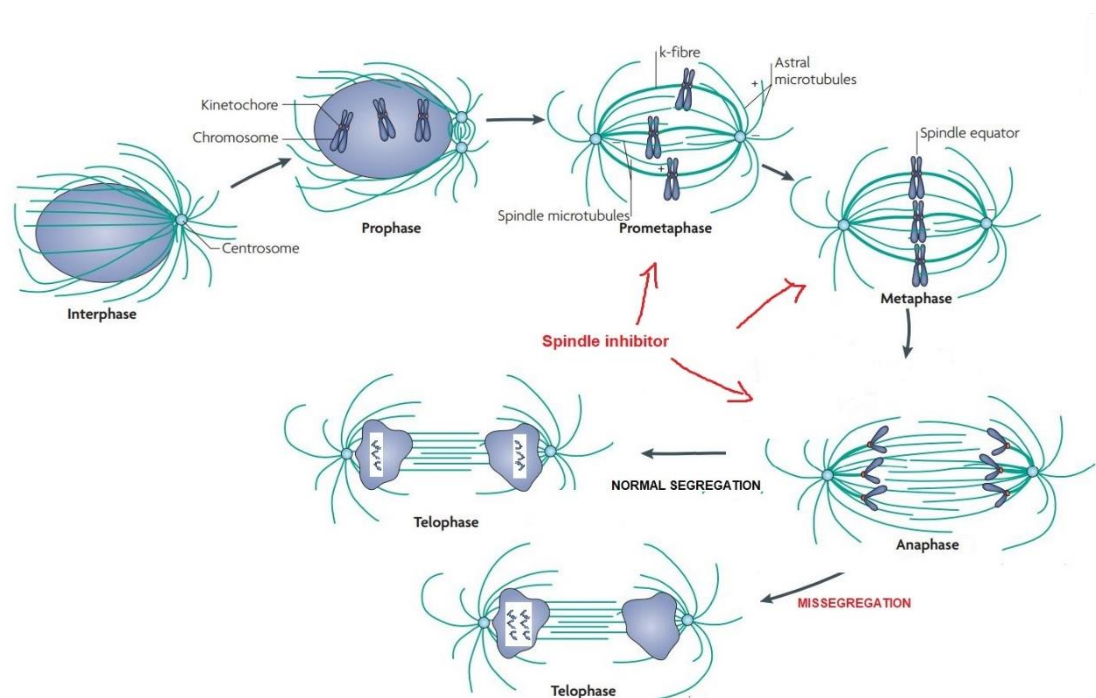

Figure 1-5 Phases of the mitosis susceptible to be affected by a spindle disruptor e.g., nitrous oxide (N_2O). The red arrows point to the mitosis phases which can be affected be a spindle disruptor. The most common consequence of the spindle disruption is the chromosome missegregation with one daughter cell having a double chromosome number while the other having zero chromosomes (nullisomic cell). Modified from Walczak et al. (2010)

1.2.5.2.3 Ploidy manipulation during meiosis

Ploidy manipulation during meiosis aims to induce unreduced gametes. The disruption could take place in meiosis I (i.e., First Division Restitution (FDR); Dewitte et al. (2012)) or during meiosis II (i.e., Second Division Restitution (SDR); Dewitte et al. (2012); Nukui et al. (2011); refer to Section 1.3.1.2). If the disruption of the meiotic spindle occurs in

prophase I, it prevents the movement of the meiotic chromosomes to the opposite ends of the cells, being retained in the cell plate or scattered within the cell. Therefore, the homologous chromosomes are not equally distributed between the two nascent cells during telophase I (Dewitte et al. (2012); Nukui et al. (2011); Figure 1-6 left). In contrast, spindle inhibition during meiosis II (SDR) allows for normal meiosis I. However, during telophase II, the sister chromatids remain together and cannot be separated to migrate to opposite poles of the cell. As consequence, the daughter cell contains half of the parental chromosomes, but each chromosome is formed by two identical sister chromatids (Figure 1-6 right; Dewitte et al. (2012); Oleszczuk et al. (2019)).

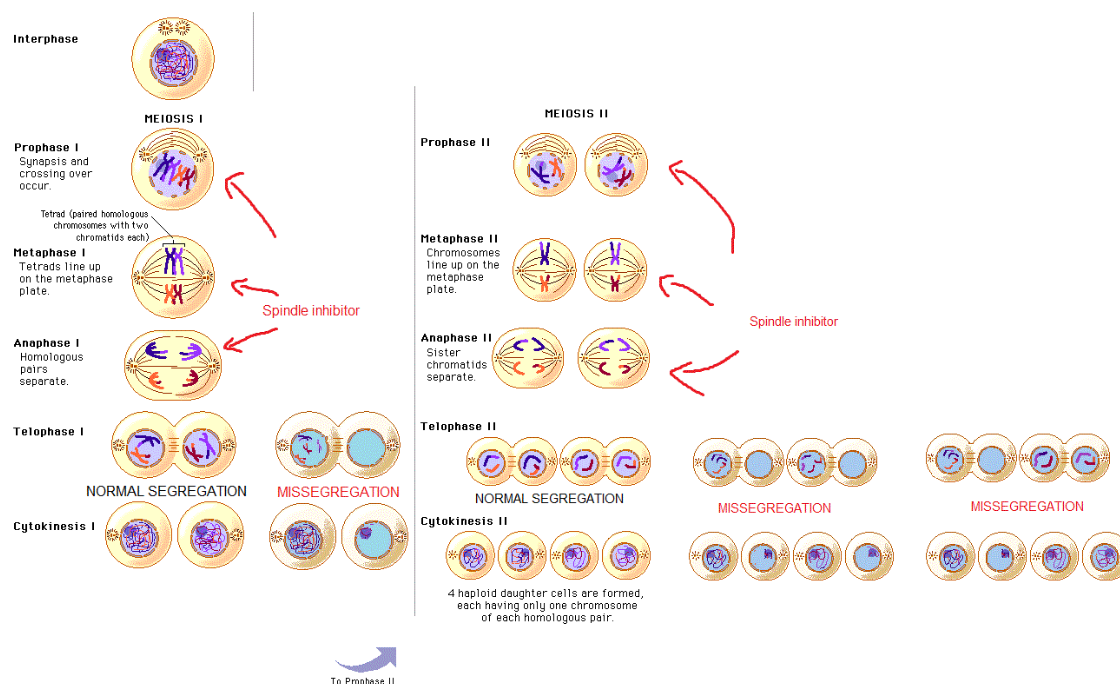

Figure 1-6 Phases of the meiosis susceptible to be affected by a meiotic spindle disruptor i.e., nitrous oxide (N_2O). The red arrows point to the meiosis phases which can be affected be a spindle disruptor. If either prophase I, metaphase I or anaphase I, is affected First Division Restitution (FDR) occurs. However, if the meiotic spindle disruptor disturbs either prophase II, metaphase II or anaphase II, Second Division Restitution (SDR) occurs. In any case, the main consequence is missegregation of chromosomes. Modified from https://celldivisionandreproduction.weebly.com/meiosis.html (date of retrieval 06/03/2024)

Ploidy manipulation during meiosis also has consequences over the number of microspores or megaspores produced which becomes evident in the type of polyads produced. For example, in interspecific Wheat-Rye hybrids, FDR results in two

unreduced cells (i.e., dyads) instead of the four expected haploid cells (i.e., tetrads; Lukaszewski (2022)); in *L. multiflorum*, SDR could produce tetrads, triads or polyads (Róis et al., 2012); and in *L. sinuatum*, the use of N_2O for induction of unreduced pollen resulted in pentads, dyads, hexads, and monads (Siregar (2021); Figure 1-7).

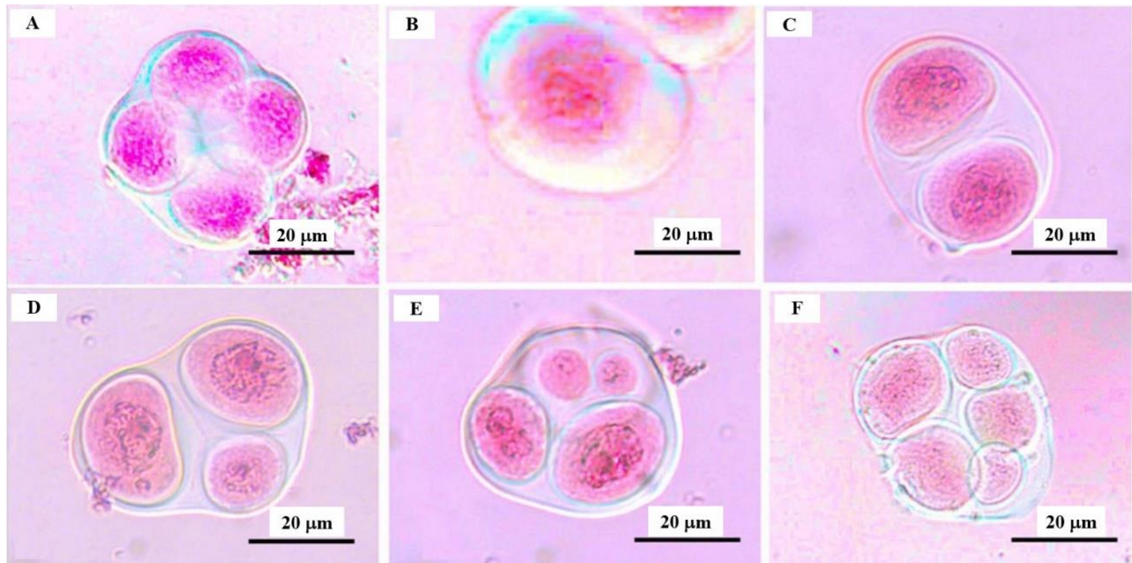

Figure 1-7 Polyads observed in *Limonium sinuatum.* The florets were fixed in Carnoy's fixative, which is a mixture of 7 parts ethanol and 3 acetic acid. The fixed florets were then stained with 2% aceto carmine (**B-F**) after treating young unopened inflorescences with nitrous oxide (N_2O) or untreated (**A**). **A.** Tetrad from a control plant. **B.** Monad. **C.** Dyad. **D.** Triad. **E.** Tetrad. **F**. Pentad. Photo modified from Siregar (2021)

1.2.5.3 Physically induced mutations – (radiation)

The techniques available to induce mutations are diverse and include physical treatments (e.g., gamma-rays or x-rays) in addition to the chemical treatments (i.e., oryzalin or N_2O) already explained for the induction of ploidy changes (refer to Section 1.2.5.2).

Physical mutagens (i.e., radiation) like gamma-rays, x-rays, or ion-beams can be used mainly to induce chromosome mutations (Mba et al., 2010; Till et al., 2003). In *Limonium*, x-rays were employed in the development of the commercial hybrid "Oceanic White" (Dai-Ichi Seed Co Ltd, 1997); gamma-rays produced six cultivars of the *Limonium* variety "Sea Pink" and the cultivars "Tall Pink Emille", "Misty Blue",

"Daifura Pink" and "Super Lady" (Nakagawa & Kato, 2017). In addition, C-ion beams were used for developing colour mutants for "Kishu Fine Lavender" and "Kishu Star" (Ogawa et al., 2014).

The production of commercial cultivars after the induction of physical mutations reveals the potential of radiation to increase the genetic diversity which is one of the objectives of the present research. However, additional investigation is still required due to the detailed experimental results published being of a contradictory nature. For example, efficacy of the irradiation for inducing colour mutations varies among genotypes with 5 Gy of C-ion beams producing colour mutation rates of 0% in "Kishu Fine Grape", 2.4% in "Kishu Fine Lavender" and 13% in "Kishu Star" and resulting in three colour mutations ('pale colour' (N84D[5]), 'deep colour (N87A[4])' and reddish colour (N81C[4]); Ogawa et al. (2014)). In another study, 20 Gy of gamma rays applied to shoots of *L. sinuatum* affected the number of leaves and the floral stem length, but did not affect the flower colour in the resulting mutant plants (Cardarelli et al., 2002). In contrast, 1.0 Gy ion-beam irradiation on *Limonium* shoots halted their development and multiplication but no mutants were reported (Chinone et al., 2007).

In summary therefore, there is evidence that new opportunities exist in *Limonium* for evaluation of radiation such as:

- Assessing the comparative efficiency of physically induced mutations (radiation) to increase the genetic diversity of *Limonium,* as compared with other strategies.
- Development of a protocol for generating physically induced mutations in *Limonium*, as none was evident within the reviewed literature.

1.2.6. Generalities about Limonium relevant to breeding

Breeding in *Limonium* can be constrained mainly by low hybridisation rate in interspecific crosses, hybrid sterility or low fertility, and limited germplasm diversity available in New Zealand. Induction of whole genome duplication (WGD) is a breeding method with potential to increase the germplasm diversity while facing the above

[5] Royal Horticultural Society, & Flower Council of Holland. (2001). *RHS colour chart* (4th ed.). Royal Horticultural Society.

mentioned hybridisation constrains by either balancing the chromosome number in hybrids or enabling the homologous chromosome pairing during meiosis as reported for *Begonia* (Dewitte et al., 2010) and *Lilium* (Nukui et al., 2011). Although reasonably simple to achieve WGD of hybrid progeny is a process that adds time to breeding programmes. As an alternative to the WGD, production of unreduced gametes could also overcome the hybrid sterility and restore hybrid fertility as well as promoting the production of polyploid plants i.e., interspecific polyploids (Dewitte et al., 2010; Nukui et al., 2011; Zeng et al., 2020).

The increase in ploidy either via WGD or production of unreduced gametes could have a direct impact on diversity as a relation between ploidy and morphology has been reported for some crops by means of the "gene dosage effect" (Birchler & Veitia, 2012; Birchler, 1993; Gordej et al., 2019). Ploidy increase also affects the reproductive performance of the infertile hybrids by restoring fertility (Luo et al., 2016; Nukui et al., 2011; Okazaki et al., 2005) and providing a source of germplasm diversity which could be introgressed into future generations or restricting the ability to be crossed by competition between reduce and unreduced pollen (Gao et al., 2019; Husband et al., 2002) or occurrence of the triploid block[6] (Akutsu et al., 2007; Morgan et al., 2021).

Another breeding strategy used in ornamental breeding to increase the genetic diversity is induction of physical mutations using e.g., gamma radiation with potential to produce colour change (Yamaguchi et al., 2008) and commercial cultivars (Nakagawa & Kato, 2017; Ogawa et al., 2014).

1.2.6.1 Environmental requirements for flowering

Flowering is affected by environmental and genetic factors (Otagaki et al., 2015). In *Limonium* particularly, flowering is associated with the daily average temperature and light integrals (i.e., photosynthetic photon flux multiply by the photoperiod; Chen et al. (2010)). Studies in siNZii™ hybrids (Chen et al., 2010) reported that daily average temperature affects the time from visible floret to floret opening (i.e., harvest maturity),

[6] The triploid block refers to a reproductive barrier which prevent the formation of seedlings due to the disruption of endosperm balance consequence of an interploidy cross Köhler, C., Mittelsten Scheid, O., & Erilova, A. (2010). The impact of the triploid block on the origin and evolution of polyploid plants. *Trends Genet., 26*(3), 142-148. https://www.sciencedirect.com/science/article/pii/S0168952509002546

but do not influence the time to first visible floret. In contrast, the daily light integrals are associated with the appearance of the first visible floret, but not with the time until floret maturity. Hence for the current research the general strategy for environmental control was to maintain the temperature in the greenhouse during the whole experiment. In this way, it was possible to limit the factors influencing flowering and to keep as constant as possible the time from first visible floret to floret maturity.

1.2.6.2 Characteristics of Limonium as a breeding model

In addition to having its own economic importance as a crop for ornamental purposes, *Limonium* was selected in the present research for undertaking scientific investigations associated with plant breeding because:

> Firstly, it is a crop with the potential to produce flowers all year around under greenhouse conditions (refer Section 1.2.6.1), and 7-8 months after germinating from seed. This is in contrast to New Zealand's more economically valuable perennial fruit crops like apple and kiwifruit, with flowering typically limited to a few weeks of the year and taking several years of growth before evaluation of breeding strategies can be completed. Hence, as undertaken in this thesis, *Limonium* offers opportunity to evaluate breeding strategies that may subsequently be transferred to these other breeding programmes.
>
> Secondly, although as a genus *Limonium* bears a high genetic diversity represented by ca. 600 species of annual plants and perennial herbs (Flora of North America Editorial Committee, 2005), that variability cannot be easily transferred in a reliable manner to any desired progeny due to reproductive barriers (refer Section 1.2.5.1), creating a range of breeding opportunities and also challenges. Hence, as undertaken in this thesis, if these challenges can be met and resolved with *Limonium*, the understanding may subsequently be transferred to other breeding programmes.
>
> Thirdly, *Limonium* is marketed internationally, primarily as a cut flower commodity, due to its long lasting, dense and abundant flowers with good shipping performance (Morgan & Funnell, 2018; Plazoleta, 2019). Additionally, the number of applications for registration of *Limonium* varieties before the Union

for the Protection of New Varieties (UPOV) increased 7% between 2005 and 2019 (UPOV, 2019), both reflecting the importance of *Limonium* in the ornamental plant market and the sustained market demand for new, genetic diversity.

1.3. Reproduction and development

1.3.1. Cell division

Cell division is the biological process that allows a cell to duplicate and produce either two identical cells with the same number of chromosomes (i.e., mitosis; Gilchrist (2023b)) or cells with half of the total number of chromosomes i.e., haploid number (i.e., meiosis; Gilchrist (2023a)). For better study and understanding of mitosis and meiosis, both have been divided in phases and sub-phases which will be briefly reviewed below.

1.3.1.1 Mitosis

Mitosis is the cell division process that results in the production of somatic cells that are genetically identical to the mother cell (Walczak et al., 2010). Even though mitosis occurs in all somatic cells, within the present PhD research, the focus was on the mitotic division that follows the fertilization of the ovule by the sperm cell, as explained below in Section 1.2.5.2.2. Irrespective of the type of cell being in mitotic division, the following are the main events within each mitotic phase (Table 1-1; Figure 1-8).

Table 1-1 Phases of the mitosis. Main events taking place in each phase (Walczak et al., 2010)

Meiosis phase	**Main event**
Interphase	• Microtubules assembly into the mitotic spindle begins at the centromeres. • Microtubules have a radial distribution from the centrosome.
Prophase	• Centrosomes separate from each other. • Nuclear envelope disappears.
Prometaphase	• Microtubules attach to the centromeres. • Chromosomes start moving toward the spindle equator.
Metaphase	• Chromosomes are aligned at the spindle equator. • Sister chromatids separate.
Anaphase	• Sister chromatids move toward the spindle poles.
Telophase	• Nuclear envelope is rebuild around the daughter nuclei. • Cytokinesis occurs.

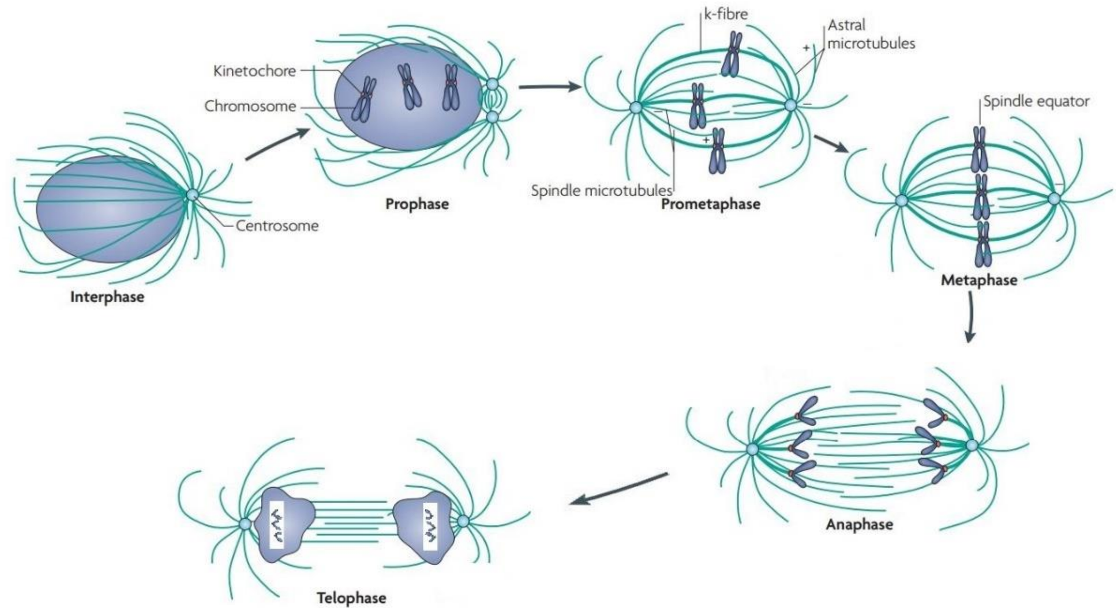

Figure 1-8 Main phases of mitosis. Assembly of the mitotic spindle during interphase and prophase. Alignment chromosomes in the spindle equator starting at prometaphase and finishing at metaphase. Movement of chromosomes toward the poles in anaphase and rebuild of the nuclear envelope in telophase. Modified from (Walczak et al., 2010)

1.3.1.2 Meiosis

Meiosis is the cell division process that results in the gametes or reproductive cells with only half the chromosome set (reviewed by Mercier et al. (2015)), and is the key driver underlying genetic variation (reviewed by Fan et al. (2019); Mercier et al. (2015)). In the current research, one of the objectives is to induce the production of unreduced gametes (refer to Section 1.6), implying meiosis must to be disrupted to restore the somatic chromosome number in the gametes (Oleszczuk et al. (2019); refer to Section 1.2.5.2.3). The principal events occurring in each phase are presented in (Table 1-2 and Figure 1-9).

Table 1-2 Phases of meiosis with the main events taking place in each phase (reviewed by Gutiérrez et al. (2021); Mercier et al. (2015))

Meiosis phase	Meiosis sub-phase	Main event
Meiosis I	Prophase I	• DNA content is already duplicated. It was duplicated in phase S. Each chromosome is formed by two sister chromatids. • Formation of bivalents (connected homologous chromosomes) containing 4 DNA molecules. • Synapsis and recombination between homolog chromosomes. • Condensation of the chromatin. The pairs of homolog chromosomes are visible (i.e., 16 pairs in *L. sinuatum* (Siregar, 2021)) • Nuclear envelope disappears.
	Metaphase I	• Assembly of the meiotic spindle from the centromeres. • Alignment of bivalents at the spindle equator.
	Anaphase I	• Separation of homologous chromosomes. • Migration of chromosomes to the meiotic spindle poles
	Telophase I	• Nuclear envelope is rebuild and two nuclei are formed.

Meiosis phase	Meiosis sub-phase	Main event
		• Cytokinesis. In some species such as *Brachiaria humidicola* (Boldrini et al., 2006) cytokinesis occurs after Telophase II
Meiosis II	Prophase II	• Nuclear envelope breaks down. • Formation of two meiotic spindles.
	Metaphase II	• Chromosomes align at the spindle equator.
	Anaphase II	• Separation of sister chromatids.
	Telophase II	• Formation of four nuclei. • Cytokinesis

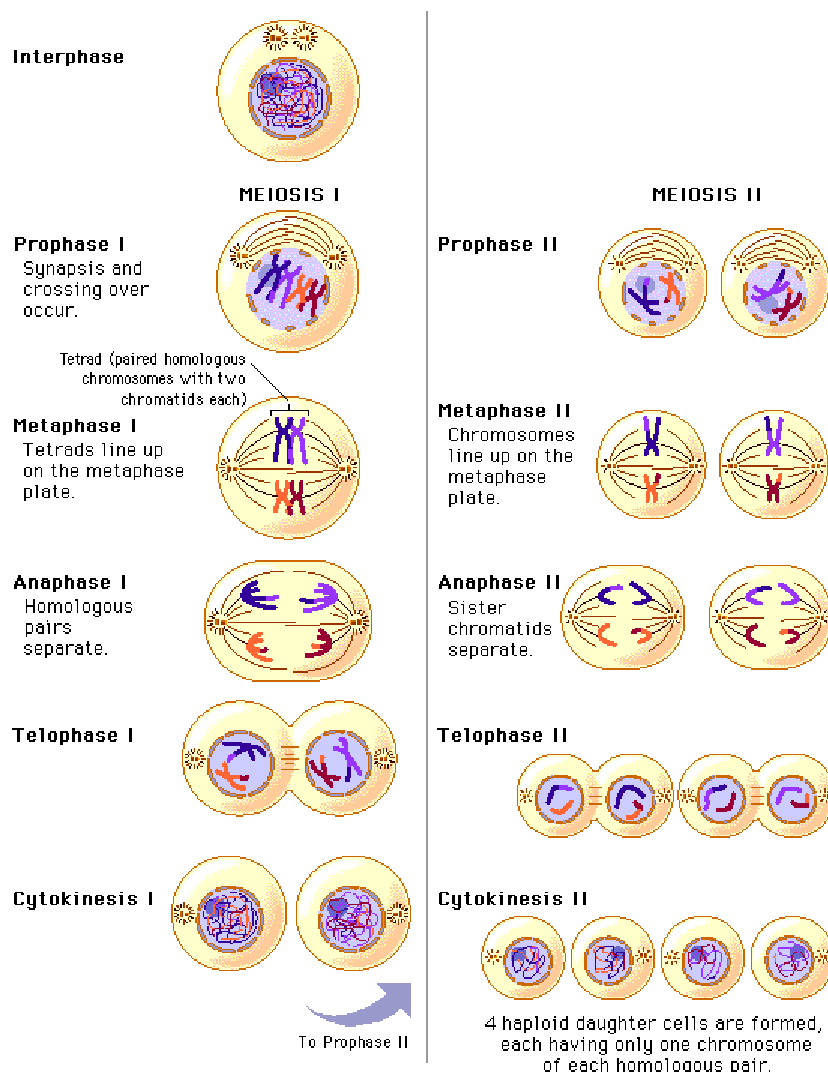

Figure 1-9 Phases of meiosis. DNA is already duplicated, bivalents formed, and synapsis and recombination occur during prophase I. In metaphase I, the meiotic spindle assembles, and homolog chromosomes align at the spindle equator. In anaphase I, homologous chromosomes separate and migrate to opposite poles of the spindle. In telophase I, nuclear envelope is rebuild, and cytokinesis occurs. In prophase II, meiotic spindle is assembled. In metaphase II, chromosomes align at the spindle equator and sister chromatids separate and migrate to opposite poles during anaphase II. In telophase II, cytokinesis occurs. Taken from https://celldivisionandreproduction.weebly.com/meiosis.html (date of retrieval 06/03/2024)

Meiosis occurs in Pollen Mother Cells (PMC) and in Megaspore Mother Cells (MMC) (Shi et al., 2015) and give rise to a polyad constituting by four haploid microspores (tetrad; Figure 1-9; Figure 1-11) or four haploid megaspores, respectively. Use of meiotic spindle disruptors (e.g., colchicine or nitrous oxide (N_2O)) and meiotic abnormalities

could result in polyads with variable number of microspores / megaspores (further detail in Section 1.2.5.2.3).

After formation and individualization of the four haploid microspores, for pollen development, each microspore undergoes two mitotic divisions (further detail in Section 1.3.2; Figure 1-11) resulting in two sperm cells and one vegetative nucleus (Gómez et al., 2015; Halbritter et al., 2018a). The development of the ovule in *Limonium* is explained in more detailed below (refer to Section 1.3.3).

Meiosis can be synchronous between PMC and MMC as reported in *Liriope spicata* (Shi et al., 2015) or asynchronous as suspected in *Limonium* (Rois, 2014; Siregar, 2021) and reported for other species where meiosis in MMC is delayed in comparison with meiosis in PMC (Chang & Sun, 2020; Koul et al., 2020; Yao et al., 2020). Meiotic asynchrony has been established in *Limonium*, revealing that various meiotic stages unfold concurrently within distinct PMCs (Siregar, 2021). Determination of the momentum of occurrence of meiosis in *Limonium* for experimental purposes (i.e., induction of unreduced gametes) could be difficult, due to the asynchrony and the small floret size. However, an association between floret size and meiosis in PMC has been reported in *L. sinuatum* (Siregar, 2021) and *L. perezii* (Figure 1-10). In *L. sinuatum*, ca. 80% of PMC undergo meiosis in unopened florets with diameters no wider than 0.8 mm and calyces protruding no more than 0.2 mm above the bract (Siregar, 2021). In the case of MMC of *Limonium*, meiosis occurs in unopened florets less than 2.5 mm length (Rois, 2014).

The time interval between male meiosis in *Limonium* and anther dehiscence is longer than 14 days with floret opening lasting just one day (Siregar, 2021). Time differences between male meiosis and floret opening occurs between florets of the same spikelet and delays between the first opened floret and the next floret in the spikelet varies between 3 and 14 days (Siregar, 2021). Each *Limonium* floret produces between 100 and more than 300 pollen grains (Siregar, 2021) and only one ovule (Rois, 2014). This means that each floret could be fertilized only once during its lifetime (24 h).

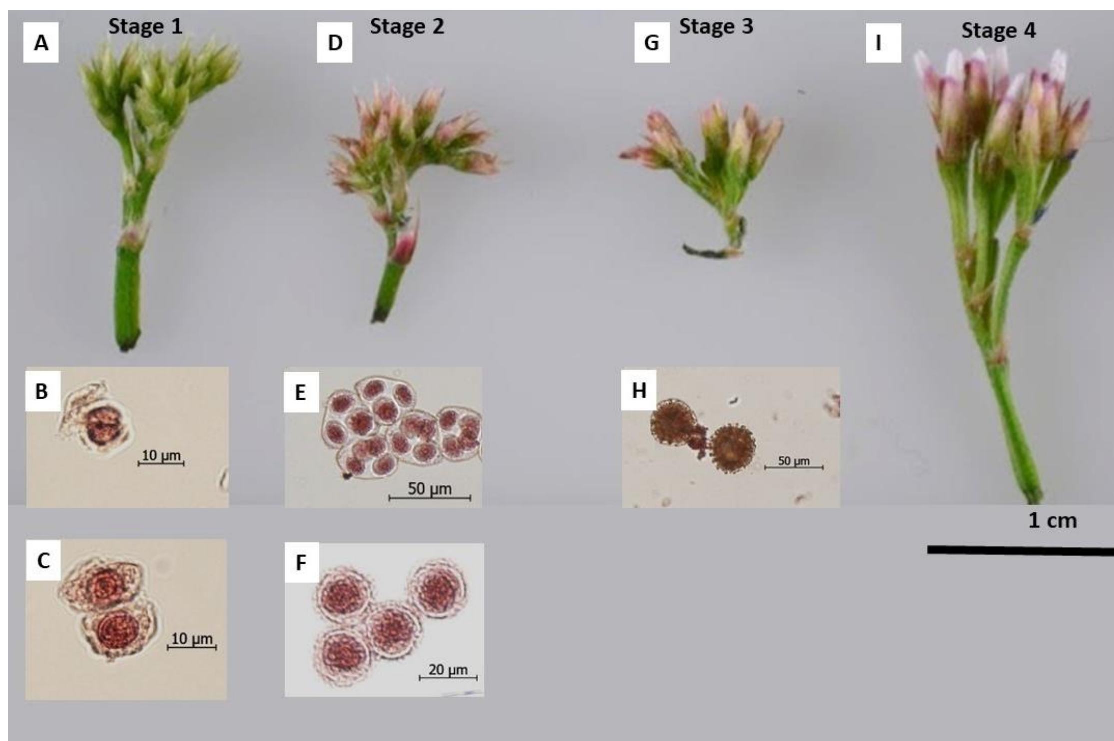

Figure 1-10 Flower and pollen development in *Limonium perezii*. **A-C**. Flower developmental stage 1. **A.** Immature flowers with only the bracts evident. **B.** Division of a sporogenous cell to produce two Pollen Mother Cells (PMC) **C.** Two PMC. **D-F.** Flower developmental stage 2. **D.** Immature flowers with elongated bracts which display their characteristic colour **E**. Tetrads of haploid microspores **F.** Released microspores **G-H**. Flower developmental stage 3 **G.** Immature flower with protruding sepals **H.** Pollen grains **I.** Flower developmental stage 4. Immature flowers with sepals fully extended. Photo taken by Cordoba-Sanchez, J.

1.3.2. Pollen development, morphology, and viability

Meiosis in the PMC, as explained above, results in tetrads containing four microspores that are individualized by a callose wall which precedes the pollen wall formation (Figure 1-11; Halbritter et al. (2018a)). The pollen cell wall distinguishes species by the number of apertures and the ornamentation of its external wall, known as exine, besides pollen shape and polarity (Halbritter et al., 2018b)

Post-meiosis development of the microspores starts after their individualization and release from the tetrads (Figure 1-11; McCormick (2004)). Microspores grow, develop a large vacuole, and go into an asymmetric pollen division i.e., pollen mitosis I (PMI) to produce one vegetative (larger cell) and one generative smaller cell. At this point the fate of both cells splits with only the generative cell going into pollen mitosis II (PMII) to

form the two sperm cells (Park et al., 1998). The occurrence (moment and place) of PMII differs between families and is associated with pollen type (i.e., binucleate or trinucleate; Figure 1-11; Halbritter et al. (2018a); Honys et al. (2006); McCormick (2004)).

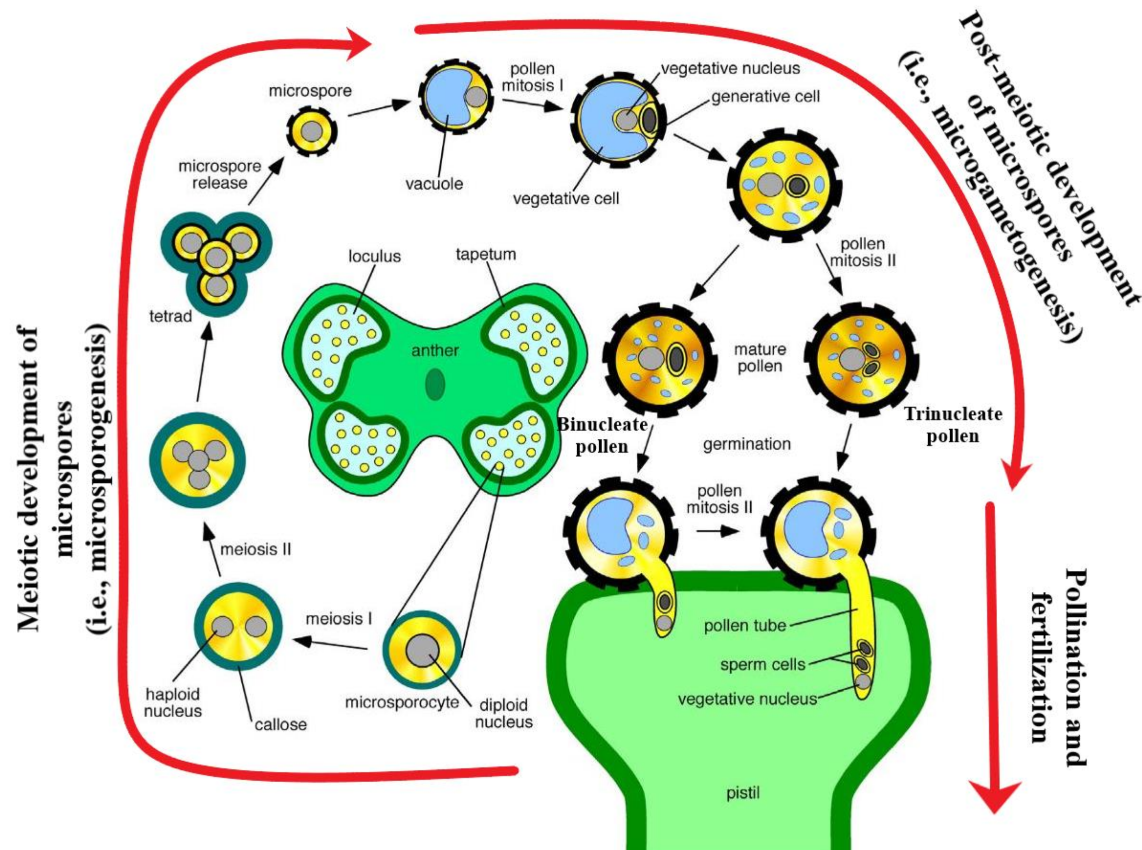

Figure 1-11 Schematic diagram illustrating key phases of pollen development, beginning with the microsporocyte formation and culminating in pollination. Trinucleate pollen, depicted on the right side of the figure, consists of one vegetative nucleus and two sperm cells. On the left side, binucleate pollen is characterized by one vegetative nucleus and one sperm cell. Modified from Honys et al., 2006

Trinucleate pollen, as the one presented in *Limonium*, presents a vegetative nucleus and two generative or sperm nuclei at anthesis meaning PMII occurs in the anther before dehiscence (Figure 1-11; Halbritter et al. (2018a)). The occurrence of three nuclei has implications for the pollen, in that it confers rapid germination, but short viability in comparison with binucleate pollen (Devrnja et al., 2012; Honys et al., 2006). In addition, trinucleate pollen has been reported as presenting difficulties for *in vitro* germination with germination around 30% germination (Pickert, 1988; Zhang et al., 1997) in comparison with binucleate pollen with at least 96% germination (Ćalić et al. (2013); Figure 1-11).

Mature pollen in *Limonium* is also characterized by presenting three apertures in the exine (Siregar, 2021), that is three portions of the pollen wall differing in their conformation to facilitate germination (Halbritter et al. (2018b); Figure 1-12). However, no apertures or more apertures than usual have been previously reported as result of treatment with N_2O (Siregar, 2021).

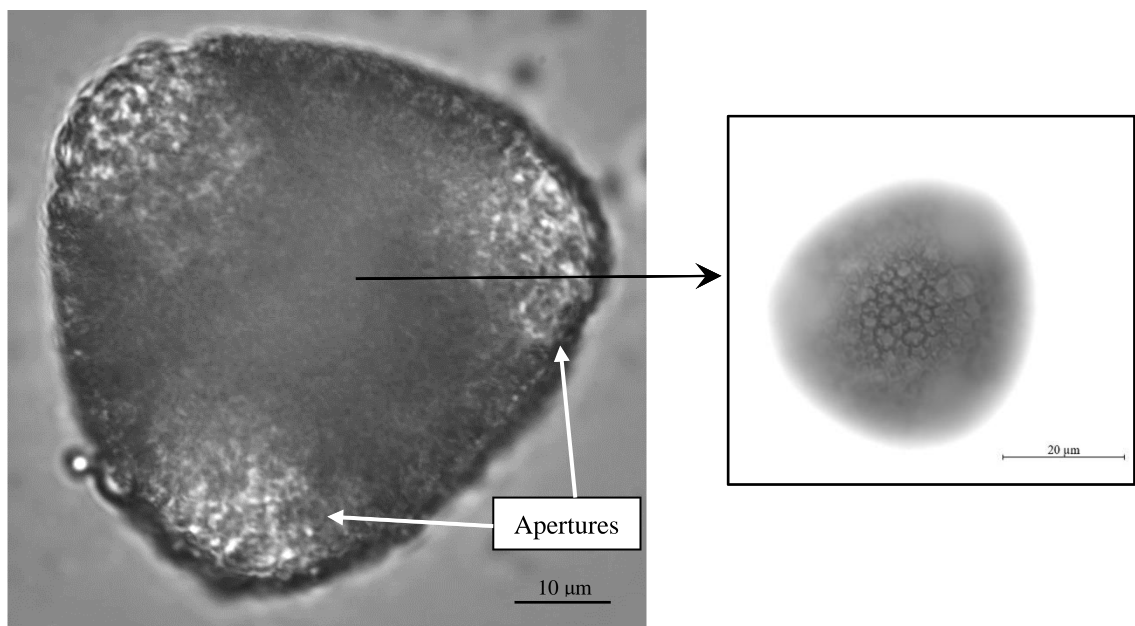

Figure 1-12 Pollen grain type B from *Limonium sinuatum* displaying the triangular shape characteristic for the species and the three apertures. At the right is the closeup of the exine presenting polygonal markings. Photos taken by Cordoba-Sanchez, J.

Pollen grains are the plant's dispersal units and, to accomplish their function, need to be able to germinate and fertilize an egg under the proper conditions (Dafni & Firmage,

2000). Pollen development and functioning are associated with defined changes in the water status of the pollen to maintain the internal stability. For instance, microspores increase their water content and become vacuolate during the development of pollen wall with subsequent pollen size increase; then pollen dehydrates during maturation and prior to dispersal; and rehydrates for germination and fertilization (reviewed by Chaturvedi et al. (2021); Firon et al. (2012)). Some modifications occur in trinucleate pollen (i.e., *Limonium* pollen) such as reduced dehydration before pollen dispersal (Williams & Brown, 2018). The regulation of the water content in pollen grains is controlled by different physiological, molecular, and structural (i.e., exine and vacuoles), physiological and molecular mechanisms (Williams & Brown, 2018). Understanding the regulation of the water content in trinucleate pollen grains is vital for standardization of *in vitro* germination protocols and staining protocols as well as understanding pollen response to external factors.

Pollen functionality and viability can be evaluated through different methods including: assessment of their ability to stain using stains such as modified Alexander's (Peterson et al., 2010), *in vitro* germination of pollen (Zhang et al., 1997), pollen size (Tatum et al., 2015), and seed set (Impe et al., 2020; Oleszczuk et al., 2019). In the present study, unless otherwise stated, pollen viability was measured by pollen staining, with non-stained pollen or blue-green stained pollen being termed aborted.

Pollen size can also potentially be used as an indirect measure of pollen viability (Tatum et al., 2015) and ploidy (Barba-Gonzalez et al., 2006; Larrosa et al., 2012; Nukui et al., 2011). In *Limonium* pollen size varies both between species (El-Garf, 2006) and within species, displaying a unimodal distribution (Siregar, 2021). As reference for this study, pollen with sizes half the average of the species size will be considered as micro-pollen (Oleszczuk et al., 2019; Tatum et al., 2015); pollen grains with sizes 1.26 times the species' average will be considered as unreduced pollen (2n) (Barba-Gonzalez et al., 2006; Larrosa et al., 2012; Nukui et al., 2011; Siregar, 2021), and sizes larger than 1.59 will be counted as 4n (Larrosa et al., 2012). For any pollen grain size, exine ornamentation (Figure 1-12) was the criterion used to discriminate between pollen grains and any other particle observed under the Zeiss microscope Imager.Z2 (Carl Zeiss Microscopy, Axio Imager Z2, Germany).

1.3.3. Megagametogenesis

Meiosis in the MMC, as explained above (refer to Section 1.3.1.2), results in four haploid nuclei. However, depending on when the cell plate formation occurs, the number of meiotic products is different from what is seen in PMC, where four microspores are produced (Halbritter et al. (2018a); refer to Section 1.3.2). The meiotic product in MMC can then be: four one-nucleate megaspores (i.e. monosporic), two two-nucleate megaspores (i.e. bisporic), or one tetra-nucleate megaspore (i.e. tetrasporic). In each of these patterns, there is only one functional megaspore (reviewed by Yadegari and Drews (2004)). For example, in the case of *L. ovalifolium*, a tetrasporic pattern *Drusa* – type has been observed (Figure 1-13;Rois (2014)).

The functional megaspore then undergoes into two or three rounds of mitosis (only two rounds in the case of the tetrasporic pattern) with subsequent cell wall development (reviewed in detail by Yadegari and Drews (2004)). Briefly, the megasporocyte has a bipolar conformation with one nucleus at one pole (i.e. micropylar pole) and three nuclei at the other (i.e. chalazal pole). The nucleus at the micropylar pole then undergoes mitosis to give rise to the egg cell and the synergids. At the same time, the three nuclei at the chalazal pole fuse and then divide to produce a tetranucleate embryo sac (Figure 1-13;Rois (2014);). In *L. ovalifolium*, the mature embryo sac is 6-nucleate and contains these types of cells: synergids, antipodals, egg cell and central cell (Berger et al., 2008; Rois, 2014).

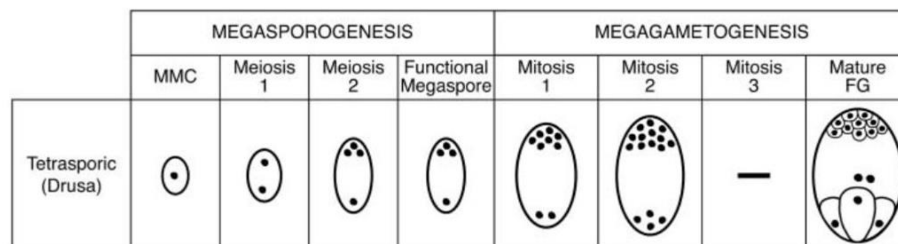

Figure 1-13 Diagram showing the main stages of megagametophyte development. First, during the megasporogenesis, meiosis occurs and a functional megaspore with tetrasporic pattern is produced. Then, during megagametogenesis, two rounds of mitosis give rise to an egg cell and two synergids at the micropylar pole, and a tetranucleate embryo sac at the chalazal end. Modified from Yadegari and Drews (2004), Rois (2014)

1.3.4. Double fertilisation

Double fertilisation is a process characteristic of angiosperms involving two female and two male gametes, first reported by Nawashin (1898) and further reviewed by several authors (e.g. Berger et al. (2008), Friedman and Floyd (2001)). Double fertilisation occurs between one sperm cell and the egg cell to produce the diploid zygote and between the other sperm cell and the central cell to produce the triploid endosperm (Nawashin, 1898).

Double fertilisation involves several steps other than the sperm cells fusion. The pollination process begins with the adherence of pollen on the stigma followed by the pollen germination and pollen tube growth to reach and penetrate the ovary (Zhang, 1995). After reaching the ovary, the pollen tube discharges two sperm cells which remain immobile between the two female gametes, and then each sperm cell fuses with one of the two female gametes as observed in *Arabidopsis thaliana* (i.e. egg cell or central cell) (Hamamura et al., 2012; Hamamura et al., 2011) and in *Nicotiana tabacum* (Sun et al., 2000).

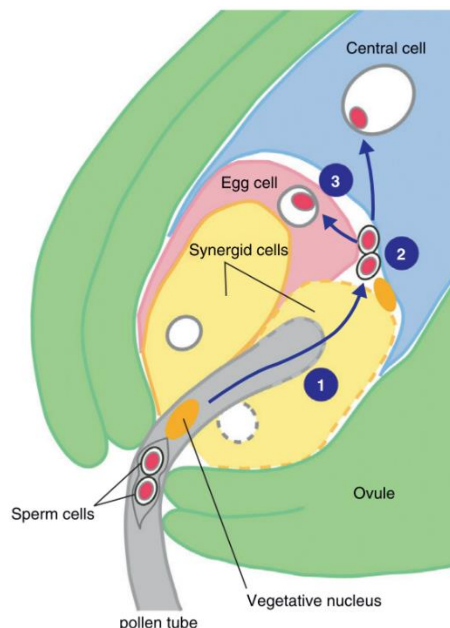

Figure 1-14. Schematic diagram illustrating the main steps of double fertilisation in *Arabidopsis thaliana*, starting with the discharge of the sperm cells (number 1 in the figure), followed by immobilisation phase during which the sperm cells remain between the two female gametes (i.e. egg cell and central cell) (number 2 in the figure), and finalising with the migration and fusion with the female gametes. Taken from Hamamura et al., 2012

Gametic fusion involves the fusion of the cytoplasm and nuclei of the male and female gametes. The cell cycles of both gametes must be synchronised for the harmonious initiation of embryo and endosperm development (reviewed by Berger et al. (2008)). It has been shown in tobacco that released sperm cells are in phase S, when DNA replicates, so that their DNA content varies between 1C and 2C. As soon as the pollen tube enters the ovary, the egg cell duplicates its DNA and 48 hours later its DNZ content is also between 1C and 2C. Therefore, fusion of the egg with a sperm results in a zygote with a DNA content of 4C, which is maintained until the first mitotic division of the zygote occurs (Tian et al., 2005).

In the case of the endosperm, there are fewer studies on how the process takes place. However, it has been reported in *N. tabacum* that the fusion between the sperm cell and the central cell is faster than the fusion between the egg cell and the sperm cell (Sun et al., 2000). Similarly, in *A. thaliana* the first nuclear division was observed to occur within the first hour after the fusion between the sperm cell and the central cell, whereas in the fertilised egg cell it started 7 to 8 hours later (Faure et al., 2002). In addition, endosperm could develop without fertilisation, as observed in *A. thaliana* (Nowack et al., 2006).

1.3.5. Embryo development

Studies of the fertilization and zygote development in *Limonium* (Zhang, 1995) and in *Arabidopsis* (Faure et al., 2002; Gooh et al., 2015) show that the pollen tube penetrates the embryo sac to perform the double fertilization and reaches its final position between 2 h (Zhang, 1995) and 8 h (Faure et al., 2002) after pollination. Between 24 h and 72 h after pollination, the first mitotic divisions from the establishment of the apical-basal axis of the zygote (ten Hove et al., 2015) to the 32-cell pro-embryonic stage (Figure 1-15) occur; and after 72 h, the embryo is in the globular stage (Faure et al., 2002; Zhang, 1995).

The mitotic divisions of the pro-embryo (before globular stage) are not synchronous with time differences observed between sister cells (Gooh et al., 2015). After the globular stage, the embryo continues its development through all the phases (globular, triangular, heart, torpedo, and mature cotyledon). In the case of *A. thaliana,* the seed development is completed in approximately 20 days (Baud et al., 2002) while in *Limonium,* seeds could be harvested after nine weeks (Conceição et al., 2018). The embryo development timing could be affected by different factors e.g., season of the year (Przyborowski &

Nlemirowicz-Szgzytt, 1994), culture media (Lippmann & Lippmann, 1993), genomic constitution (Chen et al., 2016), or ploidy (Köhler et al., 2010) and can be arrested, accelerated or delayed.

1.3.6. Embryo development without fertilisation

Embryo development in plants can also take place without prior fertilization and the seedlings obtained are genetically identical to the seed-bearing genotype (i.e., clones) as observed previously *Limonium* (Conceição et al., 2018). In this species, the asexual development of seeds (i.e., apomictic seeds) seems to be associated with polyploidy (Conceição et al., 2021) and geographical distribution (Rois et al., 2016).

Identification of seedlings developed via apomictic seeds could be done using molecular markers with seedlings displaying the same DNA markers as the seed-bearing genotype likely being apomictic seeds (Conceição et al., 2018). Flow cytometry could also be used for differentiation of interspecific hybrids from non-hybrids or apomictic seeds (Samaniego et al., 2018). In that case, the DNA content from both parents needs to be different, so the comparison of DNA content in 2C nuclei from the seedlings and the parents can be conclusive. For example, in *L. sinuatum* DNA content is 6.42 pg, in *L. perezii* is 8.69 pg and their interspecific hybrids present an average content of 7.59 pg (Morgan et al., 1998); in this example seedlings from this cross with a DNA content close either to 6.42 pg or 7.59 pg (depending on cross direction) could be considered apomictic though this would be confirmed using molecular methods.

1.3.7. The triploid block and endosperm balance number

The induction of ploidy manipulation via production of unreduced gametes or WGD meets challenges associated with interploidy crossing, e.g., occurrence of the triploid block and endosperm failure (Morgan et al., 2021), resulting in aborted hybrid seeds (Lafon-Placette & Köhler, 2016; Morgan et al., 2021). One way of predicting the success of a specific cross is to use the Endosperm Balance Number (EBN) theory which states that normal seed development depends on the ratio of two female genomes (maternal) to one male genome (paternal; Johnston and Hanneman (1980)). However, the maternal to paternal ratio is not always associated with the organism's ploidy (Johnston & Hanneman, 1980). In potato different crosses that do not follow the 2 maternal :1 paternal ratio have

been successful, suggesting a ratio of 4 maternal to 2 paternal could be equally effective as the desired 2 maternal to 1 paternal, and allow embryo development (Lin, 1984; Ortiz & Ehlenfeldt, 1992).

If EBN limitation to seedling production occurs in *Limonium*, as previously suspected (Siregar, 2021), and confirmed for other angiosperms (Carputo et al., 1999), four possible EBN scenarios could occur after induction of unreduced gametes (CHAPTER 5) or interploidy crosses involving tetraploid plants produced by WGD (CHAPTER 7):

- First, unchanged ploidy of the gametes (i.e., pollen or egg) keeping the EBN maternal:paternal genome ratio as 2:1 and producing viable seed. The endosperm would be triploid and the embryo diploid.
- Second, an unreduced egg is fertilized by a reduced pollen, with the EBN maternal:paternal genome ratio equal to 4:1 and zygotes would be lost. The endosperm would be pentaploid and the embryo triploid.
- Third, a reduced egg is fertilized by unreduced pollen, with the EBN maternal:paternal genome ratio at 2:2 and zygotes would be lost. The endosperm would be tetraploid and the embryo triploid.
- Fourth, an unreduced egg is fertilized by an unreduced pollen (bilateral sexual ploidization), the EBN maternal:paternal ratio is 4:2 and the seedlings could be produced, as seen in potato (Carputo et al., 1999; Ortiz & Ehlenfeldt, 1992) and maize (Lin, 1984). The endosperm would be hexaploidy and the embryo tetraploid.

In the present research, to restrict the occurrence of the triploid block two actions were taken:

First, treatment with N_2O was applied to both the seed-bearing parent as well as the pollen-donor for induction of unreduced gametes (CHAPTER 5 and APPENDIX I).

Second, homodiploid crosses were preferred over heteroploid crosses whenever possible (CHAPTER 7).

The hypothesis was that keeping the Endosperm Balance Number (EBN) in a proportion of 4:2 in *Limonium*, could be “equivalent” to the 2:1 as reported in potato (Ortiz & Ehlenfeldt, 1992) and maize (Lin, 1984).

1.4. Plant tissue culture

1.4.1. Embryo rescue

Zygote and embryo development could be impeded by post-zygotic barriers i.e., abortion or developmental arrest phenomena. Those phenomena are more frequently observed after interspecific crosses as consequence of incompatibilities between parental genomes or abnormalities during endosperm development (Chen et al., 2016). Ovule/embryo rescue can be an effective approach to avoid post-zygotic embryo abortion (Okazaki, 2005).

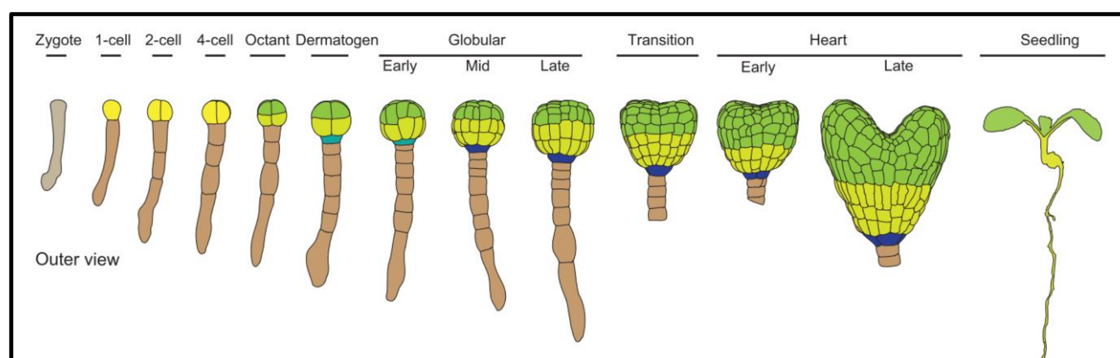

Figure 1-15 *Arabidopsis* embryo development begins with the zygote and progresses through the proembryo stage (from one to 16 cells at the dermatogen stage), followed by the globular, transition (also known as triangular stage), heart, and torpedo stages, culminating in the mature cotyledon and seedling.. Modified from ten Hove et al., 2015

Embryo rescue refers to the extraction of the embryo from the ovule and its sowing in a plant tissue culture medium (Okazaki, 2005). The aim of embryo rescue is to avoid the abortion of the embryo (Bhojwani & Dantu, 2013; Loyola-Vargas & Ochoa-Alejo, 2018) or speed up its development and subsequently the breeding cycle (Bermejo et al., 2016; Bhojwani & Dantu, 2013). In comparison, ovule rescue allows the rescue of younger abortive embryos inside the ovule (Ishizaka, 2018; Okazaki et al., 2005). In *Limonium*, for example, ovule rescue is done between 12 and 16 days after interspecific crossing and

two weeks later, the embryo is extracted from the ovule and subculture (Morgan et al., 1998). Ovule rescue in *Limonium* allowed recovery of 1% of the *L. perezii* × *L. sinuatum* hybrids (Morgan et al., 1998) and 2% of *L. perigrinum* × *L. purpuratum* hybrids (Seelye et al., 2000). In the absence of embryo/ovule culture, no interspecific hybrid seedlings were produced (Morgan et al., 1998; Seelye et al., 2000).

In vitro culture of ovules and/or embryos requires the culture medium to provide the nourishment normally provided by the endosperm to the embryo. Different medium formulations are available some examples could be found in (Bhojwani & Dantu, 2013; Deng et al., 2011; Ishizaka, 2018) which consist basically of:

- a salt formulation e.g., complete or half-strength MS (Bermejo et al., 2016; Murashige & Skoog, 1962; Okazaki, 2005) or B5 medium (Gamborg et al., 1968)
- plant growth regulators (PGRs) e.g., cytokines (e.g., 0-1mg/1 BA) and/or auxins (e.g., either 0-0.1mg/L NAA, or 0-0.175 mg/L IAA)
- sucrose (1%-3%)
- gelling agent (e.g., agar (0.6%, 10%) (Bermejo et al., 2016) or gellant gum (0.2%)) (Okazaki, 2005).

In addition, embryo culture of some species utilises additional nutrient sources such as coconut water (Souza et al., 2013) or cucumber juice (Przywara et al., 1989). In *Limonium,* the basic culture medium for ovule/embryo culture contains B5 salts (Gamborg et al., 1968), Kao and Michayluk vitamins (Kao et al., 1974), sucrose (1%-3%), coconut water (2%), and casein hydrolysate (250mg/L; Morgan et al. (1998)). However, some modifications were done in the current research (CHAPTER 2).

1.5. Opportunities identified for the present research project

In this research, ploidy manipulation and physically induced mutations were proposed as breeding strategies to approach the *Limonium* breeding problems: limited availability of genetic germplasm, interspecific hybridisation rates lower than 2% reported in *L. perezii* × *L. sinuatum* and sterility of interspecific hybrids.

Those breeding strategies have already been evaluated in *Limonium*, but some areas requiring further research were identified:

- No published information about the efficacy of ploidy manipulation via induction of unreduced gametes for increasing the interspecific hybridisation rate and interspecific hybrid fertility in *Limonium*.
- The efficacy of N_2O for induction of whole genome duplication (WGD) in *Limonium* has not been assessed.
- Contradictory results about the efficacy of radiation to induce mutations and increase the genetic diversity in *Limonium.*
- No published protocol for the use of physically induced mutations as a breeding strategy in *Limonium*.

1.6. Research objectives and thesis structure

The research done during my PhD studies was part of the ongoing research of the Hybridisation and Introgression team at The New Zealand Institute for Plant & Food Research Ltd (PFR) with the goals of:

- Developing breeding technologies for producing *"better crops faster"*.
- Increasing germplasm diversity in different crops.

The general research objectives (Figure 1-16) pursued in the current PhD research were, using the genus *Limonium* as a case of study to:

- Increase germplasm diversity.
- Perform ploidy manipulation using N_2O and evaluate its efficacy for increasing the hybridisation rate in interspecific crosses, restoring fertility in interspecific hybrids, and increasing the genetic diversity in general.
- Induce physical mutations in selected genotypes to determine the efficacy of the strategy to increase the genetic diversity of PFR's germplasm bank and develop a standardized protocol.

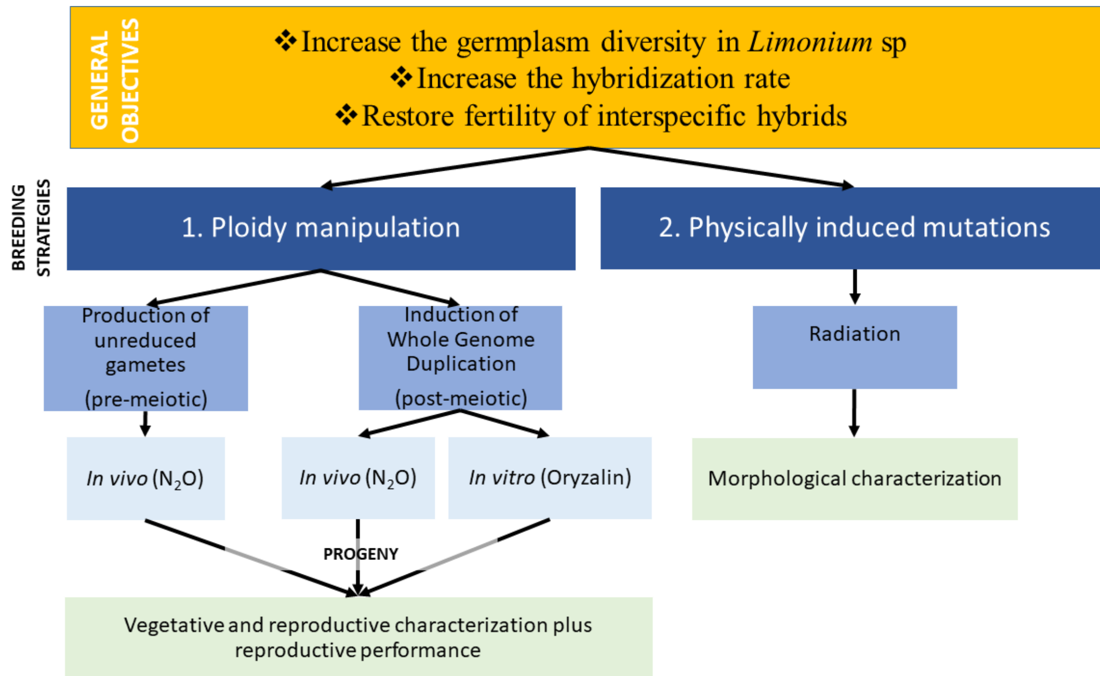

Figure 1-16 Conceptual schematic of thesis as a document, and interrelationships between topics. At the top, in the yellow rectangle, are the three general objectives. The statements in dark blue are the two breeding strategies evaluated to address the general objectives. As highlighted in mid-blue tones, implementation of the ploidy manipulation strategy could be done to obtain unreduced gametes or to induce whole genome duplication. In the present research, production of unreduced gametes was accomplished using nitrous oxide (N_2O) (light blue highlight) while whole genome duplication was induced *in vivo* using N_2O or *in vitro* with oryzalin (light blue highlight). The seedlings obtained after any of the ploidy manipulation strategies was vegetatively and reproductively characterized (light green highlight). The breeding strategy of physically induced mutations was evaluated using physical radiation (dark blue and mid-blue highlight) and the mutated plants were intended to be morphologically evaluated (light green highlight)

The research objectives, and the chapter in which they were addressed, were:

- To induce whole genome duplication (WGD) in *Limonium* using N_2O during the early developmental stages of the embryo (i.e., ploidy manipulation after pollination; CHAPTER 2, CHAPTER 3, APPENDIX F; Figure 1-17).
- To quantitatively evaluate if unreduced gametes can be produced in *L. sinuatum* and *L. perezii* using N_2O, during the early stages of the flower development (i.e., ploidy manipulation before the planned pollinations; CHAPTER 4, CHAPTER 5, APPENDIX I, APPENDIX N; Figure 1-18).

- To induce whole genome duplication in *Limonium* using oryzalin as a spindle disruptor (CHAPTER 6).
- Production of polyploid interspecific *L. sinuatum* × *L. perezii* hybrids (CHAPTER 5, CHAPTER 6, CHAPTER 7)
- To quantitatively compare morphologically and reproductively the polyploid *Limonium* plants obtained with their diploid counterparts (CHAPTER 7).
- To quantitatively evaluate the efficacy of physically induced mutations for increasing *Limonium* germplasm diversity (CHAPTER 8).

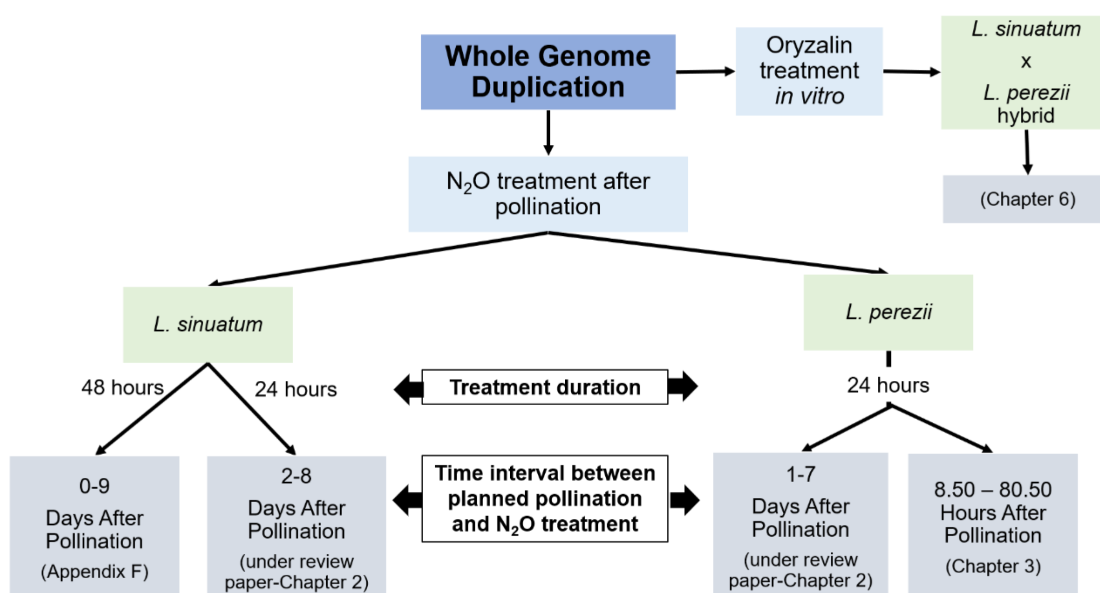

Figure 1-17 Schematic representation of the experiments done (light grey highlight) for induction of Whole Genome Duplication (WGD) in *L. sinuatum*, *L. perezii*, and *L. sinuatum* × *L. perezii* interspecific hybrids (light green highlight) using either oryzalin or nitrous oxide (N_2O) (light blue highlight) with different treatment duration and time intervals between the planned pollinations and the N_2O treatment

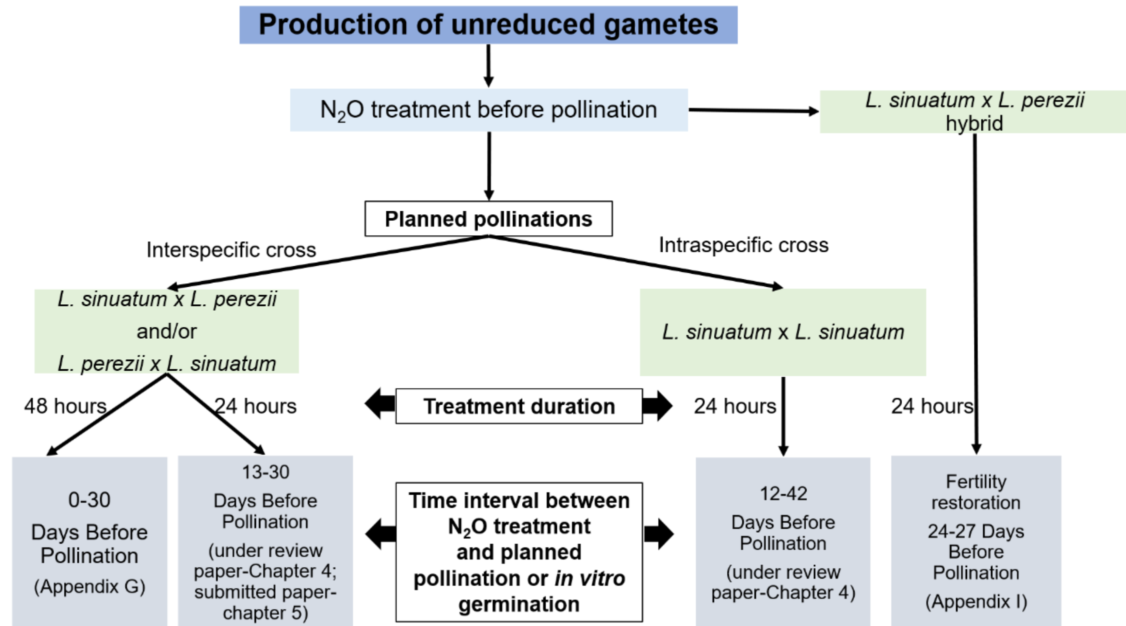

Figure 1-18 Schematic representation of the experiments (light grey highlight) performed using *L. sinuatum*, *L. perezii*, and/or *L. sinuatum* × *L. perezii* hybrids, for production of unreduced gametes using nitrous oxide (N_2O) with different treatment duration and time intervals between the N_2O treatment and the planned pollination followed by intra or interspecific crosses performed to target polyploid production of seedlings (first three (light grey highlight boxes from left to right), or for fertility restoration in hybrids (right most light grey highlight box)

In addition to the above referenced chapters (i.e., CHAPTER 2 to CHAPTER 8), the present document includes a general discussion and perspectives (CHAPTER 9), presenting a summary of the main findings and their importance, conclusions and future opportunities in the breeding of *Limonium.* This thesis document also includes appendices documenting additional experiments required for the success of the research, but do not necessarily address the above listed objectives.

1.6.1. Experimental design and data analysis

Three experimental designs were used throughout the PhD experimentation: completely randomized design (CRD), randomized complete block design (RCBD), and a mixture-amount design. For any of the designs, replications (i.e., treatments which are repeated at least two times) are compulsory to estimate the experimental error (Compton, 1994). Each unit which receives the treatment (e.g., culture vessel, day when pollinations are performed) is known as experimental unit. In any of the experimental designs, sub-

sampling could be used when within an experimental unit there is more than one observational unit (Compton, 1994). In the current research, for example, if 10 planned crosses are performed daily for three days, the days are the replicates, and the crosses are the sub-samples.

The CRD is the simplest design, and is recommended when little variation occurs between experimental units (Compton, 1994). In this design, the factor under evaluation has different levels. For example, in this research the factor use of nitrous oxide has two levels: treated and untreated (i.e., control). When differences between experimental units occur (e.g., leaves from plants from different species), it is recommended to use a RCBD as the statistical precision decreased (Compton, 1994). In the RCBD the blocks represent homogenous groups (e.g., germplasm groups (i.e., *L. sinuatum*, *L. perezii*-white, *L. perezii*-purple, interspecific hybrids)) to which the experimental units are randomly assigned. For CRD and RCBD, the levels of the factors under assessment could be arranged in a factorial design allowing the evaluation of all possible combinations between the levels (Compton, 1994).

The other experimental design used in the research was a mixture-amount design which allows to separate the effects of proportion (i.e., ratio) and concentration (Evens & Niedz, 2010). This experimental design is recommended when the aim is to standardize e.g., a culture medium which is composed of different nutrients, plant growth regulators, etc with varying concentrations and ratios between the components. In this case, the amount of each component as well as the interaction between them affects the response variable e.g., shoot induction (Nezami-Alanagh et al., 2017).

The selection of the model to analyse the data depends on the data distribution (e.g., normal, binomial, Poisson). In this PhD research, the response variables evaluated followed one of three distributions: binomial, Poisson, or normal. Variables with values with only two possible outcomes e.g., callus induction (yes/no) follow a binomial distribution; variables with values corresponding to a count (e.g., number of roots per explant) follow a Poisson distribution; and variables with a continuous distribution follow a normal distribution if the data close to the mean is more frequent in comparison with data distant from the mean (i.e., bell shape). So as to confirm continuous data follow a

normal distribution at least two assumptions need to be validated: normal distribution of the residuals and that observations are independent of each other.

Modelling of data with either binomial distribution or Poisson distribution results in an estimated mean with a confidence interval calculated from theory without need of replications (Daniel & Chad, 2018). However, if replications are available, they allow to check whether the data behaves in that way or if other source of variability could be involved. The mean in a binomial distribution represents the probability of occurrence of a success (e.g., induced callus) while in a Poisson distribution the probability calculated is for the average count (e.g., 10 roots per explant).

In the case of normally distributed data, modelling results in a calculated mean and a confidence interval for that mean, but unlike Poisson and binomial distributions, replicates are compulsory for those calculations. In the current research, for data following a normal distribution (e.g., pollen size), the estimated effect (e.g., mean size for pollen grain) and the estimated variability (e.g., standard deviation for pollen size calculated from reps) are used for performing a t-test on whether the means of two samples are equal. Probability of the t-value (calculated in the t-test) being higher than 0.05 represent no differences between means. That is the reason for selecting a *p-value* equal to 0.05 to discriminate between two sample means being equal or not.

CHAPTER 2. Nitrous oxide treatment after pollination induces ploidy changes in *Limonium* sp.

As detailed below, this chapter has been submitted for publication in a peer reviewed journal. As such, in comparison to other chapters the reader may note some differences in writing style and detail, reflecting the journal's requirements, and the targeted audience for that journal.

Details of the publication are:

Cordoba-Sanchez, J., Funnell, K., Hedderley, D., Roskruge, N. Morgan, E. 2023. Nitrous oxide treatment after pollination induces ploidy changes in *Limonium* sp. Brazilian Journal of Botany (under review).

2.1. Abstract

Production of statice (*Limonium* sp.) plants with higher ploidy through induction of whole-genome duplication (WGD) via the spindle disrupter N_2O, was examined as a strategy to increase the germplasm diversity of the species. Intraspecific crosses between two *Limonium sinuatum* Mill (sin16 × sin18 and vice versa) and two *L. perezii* (Stapf) Hubb. plants (per11 × per273 and vice versa) were conducted daily for seven consecutive days, with subsequent exposure to N_2O (608 kPa) for 24 h. N_2O induced between 16% and 35% of polyploid plants in the resulting progeny when applied between one and four days after pollination. Morphological characterisation of two *L. sinuatum* genotypes (sin15 and sin16) and their either diploid or tetraploid progeny was also carried out to evaluate the effect of ploidy. Compared to their diploid counterparts, the tetraploid plants confirmed by flow cytometry had: pollen grains 1.2 times larger in plan area; guard cell length and main floral stem diameter 1.4 times greater; leaves 1.5 times thicker and 1.6 times larger in area; and stomatal density 0.6 times lower. In conclusion, the production of tetraploid *Limonium* plants using N_2O shortly after pollination creates new diversity for breeding.

2.2. Key Words

Autopolyploid, gene dosage effect, mitotic disruption, ornamental plants, polyploidization, whole-genome duplication.

2.3. 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 (International Association of Horticultural Producers, 2022) resulting in significant interest in delivering new cultivars. Hybridisation offers opportunity to increase the diversity by introducing new characteristics and has been frequently used to generate new cultivars, e.g., ‘Ocean blue’ (Sato et al., 1993) and the ‘siNZii™’ series (RioRoses, 2021). Nevertheless, in interspecific hybrids, sterility and infertility are frequent, limiting the further use of the hybrids with breeding purposes (Morgan et al., 2001). 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).

WGD contributes to increasing germplasm diversity by means of producing plants of different ploidies (Van de Peer et al., 2017) with novel morphological features (Bhattarai et al., 2021; Chansler et al., 2016; Kumar & Yadav, 2020). 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 (Birchler & Veitia, 2012). Therefore, morphological changes such as bigger organs and lower stomatal density are expected to occur in polyploid plants (Gordej et al., 2019; Mori et al., 2016; Róis et al., 2012).

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 (Molenaar et al., 2018). In the research described here, the disruption of the mitotic spindle was attempted in single-celled zygotes using nitrous oxide (N_2O) (Gordej et al., 2019). As reported for wheat (Hansen et al., 1988), 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* (Siregar, 2021), successful generation of progeny of increased ploidy by means of inducing WGD using N_2O has not previously been reported. Therefore, the aims of this study were: to demonstrate that treatment with N_2O of zygotes of *Limonium sinuatum* (L.) Mill and *Limonium perezii*

(Stapf) Hubb. would result in plants with increased ploidy, and to describe the effects of WGD on the phenotypes of the *L. sinuatum* progeny.

2.4. Materials and methods

2.4.1. Plant material and cultivation

Three diploid *L. sinuatum* ($2n = 16$) genotypes (i.e., sin15, sin16, and sin18) and two *L. perezii* genotypes (i.e., per11 and per273) were used in this 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 pollen/stigma dimorphism (Baker, 1953a). 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 (Dalton's™ Base growing medium (CAN Fines A Grade 50%; fiber 30%); 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.

2.4.2. 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. On day zero (D_0) of the pollination series, whole plants carrying the pollinated flowers were treated with N_2O within a pressure-tolerant cylinder for 24 h at c. 600 kPa (Barba-Gonzalez et al., 2006). After application of N_2O, the plants were returned to the

greenhouse conditions until ovule/embryo rescue was undertaken at one month after pollination.

In the first experiment, *L. sinuatum* genotypes were used with an experimental design comprising a 2 x 7 arrangement of treatment factors: seed-bearing genotype (i.e., sin16 or sin18) and days after pollination (DAP 2 to 8). In a subsequent experiment, *L. perezii* genotypes were used, which consisted of a 2 x 7 arrangement of factors: seed-bearing genotype (per11 or per273) and DAP (1 to 7) following application of N_2O.

In both experiments a completely randomized design was utilized. Within each species each genotype served as both pollen-donor genotypes and seed-bearing genotype. 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 x sin18; and per11 x 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 which successfully produced zygotes (i.e., zygote success), ploidy of seedlings derived (and the proportion of tetraploid seedlings or seedling with tetraploid tissues in the case of mixoploid plants), plan area of pollen grains, guard cell length, stomatal density, leaf thickness and area, main stem diameter, and width of main ‘stem wings’ (Karis, 2004).

2.4.3. 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 (Murashige & Skoog, 1962), Gamborg B5 vitamins (Gamborg et al., 1968), 30 g/L sucrose, 7.5 g/L agar (Morgan et al. 1998), 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 (Morgan et al., 1998) for further growth. Finally, the plants were transferred to a root induction medium

comprising the base medium amended with Gamborg B5 vitamins (Gamborg et al., 1968) 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 0.1M NaOH or 0.1M HCl prior to autoclaving at 121 °C at 103 kPa for 15 minutes. 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.

2.4.4. Morphological evaluation of L. sinuatum

Rooted plants were deflasked and grown under greenhouse conditions until flowering and, when at least 80% of the calyces were open (Karis, 2004), morphological evaluation was undertaken for the parental genotypes (sin15 and sin16) as well as their corresponding diploid and tetraploid progeny.

2.4.4.1 Floral characteristics

Using a Vernier caliper (0-150 mm, Protect, New Zealand, 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 (Hammer et al., 2015; Hammer et al., 2011) 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 (Peterson et al., 2010) and observed using a Zeiss microscope Imager.Z2 (Carl Zeiss Microscopy, Axio Imager Z2, Germany). Pollen grains were photographed at 50x magnification using a digital camera (Axiocam 305 color, Carl Zeiss Microscopy GmbH, Germany) and the software ZEN2.6 (blue edition) (Carl Zeiss Microscopy GmbH, 2018). The plan area of each of 100 grains was measured and recorded using the software Fiji (Schindelin et al., 2012)(APPENDIX A). Purple-stained pollen was considered viable.

2.4.4.2 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 calliper (0-150 mm, Protect 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) were obtained and processed using the software Fiji (Schindelin et al., 2012) (APPENDIX A).

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 (Cohen & Yao, 1996). Guard cells were observed at 100x magnification (Zeiss imager.Z2, Carl Zeiss Microscopy, Axio Imager Z2, 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 100x magnification using a digital camera (Axiocam 305 colour, Carl Zeiss Microscopy GmbH, Germany) and the software ZEN2.6 (blue edition; Carl Zeiss Microscopy GmbH (2018)). Photographs were processed using the software Fiji (Schindelin et al., 2012), 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.

2.4.5. 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 analysed using flow cytometry, utilizing the methodology of Otto (1990). 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, United States). 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 (CyPAD Windows^{TM}-based software, Sysmex, United States) was used for data acquisition and analysis.

2.4.6. Statistical analysis

Data analysis was conducted using the software R (R Core Team, 2018) with ggplot 2 (Wickham, 2016) utilized for graphic outputs.

The response variable zygote 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; R Core Team (2018)) 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 R; R Core Team (2018)).

Pairwise differences between means were tested using Least Significant Difference (LSD; Mendiburu and Yaseen (2020)) adjusting the *p-value*s by the Bonferroni method (Lyman & Longnecker, 2001). For the generalized linear models (glm), pairwise likelihood ratio tests were used to compare between the levels of factors (similar to LRPAIR in Goedhart (2018)). 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 for the variables ploidy and the seven morphological characteristics evaluated. The PCA utilized the software R (R Core Team, 2018) using the packages FactoMineR (Sebastien et al., 2008) and factoextra (Kassambara & Fabian Mundt, 2020). The classical means of data summarizing, dimensionality reduction and visualization for a multi-component analysis were used.

2.5. Results

2.5.1. Plants obtained

The proportion of embryos/ovules rescued varied from seven to 53 for the treated plants (APPENDIX A), representing between 10% and 75% cross success. A lower number of zygotes occurred for *L. perezii* in comparison with *L. sinuatum.* In tissue culture, embryo mortality was between 15% for *L. sinuatum* and 85% for *L. perezii* genotypes.

2.5.2. Effect of N_2O treatment on ploidy

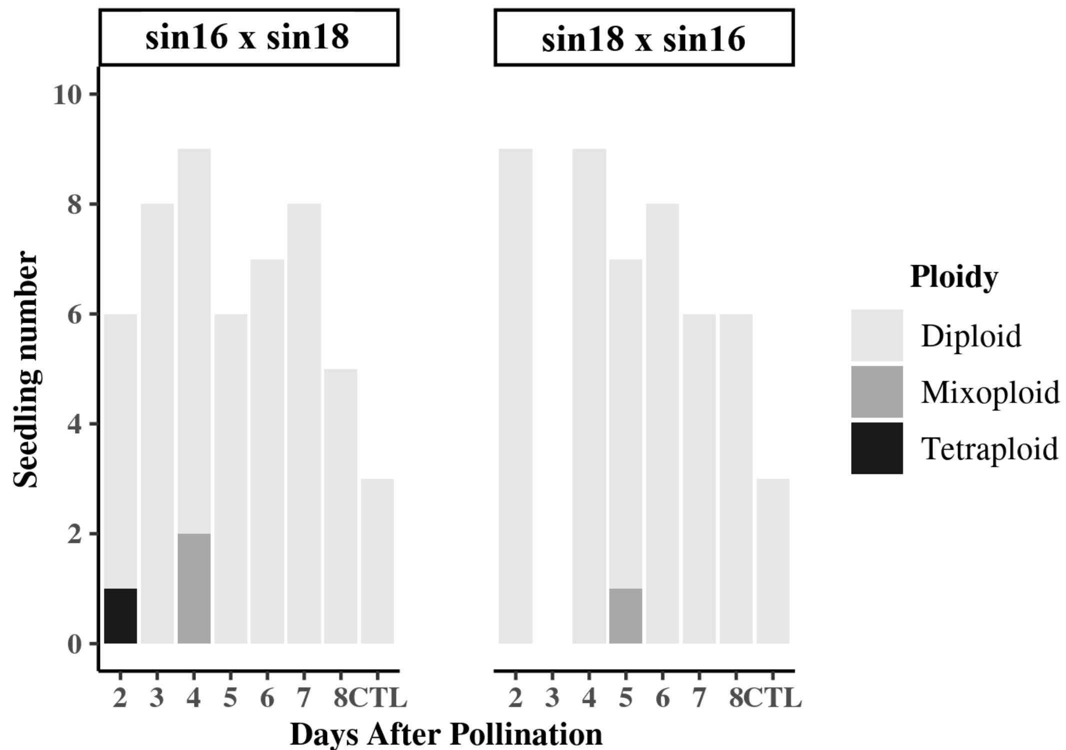

Figure 2-1 Number of diploid, tetraploid or mixoploid progeny obtained in two crosses between *Limonium sinuatum* genotypes for each Day After Pollination (DAP) when N_2O was applied

The treatment of *L. sinuatum* with N_2O for 24 h after pollination 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 2-1). 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).

The success of zygotes 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 ($p > 0.05$).

In the case of *L. perezii,* N_2O treatment for 24 h induced WGD (five plants) when it was applied between 1 and 3 DAP, with only diploids (five plants) and mixoploids (three plants) resulting when applied at other days after pollination (Figure 2-2). The cumulative

proportion of progeny with N_2O induced doubling obtained between 1 DAP and 3 DAP in *L. perezii* was, therefore, 35.7%.

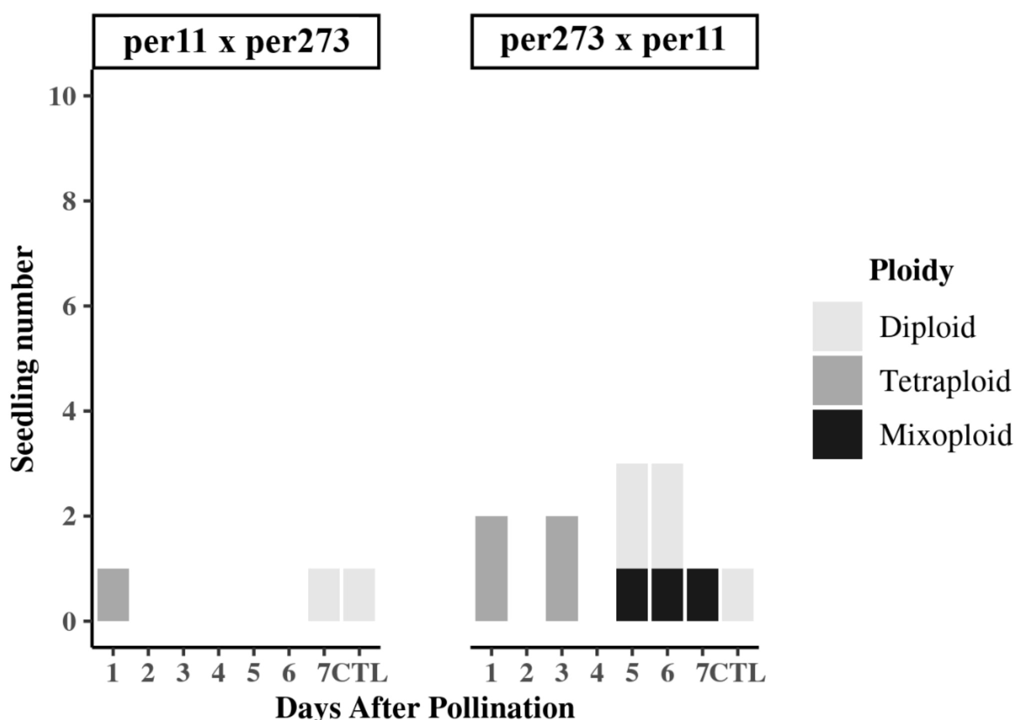

Figure 2-2 Number of diploid, tetraploid or mixoploid progeny obtained in two crosses between *Limonium perezii* genotypes for each Day After Pollination (DAP) following N_2O application. Gaps in the data means zero seedlings survived after tissue culture

2.5.3. Morphological evaluation

2.5.3.1 Pollen grain size

Across all *L. sinuatum* genotypes examined (i.e., sin15, sin16, and their progeny), which included diploids and tetraploids, the plan area of individual purple-stained (viable) (modified Alexander's stain) pollen grains ranged between 1195 µm^2 and 4500 µm^2, and the differences observed were associated with the genotype and the ploidy ($p< 0.05$). For example, between the diploid parental genotypes, the mean size of purple-stained pollen size was 0.25 times larger for sin15 compared with sin16 (Table 2-1). In the case of the progeny, the pollen grain area varied from 1854 µm^2 (diploid genotype sin192) to 2670 µm^2 (tetraploid genotype sin201), with the difference primarily attributed to the ploidy

(Table 2-1). In summary, the pollen plan grain area of tetraploid progeny of *L. sinuatum* was typically 1.2 times larger in comparison with their diploid counterparts.

Table 2-1 Plan area (µm^2) of purple-stained pollen, guard cell length (µm) and stomatal density (number of stomata per mm^2) 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 hours. Means followed by a common letter are not significantly different by the LSD test at the 0.05% significance level

Genotype	Ploidy	Area (µm^2) †	Length (µm) of guard cells †	Stomatal density ‡ §
sin15 (parent)	Diploid	2200 ± 212^b	23.8 ± 4.1^e	2.2 ± 1.4^a
sin16 (parent)	Diploid	1811 ± 274^c	24.0 ± 4.9^{de}	2.3 ± 1.3^a
sin191 (progeny)	Diploid	1946 ± 180^c	26.3 ± 3.2^e	1.8 ± 1.0^b
sin192 (progeny)	Diploid	1854 ± 363^c	30.3 ± 3.7^d	1.3 ± 0.4^c
sin193 (progeny)	Tetraploid	2551 ± 407^a	31.2 ± 4.6^c	1.7 ± 1.6^c
sin194 (progeny)	Tetraploid	2597 ± 353^a	33.7 ± 3.9^c	1.4 ± 0.6^e
sin201 (progeny)	Tetraploid	2670 ± 329^a	42.8 ± 5.8^b	1.0 ± 0.6^f
sin202 (progeny)	Tetraploid	2439 ± 336^a	38.2 ± 5.3^c	1.3 ± 1.1^g
sin203 (progeny)	Tetraploid	2437 ± 276^a	47.5 ± 5.6^a	1.1 ± 1.0^h
sin204 (progeny)	Tetraploid	2543 ± 528^a	45.1 ± 4.8^b	1.0 ± 0.9^g

† LSD test was performed for the pairwise differences between means. Different letters represent different mean groups (p = 0.05)
‡ Number of stomata per mm^2
§ LRPAIR test grouping was performed for the stomata density mean. Different letters represent different mean groups (p = 0.05)

2.5.3.2 Guard cell length and stomatal density

The length of guard cells varied, across all genotypes, from 17 to 57 µm, and differences observed were associated with both ploidy and genotype ($p<0.05$). The guard cells from

the tetraploid genotypes were 1.4 times larger in comparison with the diploids, displaying a mean value of 39.47 ± 7.65 versus 27.37 ± 4.15 (Table 2-1).

Across genotypes, stomatal density varied between 2.32 and 10.5 stomata per mm^2, and the differences observed were associated with both ploidy and genotype ($p < 0.05$). The average density of stomata was 0.6 times lower in tetraploid plants in comparison with diploids, showing densities between 1 and 1.72 stomata per mm^2, while the densities for diploids ranged between 1.27 and 2.22 stomata per mm^2 (Table 2-1). Nevertheless, some diploid and tetraploid genotypes displayed similar stomatal densities regardless of their ploidy (Table 2-1; e.g., sin192 (diploid) and sin202 (tetraploid)).

2.5.3.3 Morphological comparison of diploid and tetraploid plants

The morphological characteristics leaf thickness and area, diameter of the main floral stem and width of main floral stem wings were all increased in the tetraploid genotypes in comparison with their diploid counterparts ($p < 0.05$; Figure 2-3). As a result, in the tetraploids, the leaves were 1.5 times thicker and 1.6 times larger in area, the diameter of the main floral stem was 1.4 times greater, and the main floral stem wings were 2.5 times wider.

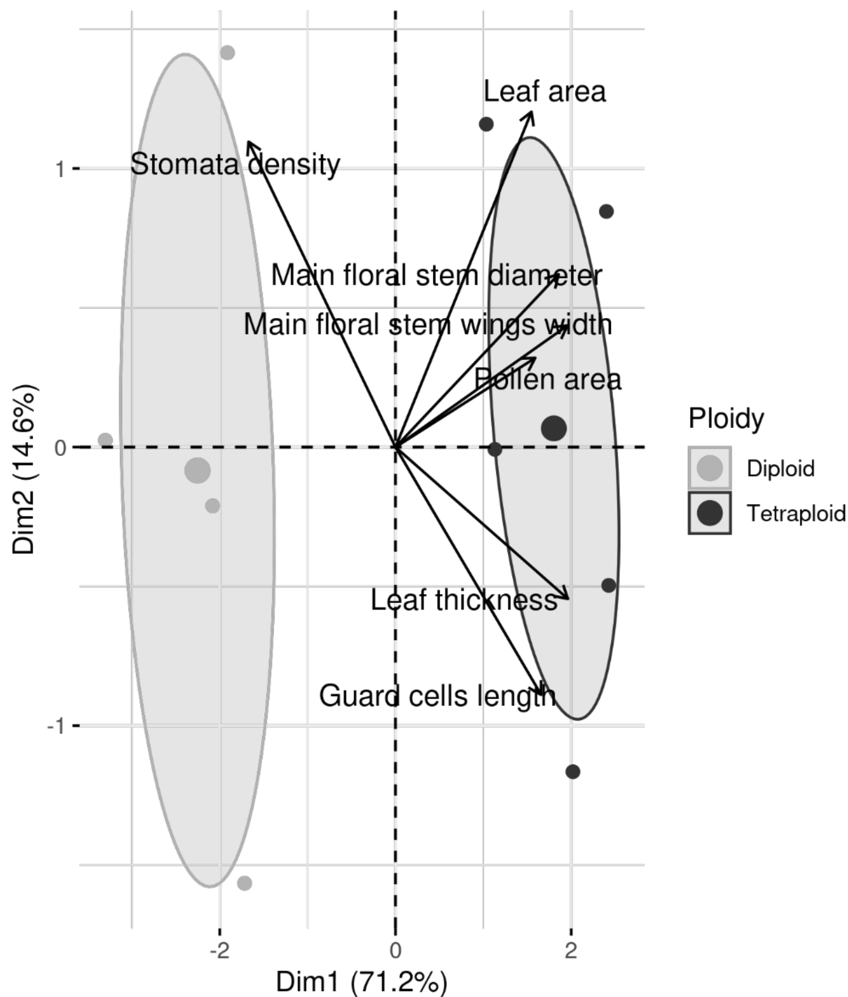

Figure 2-3 Principal Component Analysis (PCA) of the morphological-response variables evaluated (pollen area, guard cell length, stomata density, leaf thickness and area, main floral stem diameter and main floral stem wings width) in diploid and tetraploid *Limonium sinuatum* genotypes. The arrows represent the eigenvectors for each variable and point in the direction of increasing magnitude of the measurement. The small dots represent the individual genotypes while the bigger dots represent the centroid of either the diploid genotypes (light grey) or the tetraploid genotypes (black). The dimensions are described as Dim1 (Dimension 1) and Dim2 (Dimension 2)

The PCA revealed two components or dimensions, which explained 85% of the observed variance in morphological measures (Figure 2-3). There were two groups of measurements, which correlated with each other. Leaf area, pollen grain plan area, diameter of the main floral stem, and width of the main floral stem wings were one group.

Leaf thickness, guard cell length and (decreasing) stomatal density were in the other group. Both were higher for the tetraploid genotypes.

2.6. Discussion

N_2O treatments were shown to induce WGD in two *Limonium* species when applied between one and three DAP. If applied later, however, e.g., 4-6 DAP, the progeny could be mixoploid or diploid (Figure 2-1 and Figure 2-2). These effects of timing agreed with those reported for rye (*Secale cereale*; Gordej et al. (2019)). Thus, when N_2O affects the mitotic spindle later in zygote development, only mixoploid or diploid progeny are obtained (Figure 2-1 and Figure 2-2). We interpret this as being due to cell divisions in multi-celled zygotes not being synchronous. As only dividing cells can be affected by the treatment, the result is a mix of diploid and tetraploid cells within the zygote.

While a genotype-specific effect of induction of WGD has been reported in rye (Gordej et al., 2019) and maize (Molenaar et al., 2018), 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 (Figure 2-1 and Figure 2-2).

In the current study, the total proportion of tetraploid progeny observed in the *L. perezii* experiment (35%) was similar to that reported for wheat (>30%; Hansen et al. (1988); *L. sinuatum* with only one tetraploid progeny was excluded from this comparison). We are confident that N_2O treatment is effective for the induction of 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 by Van de Peer et al. (2017) through 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 in *L. sinuatum* increased between 1.2 and 1.6 times in the tetraploid plants in comparison with their diploid counterparts (Figure 2-3). The increased organ size noted in this research corroborates the “gene dosage effect” (Birchler & Veitia, 2012) and findings reported in other *Limonium* species, such as *L. bellidifolium* (Mori et al., 2016), and other crops, such as *Phlox amabilis* (Chansler et al., 2016), *Gerbera*

hybrida (Bhattarai et al., 2021) and *Brassica* (Kumar & Yadav, 2020). In the case of pollen size, the increase in size with increased ploidy here in *L. sinuatum* agrees with previous reports on *L. sinuatum* (Siregar, 2021), but contrasts to *L. ovalifolium* and *L. multiflorum*, which did not display the direct ploidy-pollen size relation observed in our study (Róis et al., 2012).

The consistency of the morphological changes observed in polyploid plants provides a simple screening protocol to minimize the number of plants that require testing using flow cytometry to confirm ploidy status. Although all the morphological variables evaluated, except stomata density, showed a direct positive association with ploidy, because of the ease of measurement, measuring pollen size and guard cell length offers the potential to be used for pre-selection of polyploid plants in *L. sinuatum*, hence reducing the resource costs within a breeding programme.

CHAPTER 3. Induction of Whole-genome duplication (WGD) in *Limonium perezii* using nitrous oxide (N_2O)

As detailed below, this chapter comprises experiments that have been presented orally at one of two conferences to my scientific peers. As such, in comparison to other chapters the reader may note some differences in writing style and detail, reflecting the particular requirements of the conference, and the targeted audience.

Details of the conferences are:

Córdoba J., Roskruge N., Funnell K, Hedderley D, Morgan E. Nitrous oxide (N_2O) and ploidy changes in a model crop. Plant Science Central Conference. Palmerston North, New Zealand. 6th – 8th July 2021.

Córdoba J., Roskruge N., Funnell K, Hedderley D, Morgan E. Uso del óxido nitroso (N_2O) para la inducción de cambios en la ploidía de un cultivo modelo. XIII Simposio Internacional de Recursos Genéticos para las Américas y el Caribe. Colombia. 30th November-3rd December 2021.

3.1. Introduction

Whole Genome Duplication (WGD) is the process of producing organisms with double number of chromosomes and can occur in nature or may be induced. In nature, four significant WGD events has been identified during the evolution and diversification of angiosperms (Van de Peer et al., 2017; Zhang et al., 2020), while in *Limonium* natural polyploids have occurred at least twice (Brullo, 1980; Evliyaoglu et al., 2008; Róis et al., 2012). Hence while WGD does occur naturally, its frequency is perhaps less than may be desired within breeding programmes. The impact of WGD as a driver of plant germplasm diversity is well documented (Chansler et al., 2016; Dewitte et al., 2012) and underlies the interest of plant breeders to include WGD in breeding programmes. As consequence, the generation of synthetic polyploids have been induced using mutagens such as nitrous oxide (N_2O), colchicine or oryzalin (Carvalho et al., 2016; Gordej et al., 2019).

The use of N_2O as a spindle disruptor during meiosis has advantages in comparison with colchicine, as its effect on treated plants is not long lasting (Hansen et al., 1988; Kato & Geiger, 2002), and no documented risk to human health. Treatment with N_2O of zygotes/proembryos aims to disrupt the mitotic spindle in the cells during the first embryonic divisions, e.g., between establishment of the apical-basal axis and the 32-cell pro-embryonic stage (refer Section 1.3.3), to increase the probability of producing

tetraploids and decreasing the probability of producing mixoploids or chimeras (Kato & Geiger, 2002). In *Limonium* these cellular divisions occur between 2 hours and 72 hours after pollination (Zhang (1995); refer Section 1.3.3) with zygotes occurring between 0 and 2 hours after pollination (HAP), proembryos between 2 and 72 HAP, and embryos after 72 HAP (refer Section 1.3.3). Treating proembryos would ideally be targeted so as to decrease the probability of producing mixoploid plants. Previous studies utilising wheat (*Triticum aestivum*) (Hansen et al., 1988) and lilium (*Lilium × formolongi*; Sato et al. (2010)) reported that the optimal time of treatment with 608 kPa of N_2O ranged between 24 h (Hansen et al., 1988) and 72 h (Sato et al., 2010) for young embryos (i.e., between 5 to 13 DAP), providing an initial range for treatment timings in the current study.

In this experiment, the time elapsed between the controlled pollinations and the N_2O treatment was decreased compared to the experiments presented in CHAPTER 2. The objective was to determine a smaller time window that optimised the WGD by treating proembryos as young as possible when a higher number of cells are undergoing their first mitotic divisions. The hypothesis was that treating proembryos younger than 80 h (~3 days) with N_2O produces a higher proportion of polyploids and a lower proportion of mixoploids.

3.2. Materials and methods

3.2.1. Plant material and cultivation

Two *L. perezii* genotypes (Y19.027.001 and Y19.027.002) were used in this experiment. The genotypes were selected based on their contrasting stigma type, synchronous flowering times, and zygote success higher than 50% in intraspecific crosses (APPENDIX A).

Plants were grown at PFR in Palmerston North, New Zealand (40.9006 °S, 174.8860 °E) in individual 10 L pots containing a commercial growing medium (APPENDIX E) and irrigated using drippers (8.5 L/h, PC Low CNL Netafim™, Hatzerim, Israel) controlled so as to maintain the medium at field capacity.

Plants were kept under greenhouse conditions with heating to maintain a minimum of 16 °C and ventilation starting at 18 °C and natural day length equal to 12 h between 1st and 4th March, 2021 when the experiment was conducted. Flower emasculation was not required as *L. perezii* is self-incompatible (Baker, 1953a).

3.2.2. Experimental design

The experimental design consisted of a 2 × 4 factorial arrangement (Compton 1994) of treatment factors; seed-bearing genotype (mother) (i.e., Y19.027.001 or Y19.027.002) and the time elapsed between pollination and when the N_2O treatment was applied (i.e., 8.5, 32.5, 56.5, or 80.5 h; HAP).

Controlled pollinations were performed manually by rubbing one dehiscent anther from the pollen-donor to the stigma of a newly opened floret from the seed-bearing genotype. Depending on the number of opened florets available daily, between 12 and 22 individual florets (replicates) were pollinated daily at the same time every day (i.e., 9:30 am) for up to four days with each floret pollinated just once. Treatment with N_2O started 8.5 h after the most recent crosses completed (i.e.,y, 6:00 pm 4th day of crosses).

The experiment was conducted as a completely randomized design, wherein each genotype served as both a seed-bearing (mother) and pollen-donor (father). The experimental unit corresponded to each pollinated floret, each with just one possible zygote.

Four response variables were evaluated:

- zygote success (number of zygotes obtained divided by the number of crosses completed). Zygote success was evaluated 30 days after pollination when the embryo rescue was done.
- ploidy of seedlings derived,
- proportion of polyploids (i.e., number of tetraploids and pentaploids seedlings produced divided by number of seedlings obtained (excluding mixoploids)),
- polyploidization rate (i.e., total number of polyploid seedling obtained in the experiment divided by the total number of seedlings produced).

Ploidy determination of the seedlings was performed as reported in CHAPTER 2, comprising the application of flow cytometry.

3.2.3. Whole genome duplication

L. perezii plants with hand-pollinated florets were treated with N_2O in a pressure tolerant cylinder for 24 h, at 608 kPa (Barba-Gonzalez et al., 2006). After treatment, plants were moved back to the above mentioned greenhouse conditions, with embryo rescue of the pollinated florets with developing embryos conducted four weeks after pollination (CHAPTER 2; Cordoba-Sanchez et al. (2023a)). This experiment did not include control plants (i.e., plants not treated with N_2O).

3.2.4. Data analysis

The statistical software package R was used for data analysis (R Core Team, 2018) and ggplot 2 (Wickham 2016) utilised for producing the resulting graphics. The response variables zygote success and proportion of polyploids were analysed using a generalized linear model (glm function from the stats package (R Core Team 2018)) with the binary distribution used to model the effects of seed-bearing genotypes (mother) and HAP. Comparison between the levels of the factors was conducted with pairwise likelihood ratio tests (similar to LRPAIR in (Goedhart, 2018)). The *p-value* for determining a treatment effect was set as 0.05 in all cases.

3.3. Results

Treatment with N_2O for 24 h of proembryos of both genotypes of *L. perezii,* induced WGD in the resulting seedlings when applied between 8.5 and 80.5 HAP (Figure 3-1), with mixoploids also appearing for Y19.027.002 at 80.50 HAP. The proportion of polyploids was between 28% and 63% with no differences detected across HAP (Figure 3-2B) or seed-bearing genotypes ($p>0.05$).

The total proportion of polyploids obtained in this experiment for *L. perezii* was between 42% and 22% for Y19.027.001 and Y19.027.002, respectively (Table 3-1).

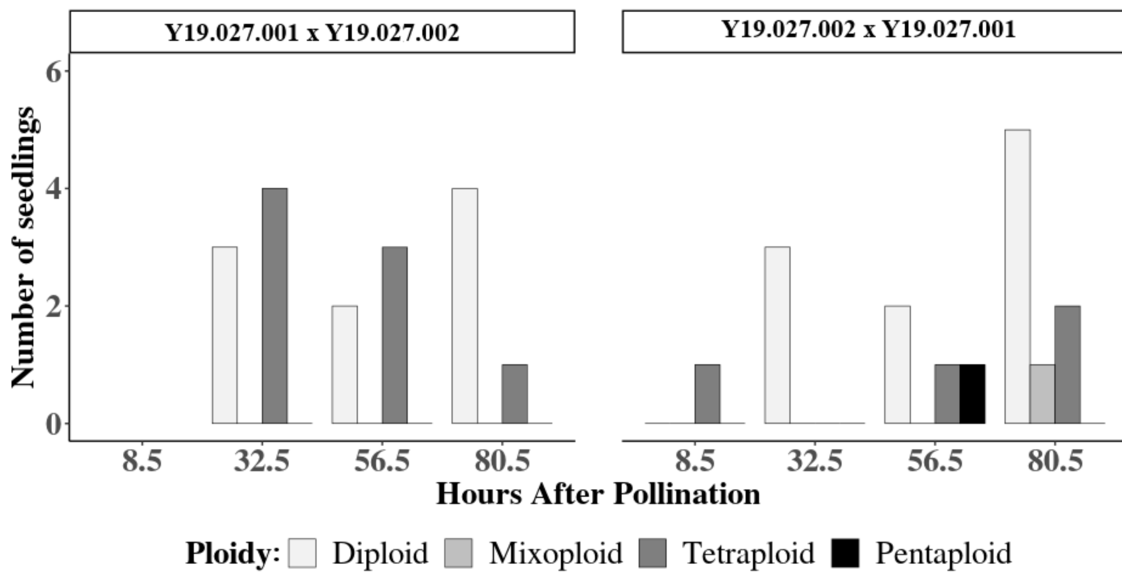

Figure 3-1 Number of diploid, tetraploid, pentaploid, or mixoploid seedlings obtained in two crosses between *Limonium perezii* genotypes for each Hour After Pollination (HAP) when the nitrous oxide (N_2O) treatment was applied

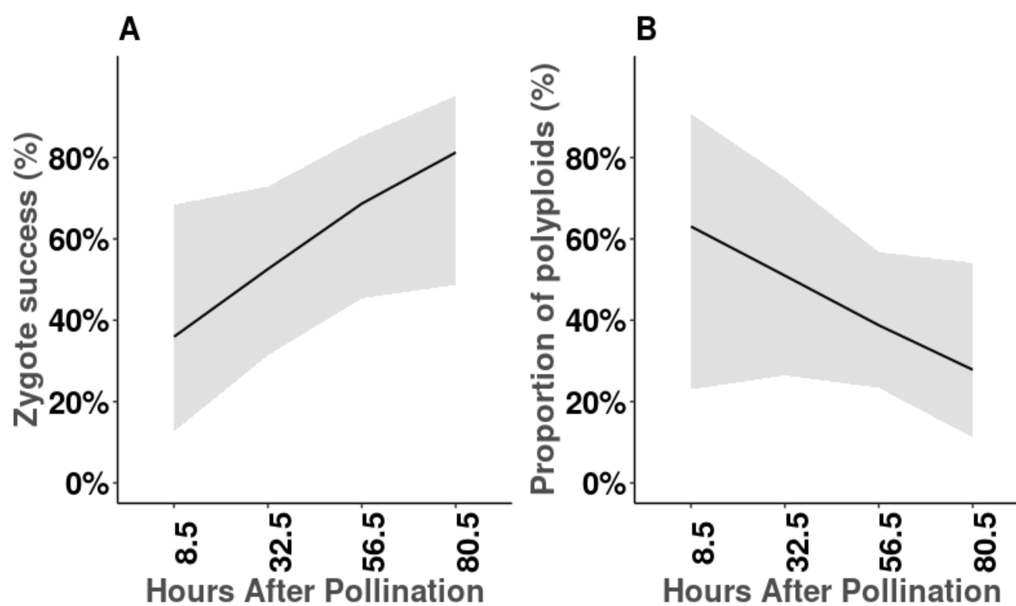

Figure 3-2 Predicted probabilities after data modelling with a generalized linear model (glm) **A.** Zygote success (number of zygotes obtained divided by the number of crosses completed) and **B.** proportion of polyploids (number of polyploids obtained divided by number of seedlings obtained (excluding mixoploids)) pooled for both *Limonium perezii* genotypes (i.e., Y19.027.001 or Y19.027.001) for each Hour After Pollination (HAP) when the nitrous oxide (N_2O) treatment was applied. Grey strip represents 95% confidence interval

The zygote success was similar across genotypes ($p>0.05$) but increased from 36% to 81% with the time elapse (i.e., HAP) between pollinations and the N_2O treatment ($p<0.05$; Figure 3-2A).

3.3.1. Comparison between experiments

Comparison between the results of WGD in *L. sinuatum* and *L. perezii* using N_2O 24 h (CHAPTER 2 and present chapter) or 48 h (APPENDIX F; Table 3-1) allowed for the determination of an increase in the polyploidization rate when the time elapsed between pollination and treatment with N_2O decreases from 10 DAP to 3 DAP (i.e., 80.5 HAP). Analysis of the number of mixoploid seedlings produced did not show a trend with values different from zero for both treatment durations and time windows between pollination and treatment with N_2O (i.e., DAP or HAP).

Table 3-1 Polyploidization rate and proportion of polyploids and mixoploids by genotype, species (*Limonium sinuatum* or *Limonium perezii*), duration of the treatment with nitrous oxide (N_2O), and time elapsed between pollination and treatment

Genotype	Species	Treatment duration (hours)	Time elapsed between pollination and N2O treatment (DAP†)	Proportion of polyploids obtained (%) ‡ (total number of seedlings)	Proportion of mixoploid seedlings (%)	Polyploidization rate‡ (%)	Reference
Y19.015.001 (sin15)	*L. sinuatum*	48	0-10	1.0 (10)	0.0	0.4	APPENDIX F
Y19.016.001 (sin16)	*L. sinuatum*	48	0-10	1.5 (68)	2.9	0.4	APPENDIX F
Y19.016.001 (sin16)	*L. sinuatum*	24	2-8	2.0 (49)	4.1	1.4	CHAPTER 2
Y19.018.001 (sin18)	*L. sinuatum*	24	2-8	0.0 (45)	2.2	0.0	CHAPTER 2
Y19.001.001 (per11)	*L. perezii*	24	1-7	33.3 (3)	0.0	1.4	CHAPTER 2
Y19.027.003 (per273)	*L. perezii*	24	1-7	36.4 (11)	27.3	5.7	CHAPTER 2
Y19.027.001	*L. perezii*	24	8-80§	47.1 (17)	0.0	11.0	CHAPTER 3
Y19.027.002	*L. perezii*	24	8-80§	31.2 (16)	6.2	7.3	CHAPTER 3

† Days after pollination

‡ Mixoploid plants were excluded from the calculation

§ Hours after pollination

3.4. Discussion

In the current study, treating proembryos younger than 80 h (~3 days) with N_2O for 24 h resulted in an increase of the proportion of polyploids and polyploidization rate in comparison with proembryos treated older i.e., starting at 2 DAP (~48 h) (Table 3-1). A possible explanation is that treating proembryos as young as 8.5 HAP resulted in the disruption of the mitotic spindle during the first embryonic divisions (Kato & Geiger, 2002) without adversely compromising them.

The impact of N_2O treatment on zygote survival (i.e., zygote success) was observed. The 24 h treatment resulted in a lower impact and higher zygote success (36% at 8.5. HAP) compared to 48 h treatment, which resulted in less than 9% embryo success before 1 DAP (24 HAP) (APPENDIX F). Then, the embryonic divisions demonstrated resilience, with the ability to either proceed without arrest or recover after arrest (Komaki & Schnittger, 2017). As a result, the proportions of polyploids increased (Table 3-1).

As evidenced here by comparing the results of inducing WGD with N_2O for 24 h (this chapter and CHAPTER 2) and 48 h (APPENDIX F) there is an inextricable link between the time interval between the pollination and the treatment with N_2O and the time of exposure to the N_2O. Longer time exposures (48h vs 24h) require zygotes to be older to be able to survive i.e., longer time intervals between the planned pollination and the N_2O treatments. In *Lilium*, for example, 72h N_2O treatment induces tetraploids from 9 and 13 DAP (Sato et al., 2010), 48h N_2O treatment in *L. sinuatum* results in tetraploids between 3 and 5 DAP (Figure A-5), and 24h N_2O treatment in *L. perezii* produces tetraploids between 8 HAP (< 1DAP) and 80 HAP (~ 3DAP; Figure 3-1).

Comparison between Figure 3-2A and Figure 3-2B confirmed that as the time elapse between pollination and treatment with N_2O increases, the zygote success increases and the proportion of polyploids decreases. The no significant effect of HAP over the proportion of polyploids ($p<0.5$) was unexpected and could be attributed to the number of seedlings obtained (i.e., between 0 and 8) more than to a biological effect. The increase in zygote success and decrease in proportion of polyploids could be associated with the embryo development with older proembryos being more tolerant to the mitotic disruption resulting in higher survival. As the proembryos develop, more cells are originated (Figure

1-15) through asynchronous mitotic divisions (Gooh et al., 2015) meaning that treatment with N_2O would affect only cells under division, but not the whole proembryo. As consequence, the chromosomic constitution of the proembryo is not duplicated resulting in a decrease in proportion of polyploids.

Despite the expected decrease in the proportion of mixoploids by treating proembryos younger than 80 h (~3 days) with N_2O, it was not possible to confirm this hypothesis and the information obtained in the experiments for WGD (Table 3-1) is insufficient to identify the possible reasons or factors involved.

3.5. Conclusions

The use of N_2O for 24-hours in proembryos between 8.5 HAP and 80.5 HAP is advisable to induce WGD in *L. perezii* with a higher number of polyploids produced per number of crosses completed (i.e., polyploidization rate).

CHAPTER 4. Nitrous oxide treatment increases the proportion of viable pollen and pollen size in *Limonium perezii*

This chapter has been submitted for publication and was orally presented in 2023. Citations:

Córdoba J., Roskruge N., Funnell K, Hedderley D, Morgan E. Nitrous oxide treatment increases the proportion of viable pollen and pollen size in *Limonium* perezii. XXVII International EUCARPIA Symposium Section Ornamentals-From Nature to Culture: Breeding Ornamentals for Sustainability. Genoa-Italy. 2nd-5th July 2023.

Córdoba J., Roskruge N., Funnell K, Hedderley D, Morgan E. Nitrous oxide treatment increases the proportion of viable pollen and pollen size in *Limonium* perezii. 2023. Acta Hortic. (accepted).

4.1. Abstract

Interspecific crosses between *L. sinuatum* and *L. perezii* have produced hybrids (*Limonium* siNZii^{TM}) utilized by the ornamental industry. The inclusion of genotypes as parents in the breeding programme depends, among other factors, on their fecundity. Two *L. perezii* genotypes, per11 and per275 with comparatively low male fertility (zygote success <40% ± 16% for both genotypes when used as pollen donors in intraspecific crosses), were selected for this study. We had observed that pollen viability and the number of zygotes obtained from the two genotypes improved after nitrous oxide (N_2O) treatment. The aim of this study was to evaluate and to understand the effect of N_2O on pollen viability, pollen size, and number of zygotes produced per number of crosses completed (zygote success) when used as pollen parents following treatment of immature inflorescences. The proportion of stained (viable) pollen increased up to 1.7 times in per11 and up to three times in per275; the size of pollen grains also increased between 1.2 and 1.5 times in both genotypes; and the zygote success was 20% higher when per11 was used as a pollen parent. These findings support the hypothesis that the improvement in zygote success following N_2O treatment is associated with an improvement in pollen viability, and an increase in pollen size. It is proposed that, when pollen viability is an issue, changes in proportions of pollen stained (viable) and size can be used to identify the optimum stage of inflorescence development to apply N_2O treatment for best pollen viability and higher zygote success.

4.2. Key Words

ornamental plants, sea lavender, statice, modified Alexander's stain, aborted pollen.

4.3. Introduction

Limonium Mill, commonly known as 'sea lavender' or 'statice', is commercialized mainly as a cut flower and demand for novelty drives numerous breeding programmes. Breeding of *Limonium* at The New Zealand Institute for Plant and Food Research Limited (PFR) has focused on the production of interspecific hybrids between *L. sinuatum* and *L. perezii*, which has led to hybrids characterized by large panicles, disease tolerance, strong colours, and perennial growth habit (BallSB, 2021). However, the production of interspecific hybrids faces some challenges as low zygote success (number of zygotes produced per number of crosses completed) of genotypes, with otherwise desirable characteristics, and limits their use. We had previously noted lower zygote success in intraspecific crosses when the *L. perezii* genotypes per11 and per275 were used as pollen donors (averaging 40% ± 16% across both genotypes) in comparison with *L. sinuatum* (e.g., sin16 and sin18 with an average zygote success equal of 78% ± 12%; unpublished data). Pre- or post-zygotic barriers, which either prevent or limit pollination, fertilization, and/or zygote success itself (Tonosaki et al., 2016), could be associated with the low zygote success apparent in *L. perezii*.

In other crop species pre-zygotic barriers arise from male sterility and partial-male sterility (Defani-Scoarize et al., 1995; Larrosa et al., 2012). Male-sterility causes phenotypic changes in plants including: no production of pollen, absence of anthers, or small non-dehiscent anthers (Defani-Scoarize et al., 1995). Contrary to male sterility, when partial male-sterility is evident in plants there is a combination of normal and abnormal anthers, with some microsporocytes having normal meiosis and, therefore, producing some viable pollen grains (Akšić et al., 2016; Defani-Scoarize et al., 1995; Tatum et al., 2015). The decrease in the number of viable pollen grains observed in partial male-sterile plants is accompanied by pollen grains of variable sizes with significantly smaller or bigger pollen grains, in comparison to average pollen grains, representing aneuploids or unreduced pollen, respectively (Larrosa et al., 2012). The variability in number of pollen grains and size can be associated with the occurrences of meiotic irregularities (Defani-Scoarize et al., 1995; Larrosa et al., 2012) and/or gametophytic

lethality (Park et al., 1998; Zhang et al., 2018). Meiotic abnormalities can affect up to 88% of meiotic cells in some species (Akšić et al., 2016; Defani-Scoarize et al., 1995) and, as a consequence, impose a variable level of constraint on the production of viable pollen grains (Oleszczuk et al., 2019). Similarly, gametophytic lethality during pollen development could affect a variable proportion of pollen grains (Park et al., 1998). Three ways to measure the production of viable versus aborted reduced gametes (n) are: via recording comparative differences in: seed set (directly related with zygote success; Impe et al. (2020); Oleszczuk et al. (2019)), pollen size (Tatum et al., 2015), and/or presence of cytoplasm as evident from staining (e.g., modified Alexanders' stain; Impe et al. (2020)).

An association between pollen size and ploidy has been reported for crops such as potato (Larrosa et al., 2012) and *Lilium* (Barba-Gonzalez et al., 2006; Nukui et al., 2011), opening the possibility of using pollen size as an indicator of pollen viability and the occurrence of meiotic abnormalities with subsequent chromosome mis-segregation (Tatum et al., 2015). Pollen grains with sizes 1.26 times the species' average are considered as unreduced pollen (2n) (Barba-Gonzalez et al., 2006; Larrosa et al., 2012; Nukui et al., 2011), while a micro-pollen diameter measuring 0.5 times that of the species average is considered as non-viable or aborted (Tatum et al., 2015).

When applied under pressure, nitrous oxide (N_2O) is a spindle disruptor and can be used as a breeding tool for inducing the production of unreduced gametes (Akutsu et al., 2007), hence restoring fertility in sterile hybrids (Barba-Gonzalez et al., 2006). Based on observations within our studies with *Limonium*, N_2O increased the zygote success of genotypes with low male fertility. With the aim of understanding the factors contributing to, or associated with this improvement, the present study investigated the effect of N_2O on pollen viability, size, and zygote success, when applied to immature inflorescences of genotypes of *L. perezii* with low male fertility. The research hypothesis was that treatment of young unopened florets with N_2O would increase the proportion of viable pollen and pollen grain size, with a subsequent improvement in zygote success.

4.4. Materials and methods

Plants of *L. perezii* genotypes per11 and per275 and *L. sinuatum* genotypes sin16 and sin18 were grown in 2 L plastic pots with a commercially sourced, bark-based growing medium. Plants were grown under greenhouse conditions at PFR in Palmerston North, New Zealand (40.9006 °S, 174.8860 °E), with air temperatures set at 18°C for heating and 21°C for venting, and natural photoperiod of 12 h in March 2021, when the experiment was conducted.

All four genotypes, developing inflorescences comprising unopened florets no wider than 0.8 mm and with their calyces protruding ca. 2 mm above the bract (a developmental stage previously identified as having a higher number of pollen mother cells undergoing meiosis (Siregar, 2021), were treated in a pressure-tolerant cylinder with N_2O for 24 h at 608 kPa (Nukui et al., 2011). The plants were subsequently transferred back to the greenhouse where the untreated plants remained. Daily, starting at 13 days after treatment (DAT) until 30 DAT (a time frame previously identified as having more meiotic cells affected by N_2O treatment), planned interspecific pollinations using *L. sinuatum* sin16 and sin18 as the seed-bearing genotypes with compatible pollen types were conducted using both per11 and per275 as the pollen-donor genotypes. At the same time pollen was collected for microscopic observation from both per11 and per275, comprising three florets from different inflorescences, with recently dehiscing anthers. As a control, pollen from untreated plants was collected on five specific days (17, 18, 22, 23 and 27 relative to the period extending from 13 DAT to 30 DAT), and either used in planned interspecific pollinations or collected for microscopic observation using a Zeiss microscope Imager.Z2 (Carl Zeiss Microscopy, Axio Imager Z2, Germany).

On each DAT the pollen collected from each of the three florets was immediately mixed and stored in a fixation solution comprising of 85% ethanol and 100% glycerol in a 3:1 proportion (Hammer et al., 2015; Hammer et al., 2011). For microscopic observations, sub-samples were prepared by adding 10 µL of modified Alexander's stain (Peterson et al., 2010) to 30 µL of the solution containing the mixed pollen. For each DAT, 100 pollen grains were photographed from both genotypes, and their plan area measured using Fiji software (Schindelin et al., 2012). For viability assessment, pollen grains stained magenta-red were documented as being viable, while blue-green or without staining (i.e.,

brown coloured) and presenting the exine pattern characteristics of the species, were documented as being aborted (Impe et al., 2020; Peterson et al., 2010).

The experimental design was completely randomized (Compton, 1994) with treatments consisting of DAT when pollen was either collected or used for crosses (from 13 DAT to 30 DAT) or their equivalent for untreated (control) plants (days 17, 18, 22, 23, 27). For the pollen analysis the experimental unit was one pollen grain with 100 replicates per treatment. In the case of zygote success, the experimental unit was each pollinated floret, and the replicates were the daily pollinated florets (i.e., 10). Each genotype (i.e., per11 and per275) was analysed separately, as initial analysis showed considerable differences between both. The response variables evaluated were pollen viability, pollen plan area and zygote success defined as the number of zygotes obtained two weeks after pollinations divided by the number of crosses completed.

Data analysis was conducted using the software R (R Core Team, 2018) with ggplot 2 (Wickham, 2016) utilized for graphic outputs. The response variable, plan area, was analysed with linear models (lm function from the stats package R (R Core Team, 2018)) followed by a Least Significant Difference (LSD; Mendiburu and Yaseen (2020)) test to detect pairwise differences between means. For the proportion of viable pollen and zygote success, a generalized linear model (glm function from the stats package (R Core Team, 2018)) with a binary distribution was used. A *p*-value of 0.05 was utilized for all analyses.

A receiver operating curve (ROC curve; Kumar and Indrayan (2011)) was calculated to identify a threshold plan area to discriminate between aborted and viable pollen collected between 13 and 30 days after treatment with N_2O, or the equivalent day in untreated (control) plants. The threshold was selected based on the highest sensitivity (i.e., probability of classifying a viable pollen grain as viable) and specificity (i.e., probability of classification an aborted pollen grain as aborted) possible for the data set.

4.5. Results and discussion

In the present study, the proportion of viable pollen sampled from plants of *L. perezii* within the control treatment was lower (29% ± 45% for per11 and 32% ± 47% for per275) in comparison with plants treated with N_2O (36% ± 48% for per11 for florets 20 DAT and 84% ± 45% for per275 for florets 14 DAT) (Figure 4-1A-B; $p<0.05$), being indicative

of the occurrence of pre-zygotic barriers normally operating, i.e., partial-male sterility (Figure 4-1). The low proportion of viable pollen in the control plants could be responsible for the differences in zygote success within the current study, with interspecific crosses between sin18 × per11 achieving a zygote success of 2.5% ± 5% for the control crosses, versus 22% ± 32% for N_2O-treated plants (Figure 4-1D). Intraspecific crosses using these genotypes of *L. perezii* as a pollen donor, achieved an average zygote success of 40% ± 16%, which was low when compared with *L. sinuatum* (78% ± 12%) (unpublished data). However, when per275 was a pollen donor in interspecific crosses (i.e., sin16 × per275), the zygote success in control crosses (88% ± 10%) was higher than in N_2O-treated plants (66% ± 20%) (Figure 4-1C; $p<0.05$) or in intraspecific *L. perezii* crosses. The increase in pollen viability observed in per11 after N_2O treatment was lower in comparison with per275 and below 50% could also suggest the occurrence of other factors, besides meiotic abnormalities, affecting pollen viability i.e., gametophytic lethality. The low proportion of viable pollen in plants within the control treatment, in comparison with plants treated with N_2O, is interpreted as suggesting the occurrence of partial-male sterility in *L. perezii* (i.e., per11 and per275) and contributes to explaining the differences in zygote success (Akšić et al., 2016; Defani-Scoarize et al., 1995; Oleszczuk et al., 2019) between *L. perezii* and *L. sinuatum* in intraspecific crosses, and between control and treated per11 plants in interspecific crosses. Similar results have been reported for *Zea mays* (Defani-Scoarize et al., 1995), *Prunus cerasus* (Akšić et al., 2016), and triticale (Guerra et al., 2013), with a low proportion of viable pollen representing partial male-sterility, and resulting from meiotic irregularities (Akšić et al., 2016; Defani-Scoarize et al., 1995; Larrosa et al., 2012).

The frequency of meiotic irregularities determines the proportion of abnormal microsporocytes and of aborted pollen (Akšić et al., 2016; Defani-Scoarize et al., 1995; Guerra et al., 2013). Therefore, if normal meiosis is restored or at least the number of cells undergoing abnormal meiosis decreases, the proportion of viable pollen could be potentially increased, as observed in the present study after treatment of *L. perezii* florets with N_2O in the pre-meiotic or meiotic stage. Application of N_2O increased the proportion of viable pollen in both genotypes ($p<0.05$; Figure 4-1) when compared with the respective controls. With the genotype per275, the application of N_2O increased the proportion of viable pollen from 32% for the control, with rather higher values from 15

DAT onwards, including up to 96% ± 27% on 29 DAT (Figure 4-1A). For per11, in comparison with the control, the positive effect of N_2O on pollen viability was observed after 21 DAT, with a maximum viability of 47% ± 50% at 25 DAT (Figure 4-1B). Unlike per275 however, not all sample periods after 20 DAT resulted in a significant increase in pollen viability compared with the control (Figure 4-1A).

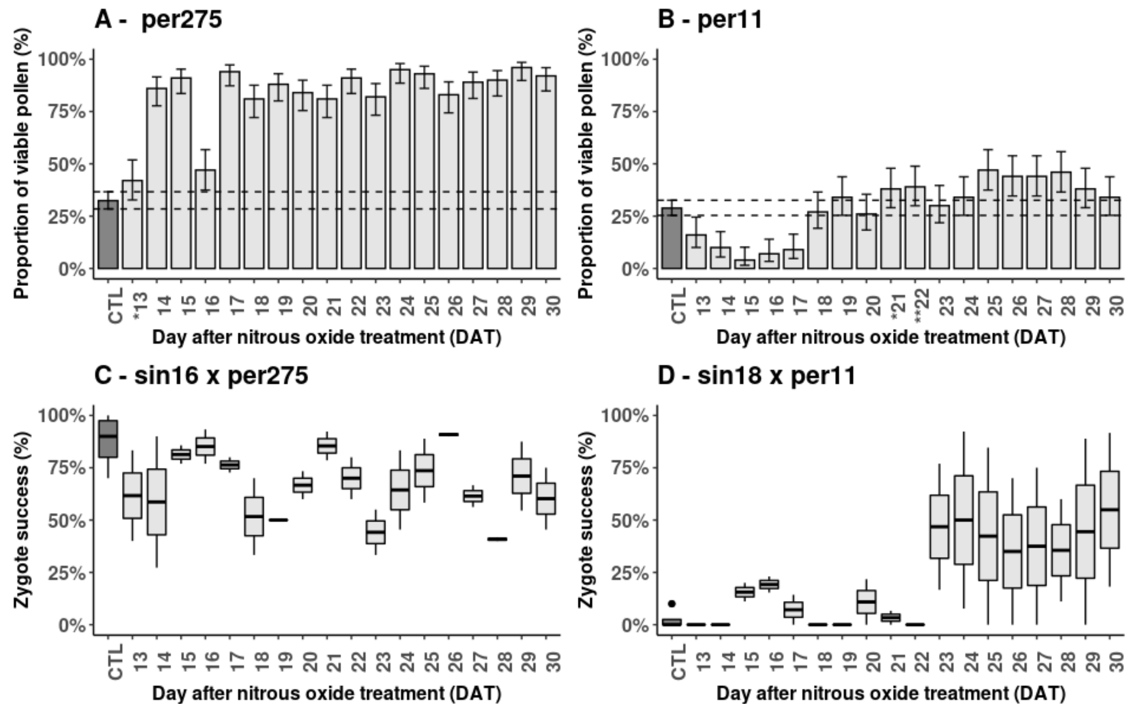

Figure 4-1 Proportion of viable pollen (%) (A and B) and zygote success (%) when pollen of treated plants were used in interspecific crosses (C and D) observed in two *Limonium perezii* genotypes per275 (A and C) and per11 (B and C)) at different times after treatment with nitrous oxide (N2O) and in untreated plants (CTL). The data presented for the untreated plants represent five specific days (17, 18, 22, 23 and 27) relative to the period extending from 13 DAT to 30 DAT. In A and B, columns represent the predicted mean values for the proportion of viable pollen. Vertical lines (error bars) represent the 95% confidence interval. Horizontal dashed lines limit the confidence interval of the proportion recorded from plants within the CTL. Error bars of the treated plants that overlap with the error bars from CTL indicate no statistical differences at $p<0.05$; n=100 pollen grains. * p-value = 0.06, ** p-value= 0.04. In C and D, the box represents the interquartile range, the horizontal line is the median, and the whiskers indicate the minimum and maximum values. The single point is an extreme datum. n=10 planned crosses for the treated plant and 5 planned crosses per day for the untreated plants

The aborted pollen identified in this study was significantly smaller in plan area than viable pollen ($p<0.05$), with average plan areas between 1200 μm2 ± 352 μm2 (per11)

and 1425 µm2 ± 456 µm2 (per275); in comparison, the plan area of viable pollen was between 2114 µm2 ± 463 µm2 (per11) and 2228 µm2 ± 384 µm2 (per275) (Figure 4-2). The ROC curve confirmed that it was possible to distinguish aborted and viable pollen based on its plan area, with a threshold equal to 1760 μm^2 for per275 (80% sensitivity and specificity; Figure 4-2A and Figure 4-2C) and 1650 μm^2 for per11 (95% sensitivity and specificity; Figure 4-2B and Figure 4-2D). The hypothesis that aneuploid pollen containing fewer chromosomes than the haploid number (Tatum et al., 2015) may be the result of meiotic abnormalities with subsequent chromosome mis-segregation (Oleszczuk et al., 2019; Tatum et al., 2015), is supported here by the reduction in the average pollen plan area between 56% (per11) and 64% (per275). Micro-pollen with a plan area less than 1000 µm2 was also observed for both genotypes for all DATs, but mainly on D15 and D16 for per11. Aneuploid pollen as well as micro-pollen are indicative of meiotic abnormalities occurring (Oleszczuk et al., 2019; Tatum et al., 2015), and while in the current study their presence is inferred, the associated lower zygote success noted for per11 (Figure 4-1B) is similarly reported as lower seed set in other crop species (Vleugels et al., 2019). However, further cytological studies are required to elucidate the chromosome constitution of the smaller pollen grains in *L. perezii*, and the possible associated meiotic abnormalities.

The mechanism of action of N_2O on the pre-meiotic or meiotic stage cells in *Limonium* is still unknown, but in *Lilium* N_2O induced meiotic restitution (Barba-Gonzalez et al., 2006) or mitotic polyploidization (Nukui et al., 2011) with consequent production of gametes with double the number of chromosomes (2n) (Barba-Gonzalez et al., 2006; Oleszczuk et al., 2019). As a result of doubling the chromosome number, pairing between homologous chromosomes was restored in *Lilium* (Barba-Gonzalez et al., 2006), and more viable pollen was produced, resulting in increased seed set in triticale (Oleszczuk et al., 2019) and also here in per11 (i.e., a 20% increment in zygote success after N_2O treatment) (Figure 4-1B). Given the increase in pollen viability (Figure 4-1) and size (Figure 4-3) observed following N_2O application, it is hypothesized that a similar mechanism could be occurring in *L. perezii*. The variable effect between DAT and both genotypes confirms the asynchrony of meiosis, with not just intra-genotype differences, but also inter-genotype differences, with cells likely presenting mixed meiotic stages when N_2O treatment was applied, as previously suggested for *L. sinuatum* (Siregar, 2021).

Nevertheless, for the purpose of developing a standardized protocol to increase the proportion of viable pollen and zygote success, targeting pollen collected from 21 DAT could be recommended for both genotypes (Figure 4-1).

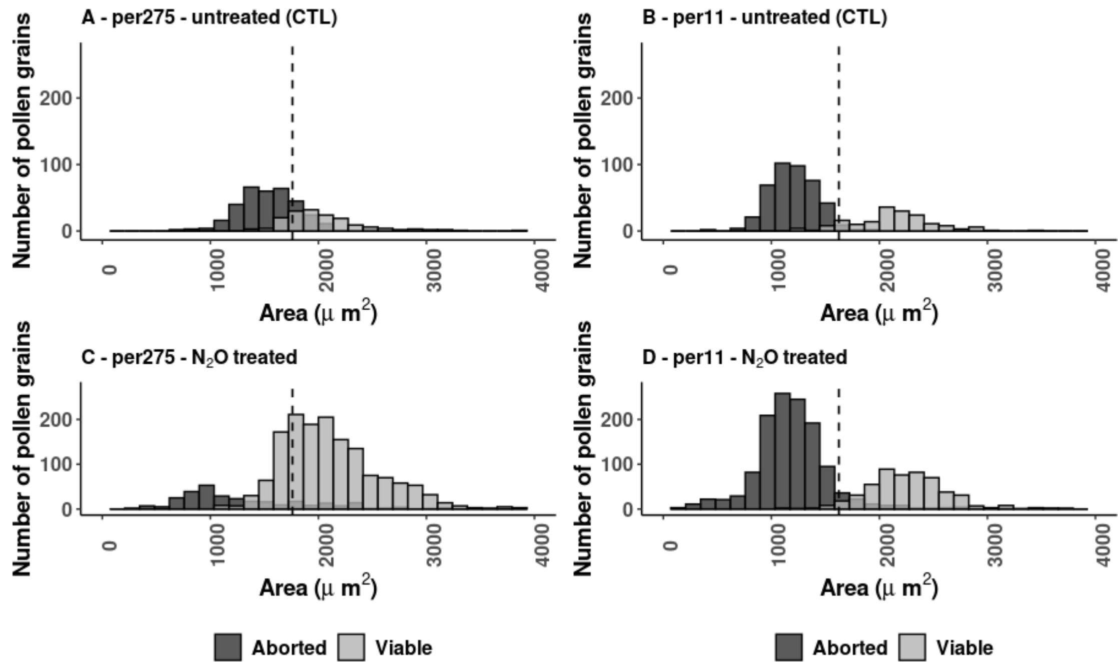

Figure 4-2 Plan area (μm^2) and number of viable and aborted pollen grains observed in untreated (CTL; **A** and **B**) and nitrous oxide (N_2O) treated (**C** and **D**) plants in two *Limonium perezii* genotypes: per275 (**A** and **C**) and per11 (**B** and **D**). Pollen was collected between 13 and 30 days after the treatment with N_2O or its equivalent in untreated plants. Dotted line represents the threshold for differentiation between aborted and viable pollen based on the pollen plan area. Based on the Receiver Operating Curve (ROC curve), for untreated and treated plants in per275 the proportion of true positives (i.e., viable pollen classified as viable) and true negatives (i.e., aborted pollen classified as aborted), which are correctly classified by the threshold (sensitivity and specificity), were both over 0.8; for samples from untreated and treated plants in per11 the threshold was 0.9. Plan area overlapped between aborted and viable pollen

The plan area of pollen, revealed a consistent trend from 21 DAT, with pollen grains of plants treated with N_2O being between 1.2 and 1.5 times larger than the mean plan area of pollen from untreated plants ($p<0.05$; Figure 4-3; an exception to this trend occurred at both 24 DAT and 29 DAT for per11 where no differences in the plan area of pollen from treated and control pollen was evident). In contrast, no trend was evident in pollen plan area when viable and aborted pollen (Figure 4-3) were analysed separately, although N_2O treatment also increased pollen size in pollen in both categories (data not shown).

The increase in pollen size observed in this study could be associated with the occurrence of unreduced pollen (2n), with the doubled number of chromosomes, in comparison with reduced (n), i.e., “normal” pollen (Barba-Gonzalez et al., 2006; Larrosa et al., 2012; Nukui et al., 2011). However, the variation in magnitude of the size increase (i.e., between 1.2- and 1.5-fold) could also suggest that chromosome duplication was not always complete, i.e., some pollen grains could also have one or more additional chromosomes, but not a complete chromosome set (Oleszczuk et al., 2019). Further cytological studies are still pending to confirm this hypothesis.

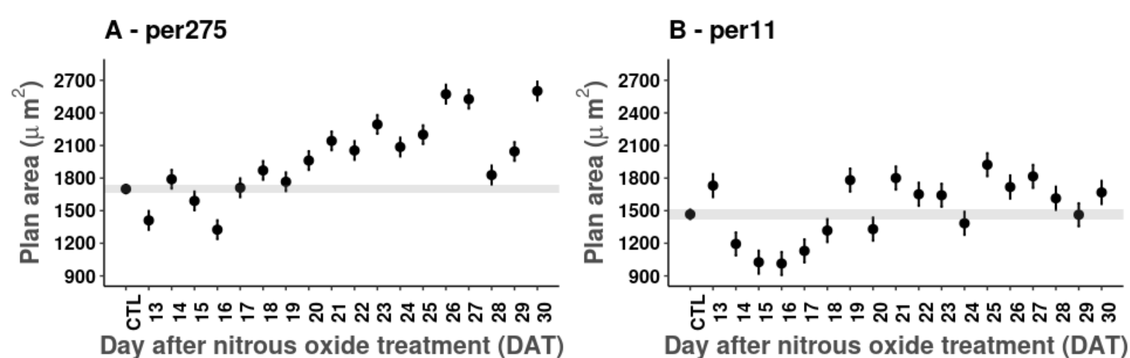

Figure 4-3 Plan area (µm2) of pollen grains collected from two *Limonium perezii* genotypes per275 (**A**) and per11 (**B**)) at different times (days) after treatment with nitrous oxide (N_2O) and in untreated plants (CTL). Dots represent the predicted mean values for pollen plan area after data modelling using a linear model. Vertical lines (error bars) represent the 95% confidence interval. Grey area indicates the 95% confidence interval for pollen collected from plants within CTL treatment. Error bars of the treated plants that overlap with the error bars (or grey area) from the CTL indicate no statistical differences $p>0.05$, n = 100 pollen grains

The increase in the proportion of viable pollen has implications for the reproductive performance of the genotype, in this case as pollen donor, with e.g., increased zygote success as observed for per11 and other crops, such as *Prunus cerasus* and *Zea mays* (Akšić et al., 2016; Defani-Scoarize et al., 1995). However, this also means that the use of N_2O for treating young inflorescences will be successful in improving the reproductive performance of the genotypes with appealing characteristics, but low pollen viability associated with meiotic abnormalities.

4.6. Conclusions

N_2O increased the zygote success following pollination using pollen from genotypes with low male fertility. This improvement is associated with the increase in pollen viability and size observed when immature inflorescences of genotypes of *L. perezii* with low male fertility were treated with N_2O.

When evaluating the effect of N_2O on pollen and optimizing the timing for crosses, we found it best to consider effects on all pollen rather than just viable pollen.

Further cytological studies are required to understand the mechanism(s) giving rise to the effect of N_2O treatment on male meiosis in *L. perezii* genotypes with low male fertility.

CHAPTER 5. Induction of unreduced pollen in *Limonium* using nitrous oxide (N_2O) and production of sexual polyploids

Part of this chapter will be submitted for publication in a peer reviewed journal. As such, in comparison to other chapters the reader may note some differences in writing style and detail, reflecting the requirements of the particular journal, and the targeted audience for that journal.

Details of the coming publication:

Cordoba-Sanchez, J., Funnell, K., Hedderley, D., Roskruge, N. Morgan, E. 2023. Induction of unreduced pollen in *Limonium* using nitrous oxide (N_2O) and production of sexual polyploids (under PFR's internal review)

5.1. Abstract

Interspecific hybrid combinations in *Limonium* breeding present challenges associated with a low hybridisation rate and hybrid sterility. The purpose of this study was to increase the frequency of unreduced pollen (2n) in both *L. sinuatum* and *L. perezii* and then evaluate the potential of 2n pollen for producing sexual polyploids, in both intra- and interspecific crosses. Nitrous oxide (N_2O) was used for the induction of unreduced pollen. *Limonium* florets treated between 23 and 30 days before anthesis exhibit a twofold higher likelihood of producing unreduced pollen compared to those florets treated either before 23 days or after 30 days before anthesis. Nitrous oxide treatment did not affect the proportion of viable (stained) pollen. In flowers of *L. sinuatum* plants crossed 23 to 30 days after treatment (DAT) and embryo cultured 14 days after pollination, 58% of the crosses resulted in diploid seedlings (81 seedling/139 crosses). In contrast, intraspecific crosses performed in *L. sinuatum* 15 or 40 DAT resulted in one triploid and one tetraploid seedling i.e., 5% of the crosses resulted in polyploids. In *L. sinuatum* × *L. perezii* interspecific crosses resulted in 5% diploid hybrids (44 seedlings/825 crosses), with no triploid nor tetraploid hybrids produced. N_2O treatments significantly increased the hybridisation rate in interspecific crosses between *L. sinuatum* and *L. perezii* to 5.3% compared to untreated plants that produced 2.8% hybrid progeny. These results demonstrated that the application of N_2O to immature florets of *L. sinuatum* and *L. perezii* had the potential to produce viable, unreduced pollen capable of producing hybrid

polyploids. Although this unreduced pollen is viable, it is prevented from producing hybrid polyploids.

5.2. Key words

Hybridisation, interspecific crosses, intraspecific crosses, meiotic doubling, pollen size, sexual polyploidization

5.3. Introduction

Limonium is typically grown as a cut flower crop, with ca. Eu$29 million in sales at wholesale auctions in Europe in 2021 (International Association of Horticultural Producers, 2022). There is thus considerable interest in developing new cultivars. Two of the fastest ways of generating variation are interspecific hybridisation and induction of polyploids (Van Tuyl & Lim, 2003). Interspecific crosses between *L. sinuatum* and *L. perezii* have resulted in hybrids which combine disease tolerance, strong colours, unwinged stems, and large panicles (BallSB, 2021). Despite the desire to combine traits from other species, hybrid breeding is frequently limited by low hybridisation rates, hybrid weakness and mortality, and sterility (Chen et al., 2016). As utilized in other breeding programmes, and explored within the current research, some of these limitations can be addressed through ploidy manipulation (Barba-Gonzalez et al., 2006).

In the case of *L. perezii* and *L. sinuatum*, and their genotypes selected in the present study, both were diploid, but with different numbers of chromosomes (Morgan et al., 1998). As a consequence *L. perezii* (2n=2x=14)× *L. sinuatum* (2n=2x=16) hybrids have an odd chromosome number (2n=2x=15) and are sterile (Morgan et al., 1998). Somatic chromosome doubling has been demonstrated to restore fertility in F1 interspecific hybrids in other genera (Zhang et al., 2017) and this was shown to apply in *Limonium* hybrids (Morgan et al., 2001) though this can add significant time to the breeding cycle and limits the number of progeny. An alternative approach is to induce the production of increased numbers of unreduced gametes for use in controlled pollinations (Barba-Gonzalez et al., 2006; Dewitte et al., 2010; Nukui et al., 2011). The use of unreduced gametes for production of polyploid hybrids has the potential to speed the breeding programme as instead of producing hybrids in one breeding cycle and then inducing the duplication of the genome in other breeding cycle, both hybridisation and

polyploidization could be done at once increasing numbers of fertile progeny for evolution of further crossing (Barba-Gonzalez et al., 2006).

Plants produce unreduced gametes at varying rates influenced by genetics and the environment (Sora et al., 2016; Zeng et al., 2020). However, these rates are typically too low, usually less than 4%, and their practical use is species-specific (Gao et al., 2019; Sora et al., 2016; Zeng et al., 2020). *Limonium* is known to produce unreduced gametes, occasionally resulting in polyploid (3x) progeny, but consistent 2n gamete-producing individuals have not been identified (Conceição et al., 2018). It is possible to treat plants to increase the frequency of unreduced gametes e.g., (Luo et al., 2016; Qu et al., 2019). The induction of unreduced gametes is usually focused on the disruption of the meiotic spindle (Akutsu et al., 2007) with a polyploid inductor (e.g., N_2O, trifluralin; Dewitte et al. (2010); Qu et al. (2019)). However, meiosis is not the only cell cycle susceptible to disruption by a polyploid inductor during anther and pollen development. Studies on anther and pollen development in *Arabidopsis* recognized 15 developmental stages, starting with mitotic division of an archesporial cell, followed by multiple mitotic divisions to produce the anther and finishing with meiosis of pollen mother cells (PMC; Gómez et al. (2015)). In *Lilium*, for example, N_2O treatment instead of inducing meiotic polyploidization, induced mitotic polyploidization in the somatic cells of the anthers (Nukui et al., 2011). Similarly, mitotic polyploidization was assumed to have occurred when *Agapanthus* plants with very immature flower buds were treated with N_2O before crossing resulting in multiple triploid progeny (Ed Morgan pers. comm.). There is a cascade of cell divisions that give rise to anther and pollen development (Cai & Zhang, 2018) and there is potential for N_2O treatment to disrupt any of the mitotic or meiotic spindles as cells divide and differentiate. In the current study we have focused on identifying and treating florets undergoing meiosis to induce an increased frequency of unreduced pollen grains. An optimal treatment time for meiotic disruption with N_2O has been proposed based on a previously developed association between floral organ development and progression of meiosis (Akutsu et al., 2007; Siregar, 2021). In *L. sinuatum*, ca. 80% of pollen mother cells (PMC) undergo meiosis in unopened florets with diameters no greater than 0.8 mm and with a calyx protruding no more than 2 mm above the bract (Siregar, 2021).

Ploidy manipulation via production of unreduced gametes could meet challenges associated with interploidy crossing (Morgan et al., 2021). For example, occurrence of the triploid block and the resulting endosperm failure (Lafon-Placette & Köhler, 2016; Morgan et al., 2021) produces hybrid seed abortion or a reduced number of progeny depending the direction of the cross (i.e., unreduced gamete × reduced gamete or vice versa; Carputo et al. (1999); Morgan et al. (2021)). In addition to endosperm failure, pollen competition between reduced and unreduced pollen may also occur as has been reported in *Rosa hybrida* (Gao et al., 2019).

Pollen function and viability can be evaluated through different methods including assessment of the pollen grain contents (e.g., lipids, polysaccharides, callose) by using stains such as modified Alexander's (Peterson et al., 2010) or seed set following controlled pollinations (Impe et al., 2020; Oleszczuk et al., 2019). In addition, pollen size can provide an indirect measure of pollen ploidy (Barba-Gonzalez et al., 2006; Cordoba-Sanchez et al., 2023b; Larrosa et al., 2012; Nukui et al., 2011). A direct relation between ploidy and pollen grain size has been reported, with pollen grains 1.26 times larger than average for the species (or genotype) considered as unreduced pollen (2n) (Barba-Gonzalez et al., 2006; Larrosa et al., 2012; Siregar, 2021). Additionally aborted pollen is smaller. In *L. perezii*, for example aborted pollen is between 0.52 and 0.62 the size of viable pollen (Cordoba-Sanchez et al., 2023b).This understanding of size differences creates a tool for identifying unreduced pollen grains, and viable and aborted pollen following treatment of flower buds with N_2O (Akutsu et al., 2007; Cordoba-Sanchez et al., 2023b).

A previous study in *Limonium* revealed the potential of N_2O for producing unreduced pollen and increasing the proportion of viable, functional pollen in treated plants (Cordoba-Sanchez et al., 2023b). Additionally, in previous experiments the fertility of unreduced gametes was confirmed in intraspecific crosses between either tetraploid *L. sinuatum* or *L. perezii* plants as well as through germination of unreduced hybrid pollen induced by treatment with N_2O. The aim of the current research was to identify the meiotic stages at which cells are more likely to polyploidise, resulting in a greater number of unreduced gametes, and to determine whether the resulting unreduced gametes could give rise to polyploid seedlings. This was investigated by first identifying the timing of

N_2O treatments in *L. sinuatum* for inducing unreduced gametes and then verifying the resulting unreduced gametes were functional through evaluation of pollen viability and production of polyploid seedlings. The results from *L. sinuatum* were also applied to interspecific crosses with *L. perezii* with the aim of generating polyploid hybrids that do not require doubling to restore fertility as needed for progeny from conventional crosses.

5.4. Materials and methods

5.4.1. Plant material and cultivation

Two diploid *L. sinuatum* (2*n* = 16) (identified as sin16 and sin18) and two *L. perezii* (2*n* = 14) (identified as per11 and per275) were selected as parents. These genotypes were selected as they had contrasting stigma types (Baker, 1953a), synchronous flowering times, and had been shown to give seed production following intraspecific pollinations at rates greater than 50% for the *L. perezii* and 70% for the *L. sinuatum* genotypes. Clonally propagated plants of each parental genotype were grown in plastic pots using a commercially available bark based growing medium (Cordoba-Sanchez et al., 2023b) under greenhouse conditions in Palmerston North, New Zealand. The greenhouse was heated from 18 °C with venting from 21 °C, and plants were grown under natural photoperiod conditions for Palmerston North, New Zealand (40.9006 °S, 174.8860 °E). The photoperiod was between 11 h and 12 h in spring and autumn, when pollinations were undertaken. When the resulting seedlings were transplanted to the greenhouse in summer, the photoperiod was 14.30 h.

5.4.1. N_2O treatment

Parental genotypes (i.e., seed-bearing and pollen-donor) were treated with N_2O during early inflorescence development when some of the florets had diameters no wider than 0.8 mm and calyces protruding ~ 2 mm above the bract (Cordoba-Sanchez et al., 2023b). Plants were placed inside a pressure-tolerant cylinder for treatment with N_2O for 24 h at 608 kPa (Nukui et al., 2011). After completion of the treatment with N_2O (day zero), the plants were returned to the greenhouse where the untreated plants had remained. Crosses (i.e., intra- or inter-specific) were performed daily as flowers opened, depending on the experiment. Ovule/embryo rescue was done 15 days after pollination, following the methodology described by Morgan et al (1998). The resulting seedlings were initially

grown under tissue culture conditions and were transplanted to the greenhouse when they had roots and at least a 2 cm length of foliage.

5.4.2. Pollinations

Daily five freshly open florets were pollinated in the untreated plants and 10 for treated plants, respectively. Hand pollination consisted of rubbing one dehiscent anther from the pollen-donor parent on the stigmas (i.e., five) of one floret from the seed-bearing genotype trying to ensure as much pollen transfer as possible. Emasculation prior to anther dehiscence was not undertaken due to the self-incompatibility reported in these *Limonium* species (Baker, 1953a).

5.4.3. Experimental design

5.4.3.1 Intraspecific crosses in L. sinuatum

Reciprocal intraspecific crosses using the *L. sinuatum* parents were undertaken. The window in developmental time between the treatment with N_2O and the planned pollination extended from 12 Days After Treatment (DAT) through to 42 DAT. For the treated plants, ten florets (replicates) were crossed per DAT, and per seed-bearing genotype. As an experimental control, five florets (replicates) from untreated plants were pollinated per day, and per seed-bearing genotype, on 17, 18, 22, 23, 27, 33, 34, 38, and 42 DAT.

The experimental design was completely randomized with a factorial arrangement of the factors (Compton, 1994): pollen-donor genotype (i.e., sin16, sin18) and treatment. The treatment levels were either N_2O (all DATs together) and control, or each DAT (i.e., from 12 to 42 DAT plus control).

5.4.3.2 Interspecific crosses

In the interspecific crosses comprising *L. sinuatum* × *L. perezii,* the timing for when the crosses were performed was determined by the results from the intraspecific pollinations in *L. sinuatum*, and was between 13 DAT and 30 DAT. In that window of developmental time, differences in pollen size had been detected (Figure 5-1) indicating higher probabilities of occurrence of unreduced pollen. In this experiment, *L. sinuatum* was used as the seed parent, while *L. perezii* was the pollen parent. Three identical plant pairs,

comprising clonally propagated plants from each parent, were used for each interspecific parental combination (*L. sinuatum* × *L. perezii*), i.e., sin16 × per275 or sin18 × per11. Two plant pairs received the N_2O treatment, and one pair was used as an untreated experimental control. Ten crosses were performed daily in the two treated plant pairs during the time spanning 13 DAT to 30 DAT. For untreated plants five crosses were performed on specific days: 17, 18, 22, 23, 27 DAT.

The experimental design was completely randomized with sub-samples and a factorial arrangement of the factors (Compton, 1994): pollen-donor genotype (per11, per275) and treatment. The treatment levels were either N_2O (all DATs together) and control, or each DAT (i.e., from 13 to 30 plus control).

5.4.4. Pollen viability and size

Pollen size data was collected for the treated plants daily or on the days specified for the control plants using a Zeiss microscope Imager.Z2 (Carl Zeiss Microscopy, Axio Imager Z2, Germany). Pollen was extracted from all five anthers of three newly opened florets at anther dehiscence and mixed to create a single sample in a fixative solution comprising 3:1 85% ethanol and 100% glycerol (Cordoba-Sanchez et al., 2023b). Viability of pollen and pollen size was recorded for 100 pollen grains per sample, following application of 10 µl of modified Alexander's stain (Peterson et al., 2010). For pollen viability, pollen grains which stained purple were recorded as viable, and those that stained brown or with doubtful coloration were recorded as aborted (Peterson et al., 2010). Pollen size was measured as plan area of pollen grains photographed at 50x magnification using a digital camera (Axiocam 305 color, Carl Zeiss Microscopy GmbH, Germany) and the software ZEN2.6 (blue edition) (Carl Zeiss Microscopy GmbH, 2018) with these images analysed using the image processing package Fiji (Schindelin et al., 2012).

For pollen of all sizes, exine ornamentation was the criterion used to discriminate between pollen grains and any other particle observed. Pollen grains with a plan area 1.2 times greater than the control were deemed as being unreduced pollen. The occurrence of putative unreduced, versus haploid, pollen was assessed using a frequency distribution of pollen grain plan areas and determining the probability of occurrence of unreduced pollen (Akutsu et al., 2007).

5.4.5. Seedling ploidy and hybrid status determination

Ploidy of parents and seedlings was confirmed by flow cytometry, utilizing the methodology of Otto (1990). Confirmation of interspecific hybrid status was based on the expected changes in DNA content of seedlings arising from crossing *L. sinuatum* × *L. perezii* (Morgan et al., 1998).

5.4.6. Statistical analysis

Data analysis of measured and calculated variables, from both intra- and inter-specific crosses, was conducted using the software R (R Core Team, 2018) with ggplot 2 (Wickham 2016) and the package sjPlot (Lüdecke et al., 2021), for the graphic outputs. Variables with values with only two possible outcomes, e.g., viable pollen (yes/no), were analysed using a binomial regression model, and other data were analysed using linear models. The residuals were checked to ensure assumptions were valid. Explanatory factors were: treated vs control, DAT, and pollen-donor genotype. For comparison between the levels of the factors for data corresponding to proportions, pairwise differences between means were tested using a pairwise likelihood ratio test (similar to LRPAIR in Goedhart (2018)). For the other data, estimated marginal means were calculated (Russell (2019); R package emmeans). Pairwise differences between means were tested; the Sidak method was used to adjust for multiple comparisons (i.e., to ensure a $p = 0.05$ family-wise error rate; Russell (2019)). For all analyses, the *p*-value was set as 0.05.

For the intraspecific pollinations, an initial statistical analysis of pollen plan area from untreated plants of *L. sinuatum* indicated differences between DAT ($p < 0.05$), grouping into either; 12 to 22 DAT, 23 to 33 DAT, or 34 to 42 DAT. Subsequent data analysis utilised these groups.

5.5. Results

5.5.1. Effect of N_2O over the plan area of pollen

In *L. sinuatum* plants treated with N_2O, pollen grains, collected between 12 to 22 DAT were 0.87 times (mean pollen area 1510 ± 364 µm^2) the size of pollen collected from the control treatment (mean pollen area 1730 ± 329 µm^2; $p < 0.05$; Figure 5-1A), with no

difference between the two genotypes (i.e., sin16 and sin18) ($p > 005$). Pollen grains collected between 23 and 33 DAT, were 1.26 times (mean plan area 1929 ± 452 µm^2) larger than pollen from the control plants (1535 ± 301 µm^2; $p < 0.05$; Figure 5-1B). During this range of DAT, there was also an interaction between the N_2O treatment and the genotype, with the increase in pollen size of sin18 being 10% smaller than those observed in sin16 ($p = 0.06$; Figure 5-1B). Additionally, pollen grains from plants treated with N_2O, but collected more than 33 DAT (Figure 5-1C), had a similar area (1617 ± 330 µm^2) to those from control plants (1593 ± 285 µm^2; $p > 0.05$).

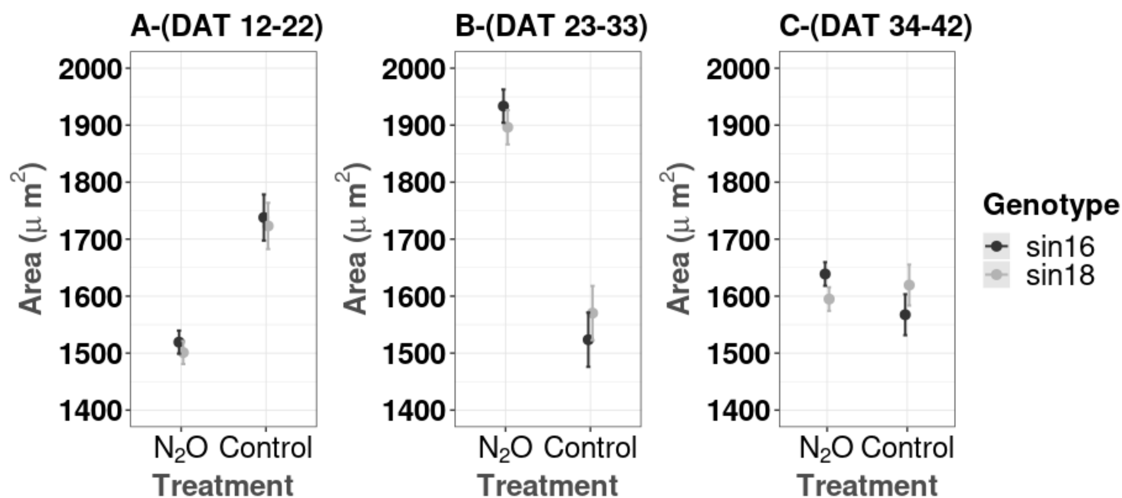

Figure 5-1 Plan area (µm^2) of the pollen grains collected from *Limonium sinuatum* parents used in the intraspecific crosses either from plants treated with nitrous oxide (N_2O) or in the control plants (untreated). Dots represent the predicted mean values for the pollen grain area after data modelling using linear models, and the vertical lines represent the 95% confidence interval. n = 100 pollen grains. p=0.05 **A.** Area of pollen grains from control plants obtained on day 17, 18, or 22 and from plants treated with N_2O and crossed between 12 and 22 days after treatment (DAT). **B.** Area of pollen grains from control plants obtained on days 23, 27, or 33 and from plants treated with N_2O and crossed between 23 DAT and 33 DAT. **C.** The plan area of pollen grains from plants in the control treatment were obtained on days 34, 38, or 42, and from plants treated with N_2O and crossed between 34 DAT and 42 DAT

In *L. perezii*, differences in pollen grain size were observed between the two genotypes in untreated control plants ($p < 0.05$; Figure 5-2). When pollen size was compared, pollen collected 13 and 17 DAT was smaller in comparison to pollen from untreated plants ($p < 0.05$). For per11, the pollen size of treated plants was 0.77 times (1104 ± 458 µm^2) that

of the control plant pollen (1432 ± 495 µm^2), and for per275 treated pollen was 0.92 times (1552 ± 485 µm^2) the size of pollen from the control plants (1684 ± 393 µm^2). Pollen grain size increased for pollen collected more than 18 DAT for per275 and more than 21 DAT for per11. Pollen from per11 was 1.14 times (1638± 542 µm^2) larger than control pollen (1432 ± 495 µm^2) while for per275 it was 1.28 times larger (2142 ± 446 µm^2) than control pollen (1684 ± 393 µm^2).

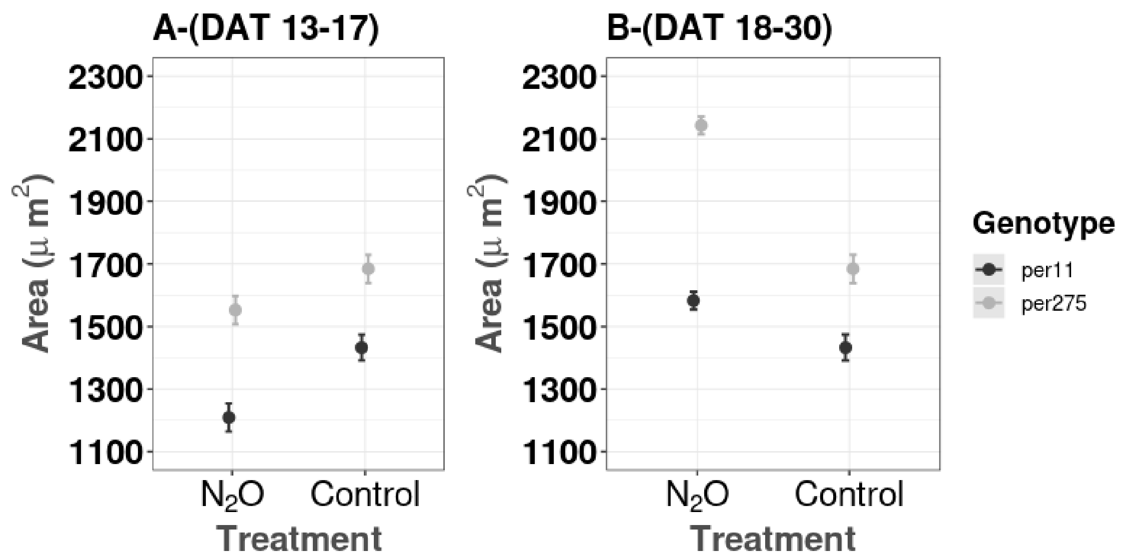

Figure 5-2 Plan area (µm^2) of the pollen grains collected from *Limonium perezii* parents used in the interspecific crosses either from plants treated with nitrous oxide (N_2O) or in the control plants (untreated). Dots represent the predicted mean values for the pollen grain area after data modelling using linear models, and the vertical lines represent the 95% confidence interval. n = 100 pollen grains. p=0.05. **A.** Area of pollen grains from plants treated with N_2O between 13 and 17 days after treatment (DAT) and from control plants **B.** Area of pollen grains plants treated with N_2O and crossed between 18 DAT and 30 DAT and from control plants

5.5.2. Proportion of unreduced pollen

With the genotypes of *L. sinuatum* used in the intra-specific pollinations, the proportion of unreduced pollen was not the same at each DAT, nor for both pollen-donor genotypes. The exception being before 22 DAT, with the proportion of unreduced pollen being lower than 7% for both genotypes (Figure 5-3). Between 23 and 33 DAT, an increase was observed in the proportion for both genotypes, however the increase was higher for sin16

with an averaged proportion of 32% in comparison with sin18 (14%). Immediately after 33 DAT the proportions of unreduced pollen dropped in both genotypes presenting an average of 15% for sin16, and 6% for sin18 (Figure 5-3).

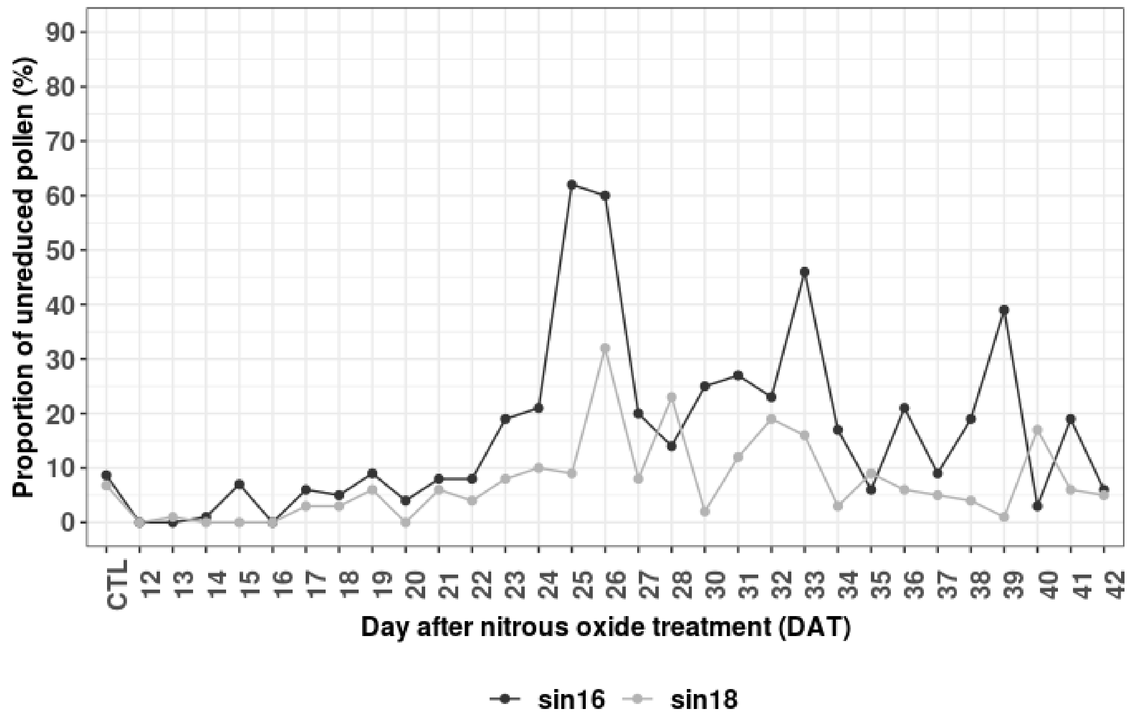

Figure 5-3. Probability of occurrence (%) of unreduced pollen in two genotypes of *Limonium sinuatum*, sin16 and sin18, at each Day After Treatment (DAT) with nitrous oxide (N_2O)

In *L. perezii* (interspecific pollinations), the proportion of unreduced pollen, while being variable, generally followed an increasing trend with increasing DAT (Figure 5-4). This increasing trend was more pronounced for per275, with a mean probability of 41% for all DAT, in comparison with a 29% for per11. In the period between 15 DAT and 20 DAT lower proportions occurred (average probability of 18% for per11, and average probability of 13% for per275).

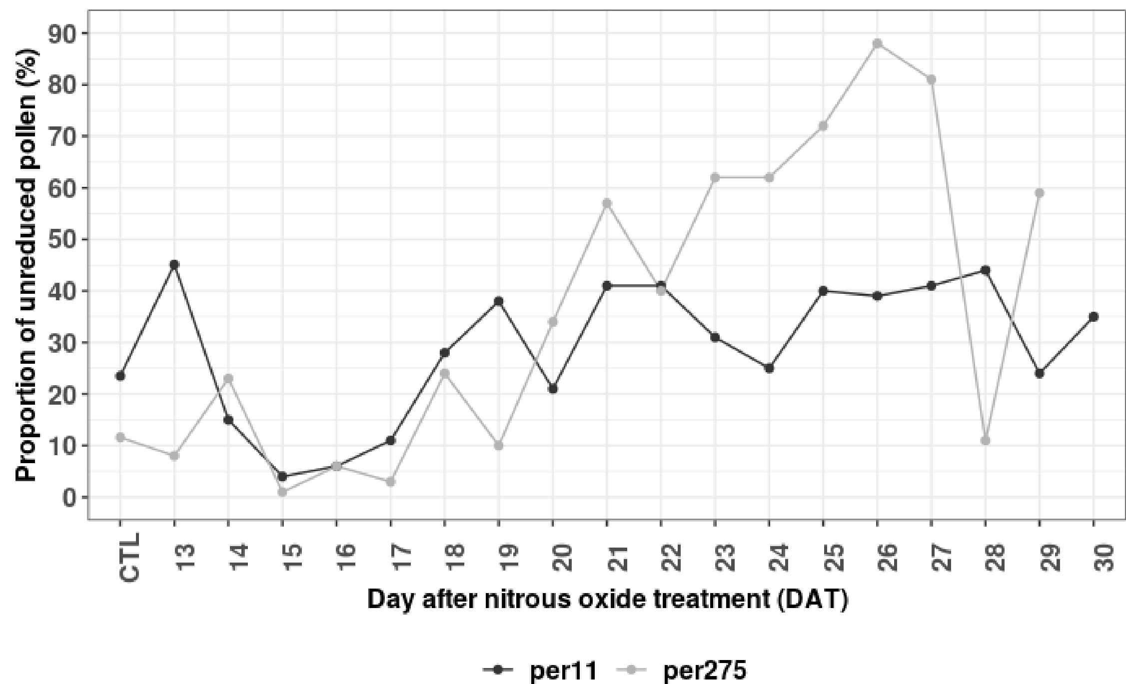

Figure 5-4. Probability of occurrence of unreduced pollen in two genotypes of *Limonium perezii* (per11 and per275) for each Day After Treatment (DAT) with nitrous oxide (N_2O)

5.5.3. Pollen viability and size

Treatment of *L. sinuatum* with N_2O did not alter the proportion of viable pollen grains ($p > 0.05$). The proportion of viable pollen from the control plants was at least five times higher (83%) than aborted pollen (17%) ($p < 0.05$).

Across both treated and untreated plants, aborted pollen was 30% smaller (1309 µm2 ± 341 μm^2) than viable pollen (1868 μm^2 ± 396 μm^2; $p < 0.05$). Nitrous oxide treatment increased the size of both aborted and viable pollen between 25% and 30%, for pollen collected between 23 and 30 DAT ($p < 0.05$), but N_2O did not affect size of pollen collected between 12 and 22 DAT ($p > 0.05$; Figure 5-3).

5.5.3.1 Production of intraspecific polyploids

The N_2O treatment negatively affected zygote success rate (number of zygotes obtained divided by the number of crosses completed) for crosses performed between 23 DAT and 33 DAT. Within this period 68% ± 20% of the pollinated flowers from plants treated with N_2O produced seedlings, in comparison with 87% ± 16% from those in the untreated control (Figure 5-5B; $p < 0.05$). There was also a difference between seed-bearing genotypes observed for crosses performed between 23 DAT and 33 DAT, with ~30% more embryos being formed when the seed-bearing parent was sin18 (84% ± 9%) in comparison with sin16 (53% ± 15%; Figure 5-5B; $p < 0.05$).

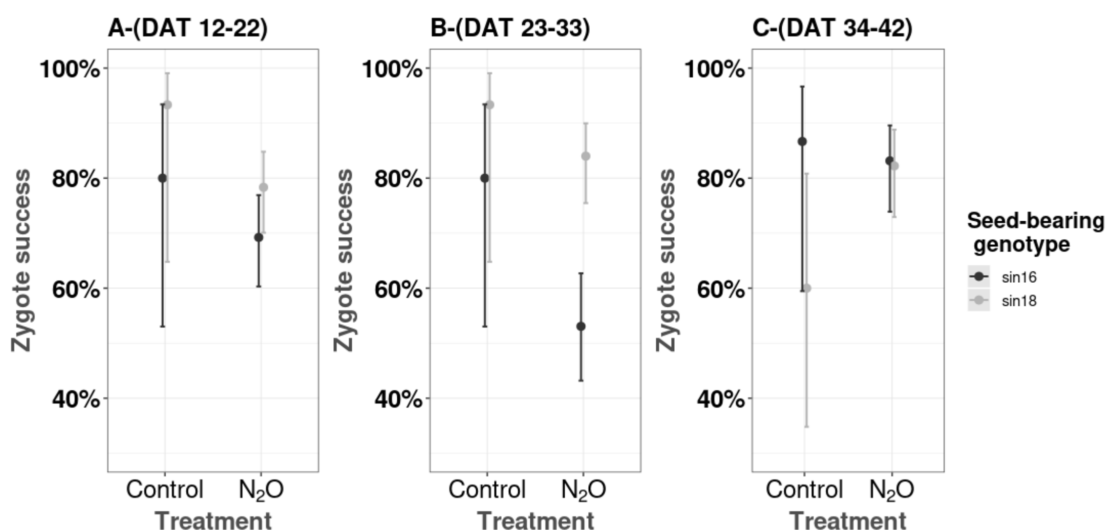

Figure 5-5. Zygote success (%) for intraspecific crosses performed in *Limonium sinuatum* treated with nitrous oxide (N_2O) or untreated (control) as affected by either: treatment (i.e., control, N_2O) or seed-bearing genotype (i.e., sin16 and sin18). Dots represent the predicted mean values for the zygote success after data modelling using generalized linear models, and the vertical lines represent the 95% confidence interval. n=5 pollination for control plants and 10 pollination for treated plants. $p = 0.05$. **A.** Zygote success for control plants on days 17, 18, and 22 of the experiment and for plants treated with N_2O collected between 12 and 22 DAT. **B.** Zygote success for control plants obtained on days 23, 27, and 33 and for plants treated with N_2O between 23 DAT and 33 DAT. **C.** Zygote success for plants in the control treatment obtained on days 34, 38, or 42, and for plants treated with N_2O between 34 to 42 DAT

The number of expected polyploids (product of the proportion of unreduced pollen and the number of seedlings obtained) for intraspecific *L. sinuatum* seedlings was between 2

and 3 (i.e., sin18 and sin16, respectively) for florets pollinated between 12 and 22 DAT; for florets pollinated between 23 and 33 DAT was between 10 and 15 expected polyploids (i.e., sin18 and sin16, respectively); and for florets pollinated after 34 DAT, the number of expected polyploids was 4 for sin18 and 9 for sin16 (APPENDIX G). The number of expected polyploid in the control plants was below 1 for both seed-bearing genotypes. The number of polyploids obtained comprised one triploid at 15 DAT for sin16 as seed-bearing genotype, and one tetraploid at 40 DAT for sin18. By comparison two triploids were recovered developed from sin16 plants within the control treatment (APPENDIX G).

5.5.4. Production of interspecific polyploids

In terms of a treatment response to N_2O, zygote success in the interspecific *L. sinuatum* × *L. perezii* crosses was influenced by the *L. perezii* genotype used as the pollen-donor. In the case of per11, the use of N_2O increased zygote success from 2% ± 4% in the control to 22% ± 32%, between 23 and 30 DAT ($p < 0.05$). In contrast, in the case of per275, the N_2O treatment caused a significant drop in zygote success from 88% ± 10% (control) to 66% ± 20% (N_2O-treated) at random DAT (i.e., 14, 18, 19, 23, 27, 28 and 30; $p < 0.05$).

The interspecific hybridisation rate increased between 160% and 500% after treatment with N_2O of the pollen-donor genotypes (i.e., per275 and per11, respectively). For the crosses between sin18 × per11, the interspecific hybridisation rate increased from 0% (control) to 5.5% (N_2O-treated). For the crosses sin16 × per275 the increment was from 3% (control) to 5% (N_2O-treated). All the hybrids were diploid.

5.6. Discussion

The N_2O-induced increment of the hybridisation rate between 160% and 500% for per275 and per11as pollen-donor parents, respectively, resulted in 84% of seedlings obtained (51) being diploid interspecific *L. sinuatum* × *L. perezii* hybrids. These results agree with the positive effect of N_2O over interspecific hybridisation and fertility restoration reported in *Begonia* (Dewitte et al., 2010) and in *Lilium* (Nukui et al., 2011) where they reported increases in hybridisation rate from 0% to 100% and 0% to 59%, respectively. While the current research does not directly allow elicitation of the mechanism involved, it could be associated with the increase in proportion of viable pollen (i.e., 6% in per 11 and 54%

in per275; Cordoba-Sanchez et al. (2023b)) after treatment with N_2O in pollen donor genotypes (i.e., per11 and per275) used. Therefore, even though it was not tested, treatment with N_2O could have led to the normalisation of meiosis with the duplication and balance of chromosome number in PMCs, resulting in: restitution of homologous chromosome pairing (Luo et al., 2016; Nukui et al., 2011) and re-establishment of intergenomic recombination. As a result, development of viable pollen occurs as reported for *Lilium* (Luo et al., 2016; Nukui et al., 2011) and suggested for *L. perezii* (Cordoba-Sanchez et al., 2023b) with subsequent increase in zygote success (Impe et al., 2020; Oleszczuk et al., 2019) and hybridisation rate. The increases in the hybridisation and pollen viability after the use of a spindle disruptor such as N_2O, support the hypothesis that in control (i.e., untreated) of *L. perezii* plants there is an imbalance in the chromosome composition of cells with a negative effect on meiosis, as also suggested for *Actinia* (Wang et al., 2017), pollen viability, zygote success, and hybridisation rate.

N_2O treatment induced the production of putative unreduced pollen in both *L. sinuatum* (Figure 5-1) and *L. perezii* (Figure 5-2), when applied between 23 and 30 DAT, inducing at least a 30% increment in their plan area, as similarly reported for *Lilium* (Barba-Gonzalez et al., 2006; Nukui et al., 2011). The occurrence of potentially unreduced pollen, but only after 23 DAT, could have arisen from either meiosis or mitotic divisions within the somatic cells of the anther (Gómez et al., 2015). As reported for *Lilium* (Nukui et al., 2011) disruption of a mitotic spindle, rather than disruption of the meiotic spindle is suspected. Supporting evidence of a mitotic disruption is evident from the association between smaller pollen grains arising in *L. sinuatum* and meiotic disruption occurring 15 DAT (Siregar, 2021). In the present study this association was also observed when N_2O was applied between 12 and 18 DAT for both *L. sinuatum* (Figure 5-1) and *L. perezii* (Figure 5-2). Regardless of when the spindle disruption happens, the occurrence of pollen grains with plan area at least 1.2 times greater than the average plan area of the species, between 23 and 30 DAT, agrees with previous observations in *L. perezii* (Cordoba-Sanchez et al., 2023b). Cytological studies are still required however, so as to offer supporting evidence on the mechanism of action of N_2O in *Limonium*.

In the present study, as reported in *Lilium* (Akutsu et al., 2007) and *Tulipa* sp. (Okazaki et al., 2005), treatment with N_2O before pollination resulted in a mixture of viable,

aborted, reduced, and unreduced pollen (refer to numeral 5.5.3, Figure 5-3 and Figure 5-4). The proportion of potentially unreduced pollen depends on the DAT and pollen-donor genotype (Figure 5-3 and Figure 5-4). Viable pollen had the potential to produce diploid, triploid, or tetraploid seedlings, depending on its own ploidy and the ploidy of the ovule. Nevertheless, in the present research no association occurred between the proportion of unreduced pollen (Figure 5-3 and Figure 5-4) and production of polyploid seedlings (i.e., two polyploids from intraspecific crosses). The low number of polyploids obtained was unexpected as, for example, in *Cymbidium* an average production of 0.76% unreduced pollen resulted in 0.3% sexual polyploids in seedlings arising (Zeng et al., 2020). In the present research, the proportion of unreduced pollen was higher (e.g., up to 80% for per275) than reported in *Cymbidium* (Zeng et al., 2020), but it did not result in sexual polyploids. The results presented here are also at odds with reports on *Lilium*, with 100% of the seedlings (211) from crosses between tetraploid plants and unreduced pollen being tetraploid (Akutsu et al., 2007), and *Begonia*, with 100% of seedlings (83) from crosses between interspecific hybrids and pollen treated with N_2O being triploid or tetraploid (Dewitte et al., 2010). However, the low number of polyploids within the current study does agree with those reported with *Tulipa sp.* (Okazaki et al., 2005), with fewer polyploids than expected obtained from crosses using unreduced pollen. As discussed further below, it is hypothesised that the occurrence of the triploid block, and pollen competition are causing the failure to produce sexual polyploid seedlings.

5.6.1. Triploid bock

The occurrence of the triploid block as a concept is supported by the production of triploid abnormal seeds in *Tulipa sp.* (Okazaki et al., 2005). Similarly in *Arabidopsis arenosa*, triploid genotypes are almost non-existent (0.14%) in regions where *A. arenosa* ecotypes with different ploidy overlaps in their natural areas of distribution (Morgan et al., 2021; Morgan et al., 2020). In the present study, occurrence of a leaky triploid block is suspected, and is supported by:

- Lower zygote success in *L. sinuatum* plants treated with N_2O in comparison with controls (i.e., 68% versus 87%; Figure 5-5B). The lower zygote success occurred mainly in the intraspecific crosses conducted between 23 DAT and 33 DAT when a higher proportion of potentially unreduced gametes occurred. The lower zygote

success could be expected if interploidy crosses (i.e., reduced ovule × unreduced pollen or vice versa) are taking place, and the triploid block occurs. Similar results have been reported for *A. arenosa* (Morgan et al., 2021), with a decrease in seed set in interploid crosses in comparison with crosses using parents of the same ploidy.

- In the present study, differences in zygote success occurred only between 23 DAT and 33 DAT depending on the seed-bearing genotype (i.e., sin16 and sin18; Figure 5-5B). Likely as reported for *A. arenosa* (Morgan et al., 2021) and other crops (Lafon-Placette & Köhler, 2016) those differences support the hypothesis of endosperm failure and occurrence of triploid block in *Limonium*.

5.6.2. Pollen competition

Another factor potentially associated with the low production of polyploids is the occurrence of a mixture of reduced and unreduced pollen (Figure 5-3 and Figure 5-4) which could result in competition between the pollen grains, as reported for *Rosa hybrida* (Gao et al., 2019) and *Chamerion angustifolium* (Husband et al., 2002). In *Limonium,* the production of less than 2% polyploid seedlings could be explained if a leaky triploid block occurs and unreduced pollen is advantageous. As reported for *Chamerion angustifolium* (Husband et al., 2002) and *Rosa hybrida* (Gao et al., 2019), pollen size and pollen tube growth are associated directly with ploidy, with a faster growth of the pollen tube in unreduced pollen, as compared with reduced pollen (Alexander, 2020; Gao et al., 2019; Husband et al., 2002). If unreduced pollen has competitive advantage for pollination over reduced pollen, in *Limonium*, interploidy crosses would take place with the leaky triploid block resulting in endosperm failure and failure of the crosses.

5.7. Conclusions

N_2O induces viable unreduced pollen in *L. sinuatum* and *L. perezii* between 23 and 30 days after treatment (DAT). Production of unreduced pollen was confirmed by an increase in the pollen plan area between 25% and 30% with 83% of viable pollen.

N_2O increases the interspecific hybridisation rate up to 5.5 % when crosses were performed between 23-30 DAT. The improvement in hybridisation could be associated

with an increase in the proportion of viable pollen in pollen-donor parent (i.e., *L. perezii*) and restitution of normal meiosis in PMC.

CHAPTER 6. Production *in vitro* of tetraploid *L. sinuatum* × *L. perezii* hybrids using oryzalin

6.1. Introduction

Interspecific hybridisation is one of the breeding strategies used for the development of new cultivars of *Limonium* (Burchi et al., 2006; Burge et al., 1995; Morgan et al., 1998; Sato et al., 1993) (further details are given in Table A-3 in the appendices). For instance, interspecific hybrids obtained by PFR utilising *L. sinuatum* and *L. perezii* and marketed as the 'siNZii™' cultivar series by BallSB (BallSB, 2021) combine the sharp and strong floret colour of *L. sinuatum,* with the unwinged stems of *L. perezii* (BallSB, 2021). In addition, 'siNZii™' is considered less susceptible to downey mildew, thrips, and slugs (BallSB, 2021) and more resistant to *Botrytis* sp. (BallSB, 2014).

The 'siNZii™' cultivars produced by breeding of interspecific hybrids of *L. sinuatum* and *L. perezii* display, as mentioned above, phenotypic traits with commercial importance. However, the initial production of interspecific hybrids (i.e., first generation (F1) of *L. sinuatum* × *L. perezii* hybrids) and their further use as breeding material is affected by low hybrid viability (i.e., seed set and/or seedling establishment) and hybrid weakness (i.e., necrosis, wilting, yellowing; Bomblies and Weigel (2007); Chen et al. (2016)). In addition, due to different chromosome numbers between both species (i.e., *L. sinuatum* has 16 chromosomes (2n=2x=16) while *L. perezii* has 14 (2n=2x=14)), the F1 interspecific hybrids have 15 chromosomes (2n=2x=15) and display sterility/low fertility (Morgan et al., 1998). Low viability, weakness, and sterility of hybrids are mainly caused by the uneven number of chromosomes (2n=15) and genetic differences or incompatibilities between the genomes from both parents (Chen et al., 2016; Husband, 2004). To address the hybrid's constraints duplication of the number of chromosome sets (i.e., whole genome duplication (WGD)) could be induced. WGD balances the chromosome number in pollen mother cells (PMC) and megaspore mother cells (MMC). Therefore, after induction of WGD, the occurrence of meiotic abnormalities is restricted (Bomblies, 2022), normal meiosis is at least partially restored, viable gametes develop (Luo et al., 2016; Nukui et al., 2011), and hybrid weakness is overcome (Husband, 2004).

In addition, the restoration of the recombination between chromosomes contributes to broadening the germplasm diversity (Liu et al., 2020; Lloyd & Bomblies, 2016).

The phenotype of F1 interspecific hybrids combine desirable and/or undesirable with intermediate traits between both parents as reported in *Limonium* (Morgan et al., 1998). To eliminate the undesirable traits repetitive cycles of hybridisation / backcrossing are needed which require the hybrids to be at least partially fertile which could be achieved, as mentioned before, by induction of WGD.

Fertility assessment could be performed indirectly through evaluation of pollen viability or germination, and/or directly by number of seeds, seed germination or fruit production after planned crosses (Sutherland & Galloway, 2017). A combination of pollen evaluation and crossing is recommended as pollen grains could be viable but unable to germinate or pollinate (Dafni & Firmage, 2000). Backcrosses were selected in the current chapter as their success could confirm the restoration of fertility after WGD while accumulating the desirable traits from the parent used for backcrossing (Cheng et al., 2011). In addition, backcrosses decrease the differences between the parental genomes in the hybrid, alleviating the low hybrid viability and weakness (Chen et al., 2016).

Backcrosses could be either homoploid (i.e., between genotypes with the same ploidy) or heteroploid (i.e., between genotypes with different ploidy) with success in producing seedlings depending on the occurrence of post-zygotic barriers (Sutherland & Galloway, 2021). Previous studies suggest that heteroploid backcrosses could be restricted by reproductive barriers such as occurrence of the triploid block resulting in the failure of endosperm formation and in low seed production or seed inviability (Sutherland & Galloway, 2017). In *Limonium*, occurrence of the triploid block has been suggested (CHAPTER 5; Siregar (2021)). As the breeding of the 'siNZii™' series has involved heteroploid backcrosses, if the triploid block exists it may not be a complete block (Morgan et al., 2021).

In *Limonium*, WGD had been induced in *L. sinuatum* and *L. perezii* using N_2O (CHAPTER 2, CHAPTER 3 and APPENDIX F), in seeds of *L. bellidifolium* with colchicine (Mori et al., 2016), and in *L. perezii* × *L. sinuatum* hybrids with oryzalin (Morgan et al., 2001). Another strategy for production of interspecific hybrids with ploidy

higher than diploid is through the induction of unreduced gametes (i.e., gametes with the somatic chromosome number). Previously, treatment with N_2O when undertaking interspecific crosses was used to produce fertile interspecific polyploids (CHAPTER 5). Although induction of unreduced gametes was detected, their use in crosses resulted in diploid interspecific hybrids only, with failure in the production of hybrids with higher ploidies (CHAPTER 5).

For *in vitro* treatments for WGD, oryzalin is preferred for its lower toxicity in comparison with e.g., colchicine or trifluralin (Carvalho et al., 2016; Podwyszyńska, 2012). Oryzalin doses used *in vitro* are up to 144 µM (Podwyszyńska, 2012; Suwanseree et al., 2011) with time of treatment extending from 24 hours (Suwanseree et al., 2011) to 14 days (Morgan et al., 2001). In *Limonium*, for WGD using oryzalin a recommended treatment includes the use of 43.3 µM oryzalin for 14 days (Morgan et al., 2001).

When grown *in vitro*, seedlings of *Limonium* display the characteristic grown pattern of the species (refer to Section 1.2.1) with the leaves forming a rosette at the base around a rhizome (Flora of North America Editorial Committee, 2005) which *in vitro* is incipient and not woody. *In vitro* multiplication of *Limonium* is by adventitious buds in culture medium (i.e., proliferation medium) containing the plant growth regulators Benzyl Amino Purine (BAP) alone or with Indole-3-Butyric Acid (IBA) (Martin & Perez, 1992; Morgan et al., 1998). After a growing cycle (i.e., 30 days) there are clumps of adventitious shoots which can be separated into individual plants. Amendment of the proliferation medium with oryzalin has been used to induce WGD (Morgan et al., 2001). When present in the medium, a proportion of the adventitious buds arise from cells undergoing WGD, and the plants developing later could be polyploids.

Ploidy confirmation could be done directly by flow cytometry which quantifies the DNA content or chromosome counting (Deepo et al., 2023); or indirectly through pollen size (CHAPTER 2) or guard cell length (Suwanseree et al. (2011); CHAPTER 2) as described in detail already. The method selected for ploidy determination is always related to the species under study. For example, chromosome counts could be difficult with small chromosomes (Deepo et al., 2023) and may not identify mixoploid plants but flow cytometry could be inadequate to detect in aneuploidy of some species (Li et al., 2021). In the latter case, flow cytometry needs to be coupled with chromosome count to confirm

the chromosome number (Li et al., 2021). When an indirect method such as guard cell length is used for the first time in a plant (e.g., interspecific hybrids in the current research) and its precision for differentiating diploid from polyploid plants is still unknown, it is recommended to use an additional method for verification of the ploidy such as chromosome counts as done in the current chapter.

To address hybrid low viability and sterility and increase the diversity of *Limonium* germplasm available, induction of WGD in interspecific hybrids was attempted using oryzalin. The aim of this component of the research was to evaluate a treatment alternative to N_2O to produce tetraploid hybrids with a view to comparing any resulting plants to those produced using N_2O in *Limonium*.

6.2. Materials and methods

6.2.1. Plant material and greenhouse cultivation

For the initial experiment evaluating oryzalin *in vitro*, four diploid, interspecific hybrids (Y19.026.001 to Y19.026.004) were used. The hybrids were obtained in October 2019 by crossing *L. sinuatum* (Y19.018.001; $2n = 2x = 16$) × *L. perezii* (Y19.001.001; $2n = 2x = 14$) that had been treated with N_2O for 48 hours (APPENDIX I). The diploid hybrid embryos were established *in vitro* through ovule/embryo rescue, following the methodology reported by Morgan et al (1998).

Plants derived both from the *in vitro* treatment with oryzalin (refer to Section 6.2.2), and those arising from the resulting backcrosses (refer below to Section 6.2.4) following transfer to the greenhouse (i.e., December 2020 and February 2022, respectively), were grown in a commercially sourced growing medium (refer to APPENDIX E). The greenhouse, located in Palmerston North, New Zealand (40.9006 °S, 174.8860 °E), provided natural light and photoperiod with controlled temperatures with heating to maintain a minimum of 16° and ventilation starting at 18 °C. Irrigation comprised the use of drippers (8.5 L/h, PC Low CNL Netafim™, Hatzerim, Israel) and was designed to provided 40 ml to 50 ml of water daily with a 10-15% drainage to keep the medium at field capacity.

6.2.2. Oryzalin treatment

After germination *in vitro*, individual interspecific hybrid seedlings are propagated in culture medium consisting of 'base medium' with the addition of 0.05 mg/L IBA, 0.3 mg/L BAP, 0.1 mg/L GA_3 (i.e., 'shoot proliferation medium'; refer to APPENDIX A). Approximately 50 mL culture medium was added to disposable polystyrene plastic culture vessels (98 mm base diameter × 60 mm deep) with snap-on lids (Alto Packaging, Hamilton, New Zealand). The adventitious shoots developed, after propagation, from *in vitro* germinated seedlings were separated into individual plants and subcultured in shoot proliferation medium amendment with 1.5 mg/L oryzalin (Sigma. Product code 36182) for WGD induction. As control, adventitious shoot proliferation medium without oryzalin was used. Plants were kept in the corresponding medium for two months with monthly sub-culturing. Each of the adventitious shoots obtained two months after either oryzalin treatment or control treatment were transferred to proliferation medium for one month and later bisected and transferred to a pre-rooting medium without PGRs (refer to APPENDIX A) for further growth and rooting. The two rooted shoots bisected from each adventitious shoot were acclimated to and grown within the greenhouse.

6.2.3. Ploidy determination and chromosome number

A pre-screening of the potential polyploid genotypes for changes in ploidy was conducted by comparison of the guard cell length of treated and control plants using the transparent impression technique (Cohen & Yao, 1996), as described previously (refer to Section 2.4.4.2), so as to eliminate diploid plants. This comprised evaluating two eight-month old plants per genotype, sampling one leaf of similar age from each plant, and measuring the length of ten guard cells measured per leaf. The sample size for the number of guard cells required to delineate between plants of differing ploidy, was calculated based on the mean and standard deviation performed previously for plants of *L. sinuatum* (refer to CHAPTER 2), with a confidence interval of 90%. A plant was determined to be a potential polyploid when guard cell length was statistically larger in comparison with the control (i.e., at least 1.2x larger). The ploidy status of the plants was confirmed by flow cytometry as described in Section 2.4.5, where samples from identified potential polyploids were compared with samples from plants established from the control treatment.

Chromosome counts were carried out specifically on plants exhibiting discrepancies in guard cell length between samples. To clarify, these discrepancies refer to instances where the guard cell length in one sample resembled the control, while in another sample, it was notably larger. This analysis took place prior to the application of flow cytometry. The protocol for counting chromosomes followed that of Morgan et al. (1995), wherein roots tips were harvested from eight-month-old, greenhouse grown plants, with samples kept in ice for 24 hours. Root samples were subsequently fixed in a solution of chloroform:ethanol:acetic acid (6:3:1) for one hour, with subsequent double staining using Feulgen stain and later 2% lactic-propionic orcein (Grant et al., 1984). The stained samples were observed using a Zeiss microscope Imager.Z2 (Carl Zeiss Microscopy, Axio Imager Z2, Germany) recording the number of condensed chromosomes present in at least three cells.

The response variables evaluated were ploidy, hybrid status, proportion of polyploids (number of polyploids obtained divided by number of adventitious shoots obtained (excluding mixoploids)); and proportion of mixoploids (number of mixoploids obtained divided by number of adventitious shoots obtained).

6.2.4. Fertility assessment

6.2.4.1 Backcrosses

Assessment of the fertility and morphology of the interspecific hybrids was achieved by conducting backcrosses between the confirmed tetraploid hybrids and the *L. sinuatum* parent (i.e., Y19.018.001; heteroploid backcrosses). As a comparative control, backcrosses were also performed between the diploid hybrids, originally derived from the control treatment *in vitro*, and the *L. sinuatum* parent (i.e., Y19.018.001; i.e., homoploid backcrosses). The backcrossing parent was selected based on compatible floret morphology. As all the hybrid seedlings had cob stigmas, the parent selected for backcrossing was *L. sinuatum* (i.e., Y19.018.001) with papillate stigmas. In both backcross types, the hybrid genotypes were used both as seed-bearing as well as pollen-donor parent to evaluate female and male fertility.

Eight hybrid genotypes were evaluated, of which four were diploids (i.e., Y19.026.001, Y19.026.002, Y19.026.003, and Y19.026.004) and four were confirmed tetraploids (i.e.,

Y19.026.006, Y19.026.009, Y19.026.010, and Y19.026.013). To conduct the backcrosses, for each hybrid genotype ten crosses (sub-samples) were conducted daily on each of three days (reps). The experimental design was completely randomized (Compton, 1994) with sub-sampling (i.e., 10 crosses), with the seed-bearing genotype as a factor (i.e., nine seed-bearing genotypes). A month after the backcrosses were completed, the zygotes still enclosed in the ovules were cultured *in vitro* (i.e., ovule/embryo rescue) as described previously (refer to Section 2.4.2).

The response variables evaluated were: zygote success (number of zygotes obtained divided by the number of crosses completed), germination rate (number of zygotes germinated *in vitro* divided by number of zygotes obtained (rescued)), cross success (number of seedlings obtained divided by number of crosses completed); fertility restoration rate (number of fertile polyploids divided by the number of polyploids produced); and ploidy and hybrid status determined by flow cytometry.

For data analysis the information corresponding to seedlings that were not hybrids was removed from the evaluation of reproductive performance, to avoid bias caused by potential apomictic events (refer to Section 1.3.6). Likewise, variables with zero as a value were excluded from the analysis, so as to avoid zero-inflated data (McCracken & Looney, 2017).

6.2.4.2 Assessment of pollen viability and germination

Pollen characterisation was performed in November 2022 and included pollen viability analysis using the tetrazolium test (Shivanna and Rangaswamy (1992); refer below to Section 6.2.4.2.2), and *in vitro* germination (refer below to Section 6.2.4.2.3).

6.2.4.2.1 Plant material

Pollen was characterized for the *L. sinuatum* parent (Y19.018.001), two diploid *L. sinuatum* × *L. perezii* hybrids (Y19.026.001 and Y19.026.003) and four tetraploid *L. sinuatum* × *L. perezii* hybrids (Y19.026.006, Y19.026.008, Y19.026.010, and Y19.026.013).

6.2.4.2.2 Pollen viability

The viability of pollen derived from florets of the parental (Y19.018.001) and hybrid genotypes (Y19.026.001, Y19.026.003, Y19.026.006, Y19.026.010, and Y19.026.013), was evaluated utilising a tetrazolium test (Shivanna & Rangaswamy, 1992). Briefly, a 30 µl drop of 0.2% tetrazolium solution consisting of 2,3,5-Triphenyltetrazolium Chloride (Sigma-Aldrich®, >98.0% (HPLC)) prepared in a 20% sucrose solution, was applied to a microslide. Pollen from 10 anthers taken from two florets was uniformly distributed within the droplet. A cover slide was placed on the top of the sample and the whole preparation put inside a humid chamber and incubated at 30°C for an hour (Shivanna & Rangaswamy, 1992). One hundred pollen grains were observed, measured, and photographed, following the protocol described previously (refer to Section 2.4.4.1). Viability was determined visually with pollen grains displaying a strong red colour counted as viable, while those either not coloured or with colour gradations, were assumed to be aborted (Shivanna & Rangaswamy, 1992).

For the purposes of evaluating the viability of pollen, the experimental design was a randomized complete block, with ploidy as the treatment (i.e., diploid or tetraploid), germplasm group blocks (i.e., *L. sinuatum* or *L. sinuatum* × *L. perezii* hybrid), utilising 100 replicates (pollen grains). The response variable was viability (yes/no).

Data was analysed with a generalized linear model (glm function from the stats package (R Core Team 2018)), with binomial distribution, utilising a *p-value* of 0.05 as a cut-off value to determine if the observed differences between treatments or blocks are due by change and not by treatment effect.

6.2.4.2.3 Pollen germination

The ability of pollen to germinate in vitro was assessed using a protocol developed by Boyd et al. (2022). The dialysis tubing method, modified for *Limonium* (Zhang et al., 1997), was used. The method involved constructing a pile of seven squares of Whatman paper N°1 (approximately 40 mm x 40 mm each) and inserting a square of dialysis tubing (SERVA, SERVAPOR dialysis tubing, MWCO 12000-14000, RC diameter 16 mm) measuring 40 mm x 40 mm between the top two layers. The pile is placed in a petri dish, and the germination media is poured over it until it is moistened. After 15 minutes, the top piece of filter paper is removed, and any excess medium is carefully wiped away from the dialysis tubing with a tissue paper. Then, dehiscent anthers are rubbed against the

dialysis tubing to scatter the pollen grains. The petri dish is then closed and incubated at 37°C for 6 hours. Finally, the pollen is observed under a Zeiss microscope Imager.Z2 (Carl Zeiss Microscopy, Axio Imager Z2, Germany).

The method utilised a modified Brewbaker and Kwack medium (Brewbaker & Kwack, 1963) with 20% PEG-20,000 added and casein hydrolysate omitted (APPENDIX J). A pollen grain was considered germinated if the length of the pollen tube was at least 1.5 times the diameter of the pollen grain (Zhang et al., 1997). For each sample, pollen from five stamens from three florets from different inflorescences was germinated. For each genotype, three replicates were performed, and 100 subsamples of pollen grains were evaluated for each replicate.

For undertaking evaluation of germination, the experimental design was a randomized complete block with sub-samples, with ploidy as the treatment (i.e., diploid or tetraploid), germplasm group as blocks (i.e., *L. sinuatum* or *L. sinuatum* × *L. perezii* hybrids). The response variables were germination (yes/no) and size measure in μm^2.

Germination data for pollen was analysed with a generalized linear model (binomial distribution) (glm function from the stats package (R Core Team 2018)). A *p-value* of 0.05 was used deciding if the observed variation in pollen germination was greater than random noise and could be attributed to the evaluated factors (i.e., ploidy, germplasm group).

6.3. Results

6.3.1. Induction of whole genome duplication

The original four interspecific *L. sinuatum* × *L. perezii* hybrid genotypes (Y19.026.001 to Y19.026.004), which were exposed to oryzalin *in vitro*, gave rise to 22 plants derived from adventitious shoots which corresponded to potential tetraploids.

A total of 26 adventitious plants (i.e., the 22 potentially tetraploids derived from plus four similar plants developed in control medium) were pre-screened using guard cell length (Figure 6-1). Twelve potential tetraploids were identified, as well as 4 diploid plants derived from those not exposed to oryzalin (i.e., control). Flow cytometry for the 12

potentially tetraploid plants confirmed: 5 tetraploid, 3 mixoploid and 4 diploid plants (Table 6-1).

The proportion of tetraploids (i.e., number of tetraploids obtained divided by number of adventitious shoots obtained) was 23%, and the proportion of mixoploids (i.e., number of mixoploids obtained divided by number of adventitious shoots) was 14%.

Table 6-1 Flow cytometry results for the 12 adventitious plants, potential tetraploids, obtained in culture medium supplemented with oryzalin and 4 adventitious plants obtained in the control medium without oryzalin. *Trifolium repens* was used as an internal reference (IR) and its DAPI relative fluorescence peak was used to compare the peak of each sample and to estimate the ploidy of the sample by calculating of the ratio between each sample peak and the peak of the IR. To determine the hybrid status of the genotypes, the peak of each sample was compared with the peak produced by the known genotypes *Limonium sinuatum* (Y19.016.001) and *Limonium perezii* (Y19.001.001).

Genotype	**Treatment**	**Peak IR (*T. repens*)**	**Peak 1 Sample**	**Ratio Peak 1 sample/Peak IR**	**Estimated ploidy**	**Peak 2 Sample**	**Ratio Peak 2/Pear IR**	**Estimated ploidy Peak 2**	**Ploidy**	**Hybrid status**
Y19.016.001	None	102	200	1.96	2.0				Diploid	No hybrid *L. sinuatum*
Y19.001.001	None	62	180	2.9	2.0				Diploid	No hybrid *L. perezii*
Y19.026.001	Control	53	128	2.42	2.01				Diploid	Confirmed
Y19.026.002	Control	53	126	2.38	1.98				Diploid	Confirmed
Y19.026.003	Control	53	127	2.40	2.00				Diploid	Confirmed
Y19.026.004	Control	53	123	2.32	1.93				Diploid	Confirmed
Y19.026.005	Oryzalin	53	130	2.45	2.04				Diploid	Confirmed
Y19.026.006	Oryzalin	55	252	4.58	3.82				Tetraploid	Confirmed
Y19.026.007	Oryzalin	53	120	2.26	1.89	244.00	4.60	3.84	Mixoploid	Confirmed
Y19.026.008	Oryzalin	56	229	4.09	3.41				Tetraploid	Confirmed
Y19.026.009	Oryzalin	56	258	4.61	3.84				Tetraploid	Confirmed
Y19.026.010	Oryzalin	55	256	4.65	3.88				Tetraploid	Confirmed
Y19.026.011	Oryzalin	56	130	2.32	1.93				Diploid	Confirmed
Y19.026.012	Oryzalin	52	127	2.44	2.04	258.00	4.96	4.13	Mixoploid	Confirmed
Y19.026.013	Oryzalin	52	265	5.10	4.25				Tetraploid	Confirmed
Y19.026.014	Oryzalin	56	130	2.32	1.93				Diploid	Confirmed

Y19.026.015	Oryzalin	55	126	2.29	1.91				Diploid	Confirmed
Y19.026.016	Oryzalin	52	127	2.44	2.04	249.00	4.79	3.99	Mixoploid	Confirmed

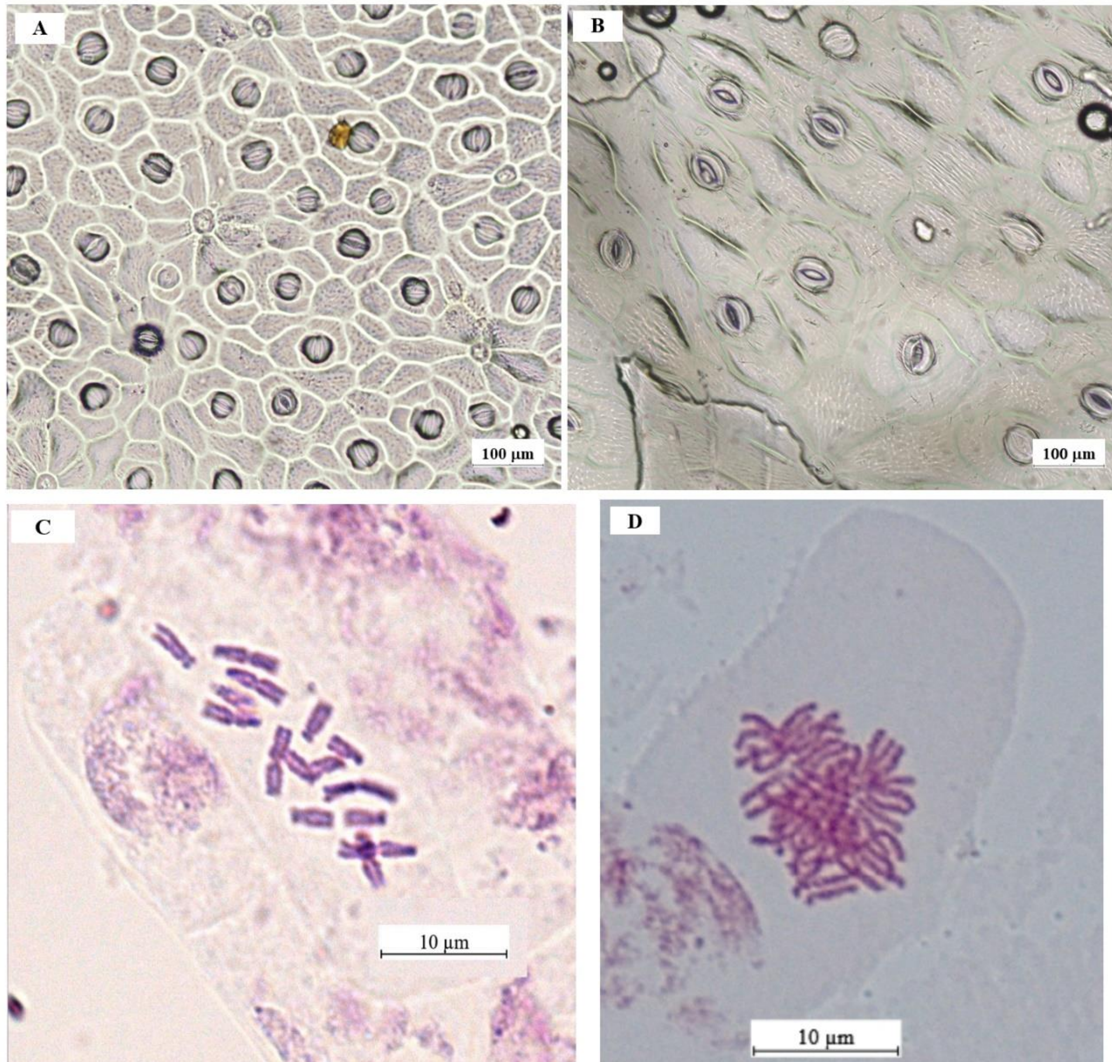

Figure 6-1 Transparent impressions, made with polyvinyl acetate wood glue (PVA), of guard cells from the abaxial surface of leaves and chromosomes from root tips of *Limonium sinuatum* × *Limonium perezii* hybrids of different ploidy. **A.** and **C.** Guard cells and chromosomes, respectively, of the original diploid hybrid (Y19.026.003), **B.** and **D.** Guard cells and chromosomes, respectively, of a tetraploid plant obtained after treatment with oryzalin (Y19.026.008)

6.3.2. Backcrosses

Two out of the five tetraploid hybrids obtained by induction of WGD utilising oryzalin produced zygotes after backcrossing.

As confirmed by flow cytometry (APPENDIX K), the backcrosses using Y19.018.001 as seed-bearing genotype resulted in one confirmed triploid hybrid, and five diploid

seedlings. In contrast, when Y19.018.001 was the pollen-donor, backcrosses resulted in 15 triploid hybrid plants with all plants produced testing as triploid.

Based on zygote success, the performance of each hybrid tetraploid as a seed-bearing parent was at least 9 times higher for both Y19.026.006 (0.46 ± 0.40) and Y19.026.009 (0.50 ± 0.10), in comparison with the diploid hybrids, Y19.026.004 (0.01 ± 0.02) and to *L. sinuatum* genotype Y19.018.001 (0.05 ± 0.08; $p<0.05$; Table 6-2).

The proportion of zygotes initially obtained (i.e., zygote success), which then germinated (i.e., germination rate) and developed into plants, decreased once the zygotes were rescued into tissue culture, with only 54% (21/39) of the seeds coming from Y19.018.001 (*L. sinuatum*), Y19.026.006, or Y19.026.009 (both hybrid tetraploids) germinating and developing into plants (Table 6-2).

As a pollen-donor, the zygote success for *L. sinuatum* was 2.4 times higher ($p<0.05$) in comparison with the diploid and tetraploid hybrids whose performance as pollen-donors was similar between them independently of the ploidy (0.05 ± 0.07 zygote success; $p>0.05$; Table 6-2).

Table 6-2 Zygote success (number of zygotes obtained divided by the number of crosses completed), germination rate (number of zygotes germinated *in vitro* divided by number of zygotes rescued), and cross success (number of seedlings obtained divided by number of crosses completed) for the backcrosses between *Limonium sinuatum* × *Limonium perezii* hybrids and their *L. sinuatum* parent (Y19.018.001). n = 10 crosses

Seed-bearing genotype	Description Seed-bearing genotype	Pollen-donor genotype	Description Pollen-donor genotype	Zygote success	Germination rate	Cross success
Y19.018.001	Hybrid's parent	Y19.026.001	Diploid hybrid	0.07 ± 0.06	0.58 ± 0.03	0.03 ± 0.06
Y19.018.001	Hybrid's parent	Y19.026.002	Diploid hybrid	0.03 ± 0.06	0 ± 0	0.00 ± 0.06
Y19.018.001	Hybrid's parent	Y19.026.003	Diploid hybrid	0.13 ± 0.06	0.58 ± 0.03	0.03 ± 0.06
Y19.018.001	Hybrid's parent	Y19.026.004	Diploid hybrid	0 ± 0	0 ± 0	0 ± 0
Y19.018.001	Hybrid's parent	Y19.026.006	Tetraploid hybrid	0.13 ± 0.15	0.51 ± 0.07	0.07 ± 0.06
Y19.018.001	Hybrid's parent	Y19.026.009	Tetraploid hybrid	0.17 ± 0.12	0.51 ± 0.07	0.07 ± 0.06
Y19.018.001	Hybrid's parent	Y19.026.010	Tetraploid hybrid	0 ± 0	0 ± 0	0 ± 0
Y19.018.001	Hybrid's parent	Y19.026.013	Tetraploid hybrid	0.03 ± 0.06	0 ± 0	0 ± 0
Y19.026.001	Diploid hybrid	Y19.018.001	Hybrid's parent	0 ± 0	0 ± 0	0 ± 0
Y19.026.002	Diploid hybrid	Y19.018.001	Hybrid's parent	0 ± 0	0 ± 0	0 ± 0
Y19.026.003	Diploid hybrid	Y19.018.001	Hybrid's parent	0 ± 0	0 ± 0	0 ± 0
Y19.026.004	Diploid hybrid	Y19.018.001	Hybrid's parent	0.03 ± 0.06	0 ± 0	0 ± 0
Y19.026.006	Tetraploid hybrid	Y19.018.001	Hybrid's parent	0.47 ± 0.40	0.29 ± 0.29	0.20 ± 0.20
Y19.026.009	Tetraploid hybrid	Y19.018.001	Hybrid's parent	0.50 ± 0.10	0.66 ± 0.44	0.30 ± 0.17
Y19.026.010	Tetraploid hybrid	Y19.018.001	Hybrid's parent	0 ± 0	0 ± 0	0 ± 0
Y19.026.013	Tetraploid hybrid	Y19.018.001	Hybrid's parent	0 ± 0	0 ± 0	0 ± 0

6.3.3. Pollen viability and germination

Pollen viability was 18 times higher for *L. sinuatum* (74% ± 45%; p<005) in comparison with the four tetraploid *L. sinuatum* × *L. perezii* hybrids evaluated (in average 4% ± 20% viable pollen) without significant differences between tetraploid hybrids (p>0.05; Figure 6-2). A similar trend was observed for *in vitro* germination of pollen with pollen germination in *L. sinuatum* being five times higher in comparison with tetraploid hybrids (25% ± 43% and 5% ± 21% germinated pollen, respectively; p>0.05; Figure 6-2). The viability and germination of pollen from diploid hybrids was zero.

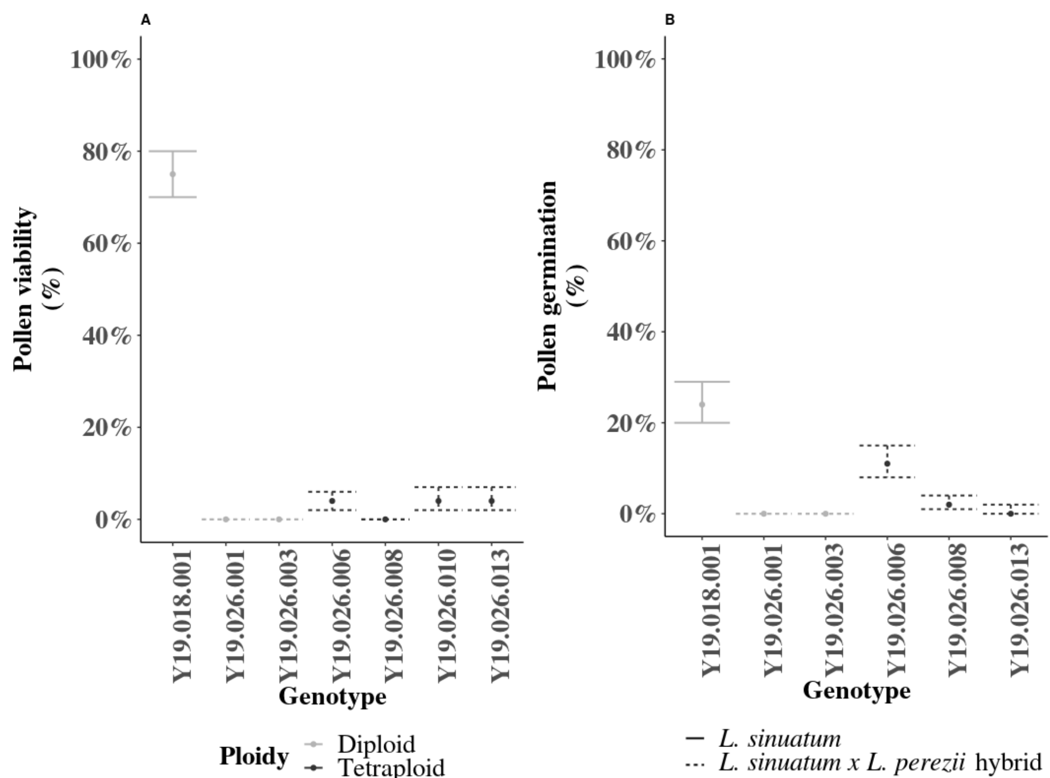

Figure 6-2 Proportion in percentage of viable pollen stained with tetrazolium **(A)** or pollen germinated *in vitro* **(B)** of the *Limonium sinuatum* parent used for backcrossing (Y19.018.001), *L. sinuatum* × *Limonium perezii* diploid hybrids (Y19.026.001, Y19.026.003), or tetraploids hybrids (Y19.026.006, Y19.026.008, Y19.026.010, Y19.026.013). Dots represent the predicted mean values for pollen plan area after data modelling using a generalized linear model. Vertical lines (error bars) represent the 95% confidence interval.

The fertility restoration rate (number of fertile polyploids divided by the number of polyploids produced) was 100% as confirmed by at least one of the following: zygote

success different from zero in backcrosses, *in vitro* germination of pollen, or production of viable pollen (Table 6-3).

Table 6-3 Summary of the results of the evaluations performed for assessing the fertility of the tetraploid hybrids produced by induction of whole genome duplication with oryzalin

Genotype	Produced zygotes as seed-bearing parent	Produced zygotes as pollen-donor parent	Viable pollen was detected	Pollen germinated *in vitro*
Y19.026.006	✓	✓	✓	✓
Y19.026.008	NA†	NA†	✗	✓
Y19.026.009	✓	✓	NA†	NA†
Y19.026.010	✗	✗	NA†	✓
Y19.026.013	✗	✗	✓	✓

† Not evaluated

✓ Successful results (yes)

✗ Negative results (no)

6.4. Discussion

Induction of WGD in *L. sinuatum* × *L. perezii* hybrids using oryzalin restored the male and/or female fertility of the hybrids addressing the hybrids' limitations: low viability / sterility and facilitated the use of the hybrids in planned crosses to increase the germplasm diversity. These results of successful production of fertile *L. sinuatum* × *L. perezii* hybrids contrast to our previous results using N_2O for production of polyploid hybrids by crossing unreduced gametes that resulted in zero hybrid polyploids.

Pollen viability and *in vitro* pollen germination did not always result in seedlings in backcrosses as observed for genotypes Y19.026.010 and Y19.026.013 (Table 6-3). The absence of seedlings as seed-bearing parents could suggest that only male fertility was restored in this genotype or that factors other than pollen fertility are impeding seedling production. A proposed factor is incompatibility between parental genomes as observed in *Lilium* (Nukui et al., 2011; Zhang et al., 2017) and suspected between some genotypes of *Limonium* with lower zygote success when backcrossed in comparison to outcrossed (refer to APPENDIX L).

Differences in the number of seedlings obtained in backcrosses when tetraploid hybrids are seed-bearing in comparison with tetraploid hybrids as pollen-donor suggest the occurrence of a parent-of-origin effect with interploidy crosses being more successful when the seed-bearing genotype is tetraploid in comparison with a diploid seed-bearing parent as also observed in *A. arenosa* (Morgan et al., 2021). These results with tetraploid × diploid crosses producing more (four) seedlings in comparison with the reverse cross diploid × tetraploid (one) suggest the occurrence of a "leaky" triploid block with *Limonium* tolerating a maternal excess (tetraploid × diploid) better than a paternal excess (diploid × tetraploid). The "leaky" triploid block would, therefore, allow gene flow in some interploidy crosses depending on the genotypes used (i.e., parent-of-origin) with less disruption probably occurring in the development of the embryo and endosperm as also reported for *A. arenosa* (Morgan et al., 2021). The success of tetraploid × diploid crosses is at odds with studies in *Lilium* with no seed production in tetraploid × diploid crosses a consequence of the triploid block (Nukui et al., 2011; Zhang et al., 2017).

In the present research, the outcomes arising from applying oryzalin contrasts with reports on other plant species where lower oryzalin concentrations and shorter duration of treatment were used. In our study, longer exposure (two months) to a higher oryzalin concentration (43.3 µM) resulted in a higher tetraploidization rate (23%) in comparison with *Tulipa gesneriana* L. (14.4 µM oryzalin; 7 days; 8 tetraploids by 50 plants obtained (16%); Podwyszyńska (2012)) and *Manihot esculenta* Crantz (3-15 µM oryzalin; 24-48 h; 2 tetraploids by 310 plants obtained (0.6%); Carvalho et al. (2016)).

Induction of WGD with oryzalin in comparison to induction of WGD with N_2O resulted in a proportion of polyploids either similar or 45% lower depending on the genotype. In contrast, there were many more mixoploids plants (280% higher) after oryzalin treatment than when N_2O was used for WGD induction (Table 6-4). As mixoploids display genetic instability this outcome is undesirable, with stable polyploids required within a typical breeding programme. An unstable mixoploid could result in ploidy reversal to its original ploidy, in this case diploid (Grosser et al., 2014; Podwyszyńska, 2012) defeating the targeted breeding outcome. Also mixoploids could be sterile, as reported for *Citrus grandis* Osbeck (Grosser et al., 2014), but sterility is not always evident (Kozar et al., 2020). Nevertheless, mixoploids could still be a potential source of a solid polyploid if a

technique for mixoploid separation (e.g., selection of single shoots following by three cycles of induction of adventitious buds) is used as reported in *Lilium* (Zhang et al., 2017).

Table 6-4 Compilation of the relevant data for induction of whole genome duplication in *Limonium* with either oryzalin (current chapter) or nitrous oxide (N_2O) (CHAPTER 3) for comparison purposes between inductors (oryzalin and N_2O)

Feature	**Oryzalin**	**N_2O**
Type of WGD treatment	*In vitro*	*In vivo*
Treatment duration	Five months¶	Up to three days
Total duration of the breeding cycle (from crossing to growing of polyploids under greenhouse conditions)	8 months	6 months
Proportion of polyploids (%)	23	22-42¥†
Proportion of mixoploids (%)	14	5†
Fertile hybrids produced	5§	0

¶It includes establishment *in vitro,* oryzalin treatment, shoot proliferation, and rooting
¥Differences by genotypes
†Information extracted from CHAPTER 3
§ Fertility restoration was assessed by backcrossing to the *Limonium sinuatum* parent, evaluation of pollen viability or *in vitro* germination of pollen

The principal drawback, at least in this research, of using oryzalin in comparison with N_2O was the requirement of first establishing the plant material to be treated in tissue culture (consuming approximately 1 month), with the subsequent exposure to oryzalin for two months, plus shoot proliferation and rooting of the plants (two extra months) extending the duration before achieving a potential polyploid plant (Table 6-4). However, a combination between N_2O treatment to increase the interspecific hybridisation rate followed by WGD using oryzalin could be potentially promising as it would eliminate the need to introduce of the genotype to *in vitro* culture. For example, the embryo resulting from the crossing of potentially unreduced gametes induced with N_2O, can be ovule/embryo rescued *in vitro* and treated with oryzalin for the induction of WGD. However, further experiments still required to validate this as a protocol.

6.5. Conclusion

Oryzalin induced WGD increased the fertility of *L. sinuatum* × *L. perezii* hybrids as evidenced by the restoration of pollen viability and *in vitro* germination, and the potential of some hybrids to produced triploid seedlings after backcrosses.

Oryzalin is an alternative to N_2O to induce WGD and produce polyploid *L. sinuatum* × *L. perezii* hybrids which required longer time and resources in comparison with WGD using N_2O.

CHAPTER 7. Morphological and reproductive characterisation of tetraploid *Limonium* plants

As detailed below, this chapter comprises experiments that have been presented orally to my scientific peers at two conferences.

Details of the conferences are:

Córdoba J., Roskruge N., Funnell K, Hedderley D, Morgan E. Nitrous oxide (N_2O) and ploidy changes in a model crop. Plant Science Central Conference. Palmerston North, New Zealand. 6th – 8th July 2021.

Córdoba J., Roskruge N., Funnell K, Hedderley D, Morgan E. Uso del óxido nitroso (N_2O) para la inducción de cambios en la ploidía de un cultivo modelo. XIII Simposio Internacional de Recursos Genéticos para las Américas y el Caribe. Colombia. 30th November-3rd December 2021.

7.1. Introduction

Diversity from Latin *dīversitās* means "various", "different". In biological terms, diversity could refer species diversity, genetic diversity, and taxonomic diversity (Petit & Thompson, 1999), among others. In the present research, one of the main objectives (refer to Section 1.6) of the breeding technologies evaluated (i.e., ploidy manipulation and physically induced mutations) was to increase the germplasm diversity; this being the diversity of the genetic material which could potentially be introgressed to future generations (Offord, 2017).

The influence of the ploidy level over diversity has been studied from different perspectives including ecological (Petit & Thompson, 1999), taxonomic (Petit & Thompson, 1999) and morphological (Birchler & Veitia, 2012). In the present study, the effect of increased ploidy after using polyploidization technologies (e.g., N_2O, oryzalin treatments) was evaluated in terms of morphological characteristics and reproductive performance of the genotypes obtained in the current research.

A relation between ploidy level and organ size, known as the "gene dosage effect", has been confirmed in *L. sinuatum* (refer to CHAPTER 2) with increased ploidy resulting in bigger pollen grains, greater guard cell length and floral stem diameter, thicker and bigger leaves, and lower stomatal density. However, the relationship between higher ploidy and

bigger organs is organ- and genotype-dependent, as observed in *Tulipa* (Podwyszyńska, 2012) or in *Brassica* (Harun et al., 2021).

An increase of a plant's ploidy, e.g., after whole genome duplication, has also consequences over its fertility and reproductive performance by: restoring fertility (Nukui et al. (2011); Okazaki et al. (2005); refer to CHAPTER 4 and CHAPTER 6); extending the genetic distance between polyploid seedlings and their corresponding diploid counterparts (Morgan et al., 2021); or promoting the occurrence of the triploid block as a consequence of an imbalance in the endosperm number (refer to Section 1.3.7).

The occurrence of morphological and/or reproductive changes in *Limonium* as a consequence of a ploidy increase has at least three implications that are attractive for a breeding programme involving ornamentals. First, as *Limonium* is the 15th most sold cut flower (International Association of Horticultural Producers, 2022), the desire expressed by the market for novelty could be met by the resulting bigger flowers or new palette colours (Manzoor et al., 2018; Mori et al., 2016; Zeng et al., 2020). Second, the further use of the artificially produced tetraploids in future crosses depends on their morphological characteristics as well as their reproductive performance (Kuligowska et al., 2016; Morgan et al., 1998). Third, the use of polyploid plants in crosses could reduce the number of breeding cycles required for combining the desired characteristics, due to faster accumulation of desired alleles via maximization of the allelic diversity (Riddle & Birchler, 2008).

Despite the above mentioned benefits of a ploidy increase, some drawbacks have been documented in neo-polyploids (i.e., artificially obtained polyploids) related mainly with sterility or low fertility as a consequence of lack of meiotic stabilization which affect e.g., homologous pairing, recombination, and chromosome segregation (Bomblies, 2022; Kato & Birchler, 2006). The impact of meiotic abnormalities after polyploidization events could represent a decrease in pollen production or in the proportion of viable pollen (Bomblies, 2022), with subsequent reduction in zygote success (Akšić et al., 2016; Defani-Scoarize et al., 1995; Tatum et al., 2015). The effect of meiotic abnormalities over megaspore fertility are far less documented, due to greater difficulties in female cytological studies in comparison with male cytological studies (Bomblies, 2022; Rois, 2014). Nevertheless, some studies reveal that meiotic abnormalities during female

meiosis are responsible for aneuploidy and apospory in *Arabidopsis* (Cao et al., 2021), and failure in the production of megaspores in *Ranunculus auricomus* (Barke et al., 2020).

For hybridisation to be successful, there must be partial or total compatibility between genotypic combinations in terms of pollen-pistil, embryo and endosperm (Larrosa et al., 2012). This partial compatibility has already been confirmed in diploid interspecific crosses between *L. sinuatum* and *L. perezii* (Morgan et al., 1998; Zhang, 1995) which have resulted after advanced breeding in commercial cultivars (BallSB, 2021). Nevertheless, the first generation of *L. sinuatum* × *L. perezii* hybrids are infertile, likely as a consequence of meiotic disruption associated with an odd chromosome number (2n=2x=15; Morgan et al. (1998)). Fertility of those hybrids could be restored in interspecific hybrids from tetraploid parents (refer to CHAPTER 6), as homologous chromosome pairing could be re-established and normal meiosis could then occur (Nukui et al. (2011); refer to CHAPTER 4).

In this study, having previously failed to produce fertile interspecific hybrids by induction of unreduced gametes (refer to CHAPTER 5), it is proposed to cross tetraploid *L. sinuatum* and *L. perezii* plants instead of diploid plants. In doing so the logic is to increase the interspecific hybridisation rate and produce fertile interspecific hybrids. The research hypothesis was that tetraploid plants produce 2n gametes which, when used in interspecific crosses, rehabilitate the paring during meiosis of homolog chromosomes facilitating the production of fertile distant hybrids (Dewitte et al., 2012; Eeckhaut et al., 2018; Younis et al., 2014).

In this chapter, the effect of higher ploidy over morphological and reproductive characteristics of two *Limonium* species: *L. sinuatum* and *L. perezii*, and their interspecific hybrids, was evaluated and compared with their diploid counterparts. In addition, the behaviour of tetraploid *L. perezii*, *L. sinuatum*, or interspecific tetraploid *L. sinuatum* × *L. perezii* hybrids, all used in intraspecific, interspecific, and backcrosses was evaluated. The intraspecific and backcrosses aimed to verify the fertility/crossability of the tetraploid genotypes, while the interspecific crosses intended to produce fertile tetraploid hybrids and evaluate if interspecific tetraploid crosses resulted in a higher number of zygotes per crosses completed in comparison with interspecific diploid crosses.

7.2. Materials and methods

7.2.1. Morphological evaluation

7.2.1.1 Plant material

Twenty-eight genotypes from *L. sinuatum, L. perezii*, or *L. sinuatum* × *L. perezii* hybrids, either diploid or tetraploid, were utilised within this study (Table 7-1). *L. perezii* genotypes were divided into two groups (*L. perezii*-white or *L. perezii*-purple) (Table 7-1) based on their floret colour (i.e., Y18.011.001, Y19.027.001, Y19.027.005) or their parents' floret colour (e.g., Y21.004.002, Y21.005.002, Y21.005.004, Y21.005.006). The division was based on previous observations of possible differences in reproductive performance, which could be associated with floret colour, but still needed confirmation.

Plants were grown under greenhouse conditions at The New Zealand Institute for Plant & Food Research Ltd in Palmerston North, New Zealand (40.9006 °S, 174.8860 °E) in 2 L solid plastic pots containing a bark-based growing medium (APPENDIX E). The set points for temperature control in the greenhouse were a minimum of 16°C with ventilation commencing at 18°C. Light conditions corresponded to natural day length for Palmerston North equal to 14h in November 2020 when the morphological evaluation of *L. sinuatum* was done, and between 11h and 9h in the period between April and June 2022 when the morphological evaluation of *L. perezii* and the interspecific hybrids was done.

The tetraploid plants of *L. sinuatum* and *L. perezii* evaluated were produced by induction of whole genome duplication (WGD) with N_2O (CHAPTER 2 and CHAPTER 3), while the tetraploid *L. sinuatum* × *L. perezii* hybrids were produce by WGD induced with oryzalin (CHAPTER 6).

Table 7-1 Germplasm group and ploidy of the *Limonium* genotypes used in the morphological evaluation and pollen characterisation

Genotype	Germplasm group	Ploidy
Y18.011.001	*L. perezii*-white	Diploid
Y21.005.002	*L. perezii*-white	Diploid
Y21.005.004	*L. perezii*-white	Tetraploid
Y21.004.002	*L. perezii*-white	Tetraploid
Y21.005.006	*L. perezii*-white	Tetraploid
Y19.027.001	*L. perezii*-purple	Diploid
Y19.027.005	*L. perezii*-purple	Diploid
Y21.008.008	*L. perezii*-purple	Diploid
Y21.009.010	*L. perezii*-purple	Diploid
Y21.008.004	*L. perezii*-purple	Tetraploid
Y21.009.001	*L. perezii*-purple	Tetraploid
Y21.008.010	*L. perezii*-purple	Tetraploid
Y19.015.001	*L. sinuatum*	Diploid
Y19.016.001	*L. sinuatum*	Diploid
Y21.003.001	*L. sinuatum*	Diploid
Y21.003.009	*L. sinuatum*	Diploid
Y19.020.004	*L. sinuatum*	Tetraploid
Y19.020.003	*L. sinuatum*	Tetraploid
Y21.001.003	*L. sinuatum*	Tetraploid
Y21.007.001	*L. sinuatum*	Tetraploid
Y19.026.001	*L. sinuatum* × *L. perezii* hybrid	Diploid
Y19.026.003	*L. sinuatum* × *L. perezii* hybrid	Diploid
Y21.010.015	*L. sinuatum* × *L. perezii* hybrid	Diploid
Y19.026.006	*L. sinuatum* × *L. perezii* hybrid	Tetraploid
Y21.011.009	*L. sinuatum* × *L. perezii* hybrid	Tetraploid
Y19.026.013	*L. sinuatum* × *L. perezii* hybrid	Tetraploid
Y19.026.008	*L. sinuatum* × *L. perezii* hybrid	Tetraploid
Y19.026.010	*L. sinuatum* × *L. perezii* hybrid	Tetraploid

7.2.1.2 Experimental design

The experimental design was a randomized complete block design (Compton, 1994) with the treatments corresponding to ploidy (diploid or tetraploid) and blocks represented by the germplasm group (i.e., either *L. perezii*-white, *L. perezii*-purple, *L. sinuatum*, or *L. sinuatum* × *L. perezii* hybrid).

The response variables evaluated were: guard cell length, stomatal density, leaf thickness and area, floral stem diameter, and stem wing width. The methods used for measurement of each trait comprised:

For leaf thickness and leaf area, three fully expanded leaves (replicates) for each genotype were sampled. Leaf thickness measures for all genotypes, and the leaf area for the *L. sinuatum* were done according to CHAPTER 2. However, the leaf area determination of *L. perezii* and the *L. sinuatum* × *L. perezii* hybrids, was done with an area meter LI-3100/1 ± .01 (LI-COR inc. Lincoln, Nebraska-United States).

For guard cell length and stomatal density, the sample size was calculated at p=0.05 and 99% power using the information about mean and standard deviation calculated for guard cell length and stomatal density for *L. sinuatum* and reported in CHAPTER 2. For the guard cell length, 30 guard cells were measured per leaf (sub-samples) in three leaves (replicates). For the stomatal count 10 different regions of each leaf were measured (sub-samples) in three leaves (replicates).

Measurement of floral stem diameter and stem wing width was conducted immediately below the first branch node (from bottom to top) on the main axis of the inflorescence, using a Vernier calliper (0-150 mm, Protect, New Zealand, TD2082). Three stems with at least 80% of the calyces open (Karis, 2004) were evaluated per plant (replicates) (Figure 7-1).

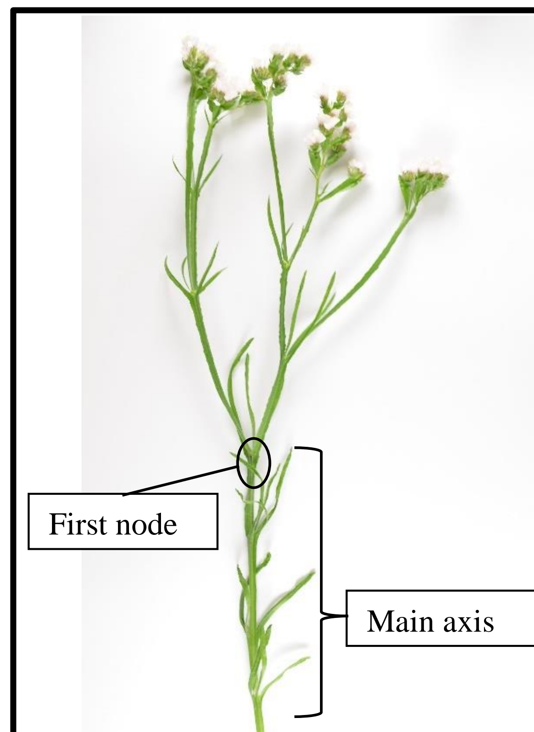

Figure 7-1 Inflorescence of *Limonium sinuatum* showing the main axis and the first node. Right below the first node was where the floral stem diameter and the stem wing width were measured

7.2.1.3 Data analysis

All the response variables: guard cell length, leaf thickness, leaf area, floral stem diameter, and stem's wing width, followed a normal distribution, so the data was analysed with linear models after validation that the residuals comply with the assumptions (i.e., normal distribution of the residuals, observations are independent of each other; lm function from the stats package R (R Core Team, 2018)). Pairwise differences between means were evaluated with the Least Significant Difference test (LSD; Mendiburu and Yaseen (2020)), utilizing a *p-value* of 0.05.

The response variable stomatal density followed a binomial distribution, so data was analysed with a generalized linear model (glm function from the stats package (R Core Team 2018)) specifying the binomial distribution. To compare the levels of the factors, pairwise likelihood ratio tests were used (similar to LRPAIR in Goedhart (2018)), utilizing a *p-value* of 0.05.

7.2.2. Pollen characterisation

Pollen characterisation was performed during April 2022 using the same genotypes used for the morphological characterisation (Table 7-1) and keeping the division of *L. perezii* in two groups (*L. perezii*-white or *L. perezii*-purple) based on their floret colour or their parents' floret colour (refer to Section 7.2.1.1). Pollen characterisation included pollen count per µl, measurement of plan area, pollen viability using tetrazolium test, and *in vitro* germination are detailed below.

7.2.2.1 Pollen count

Pollen from two florets per genotype were mixed in 100 µl of an ethanol:glycerol fixation solution containing 85% ethanol and 100% glycerol in a 3:1 proportion (Hammer et al., 2015; Hammer et al., 2011). The number of pollen grains per µl was counted with the aid of a hemocytometer (Bright-Line. Hausser Scientific, Horsham-USA). To do so, 20 µl of the solution containing the pollen were added between the hemocytometer and the cover glass (UCDavis, 2021). The pollen grains located completely inside of five major squares (four corners and the central square) were subsequently counted and averaged to become a sample (Figure 7-2). Four pollen samples (replicates) were evaluated for each genotype. As each square measured 1 mm × 1 mm, and the distance between the hemocytometer and the cover slide was 0.1 mm, the volume of each chamber was 0.1 mm^3 or 0.1 µl (UCDavis, 2021).

The experimental design for determining pollen grain counts per volume (µl), was a randomized complete block design, with ploidy as the treatment and germplasm group as blocks (i.e., *L. perezii*-white, *L. perezii*-purple, *L. sinuatum*, or *L. sinuatum* × *L. perezii* hybrid), utilising four replicates (i.e., pollen samples). The response variable was number of pollen grains per µl.

Data was analysed with a generalized linear model (glm function from the stats package (R Core Team 2018)), specifying the Poisson distribution. A *p-value* of 0.05 used as cut-off value to determine if differences in the number of pollen grains per µl were the result of treatments rather than random factors.

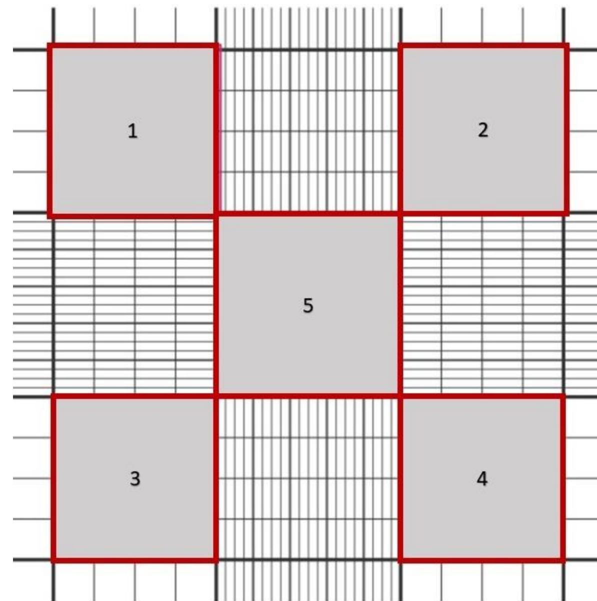

Figure 7-2 Diagrammatic illustration of a hemocytometer, showing the gridlines. The red coloured squares were those used for counting the number of pollen grains. Each square measured 1 mm^2. Modified from (UCDavis, 2021)

7.2.2.2 Pollen size, viability, germination, and bursting

Pollen viability was conducted as described in Section 6.2.4.2.2 using tetrazolium. Pollen grains stained dark red were recorded as viable while pollen grains colourless or with doubtful coloration were recorded as aborted (Shivanna & Rangaswamy, 1992). Two samples were done per genotypes (replicates) with 100 pollen grains (sub-samples) evaluated per sample.

Pollen germination was performed as described in Section 6.2.4.2.3 using the modified dialysis tubing method (Zhang et al., 1997) with Brewbaker and Kwack medium (Brewbaker & Kwack, 1963) amended with 20% PEG-20,000 (APPENDIX J). As the same time as evaluating pollen germination, bursting of pollen grains were recorded. Both response variables were mutually exclusive. Three samples were done per genotype (reps) with 100 pollen grains (sub-samples) evaluated per sample.

The same samples (i.e., three) prepared for pollen germination were used for measurement of pollen size. One hundred pollen grains (sub-samples) per sample were

observed, photographed, and measured using a Zeiss microscope Imager.Z2 (Carl Zeiss Microscopy, Axio Imager Z2, Germany) using a digital camera (Axiocam 305 color, Carl Zeiss Microscopy GmbH, Germany) and the software ZEN2.6 (blue edition) (Carl Zeiss Microscopy GmbH, 2018) as detailed in CHAPTER 2.

The experimental design was a randomized complete block design with sub-samples (Compton, 1994) with ploidy as treatment (i.e., diploid or tetraploid) and germplasm group as blocks (i.e., *L. perezii*-white, *L. perezii*-purple, *L. sinuatum*, or *L. sinuatum* × *L. perezii* hybrid). The response variables were pollen viability (yes/no), pollen germination (yes/no), and pollen plan area.

The pollen plan area was analysed with a linear model (lm function from the stats package R (R Core Team, 2018)) after validation of the assumptions (i.e., normal distribution of the residuals, observations are independent of each other). Pairwise comparison for the means were done using Least Significant Difference test (LSD; Mendiburu and Yaseen (2020)). The *p-value* used was 0.05.

Data analysis for the variables with binomial distribution (i.e., pollen count, viability, and germination) was performed with a generalized linear model (glm function from the stats package (R Core Team 2018)). Likelihood ratio tests were used (similar to LRPAIR in Goedhart (2018)) for comparison of the factors' levels. A *p-value* equal to 0.05 was used to determinate if the differences between treatments or blocks were random.

Receiver operating characteristic curves (ROC curve) (Kumar & Indrayan, 2011) were used to evaluate the performance of two classification models:

- One model to classify pollen according to their ploidy which allowed the identification of a threshold plan area to differentiate between pollen collected from diploid or tetraploid plants.
- Another model to classify pollen based on their viability after tetrazolium test which facilitate the identification of a threshold plan area to differentiate between viable and aborted pollen. ROC curve was calculated for diploid and tetraploid genotypes independently.

The ROC curves were calculated per germplasm group, as significant differences were noted during preliminary evaluation.

7.2.3. Reproductive performance

7.2.3.1 Plant material

The 21 genotypes used for evaluation of reproductive performance (Table 7-2) were selected based on their: ploidy, contrasting stigma, and synchronous timing of flowering. As a result, the genotypes selected differed from those listed in Table 7-1. The selected genotypes were either diploid or tetraploid plants, of either *L. perezii, L. sinuatum*, or *L. sinuatum* × *L. perezii* hybrids (Table 7-2).

Table 7-2 Information about the germplasm group, ploidy, and breeding method used for ploidy increase (i.e., in tetraploid genotypes) for the genotypes included in the reproductive performance evaluation

Genotype	Germplasm group	Ploidy	Breeding method used for ploidy increase	Reference
Y21.009.001	*L. perezii*	Tetraploid	WGD with N_2O	CHAPTER 3
Y21.008.010	*L. perezii*	Tetraploid	WGD with N_2O	CHAPTER 3
Y21.008.009	*L. perezii*	Tetraploid	WGD with N_2O	CHAPTER 3
Y21.008.004	*L. perezii*	Tetraploid	WGD with N_2O	CHAPTER 3
Y21.005.004	*L. perezii*	Tetraploid	WGD with N_2O	CHAPTER 3
Y19.019.009	*L. sinuatum*	Tetraploid	WGD with N_2O	APPENDIX F
Y19.019.017	*L. sinuatum*	Tetraploid	WGD with N_2O	APPENDIX F
Y19.019.018	*L. sinuatum*	Tetraploid	WGD with N_2O	APPENDIX F
Y19.020.003	*L. sinuatum*	Tetraploid	WGD with N_2O	APPENDIX F
Y19.020.004	*L. sinuatum*	Tetraploid	WGD with N_2O	APPENDIX F
Y19.020.014	*L. sinuatum*	Tetraploid	WGD with N_2O	APPENDIX F
Y21.007.001	*L. sinuatum*	Tetraploid	WGD with N_2O	CHAPTER 2
Y17.006.001	*L. sinuatum* × *L. perezii* hybrids	Tetraploid	WGD with oryzalin	(Morgan et al., 2001)
Y19.026.006	*L. sinuatum* × *L. perezii* hybrids	Tetraploid	WGD with oryzalin	CHAPTER 6
Y21.008.008	*L. perezii*	Diploid	None	CHAPTER 3

Genotype	Germplasm group	Ploidy	Breeding method used for ploidy increase	Reference
Y21.009.010	*L. perezii*	Diploid	None	CHAPTER 3
Y21.001.002	*L. sinuatum*	Diploid	None	CHAPTER 2
Y21.001.005	*L. sinuatum*	Diploid	None	CHAPTER 2
Y21.003.007	*L. sinuatum*	Diploid	None	APPENDIX F
Y21.011.009	*L. sinuatum* × *L. perezii* hybrids	Diploid	None	CHAPTER 5
Y21.010.015	*L. sinuatum* × *L. perezii* hybrids	Diploid	None	CHAPTER 5

The plants were grown in 10 L solid plastic pots under greenhouse conditions at PFR in Palmerston North, New Zealand (40.9006 °S, 174.8860 °E) during summer 2021/2022. Crosses were performed between March and May 2022. Greenhouse conditions included temperature control with heating to maintain a minimum of 16 °C and ventilation commencing at 18 °C with natural day length equal to 14.30h in summer and between 12 h in March and 9 h in May. Medium was kept at field capacity by a drip irrigation system (8.5 L/h, PC Low CNL Netafim™, Hatzerim, Israel).

7.2.3.2 Experimental design and response variables recorded

The experimental design was a randomized complete block (Compton, 1994) with the treatments corresponding to cross type (intraspecific, interspecific, or backcross) and the blocks to ploidy of the parents (diploid or tetraploid). Ten individual florets (sub-samples) were pollinated daily for three days (replicates). Each genotype was used both as a pollen-donor and seed-bearing parent, and each floret was pollinated only once. Two weeks after pollination ovule/embryo rescued was undertaken, following the method described by Morgan et al. (1998).

All the backcrosses performed in this chapter, in contrast to some of the backcrosses in CHAPTER 6 (refer to Section 6.2.4.1), were homoploid, i.e., pollen-donor and seed-bearing parent had the same ploidy, either diploid or tetraploid. Backcrosses were performed between *L. sinuatum* × *L. perezii* hybrids and an individual, genetically similar to either of the parents i.e., *L. sinuatum* or *L. perezii*.

The response variables evaluated were zygote success (number of zygotes obtained divided by the number of crosses completed), germination rate (number of zygotes germinated *in vitro* by number of zygotes obtained (rescued)), and cross success (Number of seedlings obtained divided by number of crosses completed). In the case of interspecific crosses and backcrosses, two additional response variables determined by flow cytometry (Cordoba-Sanchez et al. (2023a); refer to CHAPTER 2) were: hybridisation rate (number of hybrids obtained by number of crosses completed) and ploidy of seedling derived from the cross.

Previous to data analysis, data from confirmed non-hybrid zygotes was eliminated to avoid bias by potential apomictic events (refer to Section 1.3.6). Hybrid status was

confirmed by flow cytometry analysis after zygotes germinated *in vitro*, meaning it was not possible to exclude non-hybrid zygotes from the zygote success calculation and, therefore, it could be over-estimated.

7.3. Results

7.3.1. Morphological characterisation

A ploidy increase was associated with 1.4 times increase in guard cell length, and a 0.5 times reduction in stomatal density for all genotypes (p<0.05; Table 7-3). In contrast, only *L. sinuatum* increase in ploidy was associated with tetraploid leaves 1.5 times thicker and 1.8 times larger in area, stem diameter 1.5 times longer, and stem wings 2.4 times wider in comparison with those of the diploids (p<0.05; Figure 7-3; Figure 7-4; Table 7-3).

The differences observed for the morphological characteristics were also associated with the germplasm group. Diploid *L. perezii* and diploid *L. sinuatum* differed for all the morphological variables other than floral stem diameter and guard cell length (p<0.05; Table 7-3). The diploid hybrids exhibited intermediate values compared to the parental plants for leaf thickness and area; for the other morphological variables evaluated were similar to either of the parents. For tetraploid plants, differences between *L. perezii, L. sinuatum,* and hybrids occurred only for stem wing width (Table 7-3).

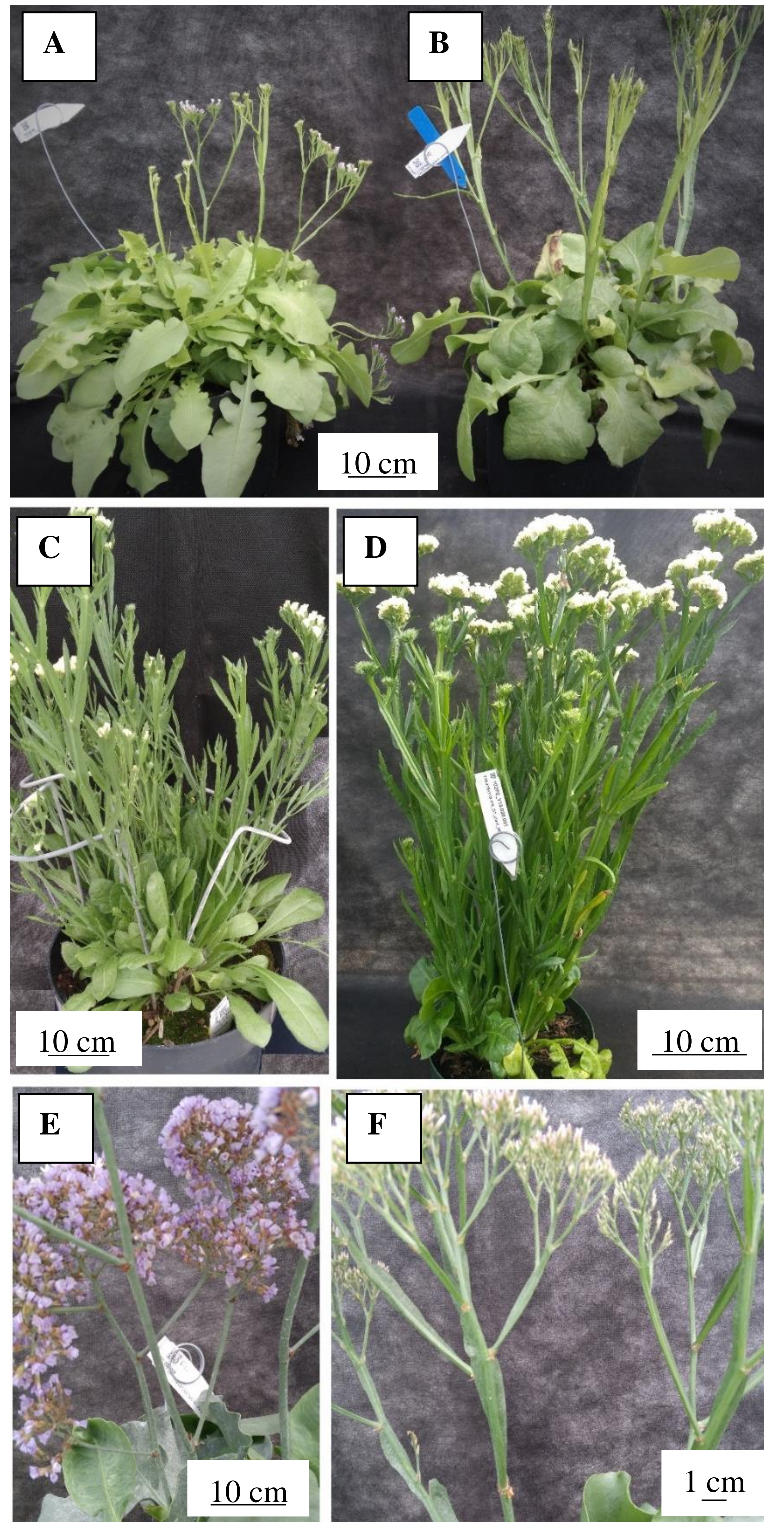

Figure 7-3 Diploid (left) and tetraploid (right) plants of interspecific *Limonium sinuatum* × *Limonium perezii* hybrids (**A** and **B**), *L. sinuatum* (**C** and **D**), and *L. perezii* (**E** and **F**). Tetraploid plants (**B, D,** and **F**) in comparison with diploid (**A, C,** and **E**) have thicker leaves and floral stems, stronger colours, and wider stem wings

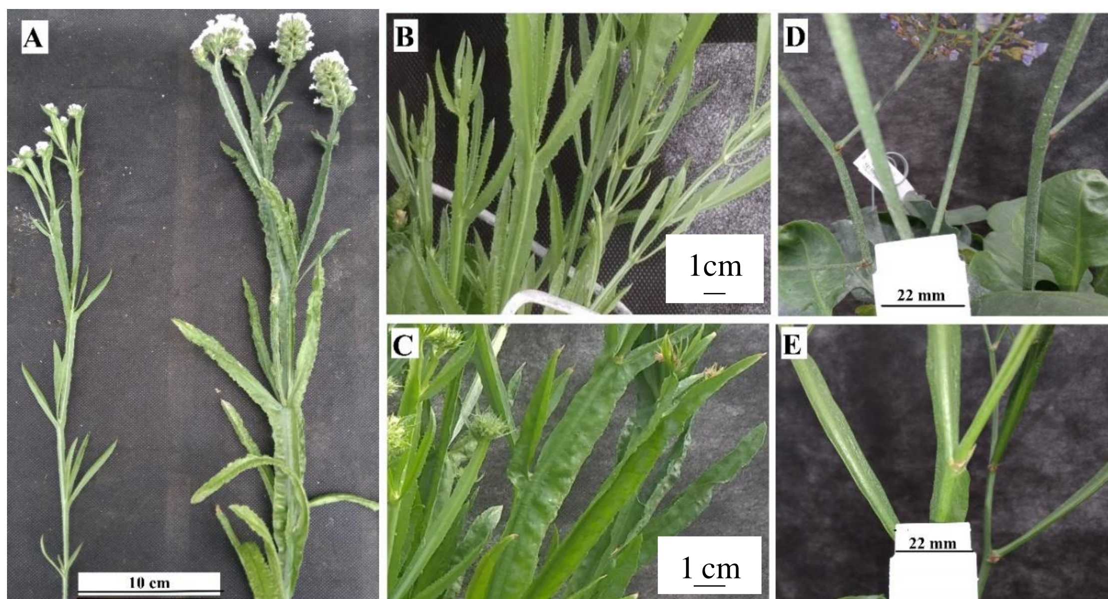

Figure 7-4 Detailed photo of the main floral stems from diploid and tetraploid plants representing *Limonium sinuatum* (**A – C**) and *Limonium perezii* (**D** and **E**). **A.** On the left a diploid floral stem thinner in diameter and with thinner wings in comparison with the tetraploid floral stem on the right. **B-C** Tetraploid (**C**) stem wings are wider and display a darker green colour in comparison with diploids. **D-E.** Floral stems from tetraploid *L. perezii* plants (**E**) present visible stem wings in comparison with diploid plants (**D**)

Table 7-3 Ploidy and germplasm group for the 21 genotypes of *Limonium* evaluated for their morphological characteristics. Means followed by a common letter are not significantly different by the LSD test at the 0.05% significance level

Germplasm group	Ploidy	Leaf thickness (mm)	Leaf area (cm^2)	Guard cell length (µm)	Stomata density†	Floral stem diameter (mm)	Stem wing width (mm)
L. perezii	Diploid	0.5 ± 0.1^{b}	81.0 ± 22.5^{bc}	30.7 ± 4.4^{a}	68.5 ± 19.0^{c}	3.6 ± 0.8^{ab‡}	0.2 ± 0.5^{a}
L. sinuatum	Diploid	0.3 ± 0.1^{a}	37.6 ± 14.5^{a}	27.5 ± 4.2^{a}	43.0 ± 15.5^{b}	3.2 ± 0.8^{a}	2.1 ± 0.7^{bc}
L. sinuatum × *L. perezii* hybrids	Diploid	0.4 ± 0.1^{ab}	52.2 ± 20.5^{ab}	27.7 ± 5.0^{a}	74.9 ± 22.9^{c}	2.7 ± 0.5^{a§}	2.4 ± 1.5^{c}
L. perezii	Tetraploid	0.5 ± 0.1^{b}	93.0 ± 27.3^{c}	41.8 ± 6.4^{b}	36.3 ± 12.3^{ab}	4.7 ± 0.7^{bc‡}	0.4 ± 0.4^{ab}
L. sinuatum	Tetraploid	0.5 ± 0.1^{b}	67.9 ± 19.6^{bc}	39.5 ± 7.6^{b}	25.0 ± 11.5^{a}	5.0 ± 0.7^{c}	5.0 ± 1.7^{d}
L. sinuatum × *L. perezii* hybrids	Tetraploid	0.5 ± 0.1^{b}	55.7 ± 21.6^{ab}	38.0 ± 6.0^{b}	43.5 ± 15.6^{b}	3.7 ± 1.1^{abc§}	2.8 ± 2.0^{c}

†Number of stomata per mm^2

‡*p*-value for the comparison between diploid and tetraploid *L. perezii* was 0.08

§ *p*-value for the comparison between diploid and tetraploid *L. sinuatum* × *L. perezii* hybrids was 0.1

7.3.2. Pollen characterisation

7.3.2.1 Pollen area

The plan area of pollen grains, including viable and aborted pollen, ranged for diploids between 185 µm^2 and 4174 µm^2; and for tetraploids between 150 µm^2 and 5798 µm^2. Differences in plan area were associated with ploidy (p<0.05), germplasm group (p<0.05), and their interaction i.e., ploidy: germplasm group (p<0.05). Pollen from tetraploid plants was at least 1.3 times larger in comparison with pollen from diploid plants (p<0.05; Table 7-4).

Table 7-4 Plan area (µm^2) of pollen from 21 genotypes representing diploid or tetraploid plants of *Limonium sinuatum, Limonium perezii* (white or purple), or *L. sinuatum* × *L. perezii* hybrids. Means followed by a common letter were not significantly different by the LSD test at the 0.05% level of significance.

Germplasm group	Plan area (µm^2)		Ratio of plan area between tetraploid and diploid	Ratio of standard deviations for plan area between tetraploid and diploid
	Diploid	Tetraploid		
L. sinuatum	1526.1 ± 424.6^b	2461.8 ± 727.3^e	1.6	1.7
L. perezii-white	1576.4 ± 399.2^b	2211.5 ± 1044.4^d	1.4	2.6
L. perezii-purple	1926.9 ± 501.9^c	2431.0 ± 1045.4^e	1.3	2.1
L. sinuatum × *L. perezii* hybrid	1068.6 ± 285.2^a	1781.3 ± 658.6^c	1.7	2.3

The differences between germplasm groups and ploidies were also observed in the distribution pattern and dispersion of the data for the plan area of pollen (Figure 7-5). For the diploid genotypes of all germplasm groups, with exception of Y21.009.010, the pollen area followed a unimodal distribution with a main peak (Figure 7-5). In comparison, the pollen plan area from the tetraploid genotypes of all germplasm groups followed a bimodal distribution with a small peak around 500 µm2 and a main peak around 2200

µm2 (Figure 7-5). In addition, the pollen areas for the tetraploid genotypes were more widely spread in a density plot (Figure 7-5) with standard deviations at least 1.7 times larger than for diploid plants (Table 7-4).

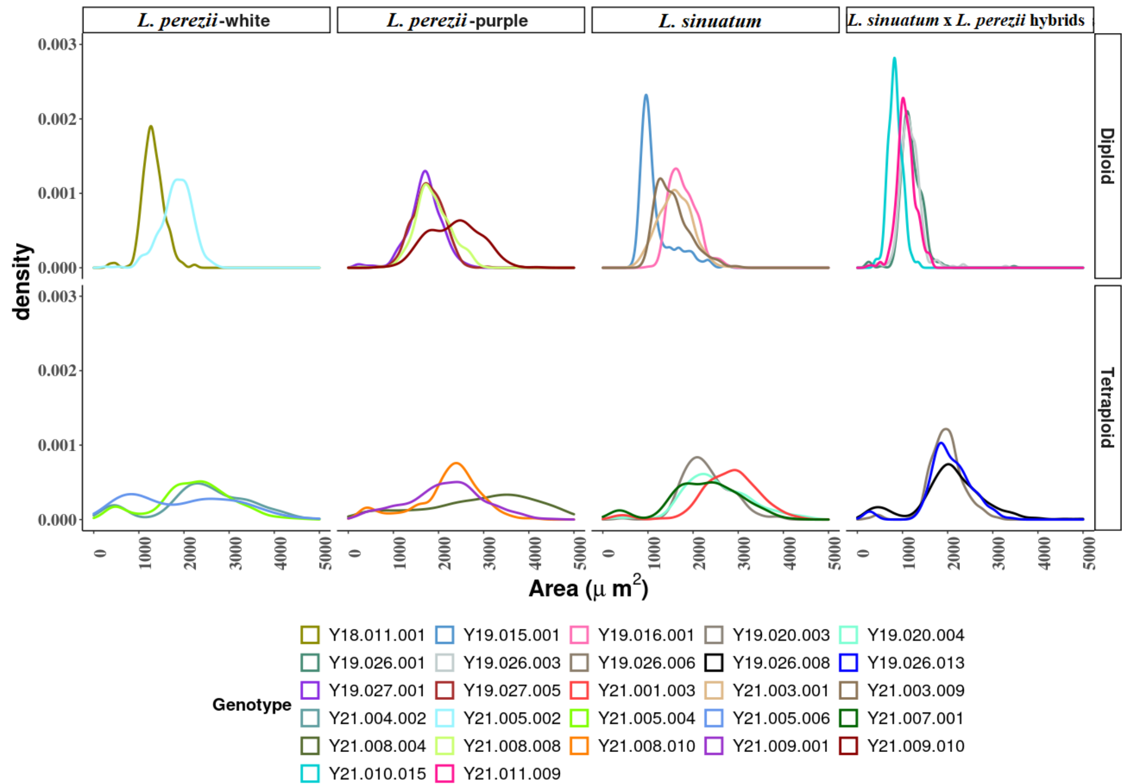

Figure 7-5 Density distribution for the plan area (µm^2) of pollen grains collected from diploid or tetraploid *Limonium sinuatum, Limonium perezii* (white or purple) or the *L. sinuatum* × *L. perezii* hybrids plants

The ROC curves calculated per germplasm group confirmed the differences, in terms of the pollen size increase associated with ploidy between them. The plan area selected as the threshold varied between 1450 µm^2 (*L. sinuatum* × *L. perezii* hybrids) and 2100 µm^2 (*L. sinuatum*) while the sensitivity and specificity were between 68% (*L. perezii* – purple) and 93% (*L. sinuatum* × *L. perezii* hybrids; Table 7-5).

Table 7-5 Pollen plan area (μm^2) selected as the threshold for differentiation between pollen from diploid and tetraploid genotypes for the germplasm groups: *Limonium sinuatum, Limonium perezii* (white or purple), and *L. sinuatum* × *L. perezii* hybrids. The corresponding sensitivity and specificity calculated by a receiver operating characteristic curve (ROC curve) means that pollen grains smaller than the threshold are potentially diploid while bigger would be potentially tetraploids

Germplasm group	Threshold (plan area μm^2)	Sensitivity and specificity (%)
L. sinuatum	1900	80
L. perezii – white	1800	70
L. perezii – purple	2100	68
L. sinuatum × *L. perezii* hybrids	1450	93

7.3.2.2 Pollen count

The number of pollen grains per μl varied between 10 ± 6 (diploid *L. perezii*-purple) and 29 ± 36 (diploid *L. sinuatum*) with differences between diploid germplasm groups ($p<0.05$; Table 7-6). Increase in ploidy increased the number of pollen grains per μl 170% ($p<0.05$) in *L. perezii*-purple but did not affect any other germplasm group (Table 7-6).

Even though the information collected for pollen count focused on the quantity of pollen grains per μl, it was observed but not quantified, that pollen quality differed between ploidies with more potentially aborted pollen grains (pollen grains smaller than the average for the corresponding germplasm group) produced by tetraploid genotypes in comparison with diploid genotypes.

Table 7-6 Number of pollen grains per μl by ploidy (diploid, tetraploid) and germplasm group (*Limonium sinuatum, Limonium perezii* (white or purple), or *L. sinuatum* × *L. perezii* hybrids). Means followed by a common letter are not significantly different by likelihood ratio test at the 0.05% level of significance

Germplasm group	Pollen grains per μl	
	Diploid	Tetraploid
L. sinuatum	29 ± 36^{d}	28 ± 14^{cd}
L. perezii-white	18 ± 16^{b}	22 ± 12^{bc}
L. perezii-purple	10 ± 6^{a}	27 ± 14^{cd}
L. sinuatum × *L. perezii* hybrid	21 ± 9^{b}	19 ± 12^{b}

7.3.2.3 Pollen viability

Pollen viability was 250% higher for all diploid germplasm groups, except for *L. sinuatum* × *L. perezii* hybrids, in comparison with tetraploids (35% ± 48% and 10% ± 30% pollen viability for diploids and tetraploids, respectively; $p<0.05$; Figure 7-6). In contrast, *L. sinuatum* × *L. perezii* hybrids had a viability increase from 0% in diploids to 4% ± 18% in tetraploids (Figure 7-6).

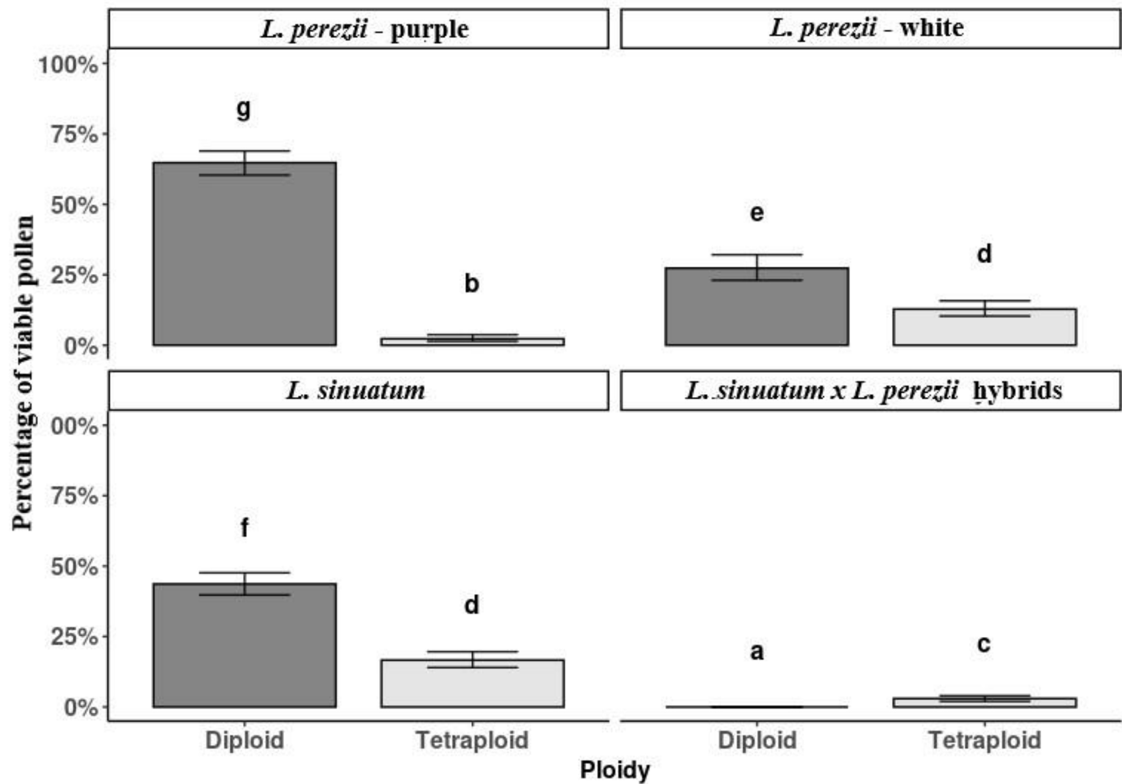

Figure 7-6 Viable pollen (%) after tetrazolium test as influenced by ploidy (diploid, tetraploid), germplasm group (*Limonium sinuatum*, *Limonium perezii* (white or purple), or *L. sinuatum* × *L. perezii* hybrid). Columns represent the predicted mean values for the proportion of viable pollen (%). Vertical lines (error bars) represent the 95% confidence interval. A likelihood ratio test was performed for the pairwise comparison between levels of the factors. Letters above the error bars in the figure denote mean groups, with different letters indicating statistically significant differences (*p*-value = 0.05). n= 100 pollen grains

An association between pollen plan area and viability was also identified with aborted pollen being between 0.8 and 0.5 times smaller in comparison with the plan area of viable pollen for diploids as well as for tetraploids ($p<0.05$; Table 7-7).

Table 7-7 Plan area (μm^2) of pollen collected from the germplasm group *Limonium sinuatum, Limonium perezii* (white or purple) and *L. sinuatum* × *L. perezii* hybrids based on their ploidy (diploid or tetraploid) and viability (viable or aborted). n = 100 pollen grains. Means followed by a common letter are not significantly different by the LSD test at the 0.05% level of significance

Germplasm group	Plan area (μm^2) Diploid		Tetraploid	
	Viable	Aborted	Viable	Aborted
L. sinuatum	2151 ± 343^{cd}	1212 ± 330^{a}	3172 ± 553^{g}	2632 ± 881^{f}
L. perezii-white	2354 ± 328d^{e}	1510 ± 278^{b}	3890 ± 1079^{h}	2098 ± 1259^{c}
L. perezii-purple	2348 ± 442^{e}	1633 ± 465^{b}	3862 ± 620^{h}	2693 ± 1041^{f}
L. sinuatum × *L. perezii* hybrid	0	1149 ± 357^{a}	3376 ± 740^{gh}	2096 ± 998^{c}

The sensitivity (i.e., probability of classifying a viable pollen grain as viable) and specificity (i.e., probability of classification an aborted pollen grain as aborted) are at least 5% higher for diploid pollen in comparison with the tetraploid pollen of the corresponding germplasm group (Table 7-8).

Table 7-8 Pollen plan area (µm^2) selected as threshold for differentiation between viable and aborted pollen and its respective sensitivity and specificity calculated by a receiver operating characteristic curve (ROC curve) in diploid and tetraploid plants from *Limonium sinuatum, Limonium perezii* (white or purple), and *L. sinuatum* × *L. perezii* hybrids. Pollen grains smaller than the threshold plan area would be aborted while bigger pollen grains would be potentially viable

Germplasm group	Diploid		Tetraploid	
	Threshold (plan area µm^2)	**Sensitivity and specificity (%)**	**Threshold (plan area µm^2)**	**Sensitivity and specificity (%)**
L. sinuatum	1750	92	3000	65
L. perezii – white	1900	90	3200	75
L. perezii – purple	2000	78	3400	74
L. sinuatum × *L. perezii* hybrids	N.A†	N.A. †	2700	77

† No apply. ROC curve was not calculated as only aborted pollen occurred in diploid *L. sinuatum* × *L. perezii* hybrids

7.3.2.4 Pollen germination and bursting

Pollen germination (Figure 7-7) was between 60% and 107% higher for diploid *L. sinuatum* and *L. perezii*-purple, respectively (p<0.05; Table 7-9). In contrast, no differences in pollen germination occurred in *L. perezii*-white (p>0.05) while in the *L. sinuatum* × *L. perezii* diploid hybrids increase in ploidy improved the pollen germination from 0% to 5% (p<0.05; Table 7-9).

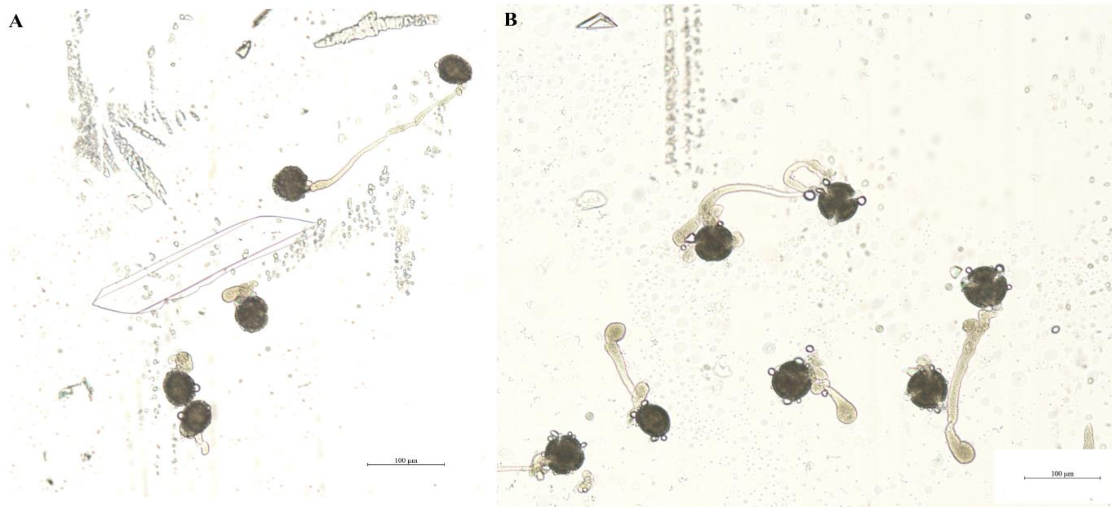

Figure 7-7 *In vitro* pollen germination. **A.** Pollen from diploid pollen from *Limonium perezii*-purple (Y19.027.005). **B.** Pollen from tetraploid pollen from *Limonium sinuatum* (Y21.007.001)

Table 7-9 Pollen grains germinated *in vitro* (%) by ploidy (diploid, tetraploid) and germplasm group (*Limonium sinuatum, Limonium perezii* (purple or white), or *L. sinuatum* × *L. perezii* hybrids). n = 100 pollen grains. Means followed by a common letter are not significantly different at the 0.05% level of significance after the likelihood ratio test was performed

Germplasm group	Pollen germination (%)	
	Diploid	Tetraploid
L. sinuatum	16 ± 37^{d}	10 ± 30^{c}
L. perezii-white	16 ± 37^{d}	16 ± 37^{d}
L. perezii-purple	27 ± 44^{e}	13 ± 33^{cd}
L. sinuatum × *L. perezii* hybrid	0 ± 0^{a}	5 ± 21^{b}

For all germplasm groups and irrespective of ploidy, except for those of diploid *L. perezii*-purple, a relation between germination and plan area of pollen grains was identified with the plan area of ungerminated pollen being between 0.6 and 0.8 times the plan area of germinated pollen ($p<0.05$; Figure 7-8). Diploid *L. perezii*-purple exhibited no size differences between germinated and ungerminated pollen ($p>0.05$; Figure 7-8).

Ploidy increase produces an increment in pollen size for all the germplasm group with tetraploid germinated pollen being 1.6 times larger than germinated diploid pollen

($p<0.05$; Figure 7-8). In the case of ungerminated pollen, tetraploid pollen was at least 1.3 times larger than ungerminated diploid pollen ($p<0.05$; Figure 7-8).

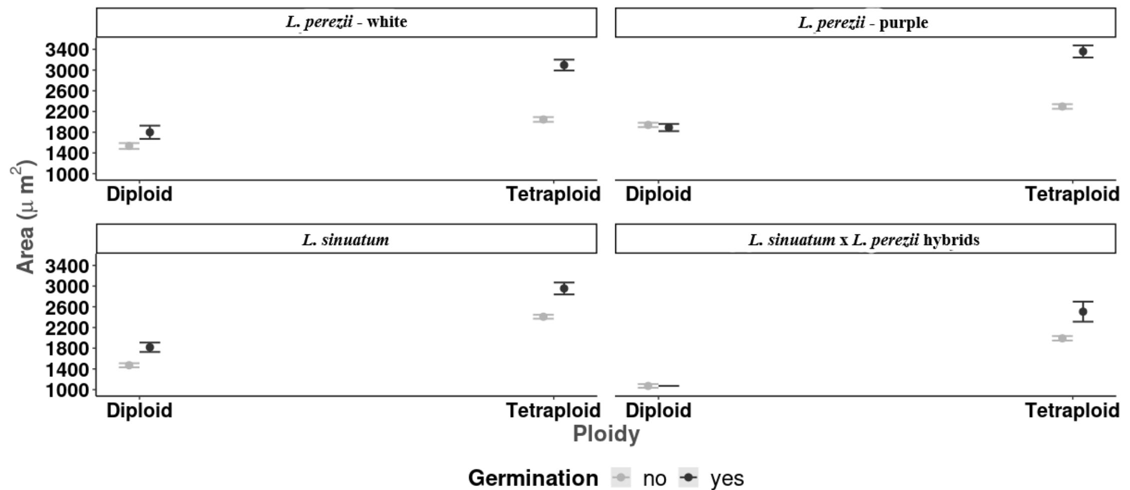

Figure 7-8 Pollen grain area after data modelling using linear modelling by ploidy (diploid or tetraploid) and germination (yes or no) for each of the germplasm groups evaluated (i.e., *Limonium perezii* (white or purple), *Limonium sinuatum,* or *L. sinuatum* × *L. perezii* hybrids) . Dots represent the predicted mean values for pollen plan area and vertical lines (error bars) represent the 95% confidence interval. n=100 pollen grains

Distribution of the pollen plan area and size variability, separately analysing for viable and unviable, as well as germinated and ungerminated, followed similar trends as described above for all the germplasm groups (refer to Section 7.3.2.1), with lower dispersion in diploid than in tetraploids, and unimodal distribution of pollen plan area for diploid genotypes (Figure 7-5). However, in the case of tetraploid genotypes, the separate analysis of viable and unviable, or germinated and ungerminated pollen, also revealed a unimodal distribution of the pollen plan area for viable or germinated pollen for most genotypes (Figure 7-9).

Bursting of pollen grains during *in vitro* germination was also observed in most genotypes (< 5% bursting), with exception of the diploid *L. sinuatum* × *L. perezii* hybrids (0% bursting).

Pollen from diploid and tetraploid plants also showed morphological differences, with pollen from the diploid plants presenting three germ pores, while pollen from tetraploid plants either displayed four germ pores or no pores at all.

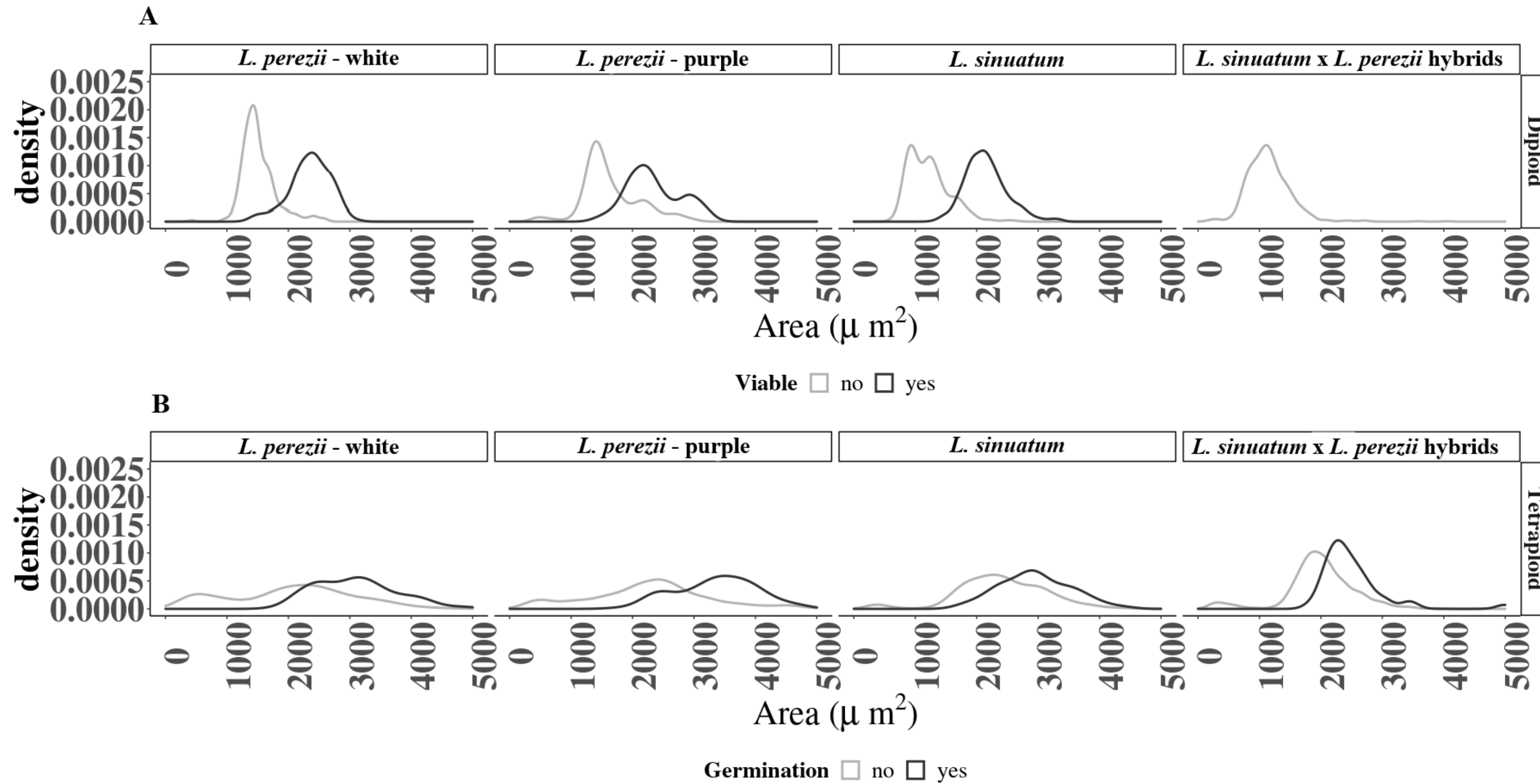

Figure 7-9 A. Density plot for the pollen size (µm^2) of viable (yes) and unviable (no) diploid pollen or **B.** germinated (yes) and ungerminated (no) tetraploid pollen collected from *Limonium perezii* (purple or white), *Limonium sinuatum* or *L. sinuatum* × *L. perezii* hybrids

7.3.3. Reproductive performance

A detailed description of the individual observations for reproductive performance of the evaluated genotypes are presented within APPENDIX M, but a succinct summary of results included, for:

- zygote success:
  - The highest value was for diploid interspecific crosses (40.8% ± 36.5%), followed by backcrosses of tetraploid genotypes to tetraploid *L. sinuatum* (28.0% ± 26.4%) and intraspecific tetraploid crosses (25.0% ± 22.6%; $p>0.05$; Table 7-10). The lowest zygote success was 0% for backcrosses of diploid hybrids ($p<0.05$; Table 7-10).
  - In tetraploid interspecific crosses zygote success was 97.5% lower in comparison with diploid interspecific crosses ($p<0.05$; Table 7-10)
  - Backcrosses between a tetraploid hybrid and an individual genetically similar to either of the parents resulted in zygote success between 15.1% (i.e., backcrosses to *L. perezii*) and 28.0% (i.e., backcrosses to *L. sinuatum*). In contrast, backcrosses involving a diploid *L. sinuatum* × *L. perezii* hybrids had a zygote success of 0% ($p<0.05$; Table 7-10).
  - In interspecific crosses, the zygote success was 700% higher for *L. sinuatum* (73.3% ± 17.5%) as seed-bearing parent in comparison with *L. perezii* (8.3% ± 9.8%; $p<0.05$; APPENDIX M).
- seed germination rate:
  - Zygotes obtained after interspecific crossing or by backcrossing tetraploid *L. sinuatum* × *L. perezii* hybrids and *L. perezii* did not germinate (Table 7-10).
  - Only zygotes from either intraspecific crosses or backcrosses between tetraploid *L. sinuatum* × *L. perezii* hybrids and *L. sinuatum* germinated, showing germination rates between 10% and 18% (Table 7-10).
- cross success:
  - Only backcrosses between tetraploid hybrids and *L. sinuatum*, and intraspecific tetraploid crosses for both *L. sinuatum* and *L. perezii* resulted in cross success different from zero with values between 2.6% and 9.2 %.

Table 7-10 Zygote success (number of zygotes obtained divided by the number of crosses completed), germination rate (number of zygotes germinated *in vitro* by number of zygotes rescued), and cross success (number of seedlings obtained divided by number of crosses completed) for intraspecific, interspecific or backcrosses performed with diploid or tetraploid genotypes of *Limonium sinuatum*, *Limonium perezii*, and *L. sinuatum* × *L. perezii* hybrids. n=30 florets. Means followed by a common letter are not significantly different at the 0.05% level of significance

Response variable	Cross type	Ploidy	
		Diploid	**Tetraploid**
Zygote success	Intraspecific (*L. perezii)*	N.A. †	14.3% ± 17.9% [c]
	Intraspecific (*L. sinuatum)*	N.A. †	25.0% ± 22.6% [cd]
	Interspecific	40.8% ± 36.5% [d]	3.0% ± 9.0% [b]
	Backcross to *L. sinuatum*	0% ± 0% [a]	28.0% ± 26.4% [cd]
	Backcross to *L. perezii*	0% ± 0% [a]	15.1% ± 18.5% [c]
Germination rate	Intraspecific (*L. perezii)*	N.A. †	10.4% ± 19.8% [b]
	Intraspecific (*L. sinuatum)*	N.A. †	18.3% ± 22.2% [b]
	Interspecific	0% ± 0% [a]	0% ± 0% [a]
	Backcross to *L. sinuatum*	0% ± 0% [a]	18.7% ± 29.7% [b]
	Backcross to *L. perezii*	0% ± 0% [a]	0% ± 0% [a]
Cross success	Intraspecific (*L. perezii)*	N.A. †	2.6% ± 4.7% [b]
	Intraspecific (*L. sinuatum)*	N.A. †	6.7% ± 8.2% [b]
	Interspecific	0% ± 0% [a]	0% ± 0% [a]

Response variable	**Cross type**	**Ploidy**	
		Diploid	**Tetraploid**
	Backcross to *L. sinuatum*	0% ± 0%[a]	9.2% ± 14.8%[b]
	Backcross to *L. perezii*	0% ± 0%[a]	0% ± 0%[a]

†N.A. No apply. These crosses were not performed in the experiment for the evaluation of reproductive performance

7.4. Discussion

7.4.1. Vegetative and reproductive characterisation

In the present research, the *Limonium* germplasm groups under study (i.e., *L. sinuatum* and *L. perezii*, and their hybrids) confirmed the asymmetrical effect of an increase in ploidy on organ size, and both the organ- and genotype-dependence of these relationships (Table 7-3), previously reported in *Brassica* (Harun et al., 2021) and tulips (Podwyszyńska et al., 2018). The morphological characteristics of potential commercial relevance, such as increased main floral stem width, as well as characteristics with potential physiological impact, such as guard cell length and stomata density, were affected by the ploidy increase in genotypes of all germplasm groups (Table 7-3). This is interpreted as meaning breeding for those characteristics could be successfully approached by polyploidization induction. Studies with *Brassica* (Harun et al., 2021) supports the hypothesis that the organ and genotype-dependence could be associated with differences in gene expression and regulation; however, in *Limonium* further investigative studies would be required to validate the applicability of this hypothesis.

In spite of the asymmetrical effect of an increase in ploidy on differences between organs and genotypes, an increase in germplasm diversification was achieved. For example, the importance of stem strength associated with floral stem diameter has been noted for F1 interspecific hybrids (Chen, 2005). Interspecific hybrids display long and slim floral stems with a 63% frequency of branch bending in comparison with 0% in *L. sinuatum* and 22% in *L. perezii* (Chen, 2005). The occurrence of thicker stems, i.e., stronger, in the tetraploid hybrids is desirable for the cut flower industry that is always looking for long and strong stems (BallSB, 2021; Farmhouse & Blooms, 2022).

Bigger guard cells and lower stomata density could be associated with an increasing tolerance to drought (Caine et al., 2019; Zhang et al., 2017).Therefore, an increase in ploidy is suspected to increase physiological diversity as well which might result in an increase in productivity in an environment with water scarcity. However, further studies could be desirable in *Limonium* for measuring specifically the physiological impact of increased ploidy and any resulting impact on horticultural yield, quality, or timing.

7.4.2. Pollen characterisation

The direct correlation between ploidy and the plan area of pollen, reported previously in *L. sinuatum* (CHAPTER 2), was validated in this study for both *L. perezii* and *L. sinuatum* × *L. perezii* hybrids (Table 7-4), and contradicts previous comparisons between the diploid *L. ovalifolium* and the tetraploid *L. multiflorum* (Róis et al., 2012). Comparison between the results of this research (Table 7-4), and the comparison between *L. ovalifolium* and *L. multiflorum* (Róis et al., 2012) support the logic of not confounding results of correlation analysis with reference to differing *Limonium* species or germplasm groups. Using the data from the current research for example, if the pollen collected from diploid *L. perezii*-purple is compared with the tetraploid pollen from the interspecific hybrids, no significant difference in plan area would have been detected (Table 7-4). In contrast however pollen collected from tetraploid *L. perezii*-purple is 1.3 times bigger than pollen from diploid *L. perezii*-purple (Table 7-4).

Pollen count did not differ between ploidies for most of the genotypes (Table 7-6); however, even though quantity was mostly constant, data from pollen germination and pollen viability confirm a decrease in pollen quality (i.e., germinability or viability) with changes in pollen germination and proportion of viable pollen (Table 7-9; Figure 7-6). The results of this study agree with previous observations done in diploid *L. ovalifolium* with high pollen viability (93%), and in the tetraploid *L. multiflorum* with low pollen viability (37%; Róis et al. (2012)). Low pollen viability/germination in tetraploids could be a consequence of abnormal meiosis which, in the case of *L. multiflorum* occurs regardless of whether the chromosome number is balanced or unbalanced (Róis et al., 2012). The occurrence of abnormal meiosis and production of aborted pollen grains is confirmed in this study by the bimodal distribution of the plan area of pollen collected from tetraploid plants (Figure 7-5) being likely derived from aborted pollen and micro-pollen presenting plan areas smaller the 0.5 times the average for the species (Oleszczuk et al., 2019; Tatum et al., 2015) and likely represented as the second small-peak observed around 500 μm^2 (Figure 7-5).

In the present study five types of pollen grains were detected:

- reduced pollen (n) with species' average pollen size and potentially viable (i.e., viable / germinated pollen from diploid plants; Table 7-7; Figure 7-8).
- unreduced pollen (2n) with 1.2 times the average pollen size of the species, potentially viable (i.e., viable / germinated pollen from tetraploid plants; Table 7-7; Figure 7-8).
- Micro-pollen deficient in chromosomes with 0.5 times the average pollen size of the species, and which are aborted (i.e., aborted pollen from diploid *L. sinuatum* and tetraploid *L. perezii*-white; Table 7-7; Figure 7-8)
- Germinated pollen greater than 1.59 times the size reported for the species (Figure 7-8).
- aneuploids corresponding to aborted pollen of variable sizes (i.e., below and above the average pollen size of the species; or above the size of potentially unreduced pollen; Table 7-4; Figure 7-5).

Out of different types of pollen grains likely identified in this study mentioned above, aneuploids are the most variable in terms of size and likely genetic constitution. Aneuploid pollen could present either: one or more extra chromosomes, be deficient in chromosomes (Potapova & Gorbsky, 2017) or contain 'laggard' chromosomes. 'Laggard' chromosomes are those that do not reach the cell's poles on time during either anaphase I or II originating micro-pollen (refer to Section 1.3.1.2; Figure 1-9; Oleszczuk et al. (2019); Tatum et al. (2015)). In other plant species pollen size for micro-pollen has been reported as 0.5 the species average (Oleszczuk et al., 2019). This agrees with the pollen size decrease detected in aborted pollen from diploid *L. sinuatum* and tetraploid *L. perezii*-white in comparison with their corresponding viable pollen (Table 7-7). In the current study it was additionally proposed than pollen grains 0.8 times the average size for the species could be considered as aneuploids deficient in chromosomes. No reports were found for aneuploid pollen grains with some extra chromosomes, but based on the experience gained in this research it is possible that the variable sizes observed for pollen bigger than the species' average (i.e., >1.3 up to 1.59 times the plan area) could represent those aneuploids with extra chromosomes. Pollen bigger than 1.59 times the species' average plan area detected by comparison of germinated and ungerminated pollen (Figure 7-7) could be considered as 4n pollen as suggested for wild potato (*Solanum* spp.; Larrosa et al. (2012)). However, in all these preceding potential scenarios suggesting causes

behind the distribution and variation in pollen grain size and functionality, cytological studies are still required to offer supporting evidence.

The occurrence of aneuploid pollen could be a consequence of meiotic abnormalities, such as chromosome mis-segregation by one or more chromosomes being unable to reach the cell's poles on time during meiosis (Larrosa et al., 2012; Oleszczuk et al., 2019). In neo-autopolyploid plants, as is the case of the tetraploids obtained in this research, the most reported factor associated with mis-segregation is occurrence of multipolar spindles (Oleszczuk et al., 2019; Potapova & Gorbsky, 2017). As a result, aborted pollen contains less chromosomes than the haploid number, i.e., aneuploid (Potapova & Gorbsky, 2017; Tatum et al., 2015) and micro-pollen contains the "laggard" chromosomes (Oleszczuk et al., 2019; Róis et al., 2012). The consequences of meiotic abnormalities depends mainly on the frequency of abnormalities affecting the pollen grains and proportion of cells presenting them, with differences reported between plants, even inflorescences within the same plant (Oleszczuk et al., 2019), and species (Bomblies & Madlung, 2014). Further investigations are required to identify if there is any applicable pathway for increasing the fertility of gametes when generating changes in ploidy in *Limonium*.

The causes of abnormal meiosis in polyploids are still unknown could be associated with reproduction mode (i.e., sexual, facultative apomictic, apomictic; Róis et al. (2012)) and the level of evolution of the polyploid (i.e., if it is newly developed or has evolved naturally; Lloyd and Bomblies (2016)). It has been proposed that apomictic reproduction is an evolutive strategy to develop stable populations when meiotic abnormalities are frequent as reported in *L. multiflorum* (Bomblies, 2022; Róis et al., 2012). So, it could be expected that plants with occurrence of meiotic abnormalities reproduce by apomixis or facultative apomixis.

As observed in this study, similarly to observations reported for maize (*Zea mays*) (Kato & Birchler, 2006), in neo-autopolyploid plants low fertility and even sterility is frequent. Different meiotic abnormalities have been previously reported in *L. multiflorum* (Róis et al., 2012) and suggested to occur in the neo-polyploid of this research such as unpaired chromosomes, chromosome bridges and 'laggard' chromosomes in anaphase I (refer to Section 1.3.1.2 for details about meiosis phases). However, cytological studies for the

Limonium neo-polyploids produced in this research are an obvious next step for future research, should resources permit.

The lack of differences in the pollen count between diploid and tetraploid plants could suggest that meiosis of the pollen mother cell (PMC) results mainly in tetrads and not in dyads or polyads. Occurrence of dyads and polyads would be expected as a consequence of some meiotic abnormalities occurring during meiosis I, which could force the cells to exit the cell cycle before meiosis II (Lukaszewski, 2022) as reported in *Limonium* (Róis et al., 2012; Siregar, 2021). However, the occurrence of tetrads does not ensure the viability of pollen, as confirmed with the viability and *in vitro* germination results discussed above.

The frequency of pollen grains bursting during *in vitro* germination was low, as expected when using a standardized protocol, and supports the hypothesis that the sucrose concentration could be further adjusted to compensate for the water potential of the pollen grain for each of the species involved (Firon et al., 2012; Zhang, 1995). However, pollen grain bursting also provides important information about the pollen grain structure and physiology (refer to Section 1.3.2). For instance, the non-bursting of pollen from the interspecific hybrids could suggest an absence of metabolic activity and/or an alteration of the exine and/or vacuoles (Firon et al., 2012; Williams & Brown, 2018) which are the processes and structures directly involved in the regulation of the water status within the pollen grain (Williams & Brown, 2018). In addition, pollen grains with such alterations are unable to increase their water content, and vacuolation during the development of the pollen walls. As consequence, the pollen grains do not have a gain in size which would also further explain its smaller size in comparison with the average size of the species (Table 7-7; Table 7-9; Firon et al. (2012)). If so the occurrence of bursting during pollen germination could indirectly suggest the pollen grains were not dead but might be just unable to germinate under the conditions given. In this case, evaluation of the pollen viability through germination could be more appropriate under natural conditions (i.e., *in vivo* germination) than *in vitro.* While in the current research programme some attempts were made to develop a reliable protocol for monitoring germination *in vivo*, resources did not permit this strategy to be explored further.

The occurrence of an additional germ pores in potentially unreduced pollen has previously been observed in this research after treating immature inflorescences with N_2O (refer to APPENDIX N). Thus, the increase in the number of chromosomes, regardless of the cause of the increase (N_2O treatment or neo-polyploid), seems to be associated with the development of an additional germ pore. The role of the additional germ pore on the competitiveness of pollen during *in vivo* germination and fertilization in *Limonium* remains to be studied, but could give insights into favouring germination and pollination as observed in *Rosa hybrida* (Gao et al., 2019) and in fireweed (*Chamerion angustifolium*; Husband et al. (2002)). However, the additional pore could also represent disadvantages such as short viability (Dajoz et al., 1991).

7.4.3. Reproductive performance

7.4.3.1 Backcrosses

Interspecific crosses between tetraploid *L. sinuatum* and *L. perezii* were unsuccessful; however, backcrosses between the tetraploid *L. sinuatum* and a tetraploid *L. sinuatum* × *L. perezii* hybrid were successful (Table 7-10). This difference in success now opens a possible strategy to produce interspecific, polyploid, hybrids which was one of the specific objectives of this programme of research (refer to Section 1.6). In addition to the successful achievement of the backcrosses (i.e., cross success), it also allows the elimination of hybrid weakness (i.e., necrosis, wilting, yellowing), as well as accumulation of the desirable characteristics of the parent or genotype used for backcrossing (i.e. plant with the form of *L. perezii*, but with the palette colours of *L. sinuatum* (Morgan et al., 2001)). With successive backcrossing it would be possible to reduce the genetic differences between the chromosomes coming from each one of the parental genotypes (i.e., *L. sinuatum* and *L. perezii*) (Husband, 2004). This reduction in genetic differences would be expected to result in a decrease in the number of mismatches and the meiotic abnormalities (Sora et al., 2016) which would have a direct impact on hybrid fitness, pollen viability (Oleszczuk et al., 2019), and potentially hybrid crossability. In the current study, diploid *L. sinuatum* × *L. perezii* hybrids were sterile and did not produce functional gametes, being unable to produce seedlings. However, the WGD of those hybrids restored pollen viability and pollen germination (Figure 7-6; Table 7-9) permitting their inclusion in planned crosses for further advance in breeding.

By achieving a 81% increase in zygote success, the homoploid crosses of this part of the research were more successful than heteroploid backcrosses (refer to Section 6.3.2) in the production of zygote (in average 27.9% versus 15.4% average zygote, respectively; APPENDIX M). A possible explanation for the higher success of homoploid backcrosses over heteroploidy lies in the interaction between the embryo and endosperm, with tetraploid by tetraploid crosses keeping the maternal to paternal ratio as 2:1 or equivalent to it, preventing the occurrence of a partial triploid block and endosperm failure (Morgan et al., 2021) as occurs in heteroploid crosses. In diploid by tetraploid crosses (i.e., heteroploid crosses) the maternal to paternal ratio would be the opposite (i.e., 1:2), altering the EBN and resulting in aborted hybrid seeds or less viable seeds (Lafon-Placette & Köhler, 2016; Morgan et al., 2021) as confirmed in this research with 6.2% ± 8.8% zygote success and only 1 viable seed (refer to APPENDIX M).

Backcrosses were successful (i.e., seed germination and cross success; Table 7-10) to produce hybrid polyploids only toward *L. sinuatum* potentially increased the germplasm diversity evidence in progeny with ploidies different from the parents (refer to APPENDIX O genotypes: Y22.002.001 to Y22.002.009; Y22.003.001 to Y22.003.006; Y22.004.001; and Y22.008.001 to Y22.008.007). The cross success only when *L. sinuatum* was the seed-bearing suggest a total incompatibility between *L. perezii* and the *L. sinuatum* × *L. perezii* hybrids and a unidirectional incompatibility when *L. sinuatum* × *L. perezii* hybrid was the seed-bearing parent and *L. sinuatum* the pollen-donor. Incompatibilities between parental genomes cause hybrid failure and impede gene flow (review by Chen et al. (2016)). In this research the mentioned incompatibilities resulted in a zygote success lower than 8% and seed germination equal to 0% (APPENDIX M). The observation of at least 8% zygote success when suspected incompatibilities occur opens the option to develop or implement techniques complementary to ovule/embryo rescue to understand the causes of the hybrid inviability and increase the performance of the crosses (e.g., *in vitro* pollination).

7.4.3.2 Intraspecific crosses

The tetraploid genotypes obtained in this research were fertile, as demonstrated by the zygote success achieved in intraspecific tetraploid crosses and backcrosses (Table 7-10). This generation of fertile tetraploids was also supported by the pollen germination and

pollen viability results, with tetraploid progeny presenting viable and/or germinated pollen (Figure 7-6; Table 7-9) and also developing seedlings. However, the fertility of tetraploids was reduced compared to diploids (Figure 7-6; Table 7-9), possibly due to meiotic abnormalities. The occurrence of meiotic abnormalities in the neo-tetraploids would explain the decrease in zygote success between 62% (*L. perezii*) and 65% (*L. sinuatum*) observed in the tetraploid intraspecific crosses compared to the diploid intraspecific crosses (APPENDIX M and APPENDIX A). The negative effect of meiotic abnormalities on pollen viability and zygote success has also been documented in *Zea mays* (Defani-Scoarize et al., 1995), *Prunus cerasus* (Akšić et al., 2016) and *Triticum* (Oleszczuk et al., 2019), with the proportion of cells affected by the meiotic abnormalities determining the extent of aborted pollen (CHAPTER 4). Further investigation would be advisable to properly identify the factors involved in the observed decrease in zygote success, in addition to identifying alternatives to improve the zygote and cross success in crosses between neo-tetraploids.

7.4.3.3 Interspecific crosses

Interspecific hybridisation between tetraploids resulted in a zygote success (3.0% ± 9.0%) that was 93 % lower than for diploid interspecific crosses (40.8% ± 36.5%), with zero germinated zygotes in both cases (Table 7-10). This result confirms the existence of reproductive pre- and post-zygotic barriers and rejects the proposed hypothesis that crosses between tetraploid *L. sinuatum* and *L. perezii* plants would increase the interspecific hybridisation rate and produce fertile seedlings.

The zygote success and cross success observed in interspecific crosses between diploid *L. sinuatum* and *L. perezii* (APPENDIX M) confirm the occurrence of partial compatibility previously reported between the two species (Zhang, 1995). However, the sharp reduction in zygote success for tetraploids compared to diploids, and the absence of zygotic germination observed in the present study, supports the hypothesis of a decrease in compatibility between *L. sinuatum* and *L. perezii* as ploidy increases. Cross-compatibility, genome stabilisation and genetic recombination are inversely related to genetic distance (Kuligowska et al., 2016), meaning that species that are more genetically distant have reduced compatibility due to the presence of more pre- and post-zygotic barriers.

The interspecific crosses between tetraploid genotypes are possibly restricted, first, by the pre-zygotic and post-zygotic barriers already identified in interspecific crosses between diploid genotypes such as abnormalities in the pollen tube growth (Zhang, 1995) and arrest of the embryo growth (Morgan et al., 1998). Second, by the meiotic abnormalities reported for neo-tetraploid genotypes (Bomblies, 2020; Potapova & Gorbsky, 2017) and suspected in this study as responsible for the low proportion of viable gametes (refer to Section 7.4.2; Figure 7-6). In addition, those barriers could be accompanied by new pollen-pistil and /or embryo-endosperm incompatibilities (Larrosa et al., 2012; Zhang, 1995), and incompatibilities between nuclear and/or cytoplasmatic genes of the male and female gametes with increased ploidy (Larrosa et al., 2012).

Occurrence of incompatibilities in plants not only reduces the number of zygotes obtained (i.e., zygote success), but also the hybrids could cease development during embryogenesis (i.e., 0% zygote germination; APPENDIX M) or, if established as a seedling may be infertile (refer to Section 6.3.2 and 6.3.3). Genetic incompatibilities in plants could be unidirectional (review by Chen et al. (2016)), as observed in diploid interspecific crosses between *L. sinuatum* and *L. perezii* (APPENDIX M) with higher zygote success when *L. sinuatum* was the seed-bearing genotype. Multiple genes and loci are involved in genetic incompatibility (review by Chen et al. (2016)) and the presence of only one of the alleles is enough for limiting the compatibility and crossability between species of other genera (Kuligowska et al., 2016; Zuellig & Sweigart, 2018). In contrast however, within the tetraploid plants produced in this research the alleles potentially associated with incompatibilities have obviously been duplicated. This duplication of alleles associated with incompatibility potentially would explains the higher interspecific incompatibility and subsequent hybrid lethality (i.e., lower zygote success) observed in tetraploid interspecific crosses of *Limonium*. Further genetic studies in *Limonium* crosses could determine to what level maternal and paternal genes are involved in determining incompatibility, with a view to alleviate the hybrid lethality via biotechnological tools.

The question about the mechanisms involved in the hybrid incompatibility between tetraploid *L. sinuatum* and *L. perezii* plants, and how to mitigate them, will remain open until incompatibility systems in *Limonium* are studied in detail. Developing an understanding of those systems and their mode of action (e.g., production of a cytotoxin

or pollen rejection (Kuligowska et al., 2016)) in *Limonium*, and specifically with regard to interspecific crosses between tetraploid genotypes, would facilitate development of an intervention to avoid the reproduction barriers (e.g., earlier ovule/embryo rescue, chemical treatment (Kuligowska et al., 2016), cut-style pollination (Barba-Gonzalez et al., 2004)).

7.5. Conclusions

- The breeding strategy of ploidy manipulation did translate into increased variability within the germplasm, through greater morphological diversity.
- Differences between germplasm groups and genotypes occurred for all the morphological characteristics evaluated. The validation that the effect of an increase in ploidy over pollen plan area and guard cell length of the germplasm groups under study was consistent, and did not display genotype-dependency, at least for *L. sinuatum*, *L. perezii*, and their interspecific hybrids. This confirmed the reliability of pollen plan area and guard cell length to be used to identify potential tetraploid plants, as suggested previously (refer to Section 2.5.3.3).
- The tetraploid genotypes obtained in this PhD research, through WGD induced by either N_2O (CHAPTER 2, CHAPTER 3, APPENDIX F) or oryzalin (refer CHAPTER 5), could be used in crosses. However, the type of cross performed (i.e., intraspecific, interspecific, or backcrosses), as well as the germplasm group and ploidy of the seed-bearing genotype, determined the cross success.
- Production of fertile, interspecific, polyploid hybrids, is possible by crossing tetraploid *L. sinuatum* and a tetraploid *L. sinuatum* × *L. perezii* hybrid.

CHAPTER 8. Physically induced mutations (Radiation)

8.1. Preface

This chapter outlines the research associated with the second research strategy undertaken within this thesis (refer to Section 1.2.5). The preceding chapters of this thesis which report on experiments, were focussed on ploidy manipulation, while this chapter is focussed on physically induced mutations. At the outset of this PhD both research strategies carried equal merit, but conjoint with COVID-19 related responses, technical roadblocks reported in the following sections resulted in a halt to developing this strategy to its full potential. The results of the experiments presented within this chapter will however provide a useful resource for those following.

8.2. Introduction

As reported previously in *Limonium*, the induction of mutations is a breeding tool which simulates the spontaneous/natural mutagenic process (refer to Section 1.2.5.3), increasing its rate of occurrence (Ogawa et al., 2014; Yuan et al., 2015). Mutations can be induced using radiation, i.e., x-rays, gamma-rays or ion beams, to produce changes in the chromosome structure which, if not lethal, may produce changes in gene function resulting in morphological or physiological variations (Lang et al., 2018; Liu et al., 2017; Thu et al., 2019). In the present research, with the objective of increasing germplasm diversity (refer to Section 1.6), gamma-radiation was selected as this is available in New Zealand and, compared to x-rays, has been reported to induce more stable DNA breakages (Ibrahim et al., 2018). Moreover, gamma irradiation has shown the highest efficiency in producing flower colour changes (Yamaguchi et al., 2008), which is one of the key phenotypic traits for the breeding programme.

Radiation could be used to treat almost any part of a plant, with seeds being the most commonly used explant in seed propagated species (Ari et al., 2015; Honda et al., 2006; Samatadze et al., 2019; Spencer-Lopes et al., 2018). However, the selection of the most suitable explant depends on the phenotypic trait the researcher wants to mutate, together with the regeneration protocols available. In *Limonium*, while irradiation of seed has been unsuccessfully trialled (Ranjith Pathirana, pers. comm) *in vitro* shoots have been used for

irradiation (Chinone et al., 2007; Nakagawa & Kato, 2017; Ogawa et al., 2014). The use of *in vitro* shoots as explants for irradiation does have the disadvantage that not all cells within the explant are mutated, potentially producing chimeras (Matsumura et al., 2010). As an alternate explant for irradiation, callus obtained *in vitro* has resulted in non-chimeric plants, though some exceptions have been noted (Mathur & Koncz, 1998; Nakano et al., 2010; Soliman et al., 2014). Within the current research, callus produced *in vitro* was selected for irradiation because of its lower probability of producing chimeras, with whole plants subsequently regenerated via indirect shoot induction.

Having chosen callus as the plant material to apply mutagenesis to, the induction of physical mutations would involve five main phases, each of which would need to be optimised. First the production of callus; second, the selection of the optimal irradiation dose; third the induction of physical mutations; fourth plant regeneration through indirect shoot induction; and finally, root induction. As explored in this chapter (refer to Sections 8.3.3 and 8.3.4), before implementing using callus as an explant for irradiation, the establishment of a protocols for callus induction, shoot induction and root induction were required.

Callus is a mass of dividing cells developed from leaves, roots, and shoots, most commonly on auxin-rich media (George, 2008; Sugimoto et al., 2010). The auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) is the PGR typically used to induce callus induction and growth *in vitro*. In dicotyledonous plants, such as *Limonium*, the suggested concentration ranges between 1 and 3 mg/L (George, 2008). Cytokinins such as 6-benzylaminopurine (BAP) and thidiazuron (TDZ) have also been employed for callus induction and growth. In *Limonium* is BAP was used to induce callus from segments of *L. perigrinum* leaves (Seelye et al., 1994) and from inflorescence stem tissue of *L. cordatum* (Casazza et al., 2002). In addition, cultivation for 24 h in culture medium with 3 mg/L TDZ induced callus growth of *L. sinuatum* × *L. perezii* embryos (Morgan et al., 1998). In the current research, 2,4-D and BAP were evaluated for the induction of callus from leaves.

An *in vitro* culture of callus can be induced to produce shoots or roots, a process known as indirect shoot/root organogenesis (George, 2008) by the application of BAP, thidiazuron (TDZ), or α-naphthaleneacetic acid (NAA). For non-specific plant species,

the recommended concentrations for BAP range between 0.2 and 2 mg/L, for TDZ between 0.01 and 0.25 mg/L (Bhojwani & Dantu, 2013) and for NAA is 0.5 mg/L (Das et al., 2018; Raju et al., 2022). Concentrations of TDZ higher than 0.025 mg/L and 0.25 mg/L have been reported to induce callus in *Echinacea purpurea* (Jones et al., 2007) and *Spirodela polyrhiza* (Yang et al., 2018), respectively. In *L. perigrinum,* shoot regeneration was obtained after culture of leaf discs on medium containing 3 mg/L TDZ for 48 hours (Seelye et al., 1994). In these experiments to induce indirect shoot organogenesis, TDZ, BAP, and NAA were selected for study.

After shoots have regenerated, roots need to be induced, in preparation for the plants to be transferred to and established for growth within a greenhouse. The PGRs commonly used for root induction in different plant species are the auxins: indol-3-acetic acid (IAA), indole-3-butyric acid (IBA) and NAA, in concentrations ranging from 0.2 mg/L to 1.5 mg/L (Bhojwani & Dantu, 2013). In *L. cordatum,* 0.2 mg/L of IBA increased the rooting rate by 13%, but the roots were shorter in comparison with media without PGR (Casazza et al., 2002). By comparison, in *L. perigrinum* Bergius × *L. purpuratum* L. hybrids root initiation was induced by cultivation on a medium with 12mg/L IBA for either 24 h (Morgan et al., 1995) or six days (Morgan et al., 1998). The PGR evaluated in this research for induction of roots, included not only different concentrations of IBA, but also the PGR: IAA.

The standardization of protocols for plant tissue culture in general (e.g., shoot induction, root induction) has mainly been based on modifying the type and concentration of PGRs. More recently, the optimization of protocols for micropropagation has started to explore the effect of the macro- and micro-nutrients of the medium (Nezami-Alanagh et al., 2018; Nezami-Alanagh et al., 2017). Statistical methods for plant tissue culture data include completely randomized designs, or randomized complete block design, with a factorial treatment design when needed (Compton, 1994; Jones et al., 2007; Takagi et al., 2011). These statistical methods analyse the effect of the factors (e.g., PGRs) and their interaction over the response variable. As used in the current experiment, for a better understanding of the relation between PGR concentration and ratios, a mixture-amount design could be more convenient as it is useful for solving proportional and concentration effects (Niedz & Evens, 2016) and allows separation of the effects of proportion (i.e., ratio) and concentration (Evens & Niedz, 2010).

C-ion beams, gamma- and x-rays, have been successfully used to induce mutations in *Limonium* (refer to Section 1.2.5.3). However, a mutagenesis protocol that allows the replication of the process to be included in breeding programmes is not yet available (Cardarelli et al., 2002; Chinone et al., 2007; Ogawa et al., 2014); callus has not been reported as an explant for mutation in *Limonium*; and the results about the potential of mutagenesis to produce diversity are contradictory (refer to Section 1.2.5.3). For those reasons, the experiments proposed in this part of the PhD. research were intended to investigate the opportunity to mutate *Limonium* callus with gamma-rays to increase germplasm diversity.

Developing a protocol for the actual mutagenesis phase comprised two steps: 1. radiosensitivity test and, 2. explant irradiation. For the radiosensitivity test, the selected explant (e.g., callus, seed, shoot) needs to be exposed to different irradiation doses and the survival rate measured (Cardarelli et al., 2002; Soliman et al., 2014). The range of radiation doses evaluated in the radiosensitivity test includes 0 Gy as control, and extends to increased dosages dependant on the species and type of explant used, e.g., an upper dose of 100 Gy for *Curcuma* rhizomes (Abdullah et al., 2009), 60 Gy for *L. sinuatum* shoots (Cardarelli et al., 2002) and *Gerbera* callus (Hasbullah et al., 2012), or 20 Gy for *Chrysanthemum* petals (Soliman et al., 2014).

As described below within the present chapter a series of experiments was performed for developing a protocol for callus induction, shoot induction and root induction prior to optimization of mutagenesis using callus as the plant material. Experiment 1 and Experiment 2 aimed to identify the medium and duration of treatment for induction and growth of callus; Experiment 3 to Experiment 5 aimed to induce shoots from callus.; and Experiment 6 was performed for identification of the medium for root induction. After protocol development, Experiment 7 was proposed for selection of an optimal irradiation dose for mutating the callus.

The objective of the experiments presented in the current chapter was to develop a standardized protocol for mutation induction in *Limonium* and determine the efficacy of mutagenesis as a breeding strategy to increase the genetic diversity of PFR's germplasm bank.

8.3. Materials and methods

8.3.1. Plant material

An interspecific hybrid of *L sinuatum* × *L. perezii* ('siNZii™ Deep Lavender' (BallSB, 2021) also known as siNZii™ Lavenderish (Ball Horticultural do Brasil, 2023)) was used in this research. Selection of the genotype was based on its international acceptance (Ball Horticultural do Brasil, 2023; BallSB, 2021) and, therefore, the possibility of mutating one phenotypic trait, e.g., floret colour, while keeping the other phenotypic traits (Ogawa et al., 2014).

Plants were either grown *in vitro* (refer to Section 8.3.2.1) or within the greenhouse. Those plants within the greenhouse were potted into 2L solid plastic pots using a commercial growing medium (APPENDIX E) and grown at The New Zealand Institute for Plant & Food Research Ltd in Palmerston North, New Zealand (40.9006 °S, 174.8860 °E), with controlled temperatures between 16 °C and 18 °C and natural day length equal to 13 h in summer 2019. Growing medium was maintained at field capacity by a drip irrigation system (8.5 L/h, PC Low CNL Netafim™, Hatzerim, Israel).

8.3.2. In vitro culture

8.3.2.1 Media preparation and culture conditions

The 'base media' used for the different experiments described below comprised Murashige and Skoog macro and micro salts, and iron (Murashige & Skoog, 1962), 30 g/L sucrose, 7.5 g/L agar, plus Gamborg B5 vitamins (Gamborg et al., 1968). As detailed within the following sections, for each experiment the 'base medium' was amended with different types and concentrations of PGRs (e.g., 2,4-D, BAP, NAA, or TDZ), depending on the aim of the experiment. Unless stated otherwise, the *in vitro* plant material used as the explant source for the all the experiments was cultured on a 'shoot proliferation medium' reported by Morgan et al (1998), with the exception that LS vitamins (Linsmaier & Skoog, 1965) were substituted by Gamborg B5 vitamins (Gamborg et al., 1968) (APPENDIX A).

For all media utilized, the pH was adjusted to 5.7 with either 0.1M NaOH or 0.1M HCl prior to autoclaving at 121°C at 103 kPa for 15 minutes. Both prior to and during each experiment, explants were held under culture room conditions of 25 ± 1°C, at a

photosynthetic photon flux density of 30 ± 5 μmol m^2/s and 16h photoperiod provided by cool-white, fluorescent tubes.

Culture vessels (replicates) were either polystyrene disposable Petri dishes (80 mm base diameter × 20 mm deep) vented with stacking ring (Alto Packing, Hamilton, New Zealand), or “tubs” that are disposable polystyrene plastic culture vessels (98 mm base diameter × 60 mm deep) with snap-on lids (Alto Packaging, Hamilton, New Zealand). When Petri dishes were used, each contained c. 25 ml of medium, while tubs contained c. 50 ml.

8.3.3. Callus and indirect shoot induction experiments

The explants comprised discs of leaf lamina including the midrib with areas of 132.7 mm^2 (i.e., 13 mm disc diameter) or 63.6 mm^2 (i.e., 9 mm disc diameter), from either greenhouse or *in vitro* leaves, respectively, which were still actively expanding. Explants derived from *in vitro* plants were used in Experiment 1 to Experiment 4 while those from greenhouse plants were only evaluated in Experiment 5 as detailed below for each experiment. Explants were planted on the culture media with the abaxial side of the leaf facing the medium.

8.3.3.1.1 Callus induction

Experiment 1. The aim of this experiment was to evaluate different culture media for the induction and growth of callus. Callus was induced from leaves harvested from plants grown *in vitro*, utilising the ‘base medium’ supplemented with concentrations of 2,4-D (0, 1, 2 mg/L) and BAP (0, 1, 2 mg/L).

The experiment was conducted as a completely randomized design with sub-sampling, utilising a factorial arrangement (3 × 3) (Compton, 1994) of the factors: 2,4-D concentration and BAP concentration. Each treatment corresponded to a combination of 2,4-D and BAP and was applied to four culture vessels (replicates) corresponding to polystyrene disposable Petri dishes, (as described within Section 8.3.2.1). Each culture vessel contained three explants (i.e., leaf segments, as sub-samples).

The efficacy of the treatment media for callus induction and growth was evaluated after 28 days that is the typical culture cycle length used for *Limonium* in the PFR’s Palmerston

North tissue culture laboratory. The response variables assessed were: callus induction (yes/no), proportion of explants forming callus, and ratio of callus area to explant area. For the measurement of callus area, the explants were observed under a stereo microscope (Leica M60, Leica Microsystems, Switzerland), with an integrated CMOS microscope camera, Leica IC90 E (Leica Microsystems, Switzerland). Photos of each explant were taken and the surface area of both the callus and explant measured using the software ImageJ (Schneider et al., 2012).

The data analysis is explained in detail below in Section 8.3.6.

Experiment 2. The aim of this experiment was to evaluate the effect of duration of treatment for callus induction and growth over callus growth. This experiment compared whether durations of 7, 14, 21 or 28 days were needed for producing callus from leaves (explants) harvested from plants previously grown *in vitro*. The treatment medium comprised either the ‘base medium’ amended with 2,4-D alone (1 mg/L) or in combination with BAP (2 mg/L of each 2,4-D and BAP), which were selected based on the results from the previous callus induction experiment (Experiment 1; refer to Section 8.3.3.1.1). The control treatment medium comprised the ‘base medium’ (i.e., without PGR). The leaf explants were kept on the ‘callus induction medium’ for 7, 14, 21 or 28 days, and later used for Experiment 3, as detailed below.

The experiment was conducted as a completely randomized design with sub-sampling, utilising a factorial treatment arrangement (3 × 4) (Compton, 1994) of the factors: treatment medium used (1 mg/L 2,4-D; 2 mg/L of 2,4-D combined with 2 mg/L BAP; no PGR) and duration of treatment (7, 14, 21 or 28 days). Each treatment combination was applied to five culture vessels (replicates) corresponding to polystyrene disposable Petri dishes, as described within Section 8.3.2.1). Each culture vessel contained three explants (i.e., leaf segments, as sub-samples).

The response variable assessed was the ratio of callus area to explant area. The corresponding data analysis is detailed in Section 8.3.6.

8.3.3.1.2 Shoot induction

Experiment 3. The aim of this experiment was to induce shoot production from callus (i.e., indirect shoot production) and to evaluate the influence of callus induction media and duration on shoot production.

For indirect shoot induction, a previously identified 'shoot induction medium' for *Limonium* (Seelye et al., 1994) was utilised (i.e., medium identification number '630', as used by PFR's Palmerston North tissue culture laboratory; APPENDIX A). In short, this medium comprised the 'base medium', with the exception that agar was substituted by phytagel (i.e., 'modified base medium'; APPENDIX A), supplemented with 3 mg/L Thidiazuron (TDZ) (Seelye et al., 1994). As a control treatment, the 'modified base medium' was used (i.e., 0 mg/L TDZ).

As callus induced within Experiment 2 was used as the source of explants for the current experiment, the callus derived from the treatment medium for either 7, 14, 21 or 28 days was transferred to the 'shoot induction medium' for 48 h following Seelye et al. (1994), and then transferred to the 'modified base medium' (APPENDIX A).

Experiment 3 utilised a randomized complete block design with sub-sampling (Compton, 1994). The treatments comprised a factorial (4 × 2) of the factors: preceding duration in the callus induction medium (7, 14, 21, or 28 days) and TDZ concentration (0 or 3 mg/L). The blocks corresponded to the callus induction treatment medium used in Experiment 2, i.e., either 1 mg/L 2,4-D or 2 mg/L of each 2,4-D and BAP. Each treatment was applied to five disposable polystyrene plastic culture vessels (refer to Section 8.3.2.1) as replicates. Each culture vessel contained three explants of callus derived from *in vitro* leaves in Experiment 2 (sub-samples). The response variables evaluated were: shoot induction (yes/no), root induction (yes/no), proportion of explants forming roots, proportion of explants forming shoots, and number of shoots. To be classified as a shoot, the organ had to be over 1 mm long.

Assessments were originally planned to be done monthly, for up to three months. However, PFR's COVID-19 response forced changes to this planned schedule of assessments. The proposed data analysis is detailed in Section 8.3.6

Experiment 4. Due to failure in Experiment 3 to induce shoots using the 'shoot induction medium' amendment with 3 mg/L TDZ, Experiment 4 was proposed with the aim of evaluating the effect of different culture media in the induction of shoots.

In this experiment, 10 shoot induction media were evaluated. The shoot induction media tested consisted of the 'base medium' (APPENDIX A) supplemented with concentrations of both BAP (between 0 and 3.20 mg/L) and NAA (between 0 and 0.5 mg/L) which resulted in different ratios between BAP and NAA (Table 8-1).

The explant used in the experiment corresponded to callus, previously induced from *in vitro* leaf segments utilising a 'callus induction medium' comprising 'base medium' supplemented with 1 mg/L 2,4-D for 28 days. This medium was noted as being best for callus induction and a reduced proportion of explants forming roots while in shoot induction medium (refer to Section 8.4.1.1 and Section 8.4.1.2).

In Experiment 4, the experimental design corresponded to a mixture-amount design (Evens & Niedz, 2010; Niedz & Evens, 2016) where the treatments were the combined concentrations of BAP (between 0 mg/L and 3.20 mg/L) and NAA (between 0 mg/L and 0.8 mg/L), and the ratio between them, i.e., (ratio BAP to NAA of either 1:1, 2:1, 4:1, 8:1, or 16:1). Each treatment was applied to four culture vessels (replicates) corresponding to disposable polystyrene plastic culture vessels (refer to Section 8.3.2.1). Each vessel contained four explants of callus, as sub-samples.

In Experiment 4, the response variables evaluated were the same as reported for Experiment 3, with changes in the assessment schedule also forced by PFR's COVID-19 response. Data analysis is explained in Section 8.3.6.

Table 8-1 Concentrations and ratios of the Plant Growth Regulators (PGRs) Benzyl Amino Purine (BAP) and α- Naphthaleneacetic acid (NAA) used in Experiment 4, targeting the induction of shoots from callus

BAP (mg/L)	NAA (mg/L)	Ratio BAP (mg/L) to NAA (mg/L)
0.94	0.06	16:1
1.78	0.22	8:1
0.44	0.06	8:1
3.20	0.80	4:1

BAP (mg/L)	NAA (mg/L)	Ratio BAP (mg/L) to NAA (mg/L)
0.80	0.20	4:1
0.20	0.05	4:1
1.33	0.67	2:1
0.33	0.17	2:1
0.50	0.50	1:1
0	0	Control

Experiment 5. Based on the results from Experiment 4 where some shoots developed at random from callus (refer to Section 8.4.1.2), Experiment 5 was proposed to examine an increased range in the concentrations of BAP and NAA and associated changes in the ratio of BAP to NAA. In addition, both in vitro and in vivo sourced leaves were compared for induction of callus.

In this experiment, 12 shoot induction media were assessed comprising the 'base medium' (APPENDIX A) supplemented with different concentrations of the PGRs: BAP (between 0 and 2 mg/L) and NAA (0.1-1 mg/L) and, therefore, different ratios of BAP to NAA (Table 8-2).

Like in Experiment 4, the callus used as the explant was produced in 'base medium' supplemented with 2 mg/L 2,4-D (i.e., 'callus induction medium'; APPENDIX A). However, unlike Experiment 1 to Experiment 4, explants used for callus induction in the current experiment corresponded to leaf discs, including midrib, from expanding leaves sourced either from *in vitro* or greenhouse grown plants. Prior to their initiation into tissue culture, leaves from greenhouse plants were surface sterilised with a 20% Sodium Hypochlorite solution (52 g/L) plus three drops of Tween® 20 (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for 10 min. Leaves were then rinsed thrice with sterile reverse osmosis water, and left to air dry within a laminar flow cabinet. To prevent bacterial infections leaves were immersed in a Ticarcillin solution (500 mg/L; Ticarcillin Disodium, Duchefa Biochemie, Netherlands) for 10 min. and left to air dry before planting onto the shoot induction media.

The experimental design of Experiment 5 corresponded to a mixture-amount design (Evens & Niedz, 2010; Niedz & Evens, 2016) where the treatments were the combined concentrations of BAP (between 0 mg/L and 2 mg/L) and NAA (between 0.1 mg/L and

1 mg/L), and the ratio between them (Table 8-2). The explant source (*in vitro* or greenhouse) corresponded to the blocks in the experimental design (Draper et al., 1993). Each treatment was applied to four culture vessels (replicates) corresponding to polystyrene disposable Petri dishes (refer to Section 8.3.2.1). Each culture vessel contained four leaf explants as sub-samples.

The response variables assessed were the same as those reported in Experiment 3. PFR's response to COVID-19 forced changes to this planned schedule of assessments with only one assessment carried out instead of the three planned assessments. Data analysis is explained in Section 8.3.6.

Table 8-2 Concentrations and ratios of the Plant Growth Regulators (PGRs) Benzyl Amino Purine (BAP) and α-Naphthaleneacetic acid (NAA) used in Experiment 5, targeting the induction of shoots from callus produced from leaf discs from either *in vitro* or greenhouse plants

BAP (mg/L)	NAA (mg/L)	Ratio BAP (mg/L) to NAA (mg/L)
0	0.1	0:0.1
0	0.5	0:0.5
0	1.0	0:1
0.5	0.1	5:1
0.5	0.5	1:1
0.5	1.0	1:2
1.0	0.1	10:1
1.0	0.5	2:1
1.0	1.0	1:1
2.0	0.1	20:1
2.0	0.5	4:1
2.0	1.0	2:1

8.3.4. Root induction experiment

Experiment 6. The aim of this experiment was to assess different culture medium for the induction of roots on shoots. Root induction medium consisted of the 'base medium' (APPENDIX A) amended with either IAA or IBA (0.01, 0.1, 0.3 or 1 mg/L). In this experiment two culture media were used as controls, comprising either the 'base medium'

without PGR or the 'rooting medium' commonly used within PFR's Palmerston North Plant Tissue Culture Laboratory for rooting *Limonium* plants. The latter comprised the base medium supplemented with 12 mg/L IBA (i.e., PFR's Palmerston North Plant Tissue Culture Laboratory medium identity number '608'; Morgan et al. (1998); Morgan et al. (1995); APPENDIX A).

The explant used corresponded to one growing shoot, devoid of roots, previously grown in tissue culture on the 'shoot proliferation medium' (APPENDIX A).

The experiment was conducted using a completely randomized design, with five replicates (Compton, 1994). Each treatment was applied to 5 culture vessels (replicates) comprising disposable polystyrene plastic culture vessels (refer to Section 8.3.2.1). Each vessel contained one explant (i.e., replicate sample).

The development of roots was evaluated after 28 days. The response variables were: root occurrence (yes/no), proportion of explants forming roots, number of roots, and length of the longest root. To be classified as a root, it had to be over 1 mm long. If the number of roots was impossible to count due to the high number, a value of 20 was assigned.

8.3.5. Induction of physical mutations

8.3.5.1 Establishment of a LD_{50} for irradiation

Experiment 7. The aim of this experiment was to identify an appropriate irradiation dose, measured as a median lethal dose (LD_{50}), for inducing mutation in callus.

Experiment 7 involved a radiation sensitivity test comprising the evaluation of five radiation doses (10, 20, 30, 40, 50 Gy) plus a control (0 Gy). However, due to the challenges faced due to PFR's responses to COVID-19, this experiment was not able to be conducted, but the planning is presented below for completeness of this chapter.

Callus was initially induced utilising the optimised 'callus induction medium' and duration (refer to Section 8.4.1.1) in Petri dishes (refer to Section 8.3.2.1).

After radiation treatments, the irradiated callus was to be transferred to Petri dishes with ca. 25 ml of the 'shoot induction medium' previously selected (refer to Section 8.4.1.2), with the effect of irradiation on shoot development evaluated.

The experimental design was completely randomized, with one factor (treatment) corresponding to the irradiation doses (i.e., 0, 10, 20, 30, 40, 50 Gy). Each treatment would be applied to 10 Petri dishes (replicates) and each Petri dish contained three calli (sub-samples). A control treatment comprising un-irradiated callus was also included.

The response variables proposed were: callus regeneration through shoot development (yes/no), time required until the first shoot was 1 mm long, and shoot number.

8.3.5.2 Irradiation and evaluation for mutation

After standardizing the appropriate tissue culture protocol for plant regeneration through indirect shoot induction and rooting, and identifying the LD_{50}, 500 calli would be irradiated under the treatment identified as optimum. Shoots recovered from independent calli would be regenerated to be rooted plants *in vitro* and transferred into the greenhouse facility for morphological evaluation.

8.3.6. Data analysis

Across all experiments within this chapter (i.e., Experiment 1 to Experiment 7) data analysis comprised modelling the effect of the factors (e.g., PGR concentration, explant source, duration of induction). For modelling effects of factors on the response variables with a binary distribution (i.e., callus, shoot and root, induction (yes/no)) or a Poisson distribution (i.e., number of shoots and number of roots) a generalized linear model (glm function, R Core Team 2018) with the relevant distribution was used. For data with either binomial or Poisson distribution, pairwise likelihood ratio tests were used to compare between the levels of factors (similar to LRPAIR in (Goedhart, 2018)).

For the response variables that were normally distributed (i.e., ratio of callus area to explant area and root length), a linear model (lm function (R Core Team, 2018)) was used to model the effects of the factors. The residuals were checked to ensure assumptions were valid (i.e., normal distribution of the residuals, observations are independent of each other), and Least Significant Difference was used for comparison between the levels of the factors (LSD.test function, Agricolae (Mendiburu & Yaseen, 2020)). For all the analyses, the *p-value* was set at 0.05.

8.4. Results

8.4.1. Plant regeneration through indirect shoot induction

8.4.1.1 Callus induction

Experiment 1. The percentage of explants forming callus depended on the use of PGR treatment, being highest (100%) with either 'base medium' supplemented with 2,4-D alone or with the combination 2 mg/L 2,4-D and 2 mg/L BAP (p<0.05; Figure 8-1). In comparison with the 'base medium' without PGRs on which 0% of explants produced callus, media with either 2,4-D alone or BAP alone increase the percentage of explants producing callus to at least 58% (p<0.05; Figure 8-1). The medium with the highest percentage of explant forming callus were the ones with: either 1 mg/L or 2 mg/L 2,4-D; or 2 mg/L 2,4-D plus 2 mg/L BAP (p<0.05; Figure 8-1).

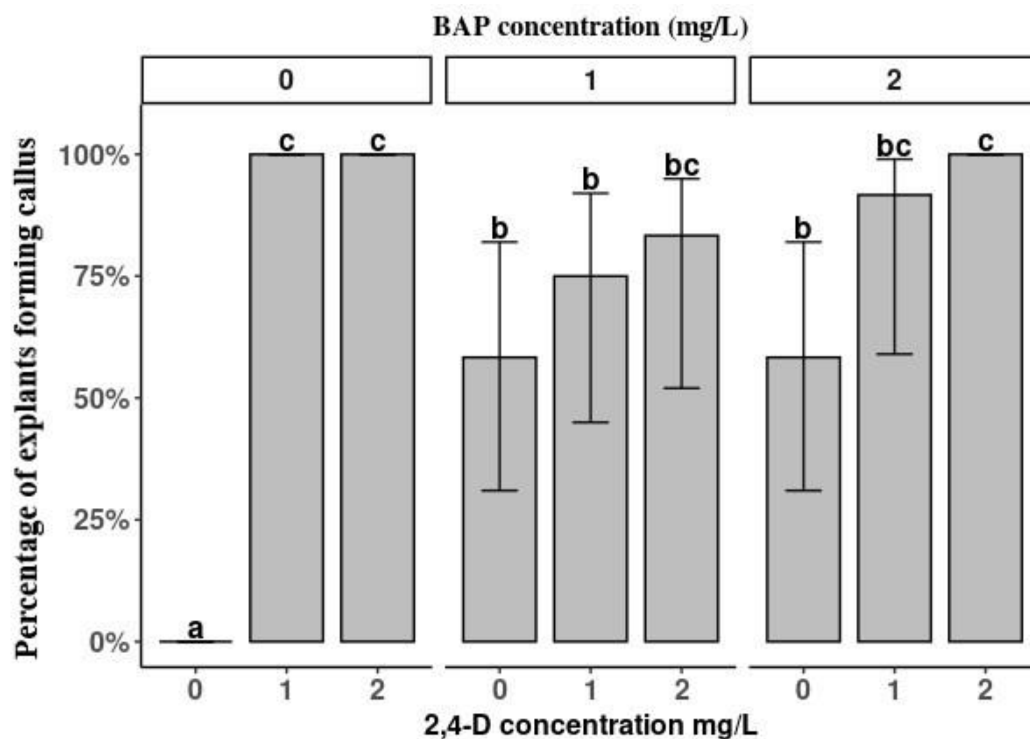

Figure 8-1 Percentage of explants of the *Limonium sinuatum* × *Limonium perezii* hybrid ('siNZii^{TM} Deep Lavender') forming callus *in vitro*, as influenced by combinations of 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D). Columns represent the predicted mean values and vertical lines (error bars) represent the 95% confidence interval. Letters above the error bars in the figure denote mean groups, after pairwise comparison with a likelihood ratio test. Different letters represent different mean groups (p = 0.05). n= 4 culture vessels

Callus growth measure as the ratio of callus area to explant area was at least 18 times higher in media amended with 2,4-D ($p<0.05$; Figure 8-2; Figure 8-3). The maximum ratio of callus area to explant area (i.e., 5.21) occurred in the medium with 2 mg/L 2,4-D and without BAP ($p<0.05$; Figure 8-2; Figure 8-3).

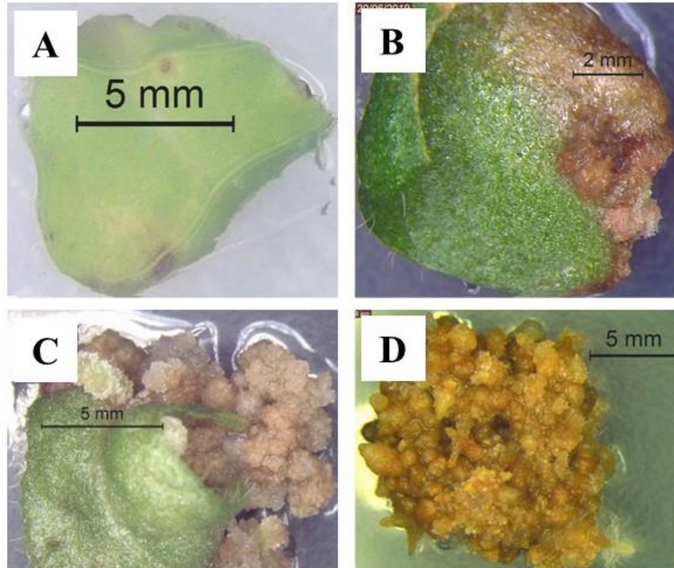

Figure 8-2 Callus growth from *in vitro* leaf explants of *Limonium sinuatum* × *Limonium perezii* hybrid (‘siNZii^{TM} Deep Lavender’) culture in medium amended with different concentrations of the plant growth regulators (PGRs) 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) or without PGR (control medium). **A.** Control medium. **B.** 1 mg/L BAP. **C.** 1 mg/L 2,4-D and 1 mg/L BAP. **D.** 2 mg/L 2,4-D

Experiment 2.

Callus was evident after 7 days and the ratio of callus area to explant area increased with time being on average 2.6 times higher at 21 days and 4.5 times higher at 28 days in comparison with 14 days ($p<0.05$; Figure 8-4). Differences in the ratio of callus area to explant area occurred after 14 days when the ratio of callus area to explant area was almost double in medium with 2,4-D and BAP in comparison with medium amended only with 2,4-D. In contrast, at 28 days, the ratio of callus area to explant area was three times higher in medium with only 2,4-D in comparison to medium with 2,4-D and BAP ($p<0.05$; Figure 8-4).

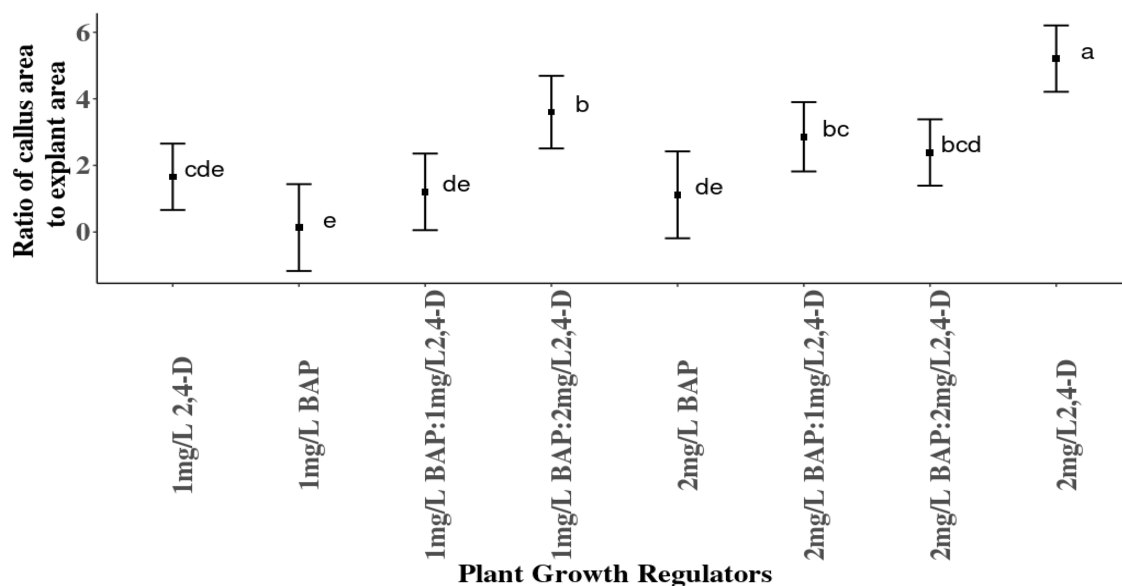

Figure 8-3 Ratio of callus area to explant area of *L. sinuatum* × *L. perezii* hybrid ('siNZii™ Deep Lavender' (BallSB, 2021) in culture medium amended with different combinations of 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D). Squares represent the predicted mean values for the ratio of callus area to explant area after data modelling using linear model, and the vertical lines represent the 95% confidence interval. Means followed by a common letter are not significantly different by the LSD test at the 0.05% significance level. n = 5 culture vessels

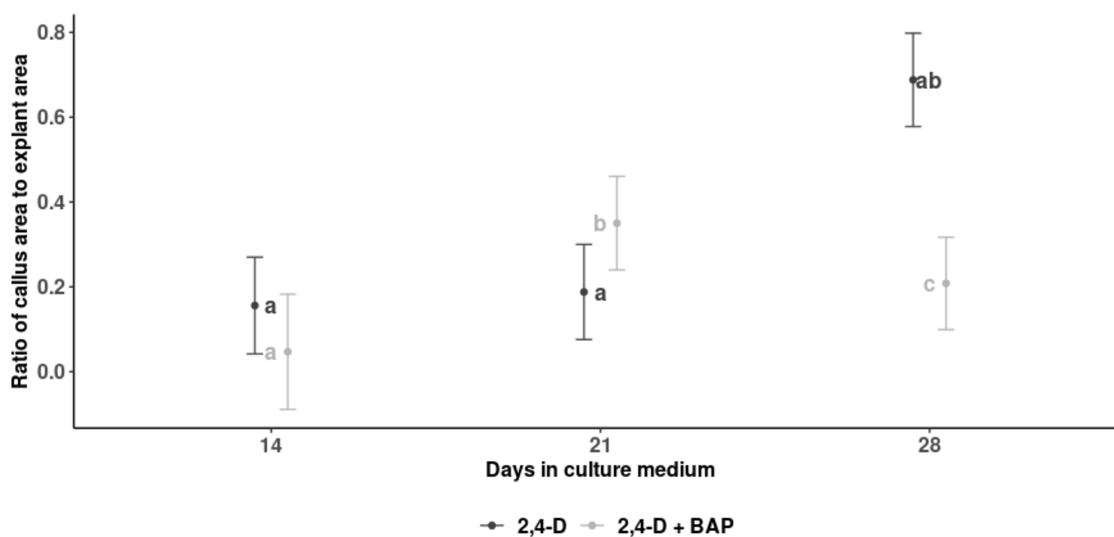

Figure 8-4 Ratio of callus area to explant area measure in culture media added with different plant growth regulators either 1 mg/L 2,4- Dichlorophenoxyacetic acid (2,4-D) or 2 mg/L 2,4-D and 2 mg/L 6-benzylaminopurine (BAP). Dots represent the predicted mean values for the ratio of callus area to explant area after data modelling using a linear model. Vertical lines (error bars) represent the 95% confidence interval. Means followed by a common letter are not significantly different by the LSD test at the 0.05% significance level. n = 5 culture vessels

8.4.1.2 Shoot induction

Experiment 3. Cultivation of callus for 48 h on medium amended with TDZ for induction of shoots resulted in zero shoots, but multiple roots.

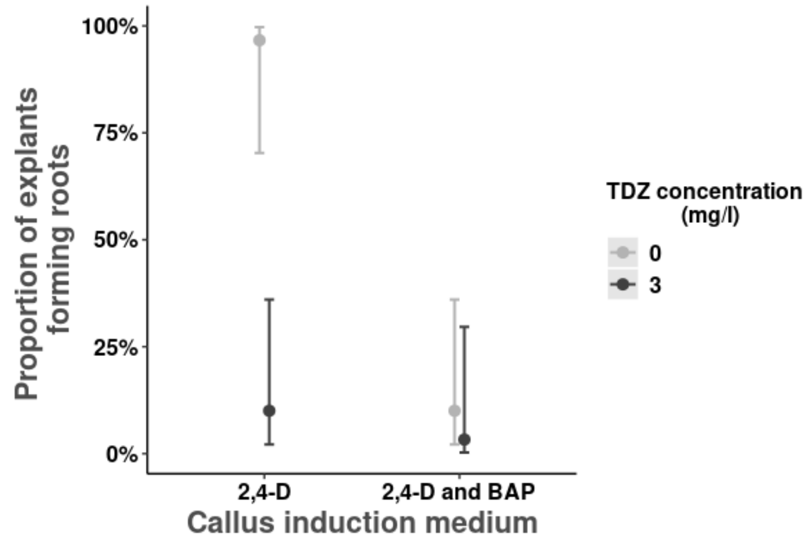

Figure 8-5 Proportion of explants forming roots by callus induction medium and thidiazuron concentration (i.e., shoot induction medium with either 0 mg/L (control) or 3 mg/L thidiazuron). Dots represent the predicted mean values for the proportion of explants forming roots after data modelling using a generalized linear model. Vertical lines (error bars) represent the 95% confidence interval. n = 5 culture vessels. *p* =

Roots were observed in most explants, including those in the control treatment for shoot induction with 0 mg/L TDZ. The occurrence of roots depended on interactions between callus induction media, shoot induction media, and the duration on the callus induction medium ($p<0.05$; Figure 8-5). The use of 2,4-D alone in the callus induction media and absence of TDZ from the shoot induction media (i.e., control for shoot induction) doubled the proportion of explants forming roots (i.e., 0.99%) in comparison with the use of 2,4-D alone in the callus induction media and TDZ (i.e., 3 mg/L) in the shoot induction medium ($p<0.05$; Figure 8-5). In contrast, when the callus induction medium was amended with 2,4-D and BAP, the use of TDZ in the shoot induction medium did not affect the proportion of explants forming roots ($p>0.05$; Figure 8-5).

Explants transferred from callus induction medium with 2,4-D (1 mg/L) to medium with TDZ (3 mg/L) had reduced the percentage of explants forming roots between 93% and

7% depending on the duration in the callus induction medium ($p<0.05$; Figure 8-6). In contrast, in callus induction medium amended with 2,4-D and BAP, the percentage of explants forming roots was not affected by either time in the callus induction medium or TDZ concentration in the shoot induction medium ($p>0.05$; Figure 8-6).

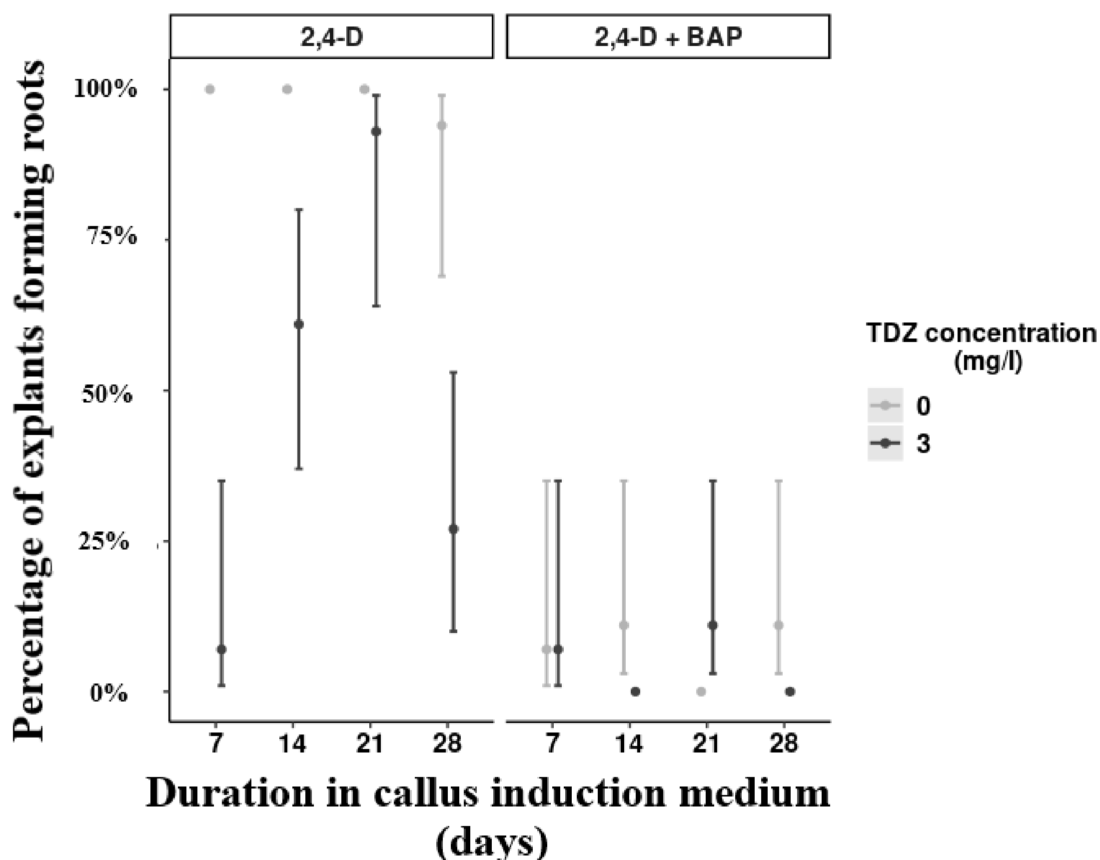

Figure 8-6 Percentage of explants forming roots by callus induction medium (i.e., callus induction medium was amended with either 2,4-D alone or 2,4-D plus BAP), duration in the callus induction medium and Thidiazuron (TDZ) concentration used in the shoot induction medium (i.e., either 0 mg/L (control) or 3 mg/L). Dots represent the predicted mean values for the proportion of explants forming roots after data modelling using a generalized linear model. Vertical lines (error bars) represent the 95% confidence interval. n = 5 culture vessels. $p = 0.05$

Experiment 4. Evaluation of different concentrations of BAP and NAA and ratio of BAP to NAA (Table 8-1) resulted in shoots in one explant from medium amended with 0.94 mg/L BAP and 0.06 mg/L NAA and one explant from medium with 0.5 mg/L BAP and 0.05 mg/L NAA. In comparison, in all the media evaluated, including the control without

PGRs, explants formed roots. However, the data collected for the number of explants forming roots and/or forming shoots was insufficient to perform any data analysis.

Experiment 5. Shoots and even roots were seen after 70 days on media containing the combination of 1 mg/L BAP and 0.5 mg/L NAA or the combination of 2 mg/L BAP and 1 mg/L NAA (Figure 8-7). Due to the long time in the same media a lot of information was lost about the explant used (either *in vitro* or greenhouse leaves) and less than 5% of the explants/shoots were still alive, so data was again insufficient to perform any statistical analysis.

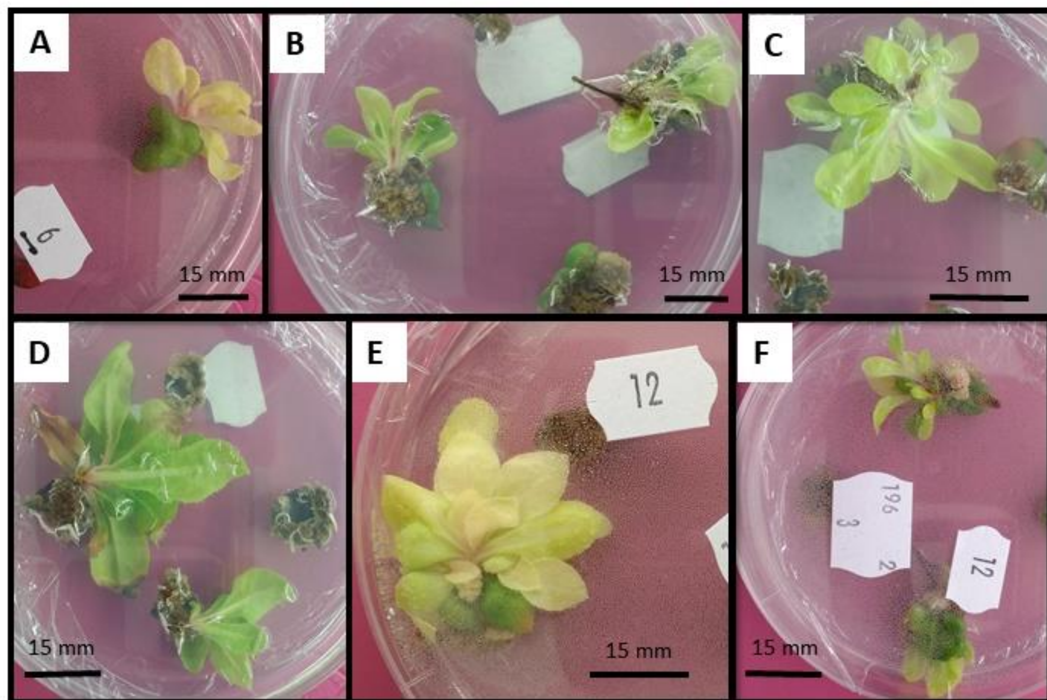

Figure 8-7 Development of shoots from callus (indirect caulogenesis) in a *L. sinuatum* × *L. perezii* hybrid grown on media supplemented with different concentrations and proportions of 6- benzyl amino purine (BAP) and 1- naphthaleneacetic acid (NAA). **A.** 0.5 mg/L BAP: 0.5 mg/L NAA (1:1). **B-D.** 1 mg/L BAP: 0.5 mg/L NAA (2:1). **E-F.** 2 mg/L BAP: 1 mg/L NAA (2:1)

8.4.1.3 Root induction

Shoots developed roots in all culture media, including both controls (0 mg/L or 12 mg/L IBA; $p>0.05$; Figure 8-8).

The number of roots per shoot ranged between 0 ± 0 (medium with either 0.1 AIA or 0.1 IBA) and 50 ± 0 (12 mg/L IBA). The number of roots per shoot was between 7 and 33 times higher in the control-1 medium without PGRs and in control-2 medium amended with 12 mg/L IBA, respectively in comparison with all the other evaluated media ($p<0.05$). The corresponding values were 50 ± 0 (i.e., control-2 with 12 mg/L IBA), 11 ± 10 (control-1 with no PGRs), and an average 1.5 ± 2.1 roots per shoot for the other media amended with different concentrations of either IAA or IBA.

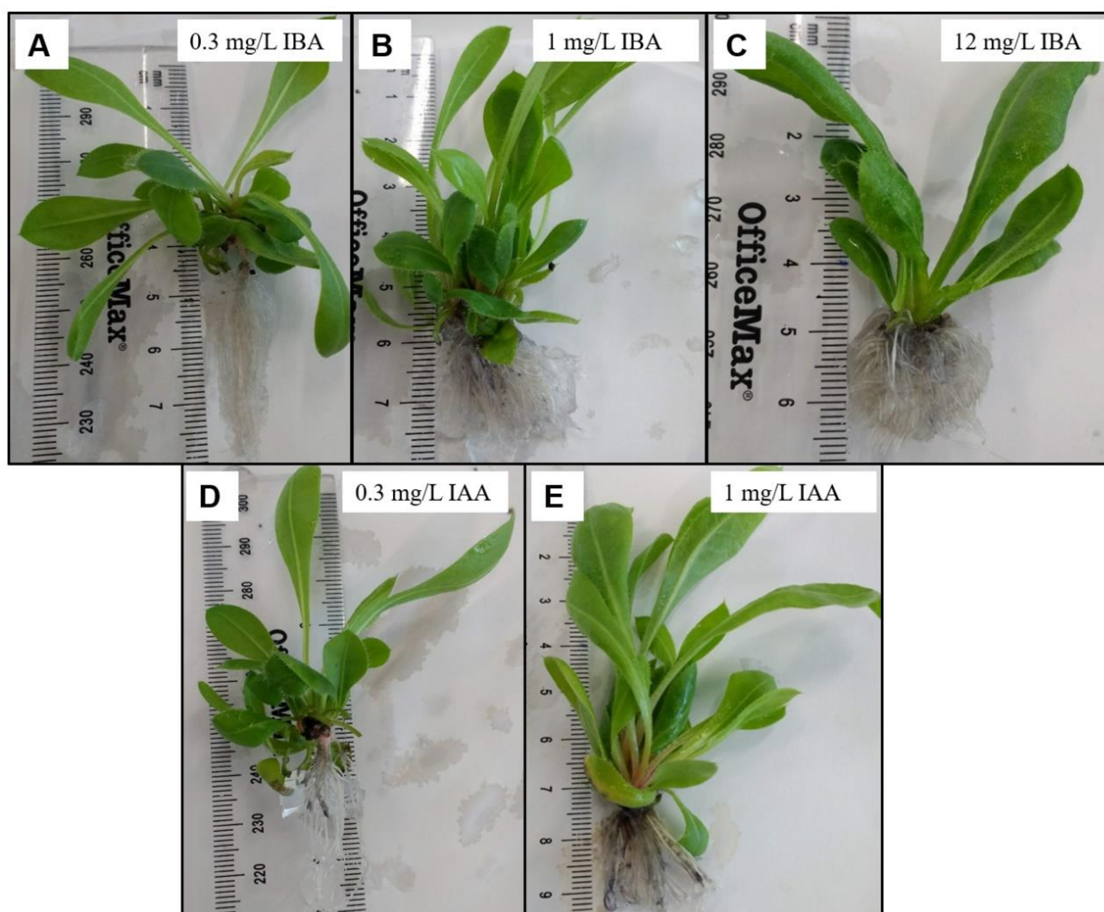

Figure 8-8 Development of roots in treatment media with different concentrations of the Plant Growth Regulators (PGR): Indole Acetic Acid (IAA) or Indole Butyric Acid (IBA). **A.** 0.3 mg/L IBA **B.** 1 mg/L IBA **C.** 12 mg/L IBA (control) **D.** 0.3 mg/L IAA **E.** 1 mg/L IAA

With regard to root length, the only medium with a significant positive effect over root length was the control treatment without PGR (i.e., control), achieving 8.2 ± 1.2 cm compared with an average of 1.7 ± 1.9 for other treatments ($p<0.05$). Compared to the control treatment without PGR (i.e., control), plants on media supplemented with PGR

had reduced root lengths, with the shortest roots evident at the higher PGR concentrations ($p<0.05$).

8.4.2. Establishment of an LD_{50} for irradiation

The experiments proposed for the induction of shoots failed (i.e., Experiments 3 and 4) or data could not be collected due to PFR's response to COVID-19 (Experiment 5). Therefore, as a consequence of not confidently being able to obtain shoots from callus, further investment of resources was redirected to the first research strategy, i.e., manipulation of ploidy.

8.5. Discussion

Callus was induced on media containing at least one PGR (2,4-D and/or BAP; Figure 8-1), with the ration of callus to total explant area being larger than the explant area after longer exposures (Figure 8-4). However, an interaction occurred between the media for callus induction and for shoot induction and, although no shoots were produced, the root development could be used to tease out the effect of the PGRs as discussed in the following paragraphs.

Rather than an independent effect of auxins and cytokinins over plant cells, the interaction between auxins and cytokinins has been for long recognized as crucial for growth and differentiation in plants. The presence of auxins as well as cytokinins is necessary to induce cell division, initiation of mitosis and cell elongation (Campanoni & Nick, 2005; Coenen & Lomax, 1997; Kurepa et al., 2019). In general terms, for the induction of callus a 1:1 ratio of auxin to cytokinin or a ratio biased slightly toward auxin is recommended (Ikeuchi et al., 2013). For shoot induction a medium amended with cytokinin is recommended, and for root development media amended with auxin are advised (Coenen & Lomax, 1997). In the current experiments, the use of TDZ in the shoot induction medium decreased the percentage of explants forming roots from callus induced on medium amended with 2,4-D alone, and additionally when the duration in callus induction medium was equal to 7 or 28 days (Figure 8-6). Those results support the hypothesis that amendment of the culture medium for shoot induction with TDZ modified the auxin to cytokinin ratio toward cytokinins, as recommended for induction of shoots (Coenen & Lomax, 1997), when the exposure to the auxin 2,4-D was shorter than 14 days.

In contrast, the decrease in the percentage of explants forming roots after 28 days in the callus induction medium could be explained by depletion of the auxins in the medium as well as in the tissue (Figure 8-6) also causing the auxin to cytokinin ratio to trend toward cytokinins.

The addition of the cytokinin BAP to the callus induction media was considered an appropriate strategy to adjust the auxin to cytokinin ration toward shoot development, and against root development, as shown by the decreased percentage of explants with roots when BAP was present, with or without TDZ (Figure 8-6). While BAP presence presumably adjusted the ratio of auxin to cytokinin, it was still insufficient to induce shoot development, with the remaining auxin concentration within the tissue possibly limiting the action of cytokinin at the levels applied (Coenen & Lomax, 1997; Kurepa et al., 2019).

The auxin concentration used in the callus induction media (i.e., 1 mg/L 2,4-D) interfered with the efficacy of the shoot induction medium. Possible mechanisms involved include reduction of the cytokinin levels by oxidative breakdown (Coenen & Lomax, 1997) or limiting the effect of the cytokinin over the cells directly (Ahmad & Faisal, 2018). However, detailed studies are needed, so as to offer supporting evidence on the mechanism of action of auxins in *Limonium* and the concentrations naturally present in the explants.

Based on the results of in Experiment 3, the next alternatives were proposed to promote shoot induction by restricting the effect of the callus induction medium on subsequent shoot induction by using a lower 2,4-D concentration (< 1 mg/L) in the callus induction medium and reducing the exposure time below 28 days; by extending the duration in callus medium beyond 28 days; by using a cytokinin such as BAP in the callus induction medium, or by replacing 2,4-D with another auxin.

The decision to change the TDZ in the induction medium for a combination of BAP and NAA was based on previous studies of success of BAP and NAA in shoot induction (Bhojwani & Dantu, 2013; Das et al., 2018; Raju et al., 2022). The results obtained in the current research with development of shoots in at least one replicate per treatment, in comparison with Experiment 3 where 3 mg/L TDZ did not result in shoots, suggest a possible positive effect of the BAP and NAA. However, these results did not provide the

information sought making it necessary to propose a new experiment (i.e., Experiment 5).

Experiment 5 focussed on shoot induction, by replacing 2,4-D with NAA, instead of lowering the 2,4-D concentration, and reflected an analysis of the reported differences in the mechanism of action between both auxins (i.e., 2,4-D and NAA). The auxin 2,4-D promotes cell division because it binds to the nuclear auxin receptors and promotes the degradation of the transcription factors responsible for the repression of the auxin-responsive genes (Peterson et al., 2016). In contrast, NAA binds to different receptors than those which bind 2,4-D (i.e., ABP1) and promotes cell elongation (Campanoni & Nick, 2005).

The replacement of 2,4-D with NAA (Experiment 5) and the use of the same media for callus and root induction, resulted in callus induction as well as shoot induction (Figure 8-7), revealing at least three situations that could be taking place in the current experiments:

1. The auxin 2,4-D might be interfering with shoot development.

2. The interaction between 2,4-D and TDZ, in both the concentrations and explant used, is antagonistic. However, the cytokinin concentration used was too low for the shoot induction requirements or both PGR act synergistically favouring root development (Coenen & Lomax, 1997; Kurepa et al., 2019).

3. The contrasting effect over shoot induction when using 2,4-D versus NAA when on the callus induction media, could be associated with differences in signalling, biosynthesis, metabolism, transport and inactivation of auxins and cytokinins, as reported for 2,4-D in comparison with IAA (Coenen & Lomax, 1997; Kurepa et al., 2019; Peterson et al., 2016).

With regard to the unsuccessful development of shoots when using TDZ, the results obtained in the current series of experiments, are in contrast to previous studies using *Limonium perigrinum* (Seelye et al., 1994). In their results TDZ induced shoot development directly (no previous callus formation). The contrasting results might suggest a possible genotype by TDZ interaction, as reported for *Limonium* in other studies

(Chamorro et al., 2007). In addition, other factors might also have contributed to the lack of shoot induction from callus with TDZ, including the concentration of TDZ used, duration of exposure, light conditions, and the concentration of other PGRs naturally present in the explant (Ahmad & Faisal, 2018). Therefore, the differences observed in our experiments could suggest species or genotype differences in the mode of action of the cytokinin TDZ.

With regard to root induction, a culture medium amended with auxins is recommended, and IAA or NAA were preferred (Bhojwani & Dantu, 2013; Casazza et al., 2002; Hsu et al., 2000). In the experiment done here for evaluation of root induction, longer and more roots were produced in the absence of PGRs, indicating the PGR naturally occurring within the *in vitro* plant material was adequate. The explants used in Experiment 6 for root induction were considered equivalent to the plants obtained by indirect shoot induction (i.e., under a scenario of successful shoot induction). Nevertheless, some interactions between the PGR used for shoot induction and root induction might occur when rooting shoots induced from callus. For example, an antagonistic effect on rooting between auxin and cytokinins could occur, with the auxins stimulating the cell division in the lateral root primordia followed by elongation (Campanoni & Nick, 2005), and cytokinins repressing the division and favouring the elongation (Coenen & Lomax, 1997). As consequence, if the explants used for root induction came from a cytokinin-enriched medium (as would be the case in indirect shoot induction) a possible addition of auxin to the root-induction medium would be required to change the ratio of auxin to cytokinin toward auxins and, therefore, to root development. Unfortunately, the resources (and time) available did not permit evaluation of the possible interactions occurring but remains a valid area for future research.

8.6. Conclusions

The interaction between auxin and cytokinin is complex and depends not only on the concentrations in the culture media but also the relative ratios between PGRs. In the case of the *L. sinuatum* × *L. perezii* hybrid evaluated in this study, the use of 2,4-D in the callus induction media is not recommended if the aim is to later produce shoots from the callus. Instead, the use of BAP, in combination with NAA, is supported, but detailed experiments are still required.

CHAPTER 9. General discussion and future research

9.1. Introduction

Plant breeding of *Limonium sp.* in New Zealand is limited by access to germplasm diversity and in the case of hybridisation, by pre- and post-zygotic barriers (i.e., low hybridisation rate, and hybrid lethality, weakness, or sterility). Those limitations added to the pollen/stigma dimorphism (refer to Section 1.2.3), differences in the number of chromosomes (refer to Section 5.3) and low male fertility for some genotypes (i.e., *L. perezii*; refer to Section 4.3) restrict the opportunities for crosses that can be performed to broaden the germplasm diversity using *L. sinuatum* and *L. perezii*. These species were used for production of the 'siNZii™' series developed by The New Zealand Institute for Plant & Food Research Ltd (PFR), and commercialized by BallSB (BallSB, 2021).

The breeding strategy of ploidy manipulation evaluated in this research using either N_2O (refer to CHAPTER 2 to CHAPTER 5; APPENDIX F, APPENDIX I, and APPENDIX N) or oryzalin (CHAPTER 6), as summarized in Figure 9-1, contributed to:

- increasing the germplasm diversity (CHAPTER 2 and CHAPTER 7),
- increasing the hybridisation rate (CHAPTER 5),
- increasing the fertility of genotypes identified as presenting low male fertility (CHAPTER 4), and
- restoring the fertility in interspecific hybrids (CHAPTER 6 and APPENDIX N).

The general research objectives pursued in this PhD research (Figure 9-1) contributed to the development and implementation of breeding technologies for producing *"better crops faster"* (refer to Section 1.6).

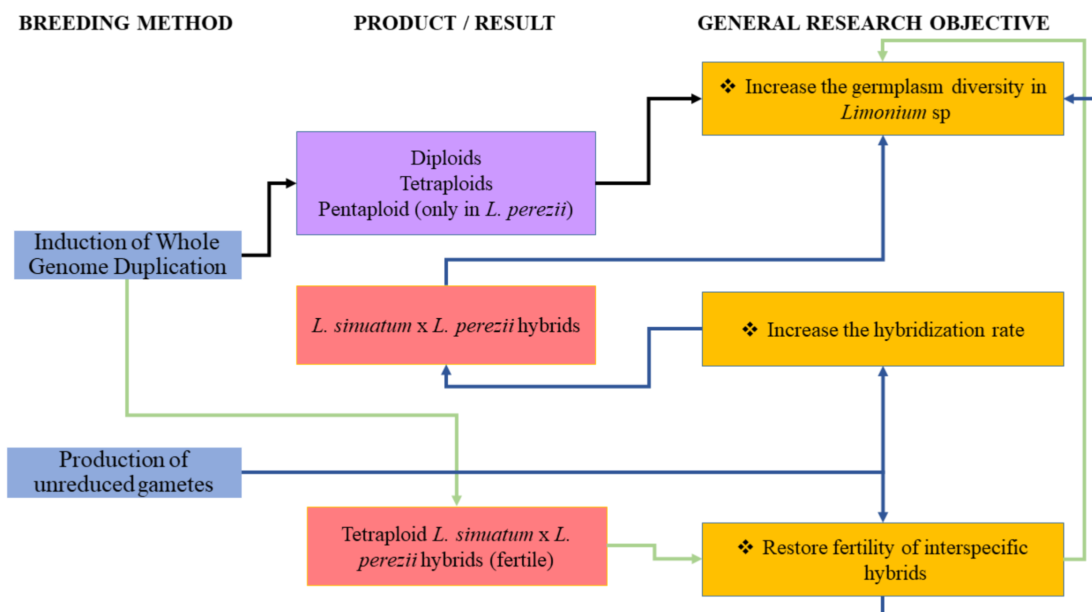

Figure 9-1 Schematic illustration of the breeding methods used within the breeding strategy of ploidy manipulation, the products / results produced by breeding method, and their contribution to the achievement of the general research objectives proposed in the current research

The limitations above mentioned for *Limonium* breeding (e.g., limited germplasm diversity, low male fertility, etc.) are not restricted to the *Limonium* genus, as explained below, therefore the findings of the current research and methodologies developed can be applied to other crops. For example, decrease in diversity (e.g., genetic erosion) is widely reported to result from intensive selection for specific phenotypic traits as reported for *Brassica* (Katche et al., 2019) and *Zea mays* (Dyer et al., 2014). Low male fertility is also present in crops such as *Zea mays* (Defani-Scoarize et al., 1995) and *Prunus cerasus* (Akšić et al., 2016). In addition, limitations in crosses associated with pollen/stigma dimorphism occur in other genus from the Statice tribe (Baker, 1948a) such as *Goniolimon* with potential to be used as an ornamental (Kaninski & Bistrichanov, 2008), *Acantholimon* with medicinal properties (Kiziltas et al., 2022) and *Armeria* which is adapted to saline and arid habitats and with potential use for soil remediation (Purmale et al., 2022).

In this chapter, the advances made in the implementation of the breeding strategies of ploidy manipulation and physically induced mutations and their contribution to the general research objectives are discussed, as well as future research questions and opportunities.

9.2. Increment of germplasm diversity

The germplasm diversity (i.e., diversity that could potentially be introgressed to future generations (Offord, 2017)) of the *Limonium* germplasm collection available at PFR was increased in at least three ways within the present research (Figure 9-1 product/result):

- Production of 10 *L. sinuatum,* 21 *L. perezii*, 5 *L. sinuatum* × *L. perezii* hybrid plants and 24 backcrossed *L. sinuatum* × *L. perezii* hybrid with higher ploidy (i.e., triploid, tetraploid, pentaploid; APPENDIX F, CHAPTER 2, CHAPTER 3, CHAPTER 6, and CHAPTER 7). They displayed higher morphological diversity represented as bigger organs, different plant forms and novel floret colours (refer to Section 7.4.1).
- Production of new 49 *L. sinuatum* × *L. perezii* diploid hybrids (APPENDIX I and CHAPTER 5) by increasing the interspecific hybridisation rate.
- Increased potential to use genotypes with low male fertility (2 genotypes; CHAPTER 4) or infertile (1 genotype; APPENDIX N) in planned crosses to further increase the germplasm diversity after inducing unreduced gametes using N_2O.
- Potential to use infertile genotypes (i.e., 4 *L. sinuatum* × *L. perezii* hybrids) in backcrosses (CHAPTER 6 and CHAPTER 7) to further increase the germplasm diversity after inducing whole genome duplication (WGD) using oryzalin.

As mentioned throughout the present document, induction of polyploids using N_2O could be done before pollination to induced unreduced gametes or after pollination for whole genome duplication (WGD) in the zygote (Figure 9-2). The use of N_2O for induction of WGD, and subsequent increase of ploidy and diversity of germplasm, was more effective in producing diversity than induction of unreduced gametes by targeting the cell division cycles of interest in this research (Figure 9-2). WGD is more accurate as the timing of treatment with N_2O is determined by the date of the controlled pollination (day 0) and no differences were detected in the progression of the embryo development at least between *L. sinuatum* and *L. perezii* (refer to Section 2.6). In contrast, for induction of unreduced gametes the moment of the treatment with N_2O is determined by the floret size, as an indirect sign of the occurrence of meiosis, which is difficult to measure precisely. In addition, the time span between meiosis/day of the treatment and pollen maturation/floret

opening/planned cross is longer in comparison with time span between planned cross and mitosis in WGD (Figure 9-2). As consequence, the induction of unreduced gametes is more susceptible to the influence of external factors which affect the floret development and ontogeny (Mason et al., 2011) as well as the production of unreduced gametes (Sora et al., 2016).

1. ANTHER DEVELOPMENT

2. POLLEN DEVELOPMENT

Mitosis

Mitosis

E
PPC
Ar

E
En
M
T
Sp

E
EN
M
T
V
PMC

Meiosis I

Meiosis II

Pollen mother cell

Chromosomal segregation

Tetrad

Microspore

Polarized microspore

Bicellular pollen

Tricellular pollen

Mature pollen

12 - 18 days – Induction of unreduced pollen (aimed spindle disruption)

20 - 30 days – Induction of unreduced pollen (spindle potentially disrupted)

3. FERTILIZATION & POLLINATION

4. EMBRYO DEVELOPMENT (*in vitro*)

5. SHOOT PROLIFERATION (*in vitro*)

Zygote

Proembryo

Globular

Transition

Heart

Seedling

Multiple mitosis

Multiple mitosis

Multiple mitosis

Embryo/ovule rescue

Approx. 4 days
Induction of whole genome duplication

Aimed target (mitotic/meiotic spindle) for N_2O treatment

Mitotic spindle potentially affected by N_2O treatment

Mitotic spindle target of oryzalin treatment

Time interval between male meiosis and pollination

Time interval between mitosis in archesporial cells and pollination

Figure 9-2 Diagram of the moments during the plant development when treatment with an inducer of polyploidy such as oryzalin or nitrous oxide (N_2O) could be used to disrupt either the mitotic or meiotic spindle. The time lapse between either mitosis in the archesporial cells or meiosis in the pollen mother cell and the pollination is shown as well as the time elapsed between pollination/fertilization and first developmental stages of the embryo (i.e., zygote and proembryo). E: Epidermis; PPC: PMC: Pollen mother cells; Ar: Archesporial cell; En: Endothecium; M: middle layer cell; T: Tapetum; Sp: Sporogenous cell; V: vasculature. Modified from Cai and Zhang (2018); Chaturvedi et al. (2021); Honys et al. (2006); ten Hove et al. (2015)

The increase in germplasm diversity is faster with the induction of WGD than with the induction of unreduced gametes because the time between the application of doubling treatments (i.e., treatment with N_2O plus planned crosses) and the recovery of a new plant is shorter and involves fewer steps for WGD. In WGD, planned crosses are performed during a smaller time window (i.e., between 8 and 72 hours after pollination (~ 3 days of crossing) before the 24 h N_2O-treatment (CHAPTER 3; Figure 9-2). This is in comparison with at least 10 days (between 20 and 30 Days Before pollination; Figure 9-2) of crossing required for recovery of plant from induced unreduced gametes (CHAPTER 5, APPENDIX N).

The selection of the breeding method, i.e., WGD induction or unreduced gamete induction, depends mainly on the advantages and disadvantages of each. Studies comparing the meiotic and mitotic polyploidization revealed that meiotic polyploids present larger genetic variability, fitness, and heterozygosity, in comparison with the mitotic polyploids (reviewed by Dewitte et al. (2012); Younis et al. (2014)). However, in the current research, no polyploid seedlings were produced by using unreduced gametes in crosses, so comparison of the diversity produced by WGD and unreduced gametes is still pending for *Limonium*. The inability to produce polyploid seedlings has been problematic for investigations requiring a detailed study of the conception and development of seedlings using unreduced gametes. Further research is needed with particular emphasis on the processes of pollination, fertilization, and embryo development. This follow-up investigation is essential to determine the underlying mechanisms or processes behind the failure to produce polyploid seedlings using induced unreduced gametes.

The polyploid seedlings produced by WGD (20 new *L. perezii* auto-tetraploids and 7 new *L. sinuatum* auto-tetraploids; APPENDIX O) may be less genetically diverse than seedlings obtained by crossing unreduced gametes. The genetic constitution of auto-tetraploid seedlings consist of four similar haploid genomes (Bohutínská et al., 2021) or two copies of the original genome (Bomblies and Madlung (2014) ; refer to Section 1.2.5.2.2). In contrast, allo-tetraploid seedlings produced by fusion of unreduced gametes allows the chromosome synapsis in prophase I-metaphase I and recombination (Table 1-2; reviewed by Lloyd & Bomblies (2016)). As result the genome of the seedlings contains two copies of the recombined parental chromosomes (refer to Section 1.2.5.2.3) as reported in *Lilium* by genomic *in situ* hybridisation (Barba-Gonzalez et al., 2004). Even though both types of seedlings (i.e., auto-tetraploid, allo-tetraploid) might contain genetically the same information (i.e., chromosomes/genes), the occurrence of recombination prior to fertilization in allo-tetraploid results in new chromosome combinations and subsequent chromosome segregation in the gametes (Villeneuve & Hillers, 2001). Meiotic recombination has been proposed to represent evolutionary advantages for the species contributing to adaptation, genetic diversity, and additionally provides new combinations of traits for selection and development of improved plant varieties (Choi et al., 2016; Lambing et al., 2017).

Besides the absence of recombined parental chromosomes in WGD, auto-tetraploid plants could present an additional reduction in the meiotic recombination during meiotic prophase I-metaphase I (Table 1-2). Moreover a reduction in pollen germination (between 38% and 52%; Table 7-9) and viability (71%; refer to Section 7.3.2.3) was observed in addition to potential negative effects on diversity, introgression of characteristics of interest in ornamental plants (Kuligowska et al., 2016), and segregation of the parental characters in *Lilium* seedlings (Barba-Gonzalez et al., 2004). That reduction in meiotic recombination is a plant’s mechanism to limit the occurrence of multivalents (refer to Section 7.4.2; Bomblies and Madlung (2014)), mis-segregation (Bohutínská et al., 2021) and aneuploidy (Bomblies, 2022) which are immediate consequences of WGD and the occurrence of four copies of each chromosome instead of the original two (Bomblies, 2022).

Despite the potential disadvantages of the newly created auto-tetraploids (i.e., neo-polyploids) explained above, if the polyploidy represents advantages for the species, for

example increasing its adaptability (Potapova & Gorbsky, 2017), and are able to deal with the additional copies of chromosomes, the neo-polyploids will adapt to WGD (Bomblies et al., 2015). The establishment of polyploid cytotypes (e.g., a tetraploid *L. sinuatum* (i.e., same species, different ploidy)) varies among species, and depends on the number of generations elapsed since WGD (e.g.,~20,000 generations is still considered as evolutionarily young in *A. arenosa* (Bohutínská et al., 2021)), and the mode of reproduction with vegetative reproduction facilitating genome stabilization as suggested for other *Limonium* species (Róis et al., 2012).

In the case of WGD induced in *L. sinuatum* × *L. perezii* hybrids using oryzalin, the diversity of the hybrids was initially, before the WGD, higher than for *L. sinuatum* or *L. perezii* alone as the hybrids formed after a hybridisation event and combined traits from *L. sinuatum* and *L. perezii* (refer to Section 7.3.1). However, after WGD in hybrid genotypes, the increase in diversity gained, represented in a ploidy increase, could be prevented from further diversification or inheritance to future generations. For example, auto-tetraploid hybrids could become “permanent hybrids” without recombination between the parental genomes and no segregation. In the “permanent hybrids” the synapsis and recombination tend to form between homologous chromosomes (i.e., from the same species) rather than between homeologous chromosomes (i.e., from different species) which could led to gene losses or aneuploidy (Bomblies, 2022; Lloyd & Bomblies, 2016) and prevent new chromosome combinations (Villeneuve & Hillers, 2001). However, recombination between homeologous chromosomes has been reported, and has contributed to new diversity and introgression as observed in wheat (Liu et al., 2020) and *Arabidopsis* (Lloyd & Bomblies, 2016). The occurrence of recombination between homeologous chromosomes is related to adaptation to polyploidy (Bomblies, 2022; Liu et al., 2020), as observed in kiwifruit (*Actinidia chinensis*; Wu et al. (2014)). In the adaptation to polyploidy, the number of generations is a determinant of the genome stabilization and reduction of the frequency of multivalents during meiosis with subsequent non-preferential pairing between chromosomes (Wu et al., 2014).

This section has focussed on utilising *Limonium* as a model crop to evaluate different strategies for increasing the diversity of germplasm. The concepts developed here for utilising N_2O and oryzalin are applicable to the breeding programmes of other important

crops such as *Brassicca* and *Zea mays* as examples where losses of germplasm diversity have been reported (Dyer et al., 2014; Katche et al., 2019).

9.2.1. Use of the germplasm diversity produced in planned crosses

The neo-polyploids obtained after WGD using either N_2O (CHAPTER 2, CHAPTER 3 and APPENDIX F) or oryzalin (CHAPTER 6) were fertile and competent for crossing, with the newly developed characteristics potentially transferable to future generations (CHAPTER 7). However, as introduced in Section 9.2 and explored further here when considering the implementation of these strategies in a future breeding programme, new challenges became apparent with the use of neo-polyploids in planned crosses, including:

- First, the lower pollen viability and pollen germination observed in tetraploid *L. sinuatum* and *L. perezii* genotypes, as compared with their corresponding diploids (refer to Sections 7.3.2.3 and 7.3.2.4), negatively impacted the number of planned crosses needed for production of seedlings. For example, in the winter season (May to July) using diploid *L. sinuatum* with a 47% pollen viability (CHAPTER 7) resulted in 23% zygote success (data not shown) with approximately 80% of those embryos growing into seedlings. In this scenario five crosses would be needed for obtaining 1 seedling. In the case of tetraploid genotypes with a pollen viability equal to 19% (CHAPTER 7), under similar conditions, 14 crosses were needed to obtain one seedling, assuming no occurrence of pre- or post-zygotic barriers.
- Second, the type of cross performed will determine the successful transmission of the phenotypic traits of interest. Backcrosses are the preferred type of cross for:
    - eliminating undesirable characteristics of the hybrids e.g., low plant vigour or low fertility(Cheng et al., 2011; Nukui et al., 2011; Tatum et al., 2015);
    - accumulating within the seedlings the desirable characteristics of the parent used for the backcross (Cheng et al., 2011);
    - reducing the genetic differences between the chromosomes coming from each of the parental genotypes (Husband, 2004) and associated with meiotic abnormalities (e.g., recombination between homeologous chromosomes (Bomblies, 2022; Lloyd & Bomblies, 2016)) and hybrid weakness (Husband, 2004).

In the present research two types of backcrosses were performed: homoploid (CHAPTER 7) and heteroploid (CHAPTER 6). Heteroploid backcrosses (i.e., diploid × tetraploid or vice versa) resulted in seedlings, independently of the ploidy of the genotype used as the seed-bearing parent (APPENDIX M). In contrast, in homoploid backcrosses (i.e., diploid × diploid; tetraploid × tetraploid) seedlings were produced only if the seed-bearing was *L. sinuatum* (APPENDIX M). These results are similar to other studies done in *Limonium* (Conceição et al., 2018) where more viable seeds were produced in heteroploid crosses in comparison with homoploid crosses (i.e., they reported 74% viable seed in heteroploid crosses versus 50% in homodiploid crosses). However, in contrast with Conceição et al. (2018), in the current study, the ploidy of the seedlings from heteroploid crosses was as expected from the two parents, i.e., triploid seedlings were produced, which confirmed that seedlings did not arise from apomixis, as reported by Conceição et al. (2018). Hence the current limitations of homoploid crosses within the *Limonium* siNZii™ breeding programme may provide a future focus for research efforts to understand and resolve.

- Third, the selection of the parental genotypes was found to be crucial for successfully achieving multiple seedlings within the breeding programme as demonstrated in this research (APPENDIX F):
  - One advantage of the breeding technologies implemented in this research is that some of the genotypes initially rejected in the present research, due to their poor performance in previous intraspecific crosses (APPENDIX A), could now potentially be used, e.g., after treating them with N_2O before pollination for induction of unreduced gametes, restoration of pollen germination, or an increase in pollen viability. However, within a breeding programme the success of using N_2O treatment will depend on the specific factors causing the poor performance of those genotypes (CHAPTER 4).
  - For the selection of parental genotypes, if crosses will be performed using *L. sinuatum* and/or *L. perezii,* it is recommended to use *L. sinuatum* as the mother whenever possible. When used as the mother *L. sinuatum* genotypes displayed at least 1.7 times higher embryo success and viable seedlings in intraspecific, interspecific, and homoploid backcrosses (APPENDIX M). In the case of heteroploid backcrosses, the embryo success appears to depend more on the

ploidy of the seed-bearing genotype than the seed-bearing species (APPENDIX M) as previously reviewed for other plant species (Lafon-Placette & Köhler, 2016; Ortiz & Ehlenfeldt, 1992). In *Limonium*, higher numbers of embryos and seedlings were produced in tetraploid × diploid crosses compared to diploid × tetraploid crosses in heteroploid crosses (APPENDIX M), as also reported for *A. arenosa* (Morgan et al., 2021). This could be associated either to the EBN ratio (refer to Section 1.3.7) with a maternal genome excess (maternal to paternal genome ratio greater than 1) being better tolerated than a paternal excess (maternal to paternal genome ratio less than 1), or to differences in the pollen fitness, with unreduced pollen being less fit than reduced pollen. Clearly further research to improve the performance of *L. perezii* as seed-bearing parent would be relevant to *Limonium* breeding.

- In addition, for the selection of the parental genotypes, it is recommended to select outcrosses that involve unrelated genotypes over backcrosses to one of the parents. This is because the sporophytic self-incompatibility system evident in both *L. sinuatum* and *L. perezii* (Baker, 1966; Conceição et al., 2018) could negatively affect embryo success and seed viability (APPENDIX L).

9.3. Increase the hybridisation rate

The use of N_2O for treating unopened young inflorescences and, therefore inducing unreduced gametes, had at least three important impacts (Figure 9-3):

- An increase in the hybridisation rate of between 160% and 500% for production of interspecific crosses between *L. sinuatum* and *L. perezii* (CHAPTER 5).
- An increase in the proportion of viable pollen between 1.7 times and three times, and up to a 20% increase in embryo production in *L. perezii* genotypes which originally had low-male fertility depending on the genotype used as pollen-donor (CHAPTER 4).
- Induction of up to 85% potentially unreduced pollen depending on the genotype (CHAPTER 5).

- Providing germination potential of previously aborted pollen from interspecific hybrids (APPENDIX N).

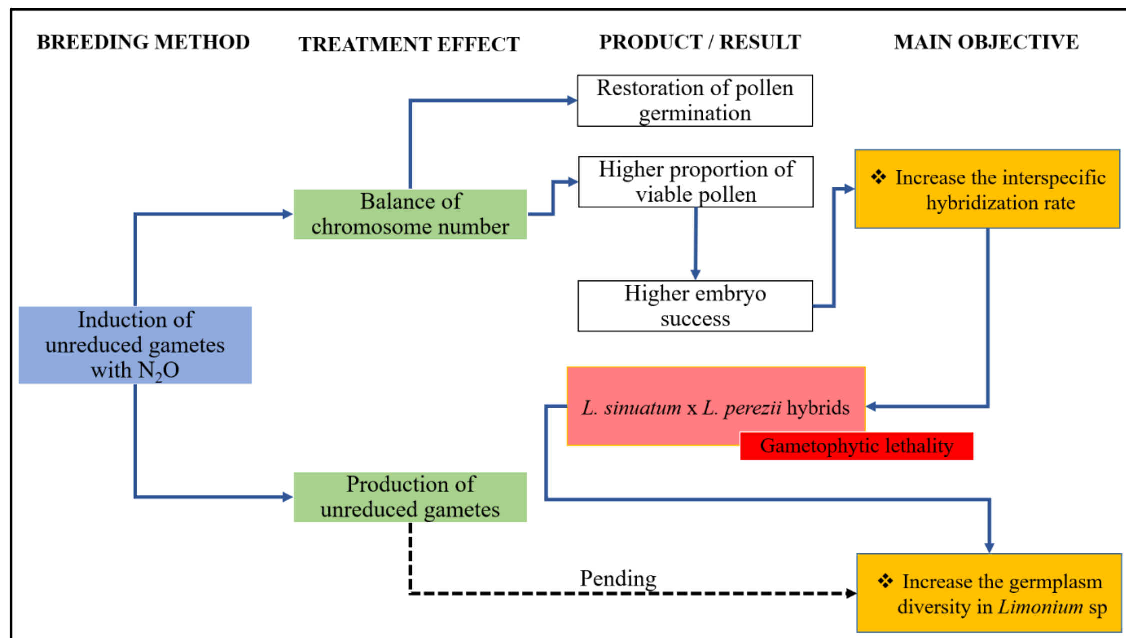

Figure 9-3 Schematic illustration of the effects of the breeding method induction of unreduced gametes with N_2O. The consequent product/result of treating young, unopened inflorescences of *Limonium* with N_2O is shown as well as the primary research goal to which they contributed. The black dashed line represents a pending activity (i.e., production of polyploid hybrids by crossing unreduced gametes), and the red box (gametophytic lethality) is a factor still potentially affecting the zygote success, but which could not be influenced with N_2O treatment

The increase in hybridisation rate observed in this study (160% - 500%; CHAPTER 5) agrees with the reported positive effect of N_2O over interspecific hybridisation and fertility restoration in pollen grains in *Tulipa* sp. (Okazaki et al., 2005) and *Lilium* (Nukui et al., 2011). This improvement in the hybridisation rate increases trait introgression between species of the same genus as in the case of *L. perezii* and *L. sinuatum* and represents a significant increase in germplasm diversity for crop improvement. Such outcomes are desirable in any crop such as potato, *Brassica* or chrysanthemum in which hybridisation is an important method for breeding and germplasm diversification (Chen et al., 2020; Cheng et al., 2011; Jansky, 2006; Katche et al., 2019).

The increase in hybridisation rate observed could in first place be a result of correcting some imbalance of the chromosome number in the pollen mother cells (PMC) of the *L. perezii* genotypes used as the pollen-donor (i.e., per275 and per11). As discussed in CHAPTER 4, the average proportion of viable pollen in treated (with N_2O) plants of per275 and per11 not treated with N_2O was ca. 30%. The proportion of viable pollen increased up to 36% ± 48% for per11 and 84% ± 45% for per275 after treatment with N_2O (refer to Section 4.5). Those increments in pollen viability after the use of N_2O support the hypothesis that the low pollen viability in untreated plants may be due to the occurrence of some form of meiotic aberration resulting in an imbalance in the chromosome composition of the cells, i.e., an odd number of chromosomes. An imbalance in the number of chromosomes in plants negatively affect meiosis, with regular meiosis being the exception in such plants (J. Wang et al., 2017). As consequence of this imbalance, an unbalanced segregation of chromosomes occurs in microspores, and later in pollen grains (refer to Sections 1.3.1.2 and 1.3.2), with some pollen grains containing the expected number of chromosomes and therefore being potentially viable, while other grains are aneuploid and potentially non-functional or aborted (J. Wang et al., 2017). Supporting the proposed hypothesis in the current research was that aborted pollen was observed to be smaller than viable pollen (refer to CHAPTER 4 and Section 7.3.2.1). The smaller pollen size has been associated in other crop species with chromosome losses or aneuploidy in the aborted pollen (Oleszczuk et al., 2019; Tatum et al., 2015), as well as the occurrence of a balanced number of chromosomes in the potentially viable pollen. Future investigations of this nature would confirm the association between pollen viability and the restoration of a balanced chromosome number in genotypes with a low-male fertility, such as per11 and per275.

The restitution of the balanced chromosome number in PMC suggested in *Limonium* after treatment with N_2O (refer to CHAPTER 4) could also be associated with restitution of homologous chromosome pairing (Luo et al., 2016; Nukui et al., 2011) and/or re-establishment of intergenomic recombination during meiosis in the unreduced gametes (Barba-Gonzalez et al., 2005; Luo et al., 2016). Both of these processes would lead to normal meiosis in PMC and subsequent development of viable pollen as observed here with significant increases in the proportion of viable pollen (CHAPTER 4) and as reported for *Lilium* (Luo et al., 2016; Nukui et al., 2011).

The increase in pollen viability in both *L. perezii* genotypes noted above, also resulted in changes in the embryo success for interspecific crosses, increasing 20% when N_2O-treated plants of per11 were used as the pollen-donor (CHAPTER 4 and CHAPTER 5). In contrast with plants of per275 the rate of embryo success changed from 88% ± 10% for plants not treated with N_2O to 66% ± 20% in treated plants. These values confirmed suspected reproductive differences between the two different genotypes of *L. perezii*, one of which phenotypically exhibited white florets (e.g., per11) with the other with purple florets (e.g., per275; refer CHAPTER 7). A possible factor underlying those differences could be the occurrence of gametophytic lethality in *L. perezii* – white per11, which could explain the low proportion of viable pollen even after N_2O treatment, i.e., values below 50%, and lower in comparison with *L. perezii* – purple per 275 (84% ± 45%; CHAPTER 4). Gametophytic lethality is a consequence of the presence of mutations that impede the development of viable pollen grains by affecting the post-meiotic development of microspores (Figure 1-11; Candela et al. (2011)). Studies in *A. thaliana* identified multiple genes associated with gametophytic lethality (Candela et al., 2011; Honys et al., 2006; Park et al., 1998; Zhang et al., 2018), but depending on the mutations present in an organism, their expression pattern, and their penetrance[7], differences between plants, inflorescences and flowers could take place (Candela et al., 2011; Park et al., 1998). As a result, a variable frequency of irregularities occurs with some microsporocytes being normal and leading to fertile pollen (Guerra et al., 2013; Koutroumpa et al., 2018). The results obtained in this study for per11, which showed no more than 50% viable pollen (Figure 4-1B), support the hypothesis that gametophytic lethality can occur in this genotype. The mutation(s) involved have an incomplete penetrance, as observed for *gem1* in *A. thaliana* (Park et al., 1998). Further genetic studies could confirm the proposed hypothesis and identify mutations affecting pollen development. In addition, a detailed macroscopic study of the morphology of the florets of *L. perezii* genotypes would be valuable to identify of the occurrence of male-sterile characteristics (e.g., occurrence of

[7] Penetrance refers to the proportion of individuals in a population with a given gene which display the corresponding/expected phenotypic trait. This means than an individual with a gene not necessarily expressed the associated phenotypic trait Kang, M. S. (2022). Penetrance. *Salem Press Encyclopedia of Health*, 1-2.

small non-dehiscent anthers, low pollen production; Defani-Scoarize et al. (1995)) associated with gametophytic lethality and/or low male fertility.

The occurrence of other pre- and post-zygotic barriers besides pollen viability could be confirmed by hybridisation rates less than 5% and similar for some interspecific crosses (i.e., sin18 × per11 and sin16 × per275; CHAPTER 5). The similarly low hybridisation rate was unexpected after obtaining embryo successes of 22% ± 32% for sin18 × per11 and 66% ± 20% for sin16 × per275, when both treated with N_2O before anthesis (CHAPTER 5). Those barriers could be associated with pollen-pistil compatibility or endosperm-embryo compatibility (Larrosa et al., 2012), with future investigations focussed on pollination, fertilization and embryo development in addition to an earlier determination of ploidy (i.e., when the embryo/ovule rescue is done) needed. Those investigation would offer the opportunity to confirm the proportion of hybrids produce before abortion occurs in case of endosperm-embryo incompatibilities and explore the causes associated with the difference in observed hybridisation rates.

This section focused on the use of *Limonium* as a model to evaluate strategies for increasing the hybridisation rate between species in the same genus, with restoration of the fertility in pollen grains being the main contributing factor to the increase. This finding is potentially applicable to crops important to New Zealand such as kiwifruit and apples with recognized low male fertility or sterility (Asakura & Hoshino, 2018; Dar et al., 2020; Qin et al., 2017; S. Wang et al., 2017) limiting the planned crosses within both these breeding programmes. In such cases consideration should be given to improving or restoring of male fertility using N_2O.

9.4. Restore the fertility of interspecific hybrids

Fertility restoration in *L. sinuatum* × *L. perezii* hybrids was possible following by WGD induced using oryzalin (CHAPTER 6) and by treatment with N_2O of young unopened inflorescences (APPENDIX N).

The mechanism involved in fertility restoration of the interspecific hybrids could be associated with restoring the balance of the number of chromosomes as discussed in Section 9.2.1. The occurrence of an even number of chromosomes in the tetraploid *L. sinuatum* × *L. perezii* hybrids (2n=4x=30) would have allowed chromosome pairing (i.e.,

homologous pairing and/or homeologous pairing), recombination and equal segregation of chromosomes between microspores with subsequent production of viable pollen (Barba-Gonzalez et al., 2005; Luo et al., 2016). However, the occurrence of a balanced number of chromosomes is not the only factor responsible for normal meiosis, and meiotic abnormalities could still be taking place. Such abnormalities would mainly be associated with occurrence of homeologous pairing instead of homologous pairing (refer to Section 9.2).

It has been proposed that one of the natural causes of polyploid formation is the occurrence of unreduced gametes (Kolář et al., 2017), whose probability of natural occurrence varies between and within species (Köhler et al., 2010). In the current research treatment with N_2O before pollination created the conditions needed to produce sexually, fertile polyploids. For example, the restoration of germination of otherwise aborted pollen was achieve confirming the potential of N_2O for fertility restoration (APPENDIX N). In addition, it was demonstrated that the treatment of young, unopened, inflorescences has the potential to produce viable, unreduced pollen (CHAPTER 4 and CHAPTER 5), as evident through:

- the increase in pollen size (plan area) by at least 20% when treated 22 days before anthesis (CHAPTER 5),
- the proportion of viable pollen exceeding that of aborted pollen for all genotypes, other than Y19.001.001 (per11;CHAPTER 4 and CHAPTER 5),
- reduction of the time of treatment from 48h to 24h translated into a higher zygote success (0.2% ± 0.7% vs 7.0% ± 9%; refer to Section 3.3.1), and
- at least 25% of potentially unreduced pollen occurred in florets with anthesis between 23 and 30 DAT for *L. sinuatum* as well as for *L. perezii* (CHAPTER 5).

Despite the above however, sexual polyploids were not produced in the present research, for yet still unconfirmed factors. Proposed hypotheses for the lack of sexual polyploids are discussed below and include:

1. Occurrence of the triploid block,
2. Pollen competition,
3. Incompatibility factors affecting interspecific crosses specifically, or

4. Asynchrony between male and female sporogenesis and/or gametogenesis.

This section centred on the use of *Limonium* for testing strategies for restoring the fertility in interspecific hybrids. The two approaches used: induction of WGD with oryzalin or induction of unreduced gametes with N_2O are applicable to other crops with hybrids, or even genotypes, displaying sterility. Hybrid sterility is frequent in wide hybridisation crosses between different species, as seen in this research, different genera and and/or different ploidies such as in *Brassicas* (Chen et al., 2020) and kiwifruit (Asakura & Hoshino, 2018).

9.4.1. The triploid block in Limonium

The occurrence of the triploid block in *Limonium* has previously been suspected, but not yet confirmed, as impeding the production of polyploid seedlings (Siregar, 2021). If the triploid block mechanism does occur in *Limonium*, as has been reported for other plant species (Akutsu et al., 2007; Morgan et al., 2021), only polyploid crosses between 4x plants and unreduced pollen (bilateral sexual polyploidization) could potentially be successful. In that case, an endosperm balance number (EBN) 4 maternal genome: 2 paternal genome would be equivalent to an EBN 2 maternal genome:1 paternal genome, as observed in some potato species (Ortiz and Ehlenfeldt (1992); refer to Section 1.3.7) and maize (Lin, 1984). Then the resultant seedlings would be tetraploids. In this research, after induction of unreduced gametes followed by intraspecific crosses, only one tetraploid seedling was obtained (CHAPTER 6, APPENDIX P), supporting either the hypothesis of the triploid block, and that a maternal to paternal endosperm ratio of 4 to 2 displays a low viability in *Limonium* compared to potato and maize, or pollen competition with unreduced pollen being less fit than reduced pollen (discussed below in Section 9.4.2).

In *Limonium*, if the triploid block occurs, any resulting triploid zygotes would mostly be unviable. The viability of the zygotes depends on the interploidy crossing direction (Carputo et al., 1999; Morgan et al., 2021). In the present research, ovule/embryo rescue was used to produce triploid seedlings after heteroploid backcrosses (CHAPTER 6). Contrary to what would be expected if the triploid block occurs (Morgan et al., 2021), the phenotype of the triploid seedlings did not display any visual signs of low fitness, such as necrosis, wilting, or yellowing (i.e., hybrid weakness). Triploid hybrid plants may not

exhibit hybrid weakness due to transgressive variation, as observed in *A. thaliana* (Duszynska et al., 2013). However, additional research is required to include a morphological characterisation of triploid hybrid plants and assess their fertility, such as zygote success and pollen production, in both F1 and F2 seedlings. It is important to consider that the triploid block effect may be long-lasting (Morgan et al., 2021) and may also be influenced by the parent-of-origin (Duszynska et al., 2013).

The results of the current research programme support the hypothesis that if the triploid block does occur in *Limonium*, it is just partial or its effect over embryos produced by interploidy crosses depends on the parent-of-origin, i.e., with an EBN with maternal to paternal genome ratio with maternal excess (i.e., greater than 1) being better tolerated than an EBN with maternal to paternal genome ratio with paternal excess (i.e., lower than 1), as reported for *A. arenosa* (Morgan et al., 2021) and in maize (Lin, 1984). The observations of the occurrence of failures of the endosperm development (i.e., precocious or delayed cellularization (Lafon-Placette & Köhler, 2016)), and arrest of the embryo development (APPENDIX H), also contribute to the hypothesis of the occurrence of the triploid block in *Limonium*, with a parent-of-origin effect. Future research undertaking detailed studies of embryo and endosperm development with *Limonium* may, therefore, enable confirmation of how relevant consideration of the triploid block may be with *Limonium*.

Currently the information presented in this thesis is not enough to confirm or deny the occurrence of the triploid block in *Limonium.* In addition, occurrence of the triploid block by itself is insufficient to explain the significant increase (i.e., between 200% and 800%) in the number of diploid seedlings, and a significant decrease (between 44% and 100%) in the number of tetraploid seedlings (APPENDIX P), observed after induction of unreduced gametes with N_2O. If not the triploid block being involved, other areas of future research someone may wish to explore may include pollen competition and incompatibility systems which will discussed below (refer to Section 9.4.2 and Section 9.4.2).

9.4.2. Pollen competition

Pollen competition could explain the low number of polyploids produced and the higher number of diploids obtained in intraspecific crosses (APPENDIX P). In *Limonium,* treatment with N_2O before pollination results in a mixture or an overlap in the presence

of reduced and unreduced pollen (CHAPTER 5), as also reported for *Lilium* (Akutsu et al., 2007), plus aborted pollen (CHAPTER 4 and CHAPTER 5). Pollen plan area and pollen tube growth are directly associated with ploidy. Tetraploid pollen is larger and achieves faster tube growth in *Rosa hybrida* (Gao et al., 2019) and *Chamerion angustifolium* (Husband et al., 2002). In addition, tetraploid pollen offers a potential advantage over diploid in some species where the success as a father is higher for tetraploid than for diploid pollen parents (Gao et al., 2019; Husband et al., 2002). Relating this to the present research, two scenarios could be possible:

1. if the same advantage occurs for unreduced pollen in *Limonium*, it would lead to a higher proportion of aborted embryos than expected under a EBN scenario; however, it would hardly explain the numerical advantage for diploid embryos unless other unknown reproductive barriers are taking place
2. Contrary to the findings in both *Chamerion angustifolium* (Husband et al., 2002) and *Rosa hybrid* (Gao et al., 2019), in *Limonium* unreduced pollen could be less fit than reduced pollen and therefore disadvantageous compared to reduced pollen.

Future investigations focussed on the total amount of pollen produce per anther and detailed analysis of pollination and fertilization using pollen mixtures could give insights into possible competitive advantages and/or interferences associated with pollen ploidy.

9.4.3. Incompatibility system

Genetic interactions between divergent genomes in hybrid plants could result in incompatibilities which cause lethality, low viability, weakness, or sterility in hybrids (Bomblies & Weigel, 2007; Chen et al., 2016). In the specific case of interspecific crosses could be the result of an "increase" in the level of genetic incompatibility. Incompatibility in interspecific crosses present "different levels", depending on the genetic distance between the genotypes and the genera under study (Moyle et al., 2004). It has been reported that incompatibility is not associated with the whole parental genome, but with specific genes or alleles (Chen et al., 2016). If specific genes or alleles are responsible for incompatibility in *Limonium*, then the genome duplication induced using N_2O could result in more copies of those genes/alleles, leading to an increase in interspecific incompatibility (refer to CHAPTER 5). Hence future research focussed on

identification of the genes/alleles responsible for incompatibility in *Limonium* and their *modus operandi* may provide further insights as to whether this is a source of the failure to produce polyploid seedlings reported in this thesis.

9.4.4. Bilateral sexual polyploidization

The last proposed hypothesis for the absence of sexually produced polyploids through bilateral sexual polyploidization i.e., crosses involving an unreduced ovule and unreduced pollen, are asynchronous development between pollen and ovule. Sexual bilateral polyploidization, originally targeted for production of tetraploid seedlings, could have failed due to pollen and ovule development (i.e., microsporogenesis and megasporogenesis respectively) being separated by an amount of time longer than 24 h (i.e., shortest duration of N_2O treatment applied in the current research). If these two developmental processes (i.e., microsporogenesis and megasporogenesis) are asynchronous in *Limonium* to this extent, then when the N_2O treatment was applied, it would potentially have affected just one of these processes and, in the case of interploidy crosses (i.e., either tetraploid × diploid or vice versa) seedling development could be prevented by the triploid block (refer to Section 1.3.7). The absence of studies in the current research to evaluate the temporal relation between microsporogenesis and megasporogenesis or to establish the relation between megasporogenesis and floral development in the species used (i.e., *L. perezii* and *L. sinuatum*) became essential for the success of bilateral sexual polyploidization. Moreover those studies would complement the studies done for the microsporogenesis in *Limonium* (Siregar, 2021). Studies done in other plant species indicate that the temporal relationship between microsporogenesis and megasporogenesis differ between plant species and even between flower types in the same plant (Wang et al., 2022). Megasporogenesis could occur synchronously with microsporogenesis (Shi et al., 2015; Wang et al., 2022), be delayed (Koul et al., 2020) by even up to a couple of days (~ 5; Chang and Sun (2020)), or even occur at different development stages in different flower buds (Yao et al., 2020). The information available in *Limonium* sp. was that pollen and ovule meiosis occurs in young and as yet unopened florets (refer to Section 1.3.1.2), with meiosis in PMC occurring mainly in florets with an average diameter of 0.8 mm (Siregar, 2021) and in MMC in florets with a length under 2.5 mm (Rois, 2014). As both these previous studies were conducted using different *Limonium* species, it is not possible to infer the extent to which microsporogenesis and

megasporogenesis are synchronous in the *Limonium* species used in the present study. This highlights a remaining need for detailed studies to be undertaken at this level of investigation.

9.5. Physically induced mutations

The breeding strategy involving physically induced mutations, was not able to be completed. However, some progress was made toward the development of protocols for *in vitro* production of callus, shoots, and roots (refer to CHAPTER 8). The investigations into root induction proved useful in the current research for inducing roots on the polyploid plants obtained after ploidy manipulation (oryzalin and/or N_2O). The information provided will also be useful for those investigating callus and shoot induction and as a documented background for any continued development of this research strategy of physical induction of mutations in *Limonium* sp.

The development of callus *in vitro* after induction of unreduced gametes using N_2O after 48 h (APPENDIX I) or 24 h treatment (CHAPTER 5) when the rescued embryo was in the heart stage, presents an opportunity to evaluate other tissue culture techniques, such as achieve shoot development from callus. As noted in the current research, the growth cessation in embryos arising from crossing *L. sinuatum* and *L. perezii* has previously been documented, with successful shoot induction using TDZ (Morgan et al., 1998). The induction of shoots from callus arising from those embryos, could open the opportunity to obtain more interspecific hybrids and, if the callus was first irradiated, open the opportunity for even more mutagenesis-induced diversity. The interspecific hybrids produced could be diploid, triploid, or tetraploid, depending on the effect of the earlier applied N_2O treatment (refer to Section 1.3.7).

The usefulness of the advances made in the induction of shoot from calluses in the further development of the breeding strategy of ploidy manipulation rely on the fact that tissue culture is genotype and organ specific. If TDZ is unsuccessful, as reported within the current research (refer to Section 8.4.1.2), an amount-concentration experiment could be set up using BAP and NAA, similar to experiment 5 presented in Section 8.3.3.1.2, the one with more promising results (refer to Section 8.4.1.2).

In this section emphasis was given to utilising *Limonium* as a crop to evaluate the induction of mutation for increasing the diversity of germplasm. The alternatives given here to use the knowledge obtained on callus, shoot and root induction are potentially applicable to the breeding programme of any of the crops with: limited access to genetic diversity mentioned in Section 9.2 (Dyer et al., 2014; Katche et al., 2019)); and/or presenting hybrid inviability or sterility as documented in wide hybridisation crosses such as kiwifruit (Asakura & Hoshino, 2018) or *Camellia oleifera* (Li et al., 2021) to provide a very few examples.

9.6. Summarised future research steps and questions

- Study in detail the development of embryos produced after inter- or intra-specific crosses with prior N_2O treatment for induction of unreduced gametes. This would be designed to determine with higher precision when the embryo development ceases, what changes does the endosperm suffer (i.e., nuclear endosperm failure or cellular endosperm failure), and if a parental dosage imbalance could be identified (Lafon-Placette & Köhler, 2016; Morgan et al., 2021). This information could lead to the identification of the effect of N_2O treatment over gametes and confirm or refute the hypothesis of a triploid block occurring (refer to Section 9.4.1), and potentially remove the requirement for ovule/embryo culture to recover seedlings.
- Evaluate different times between the planned pollination and the embryo/ovule rescue to identify if it is possible to rescue embryos earlier than 15 days after pollination and avoid embryo arrest caused by the imbalance of EBN or other post-zygotic barriers. The development of a protocol for earlier ovule/embryo rescue could facilitate the production of sexual polyploids.
- Use normally infertile hybrids with desired traits, whose young florets have been treated with N_2O in planned intra- and inter-specific crosses, so as to confirm if the pollen germination observed after treatment with N_2O (i.e., treatment of unopened florets with calyces protruding less than 2 mm above the bract and floret diameters ca. 0.8 mm (refer APPENDIX N)) translates into interspecific seedlings being produced without the requirement for WGD to restore fertility.

- Evaluate if the asymmetrical effect of ploidy increase, observed in *Limonium* with differences between organs and species (refer to Section 7.3.1), is associated with differences in gene expression and regulation in *Limonium*, as reported in *Brassica* (Harun et al., 2021).
- Detailed cytological studies of:
    - Comparative study of meiosis in neo-autopolyploid plants and diploid plants, to understand the effect of induction of ploidy increase over the development of gametes and potentially over reproductive performance of the neo-polyploids.
    - meiosis in pollen mother cells after treatment with N_2O, to understand the mechanism responsible for:
        - the restoration of the pollen viability and germination in what would normally be aborted pollen in hybrid plants (APPENDIX N), as well as
        - the increase in the proportion of viable pollen from genotypes with comparatively low male fertility (CHAPTER 4).
- Evaluation of the performance of unreduced pollen versus reduced pollen in planned crosses involving a mixture of both to:
    - evaluate the performance of pollen collected from plants with different ploidies regarding the number, germination rate and, pollen competitiveness (Gao et al., 2019; Husband et al., 2002).
    - determine if a competitive advantage of reduced pollen over unreduced pollen could explain the failure to produce polyploid, interspecific hybrids, after induction of unreduced gametes (CHAPTER 5).
    - Better determine any difference in efficacy of pollen grains with different numbers of germination pores arising from potentially unreduced pollen following N_2O treatment of immature florets (APPENDIX N).

Answering these questions will allow the prediction of what to expect from a pollen mixture containing reduced and unreduced pollen, i.e., after the induction of unreduced gametes. Once the comparative efficacy was known, then grading and separation of pollen types could then allow for a higher siring success for unreduced pollen potentially resulting in bilateral sexual polyploids.

- Evaluation of either the temporal relationship between microsporogenesis and megasporogenesis in *Limonium* or the relation between megasporogenesis and floral development in *Limonium* as a complement to the studies done in the microsporogenesis in *Limonium* (Siregar, 2021). The aim is to confirm if temporal differences (i.e., asynchrony) between both events could be responsible of the absence of bilateral sexual polyploids produced in this work. If so the information collected will allow the adjustment of the treatment with N_2O according to the most appropriate floret developmental stage to disrupt the meiotic spindles in both PMC and MMC.
- In future experiments pollen count (refer to Section 7.2.2.1) should accompany an evaluation of pollen plan area and/or viability, through either pollen germination or tetrazolium testing, as an indirect way to determine if abnormal meiosis is occurring resulting in tetrads with unreduced pollen, reduced pollen, micro-pollen, or a combination between any of them (Mason et al., 2011).
- In the current research, the induction of WGD has opened the possibility to produce *L. sinuatum, L. perezii* and interspecific hybrids plants with stronger stems (refer to Section 7.3.1). However, it is unclear whether increased ploidy affects the colour of *Limonium*'s florets, which is an important trait for the ornamental industry. It is necessary to conduct further investigation to determine whether an increase in ploidy leads to stronger colours, as reported in other ornamental plants such as *Gladiolus grandifloras* (Manzoor et al., 2018; Zeng et al., 2020). Additionally, it should be investigated whether ploidy increase combined with hybridisation results in new colours, as reported for *Nicotiana* (McCarthy et al., 2015) and *Actinia chinensis* (McCarthy et al., 2015; Wu et al., 2013).
- In this PhD programme of research, a first and second generation of tetraploids and hybrids were obtained. Nevertheless, different studies suggest that genome stability and the occurrence of chromosome recombination depend on the evolution of the genotypes (Bomblies, 2022; Róis et al., 2012). In breeding terms, this would require a number of generations to achieve. The newly obtained polyploids therefore, likely need to be included in new crosses and/or be propagated vegetatively with the objective of reaching genome stability and

occurrence of normal meiosis and homologous chromosome recombination (Bomblies, 2022; Róis et al., 2012).

- The first-generation backcrosses obtained in this research, and corresponding to interspecific hybrids, could be used as a "triploid bridge" for crossing with either tetraploid *L. perezii* or *L. sinuatum*, so as to finally obtain a hybrid neo-polyploid (Hojsgaard, 2018; Mason et al., 2011). One option could be to allow open pollination between diploids and tetraploids, with triploids acting as a "triploid bridge", as a way of fixing the tetraploids in the population as proposed by Husband (2004), and to improve the fertility of the neo-polyploid by genome stabilization (Bomblies, 2022).
- To enable the continued creation of germplasm diversity, the use of N_2O is highly recommended. The decision about whether it should be used for induction of WGD or unreduced gametes, may be based on the aim of the targeted breeding endpoint and genotypes available. However, the option of combining both induction of unreduced gametes for increasing the hybridisation rate, by restoring fertility, or increasing pollen viability followed by WGD induction, after the resulting restoration of crossing potential fertility in the potential hybrids, remains open for debate.

The detailed recommended strategy for production of polyploid hybrids of the siNZii™ *Limonium* programme is:

1. Induction of unreduced pollen using N_2O for 24h
2. Perform planned crosses between 21 and 30 DAT. It is recommended the verification of the effect of N_2O over pollen can be readily achieved by comparison of the average pollen size for the species with that of pollen collected from treated plants. This verification could be used to reduce the number of days for performing the crosses and, therefore, conserve resources.
3. A second treatment with N_2O for 24 h, starting at 8 hours and up to 72 hours after manual crossing.
4. Ovule/embryo rescue two weeks after crossing, or earlier if this proves possible.

An alternative option for production of fertile polyploid hybrids, if double treatment with N_2O does not work, is using oryzalin. In that case, the next steps after ovule/embryo rescue would be:

1. After zygote germination, induction of WGD using oryzalin for two months with a monthly media replacement.
2. Individualization of the shoots arising.
3. Screening and selection of polyploid shoots.

This section summarized the questions which emerged in the current research and whose answers will contribute to the improved opportunities for breeding in *Limonium.* Nevertheless, neither the concepts develop here for ploidy manipulation and their effect over diversity, hybridisation, genome stabilization, meiosis, and plant fertility nor the proposed questions and future research are exclusive to *Limonium.* Potentially both the proposed concepts and questions are applicable to enhance breeding of any crop with identified barriers (e.g., genetic erosion, low male fertility, failure of bisexual polyploidization) limiting their breeding.

9.7. Protocol improvement

In addition to the impact of the current research itself, during its development it was possible to contribute to the improvement of existing protocols as listed:

- With the support of a summer student, Alice Boyd, it was possible to improve the protocol used for pollen germination (Boyd et al., 2022).
- Prior to this research there was not a method to measure pollen size in a rapid and effective way (APPENDIX A), so as to identify the point in developmental time for performing planned crosses for:
    - best pollen viability (CHAPTER 4),
    - higher zygote success (CHAPTER 4), or
    - germination restoration (APPENDIX N).

The rapid and simple protocol to analyse pollen size saves resources, in terms of time and money, as more crosses could be performed on the critical dates, i.e., with the occurrence of potentially unreduced pollen instead of doing less crosses daily for more days. In addition, having information about the various genotypes of the

two species used in PFR's breeding programme allows the reduction in size of pollen sample required for accurate size determination (APPENDIX N).

- Some of the interspecific *L. sinuatum* × *L. perezii* hybrid genotypes obtained in this research are already at the third generation level (APPENDIX O), meaning they could potentially be included in further crosses to produced new cultivars for the siNZii™ series.

APPENDICES

APPENDIX A. Macro for pollen analysis

For the physical measurement of pollen grains, in terms of the: plan area, perimeter, minor and major axis, a macro was developed and run as a batch process. The objective of developing this macro was to speed up the processing of images derived from multiple samples of pollen.

The macro developed utilised an open-sourced image processing software Fiji (Schindelin et al., 2012). The macro segmented the image and identified the objects (i.e., pollen grains) within each segment. Once implemented, the macro allowed the scale to be initially set, according to the microscope magnification used, with the following detailed steps involved in image analysis:

1. Image conversion to an 8-bit type.
2. Auto Threshold setting the method as "default" to convert the image into a binary black and white image.
3. The white and black colours were inverted, and the visible particles identified analysed. For the particle analysis to capture those associated with pollen grains, the size range selected was between 180-5000 μm^2, and a circularity of between 0.5 and 1 was used.
4. Finally, a .tiff file was obtained from the processed image, and a .csv file containing the information about photo name, area, and perimeter of each one of the analysed particles identified as pollen grains. The quality control of the processed images was done manually by visually comparing the .tiff file with the original photo.

APPENDIX B. Macro for leaf area

As described in CHAPTER 2 and CHAPTER 7, characterisation of morphological features of *Limonium* plants included comparative measurement of leaf area. To facilitate speeding the process of collecting this data, a macro was developed.

The macro was developed in the open-sourced image processing software Fiji (Schindelin et al., 2012) and segmented the image and identified the objects (i.e., leaf) within each segment. These are the detailed steps performed automatically by the macro:

1. The conversion of the photo including a leaf to an 8-bit image type.
2. Auto Threshold setting with the method set as “Mean ignoring the white” to convert the image into a binary black and white image.
3. The white and black colours were inverted and the particles analysed.
4. For particle analysis, the size range selected was between 20 μm^2 and infinity.
5. Finally, a .tiff file was obtained from the processed image and a .csv file containing the information about photo name, area, and perimeter of each analysed leaf.

APPENDIX C. Supplementary information from Section 2.5.1

Table A-1 Number of crosses completed, embryos/ovules rescued, and seedlings resulting from genotypes of *L. sinuatum* or *L. perezii* treated with nitrous oxide (N_2O) or untreated (control). Zygote success (i.e. number of zygotes obtained divided by the number of crosses completed) and the cross success (i.e. number of seedlings obtained divided by number of crosses completed) was also calculated

Genotype	Treatment	Crosses completed	Embryo/ovules rescued	Zygote success (%)	Seedlings after tissue culture	Cross success (%)
sin16	N_2O	70	49	70	49	70
sin18	N_2O	70	53	75	45	64
sin16	control	5	4	80	4	80
sin18	control	5	3	60	3	60
per273	N_2O	70	27	38	11	16
per11	N_2O	70	7	10	2	2.8
per11	control	5	1	20	1	20
per273	control	5	1	20	1	20

APPENDIX D. Selection of parental genotypes

INTRODUCTION

Identification of the most suitable parents for crossing among the available genotypes is one of the key decisions plant breeders need to make, so as to ensure the success of a breeding programme. The selection of the parental genotypes depends on the aim of the breeding programme, i.e., genetic distance (Georgieva et al., 2016; Harlan & De Wet, 1971), the genetic gene pool to which the genotypes belong, and their combinatory ability if available (Fasahat et al., 2016). However, independent of the criteria used for the parental selection, the goal is that genotypes cross well with each other, producing viable seedlings (Kuligowska et al., 2016).

In this programme of research, the adequate selection of the parents was found necessary for consideration after the results presented in Section APPENDIX F and APPENDIX I, where production of hybrids and zygotes, respectively, were negatively affected by the parental genotypes used. As consequence, the evaluation of the effect of nitrous oxide (N_2O) over polyploid induction, zygote success (number of zygotes obtained divided by the number of crosses completed) or hybridisation rate (number of hybrids obtained by number of crosses completed) was initially jeopardized. To subsequently limit the factors affecting the response variables selected for the evaluation of the effectiveness of N_2O over ploidy, an experiment for conducting preselection of the genotypes to be used as parents was done.

The selection of parental genotypes for crosses was done taking various aspects into account:

First, *Limonium* is a plant genus exhibiting pollen-stigma dimorphism and self-incompatibility (Baker, 1953a), so the first criterion to be used when planning crosses was the pollen/stigma type.

Second, as interspecific crosses were intended in some of the experiments, the identification of the gene pool for a given species was considered so as to detect levels of infertility between taxa, given that the closer species are the easier their crossing (Harlan & De Wet, 1971; Kuligowska et al., 2016).

Finally, the performance of previously used genotypes in the production of hybrids and/or commercialized *Limonium* varieties was contemplated.

MATERIALS AND METHODS

Plant material

In the year 2020, information about the genotypes available in Plant and Food Research, which could be potentially used in crosses for the PhD, was collected and is presented in Table A-2.

Table A-2 Information about the taxonomic classification, number of genotypes available at The New Zealand Institute for Plant & Food Research Ltd, and reference for the *Limonium* species available at the beginning of the research project

Genus	Subgenus	Clade	Sect.	Subsect.	Species	Number of available genotypes	Reference
Limonium	Pteroclados	Pteroclados	Pteroclados	Odontolepidae	*sinuatum*	28	Karis (2004)
Limonium	Pteroclados	Pteroclados	Pteroclados	Nobiles	*perezii*	10	Karis (2004)
Limonium	Pteroclados	Pteroclados	Pteroclados	unknown	*sinuatum* × *perezii* / *perezii* × *sinuatum*	42	E-Brida within The New Zealand Institute for Plant & Food Research Ltd
Limonium			Limonium	Genuinae	*latifolium*	1	Koutroumpa et al. (2018)
Limonium	Limonium	reniforme	Limonium		*caspium* (caspia)	5	Akhani et al. (2013); Lledó et al. (2005)
Limonium	Limonium	Limonium	Limonium	Genuinae	*gmelinii*	8	Akhani et al. (2013); Koutroumpa et al. (2018); Malekmohammadi et al. (2017)
Goniolimon			Arthrolinum		*tartaricum* (*Goniolimon tartaricum*)	9	https://www.owairakaseeds.co.nz/product/*Limonium*-tartaricum-*Limonium*-dumosum-german-statice/ (date of retrieval 06/03/2024), https://cpvo.europa.eu/sites/default/files/documents/limonium_1.pdf (date of retrieval 06/03/2024)

The selection criteria used for the parents were:

- First, the taxonomic proximity, assuming the taxonomically closer species are easier to cross, though we are aware that exceptions do occur (Harlan & De Wet, 1971; Kuligowska et al., 2016).
- Second, any evidence of successful previous results for producing hybrids with outstanding characteristics (Table A-3).
- Third, the number of genotypes (plants) available. Therefore, twenty-eight *L. sinuatum* and ten *L. perezii* genotypes were selected for parental evaluation.

Table A-3 Information about seed-bearing genotype, pollen-donor genotype, and reference material for *Limonium* hybrids previously produced, and available for inclusion in this PhD research

Seed-bearing species	Pollen-donor species	Crossing Result	Reference
perigrinum	*purpuratum*	15 hybrids	(Morgan et al., 1995)
latifolium	*caspium*	Hybrids	(Burchi et al., 2006)
latifolium	*caspium*	Ocean blue	(Sato et al., 1993)
perezii	*sinuatum*	siNZii™ series	(Morgan et al., 1998)
sinuatum	*perezii*	42 hybrids	E-Brida within The New Zealand Institute for Plant & Food Research Ltd

The crosses were subsequently planned based on contrasting stigmas and synchronous flowering times. Emasculation was not required as *L. sinuatum* and *L. perezii* are self-incompatible (Baker, 1953a). All genotypes were used as seed-bearing as well as pollen-donor. Some genotypes participated in more than one cross, depending on the number of florets available. Intraspecific crosses involving *L. sinuatum* were performed in February, June-July, or August-September 2020, depending on when the plant started flowering. In *L. perezii,* the intraspecific crosses were done between July and September 2020. The genotypes selected as “good seed-bearing genotype” and/or “good pollen-donor genotype” (i.e., highest values for zygote success) were clonally propagated for future experiments according to Morgan et al. (1998).

Experimental design

At least 10 crosses were performed daily (sub-samples) for three days in a row (reps). The factors evaluated were: either genotype used as seed-bearing and genotype used as pollen-donor, or cross. The response variable was zygote success (number of zygotes obtained divided by the number of crosses completed) evaluated 30 days after pollination.

Data analysis

Data analysis was conducted using the software R (R Core Team, 2018) with ggplot 2 (Wickham 2016) utilised for graphic outputs. A generalized linear model (glm function from the stats package (R Core Team 2018)) with a binary distribution used for analysis of the response variable. Pairwise likelihood ratio tests were used to compare between the levels of the factor, i.e., seed-bearing or pollen-donor genotype (similar to LRPAIR in (Goedhart, 2018)).

For the selection of the best genotypes to be used as mother/father, the data was analysed independently for February, June-July, and August-September.

RESULTS

Limonium sinuatum

A general analysis of the response variable zygote success revealed that even with the plants grown under heated greenhouse conditions, the flowering was affected by the season. As a consequence, both floret production (not recorded) and zygote success decreased 36% during winter (June-July) in comparison with summer (February) and spring (September; $p<0.05$).

Zygote success depended on the genotype used as the seed-bearing, as well as the pollen-donor, and ranged between 0% for 5 out of the 28 evaluated genotypes, and 93% (Figure A-1 and Figure A-2). The genotypes with seed set statistically higher than 70% (i.e., Y19.018.001, Y19.016.001, Y19.014.001, Y19.012.001, Y19.008.001 and *L. sinuatum 46* (2018); $p<0.05$; Figure A-1) were selected as “good seed-bearing genotypes”.

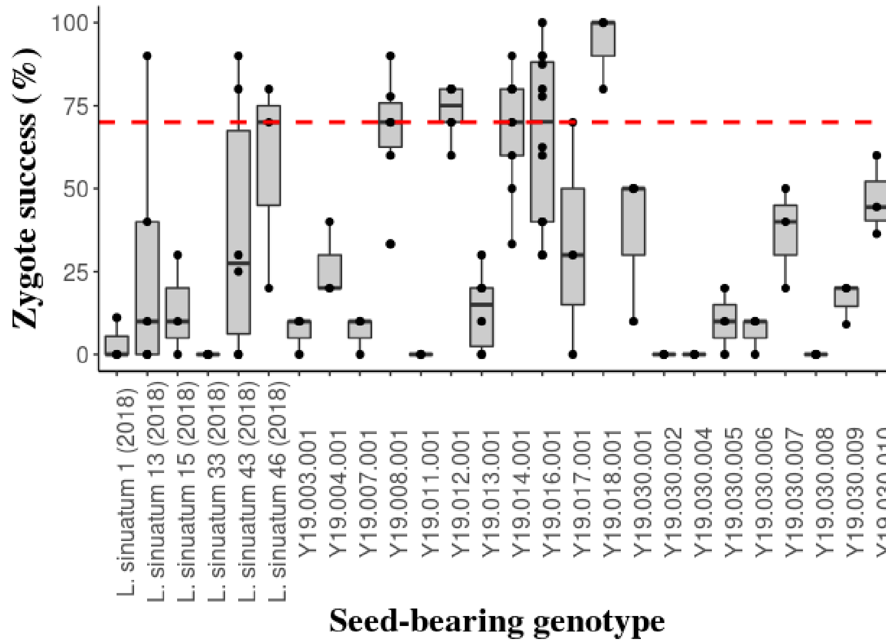

Figure A-1 *L. sinuatum* genotypes evaluated as seed-bearing parent. The box represents the interquartile range, the horizontal line is the median, and the whiskers indicate the minimum and maximum values. The single point is an extreme datum. n>10 planned crosses. The dashed red line delineates the minimum zygote success percentage set to be considered as a "good seed-bearing genotype"

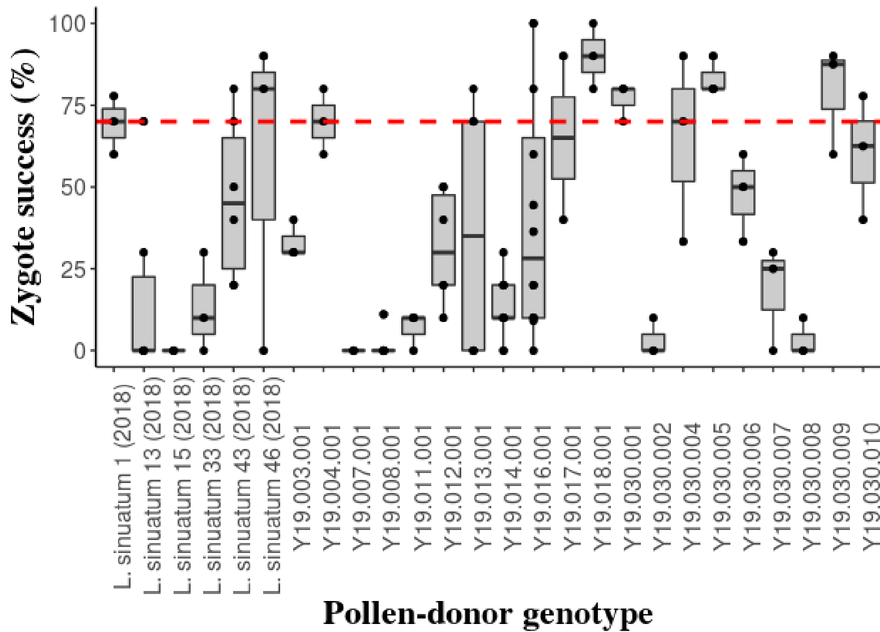

Figure A-2 *L. sinuatum* genotypes evaluated as pollen-donor genotypes. The box represents the interquartile range, the horizontal line is the median, and the whiskers indicate the minimum and maximum values. The single point is an extreme datum. n>10 planned crosses. The dashed red line delineates the minimum zygote success percentage set to be considered as a "good pollen-donor genotype"

For the genotypes used as pollen-donors, the seed set values were between 0% and 90%. Eight genotypes presented mean zygote success values above 70% and were considered as “good pollen-donors genotypes” (i.e., *L. sinuatum* 1 (2018), *L. sinuatum* 46 (2018), Y19.004.001, Y19.018.001, Y19.030.001, Y19.030.004, Y19.030.005 and Y19.030.009; $p<0.05$; Figure A-2).

In brief, a total of 12 *L. sinuatum* genotypes were selected from the 28 genotypes evaluated, as “good seed-bearing genotype”, “good pollen-donor genotype” or both (i.e., *L. sinuatum* 46 (2018) and Y19.018.001). Six genotypes presented their stigma as being cob, and six as papillate.

Limonium perezii

Intraspecific crosses utilising *L. perezii* genotypes which were used as seed-bearing, with a zygote success that were statistically higher than others, presented values between 56% and a maximum of 72% ($p<0.05$; Figure A-4). Due to the limited number of genotypes available, and few achieving over 70% zygote success used for genotypes of *L. sinuatum*, the genotypes selected as a “good seed-bearing genotype” were: Y19.027.002, Y19.027.001, and Y19.027.006. These genotypes achieved ≥ 50% zygote success.

The pollen-donor genotypes with zygote success values between 66% and 72% were selected as “good pollen-donor genotype”, and corresponded to: Y19.027.001, Y19.027.002 and Y19.027.005 ($p<0.05$; Figure A-4).

For *L. perezii*, genotypes Y19.027.001 and Y19.027.002 were good as seed-bearing as well as pollen-donor, and four out of the ten evaluated genotypes were selected for further experiments. Two genotypes presented a cob stigma and two papillate.

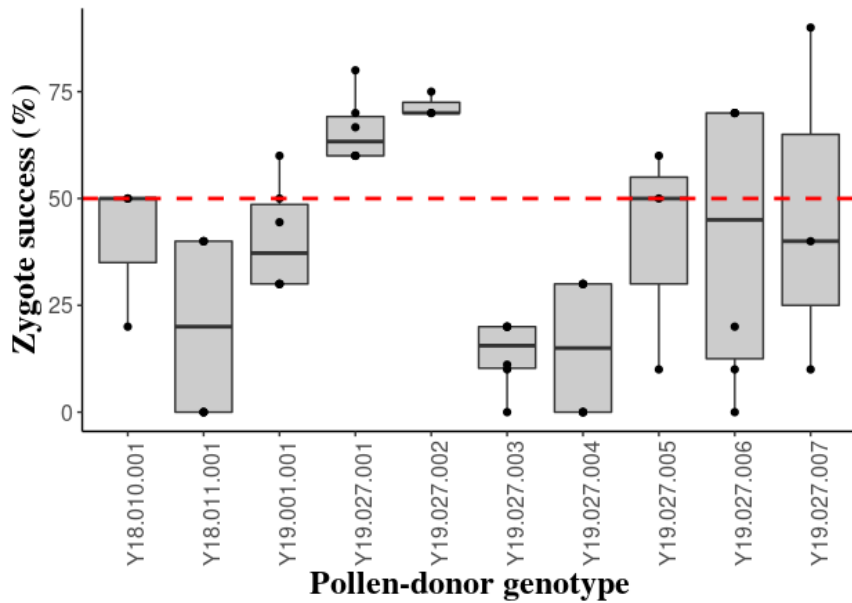

Figure A-4 *L. perezii* genotypes evaluated as pollen-donor between July-September 2019. The box represents the interquartile range, the horizontal line is the median, and the whiskers indicate the minimum and maximum values. The single point is an extreme datum. n>10 planned crosses. The dashed red line delineates the minimum zygote success percentage set to be considered as a "good pollen-donor genotype". Genotypes Y18.010.001 and Y19.001.001 had white florets, while all others had purple florets

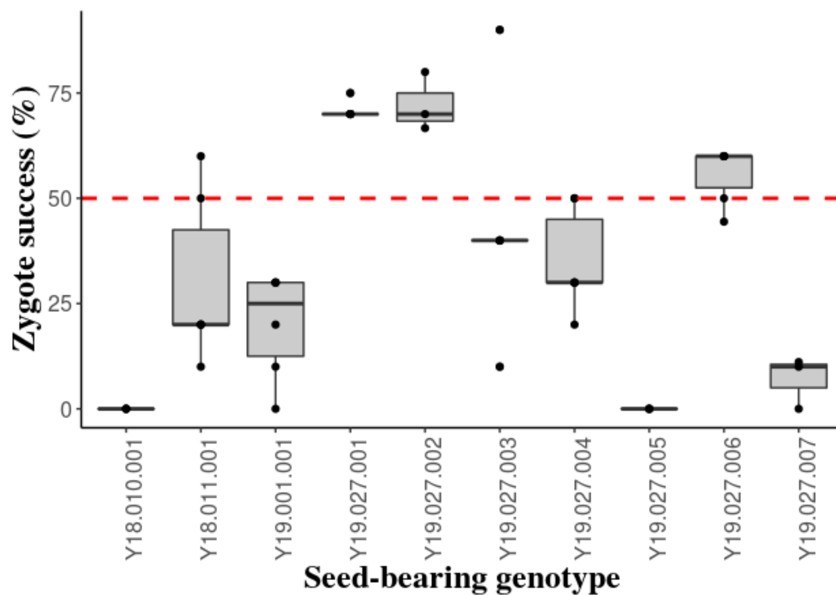

Figure A-4 *L. perezii* genotypes evaluated as seed-bearing between July and September 2019. The box represents the interquartile range, the horizontal line is the median, and the whiskers indicate the minimum and maximum values. The single point is an extreme datum. n>10 planned crosses. The dashed red line delineates the minimum zygote success percentage set to be considered as a "good pollen-donor genotype". Genotypes Y18.010.001 and Y19.001.001 had white florets, while all others had purple florets

CONCLUSIONS

The genotypes for use in subsequent experiments of the PhD project were selected based on: their close taxonomic classification, being both in the Section Pteroclados; had been successfully used in the production of interspecific hybrids; their seed formation in intraspecific crosses was higher than 70% in the case of *L. sinuatum* and 50% for *L. perezii*, and they included multiple examples of both stigma types.

APPENDIX E. Greenhouse growing medium composition

The growing medium used in the greenhouse consisted of: A-grade bark C.A.N. fines (50%) (Daltons, New Zealand), bark fibre (15%; Daltons, New Zealand), 7 mm-pumice (20%) (Daltons, New Zealand), supplemented with fertiliser comprising: 5 kg/m^3 8-9 month Osmocote Exact (Standard 8-9M; ICL Specialty fertilizers, Australia/New Zealand), 1.6 kg/m^3 dolomite (Daltons, New Zealand), 500 g/m^3 Superphosphate (0-9 P-0-12 S, Daltons, New Zealand), 500 g/m^3 Potassium sulphate (Daltons, New Zealand), 333 g/m^3 Calcium Ammonium Nitrate (CALCINIT, YaraTera, New Zealand), and 666 g/m^3 Gypsum (Daltons, New Zealand).

APPENDIX F. Whole genome duplication induction using nitrous oxide for 48 hours

As introduced within Chapter 1, studies in different crops (e.g., *Triticum aestivum* (Hansen et al., 1988); *Phalaris canariensis* (Ostergren, 1957); *Secale cereale* (Gordej et al., 2019)) reported for induction of WGD with N_2O: pressures between 608 Kpa and 1013 Kpa, treatment duration from 4 h to 72 h and zygote/embryo developmental stages between 5 to 13 days after pollination (DAP). After analysis of the results obtained in the above-mentioned studies, in addition to the information on the embryo development of *Limonium* (Zhang, 1995) and *Arabidopsis thaliana* (Faure et al., 2002; Gooh et al., 2015); and the unpublished data of experiments conducted at PFR using N_2O, a preliminary experiment was undertaken to induce WGD in *Limonium* by treating with N_2O for 48 h whole plants containing zygotes/embryos.

MATERIAL AND METHODS

Plant material

Two *L. sinuatum* ($2n$ = 16) genotypes (Y19.015.001, Y19.016.001(sin16)), selected for their contrasting stigma and synchronous flowering times, were used. Individual *L. sinuatum* florets were manually pollinated each day during spring, between 26th August and 9th September 2019, without prior floret emasculation as *L. sinuatum* is self-incompatible (Baker, 1953a).

Plants were grown under greenhouse conditions in PFR, Palmerston North, New Zealand (40.9006 °S, 174.8860 °E) in 10 L pots containing commercially growing medium (APPENDIX E), and irrigated three times daily with 13 to 17 ml of water plus a drainage of 10%-15% with the aim of maintaining the soil at field capacity. Greenhouse conditions included heating to maintain a minimum of 16 °C, ventilation starting at 18 °C and natural day length equal to 11 h.

Whole genome duplication induction

At least 10 individual florets of *L. sinuatum* of the selected genotypes were pollinated daily depending on the number of opened florets available each day. Whole plants were subsequently treated with N_2O in a pressure tolerant cylinder for 48 h at 608 Kpa (Barba-Gonzalez et al., 2006), and returned to greenhouse's conditions after treatment. Ovule/embryo rescued of the pollinated florets was undertaken four weeks after pollination following (Morgan et al., 1998).

Ploidy determination of resulting seedlings was done by flow cytometry using three-month old plants, following the protocol described by Cordoba-Sanchez et al. (2023a)(CHAPTER 2).

Experimental design

The experimental design comprised a 2 × 10 factorial arrangement (Compton, 1994) of treatment factors: seed-bearing genotype (i.e., Y19.016.001 or Y19.015.001) and the days after pollination (DAP) when the N_2O treatment was applied (each of 0 to 9 days).

The experiment was operated as a completely randomized design, and each genotype was used as both seed-bearing and pollen-donor. The experimental unit was the pollinated floret, and the replicates comprised the daily pollinated florets.

The response variables were: zygote success (number of zygotes obtained divided by the number of crosses completed), ploidy of seedlings derived, and proportion of polyploids (number of polyploids obtained divided by number of seedlings obtained (excluding mixoploids)), and polyploidization rate (total number of polyploid seedling obtained in the experiment divided by the total number of pollinations performed).

Data analysis

A generalized linear model (glm function from the stats package (R Core Team 2018)) was used for modelling the effect of the factors with comparison between the levels of the factors performed with pairwise likelihood ratio tests (similar to LRPAIR in (Goedhart, 2018)). The *p-value* for the statistical analysis was set as 0.05 in all cases.

RESULTS

Treatment with N_2O for 48 h induced the development of tetraploids and mixoploids. Tetraploids developed when the N_2O treatment was applied 3 or 5 DAP for Y19.015.001 or Y19.016.001, respectively. In contrast, mixoploid plants developed only for genotype Y19.016.001, when N_2O treatment was applied 2 or 7 DAP.

The proportion of polyploids (number of polyploids obtained divided by number of seedlings obtained (excluding mixoploids)) was between 7.7% (for 5 DAP) and 9% (for 3 DAP; Figure A-5). The polyploidization rate (total number of polyploid seedlings obtained divided by the total number of pollinations performed) ranged between 0.37% and 0.43% for Y19.016.001 and Y19.015.001, respectively.

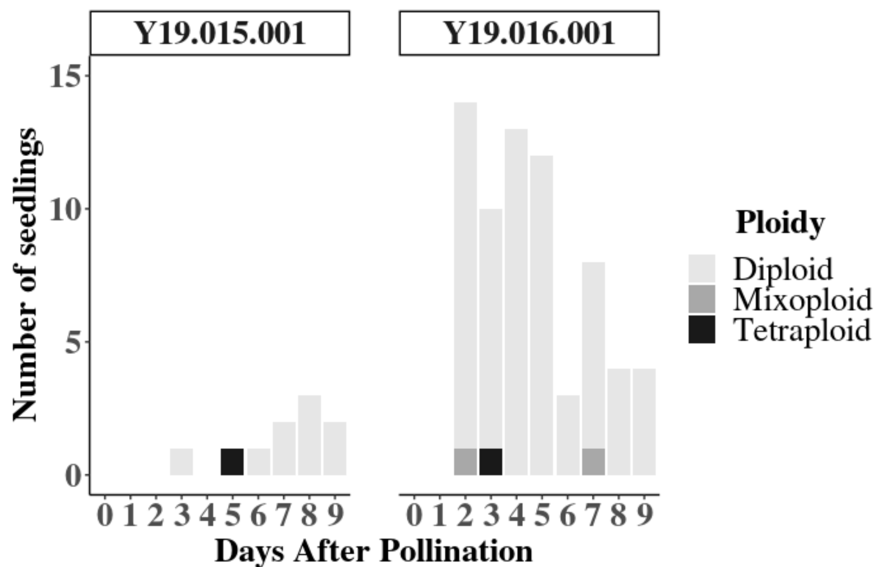

Figure A-5 Number of diploid, tetraploid, or mixoploid seedlings obtained from each of two *L. sinuatum* genotypes used as seed-bearing (Y19.05.001 and Y19.016.001) for each Day After Pollination (DAP), between 0 and 9, when N_2O treatment was applied for 48 h

The effect of N_2O over zygote success (i.e., number of zygotes obtained divided by the number of crosses completed) was inverse to the time interval (number of days) between the planned cross and the treatment, with higher zygote success at a longer time interval ($p < 0.001$; Table A-4). Higher zygote success was observed at least three days after pollination (DAP), reaching a maximum at 5 DAP with values of 96% zygote success for Y19.016.001 and 28% for Y19.015.001 (Table A-4). Differences in zygote success varied also between genotypes, wherein when Y19.016.001 was the seed-bearing, zygote success was five-times higher (59%) in comparison with Y19.015.001 (11%; $p < 0.05$; Table A-4).

Table A-4 Zygote success (number of zygotes obtained divided by the number of crosses completed) in *L. sinuatum* by seed-bearing genotype (Y19.015.001 or Y19.016.001) and Day After Pollination (DAP) when the 48 hours N_2O treatment was applied

DAP[†] when the N_2O was applied	Seed-bearing genotype Y19.015.001 Zygote success (%)[‡§]	Seed-bearing genotype Y19.016.001 Zygote success (%)[‡§]	Zygote success pooled (%)
0	**0[ab]**	**0[a]**	**0[a]**
1	**0[a]**	**19[b]**	**9[b]**
2	**0[a]**	**82[cd]**	**41[cde]**
3	**17[bcd]**	**77[c]**	**47[cd]**
4	**7[abc]**	**65[ce]**	**36[ce]**
5	**28[d]**	**96[d]**	**62[f]**
6	**22[bcd]**	**71[ce]**	**46[cdef]**
7	**18[bcd]**	**61[ce]**	**39[cde]**
8	**20[cd]**	**82[cd]**	**51[df]**
9	**11[abcd]**	**60[ce]**	**35[ce]**
Control	**9[abc]**	**53[e]**	**31[e]**
Mean	**10.7**	**60.5**	**36.1**

[†] Day After Pollination
[‡] Predicted mean values after modelling using a generalized linear model
[§] Mean separation conducted by LRPAIR, different letters represent different mean groups (*p-value* = 0.05)

DISCUSION

Treatment of zygotes with N_2O for 48 h resulted in only two tetraploids, which represented a low polyploidization proportion (i.e., 0.37%-0.43%), in comparison with reports with wheat of more than 30% (Hansen et al., 1988), rye at over 51% (Gordej et al., 2019) and *Lilium* at more than 55% (Sato et al., 2010). The low polyploidization proportion could be associated with a detrimental effect of the long exposure to N_2O that prevented the development of embryos treated up to 2 DAP, and requiring embryos to be older for the chromosome duplication to occur and be able to survive (Table A-4), as have been also reported in *Lilium* (Sato et al., 2010) and rye (Gordej et al., 2019).

In my opinion, the negative effect of 48 h N_2O treatment on zygote success and WGD induction could be due to the treated zygotes/proembryos' inability to recover from the treatment, resulting in low embryo success. Studies on the Spindle Assembly Checkpoint (SAC) in plants have shown that SAC is crucial for the equal distribution of chromosomes during cell reproduction (Komaki & Schnittger, 2016). Studies in *Arabidopsis* show that the mitotic arrest or SAC control lasts up to two hours. If the spindle disruption lasts longer, the cell cycle is reset. Cells do not divide, and they rebuild the nuclear envelope, but now with double the number of chromosomes (Komaki & Schnittger, 2017). The duration of the mitotic arrest determines whether it can be overcome, either 24 or 48 hours. The experiment suggests that *Limonium* may be hypersensitive to 48 h N_2O treatments, similar to some *Arabidopsis* genotypes treated with colchicine (Komaki & Schnittger, 2017). Consequently, zygotes of *Limonium sinuatum* may not fully recover from the mitotic arrest and reset the cell cycle. Alternatively, the zygote may die after the mitotic exit. This phenomenon has been observed in mammals but has not yet been confirmed in plants (Giladi et al., 2015; Komaki & Schnittger, 2017).

The long exposure to N_2O (48 h) compromised the WGD in zygotes and the proembryos (before the globular developmental stage (refer to Section 1.3.3; Figure 1-15)). This increased the likelihood of tetraploids instead of mixoploids, so it is recommended to increase the time between the planned cross and N_2O treatment to obtain seedlings.

Resources did not permit a subsequent experiment to compare 48 h and 24 h treatments. However, as noted within experiments reported within CHAPTER 2 and CHAPTER 3 once 24 h was used the efficacy improved and became reliable.

CONCLUSIONS

For induction of WGD with a 48 h treatment with N_2O, *L. sinuatum*'s zygotes should be older than 3 DAP (i.e., time interval between the planned pollination and the N_2O treatment $\geq$ 3 DAP).

APPENDIX G. Supplementary information for section 5.5.3.1

Table A-5 Number of expected polyploids and produced polyploid in intraspecific crosses in *Limonium sinuatum* by seed-bearing genotype and day after treatment (DAT) for plants treated with nitrous oxide (N_2O) or untreated (control) plants. The number of expected polyploids was calculated by multiplying the proportion of unreduced pollen by the number of seedlings obtained from intraspecific crosses.

Seed-bearing genotype	Day after treatment	Number of polyploids expected	Number of polyploids produced
sin16	Control	0.8	2
sin16	12	0.0	0
sin16	13	0.0	0
sin16	14	0.0	0
sin16	15	0.5	1
sin16	16	0.0	0
sin16	17	0.2	0
sin16	18	0.2	0
sin16	19	0.5	0
sin16	20	0.3	0
sin16	21	0.6	0
sin16	22	0.6	0
sin16	23	0.8	0
sin16	24	1.1	0
sin16	25	3.1	0
sin16	26	3.0	0
sin16	27	1.0	0
sin16	28	1.4	0
sin16	30	1.0	0
sin16	31	1.1	0
sin16	32	1.2	0
sin16	33	1.8	0
sin16	34	1.7	0
sin16	35	0.5	0
sin16	36	1.7	0
sin16	37	0.8	0
sin16	38	0.6	0
sin16	39	1.6	0
sin16	40	0.3	0
sin16	41	1.5	0
sin16	42	0.4	0
sin18	Control	0.6	0

Seed-bearing genotype	**Day after treatment**	**Number of polyploids expected**	**Number of polyploids produced**
sin18	12	0.0	0
sin18	13	0.0	0
sin18	14	0.0	0
sin18	15	0.0	0
sin18	16	0.0	0
sin18	17	0.2	0
sin18	18	0.3	0
sin18	19	0.6	0
sin18	20	0.0	0
sin18	21	0.4	0
sin18	22	0.3	0
sin18	23	0.4	0
sin18	24	0.8	0
sin18	25	0.9	0
sin18	26	1.9	0
sin18	27	0.6	0
sin18	28	1.6	0
sin18	30	0.2	0
sin18	31	1.0	0
sin18	32	1.7	0
sin18	33	1.3	0
sin18	34	0.3	0
sin18	35	0.7	0
sin18	36	0.4	0
sin18	37	0.5	0
sin18	38	0.2	0
sin18	39	0.1	0
sin18	40	1.4	1
sin18	41	0.5	0
sin18	42	0.5	0

APPENDIX H. Culture media

Table A-6 Components of the different culture media used in the research. The abbreviation of each component is accompanied by the amount used. Abbreviations are detailed below the table

Medium	Macro	Micro	Iron	Vitamins	Auxins (mg/l)	Cytokinins (mg/l)	Gibberellins (mg/l)	Sucrose (g/l)	Gelling agent (g/l)	Other (mg/l)	Identification number[††††]	Reference
Base	MSA[*]	MSI[†]	MSF[§]	B_5V[¶]				SUC[‡] 30	AGD[**] 7.5		13	
Modified base	MSA[*]	MSI[†]	MSF[§]	B_5V[¶]				SUC[‡] 30	PHY[****] 7.5		537	
Ovule/embryo rescue	MSA[*]	MSI[†]	MSF[§]	B_5V[¶]	IBA[††] 0.05	BAP[§§] 0.3	GA_3[¶¶] 0.1	SUC[‡] 30	AGD[**] 2.5	KTH[‡‡] 0.03	2070	Modified from (Morgan et al., 1998)
Shoot proliferation	MSA[*]	MSI[†]	MSF[§]	B_5V[¶]	IBA[††] 0.05	BAP[§§] 0.3	GA_3[¶¶] 0.1	SUC[‡] 30	AGD[**] 7.5		4	Modified from (Morgan et al., 1998)
Callus induction	MSA[*]	MSI[†]	MSF[§]	B_5V[¶]	24D[***] 2			SUC[‡] 30	AGD[**] 7.5		453	
Shoot induction	MSA[*]	MSI[†]	MSF[§]	B_5V[¶]		TDZ[†††] 3		SUC[‡] 30	PHY[****] 2.5		630	(Seelye et al., 1994)
Pre-rooting	MSA[*]	MSI[†]	MSF[§]	LSV[¶¶¶]				SUC[‡] 30	AGD[**] 7.5		1	
Rooting	MSA[*]	MSI[†]	MSF[¥]	B_5V[¶]	IBA[††] 12			SUC[‡] 30	AGD[**] 7.5		608	
Oryzalin	MSA[*]	MSI[†]	MSF[§]	B_5V[¶]	IBA[††] 0.05	BAP[§§] 0.3	GA_3[¶¶] 0.1	SUC[‡] 30	AGD[**] 7.5	ORY[‡‡‡] 1.5	2962	

[*] MSA: Murashige and Skoog macronutrients solution (Murashige & Skoog, 1962) containing calcium chloride dihydrate (440 mg/L), potassium dihydrogen ortho phosphate (170 mg/L), magnesium sulphate (370 mg/L), ammonium nitrate (1650 mg/L), and potassium nitrate (1900 mg/L)

[†] MSI: Murashige and Skoog micronutrients solution (Murashige & Skoog, 1962) containing boric acid (6.2 mg/L), manganese sulphate (monohydrate) (15.1 mg/L), zinc sulphate (8.6 mg/L), potassium iodide (0.83 mg/L), copper sulphate (0.025 mg/L), molybdic acid (0.25 mg/L), and cobalt chloride (0.025 mg/L)

[§] MSF: Murashige and Skoog Iron (Murashige & Skoog, 1962) containing ferric sodium EDTA (36.7 mg/L)

[¥] MSF: Murashige and Skoog Iron (Murashige & Skoog, 1962) containing ferric sodium EDTA (73.4 mg/L)

[¶] B5V: Gamborg B5 vitamins solution (Gamborg et al., 1968) containing inositol (myo) (100 mg/L), thiamine hydrochloride (Vitamin B1) (10 mg/L), nicotinic acid (1 mg/L), and pyridoxine hydrochloride (Vitamin B6) (1 mg/L)

[‡] SUC: Sucrose, Chelsea™ white sugar

[**] AGD: Bacteriological Agar. New Zealand Seaweeds

[††] IBA: Indole-3-Butyric Acid. Sigma. Product code I1875

[§§] BAP: Benzyl-amino-purine. Sigma. Product code B3408

[‡‡] KTH: Kathon® LXE (active ingredient chloromethylisothiazolinone/methylisothiazolinone)

[¶¶] GA_3: Gibberellic acid. Sigma. Product code G7645

[***] 24D: 2,4-dichlorophenoxyacetic acid. Sigma. Product code D8407

[†††] TDZ: Thidiazuron. Sigma. Product code P6186

[¶¶¶] LSV: LS vitamin solution (Linsmaier & Skoog, 1965) containing inositol (myo) (100 mg/L), thiamine.HCl (Vitamin B1) (0.4 mg/L)

[‡‡‡] ORY: Oryzalin. Sigma. Product code 36182

[****] PHY: Phytagel. Sigma. Product code P8169

[††††] Identification number used by The New Zealand Institute for Plant & Food Research Ltd, Palmerston North Tissue Culture Laboratory

APPENDIX I. Induction of unreduced gametes using nitrous oxide (N_2O) for 48 hours

INTRODUCTION

Induction of unreduced gametes has been achieved in crops such as *Lilium* (Barba-Gonzalez et al., 2006) and *Tulipa* (Qu et al., 2019). The treatment conditions applied in these reports were 600 KPa of pressure of N_2O; however, differed regarding duration of treatment (24 h, 48 h). Therefore, a preliminary experiment was done using different genotypes of *L. sinuatum* as well as *L. perezii*, which corresponded to the genotypes used in PFR for production of interspecific hybrids with potential commercial interest (BallSB, 2021). The aims of this experiment were:

- Induction of unreduced gametes in *L. sinuatum* and *L. perezii* using 48 h treatment duration with N_2O.
- Production of sexual interspecific polyploids

MATERIALS AND METHODS

Plant material

Four *L. sinuatum* genotypes (Y19.015.001, Y19.016.001 (sin16), Y19.018.001 (sin18), and *L. sinuatum* 13), and two *L. perezii* genotypes (Y18.011.001, Y19.001.001 (per11)) were used in this experiment. The genotypes were selected based on their synchronous flowering times and contrasting stigma types. One plant was available from each genotype, with exception of Y19.018.001 and Y19.001.001, with three plants each.

The growth period began in September 2019 and lasted for several months. The greenhouse was heated to 16°C and ventilated to 18°C, with 14 hours of natural daylight each day. The plants were grown in 10 L solid plastic pots filled with a commercial growing medium (APPENDIX E) under greenhouse conditions at The New Zealand Institute for Plant & Food Research Ltd in Palmerston North, New Zealand (40.9006 °S, 174.8860 °E). The plants were irrigated daily with a Netafim irrigation system that provided between 40 ml to 50 ml of water daily, with a 10-15% drainage to keep the soil at field capacity.

Nitrous oxide treatment

For the induction of unreduced gametes, florets with sepals longer than 2 mm were carefully removed and discarded from each selected *L. sinuatum* and *L. perezii* genotype to ensure that only young florets which were likely to have pollen mother cells (PMC) undergoing meiosis (refer to Section 1.3.1.2) were treated with N_2O. For N_2O treatment, one plant from each genotype was put inside a pressure-tolerant cylinder for 48 h and N_2O pressure set at 608 KPa (Barba-Gonzalez et al., 2006) (Figure A-6). After the treatment (day zero), the plants were returned to the above-mentioned greenhouse conditions.

Figure A-6 Equipment utilised for treating plants with nitrous oxide (N_2O), which included a pressure-tolerant cylinder (inside diameter 45 cm; height 110 cm), a N_2O source, and a manometer. Photo taken by Steven Ray.

Planned crosses were performed after day zero, i.e., date of removal of the plants from N_2O treatment (0 DAT). The first cross occurred 4 days after treatment (DAT) and continued daily for 30 days (i.e., between 18th October and 15th November 2019; refer to Results Section for further description as to why the first crosses occurred at 4 DAT). On each day of pollination, crosses were performed when the florets opened and within a few

hours of an individual floret opening and exhibiting anther dehiscence. Individual florets were pollinated once. Each genotype was used as a seed-bearing as well as pollen-donor parent and some genotypes were used in more than one cross (Table A-7).

Plants that were non-treated, i.e., control, only available for genotypes Y19.018.001 (*L. sinuatum*) and Y19.001.001 (*L. perezii*), remained in the greenhouse and pollinations were performed on four randomly selected days between the 4 DAT and 30 DAT period for each of two plant pairs available (i.e., Y19.018.001 × Y19.001.001).

The number of crosses performed each DAT ranged between 7 and 71, depending on the number of freshly opened florets available (Table A-7), and was followed by ovule rescue 15 days after pollination (Morgan et al., 1998). Emasculation before pollination was not necessary as *L. sinuatum* and *L. perezii* are self-incompatible (Baker, 1953a).

Ploidy and hybrid status determination of the seedling obtained was performed using flow cytometry as described in CHAPTER 2 (Cordoba-Sanchez et al., 2023a).

Table A-7 Crossing plan of interspecific crosses performed between *Limonium perezii* and *Limonium sinuatum* genotypes treated with nitrous oxide (N_2O) for 48 h or untreated (control)

Seed-bearing		**Pollen-donor**		**Treatment**
Genotype	**Species**	**Genotype**	**Species**	
L. sinuatum 13 (2018)	*L. sinuatum*	Y19.001.001	*L. perezii*	N_2O
Y19.015.001	*L. sinuatum*	Y18.011.001	*L. perezii*	N_2O
Y19.018.001	*L. sinuatum*	Y19.001.001	*L. perezii*	N_2O
Y19.018.001	*L. sinuatum*	Y19.001.001	*L. perezii*	control
Y18.011.001	*L. perezii*	Y19.015.001	*L. sinuatum*	N_2O
Y19.001.001	*L. perezii*	*L. sinuatum* 13 (2018)	*L. sinuatum*	N_2O
Y19.001.001	*L. perezii*	Y19.018.001	*L. sinuatum*	N_2O

Experimental design

The experimental design used was a randomized complete block, with a 5 × 26 arrangement of the factors: seed-bearing genotype (Y19.015.001, Y19.016.001, Y19.018.001, Y18.011.001 or Y19.001.001), and DAT (from 4 to 30 DAT) when the plants were pollinated. The crosses per plant pair, done on each day, corresponded to the replicates.

Response variables

The response variables evaluated for parents and derived seedlings were:

- zygote success (number of zygotes obtained divided by the number of crosses completed)
- cross success (number of seedlings obtained divided by number of crosses completed)
- germination rate (number of zygotes germinated *in vitro* by number of zygotes obtained (rescued))
- ploidy of derived seedlings
- proportion of polyploids (number of polyploids obtained divided by number of seedlings obtained (excluding mixoploids))
- polyploidization rate (total number of polyploid seedling obtained in the experiment divided by the total number of pollinations performed)
- hybridisation rate (number of hybrids obtained by number of crosses completed)

Data analysis

The effect of the factors, seed-bearing genotype and DAT, were modelled using a generalized linear model (glm function from the stats package (R Core Team 2018)) with a binomial distribution. Pairwise likelihood ratio tests (similar to LRPAIR in (Goedhart, 2018)) were subsequently applied for comparison between the factor levels. The *p-value* for the statistical analysis was set as 0.05 in all cases.

RESULTS

No polyploid plants were detected from seedlings arising in this experiment. Out of the 2584 crosses performed after N_2O treatment, after ovule rescue only 9 diploid seedlings were obtained (0.35% cross success); out of which only 4 were hybrids from Y19.018.001

× Y19.001.001 (0.43% hybridisation rate for the cross). For the control plants, the 80 crosses performed resulted in 2 diploids progeny similar to *L. sinuatum* (2.5% cross success; 0% hybridisation rate; Figure A-7). In addition, no florets were produced during the first four to five days after treatment with N_2O, depending on the genotype (data not presented), and the first florets that did open presented an abnormal appearance, being withered/malformed and stunted. Hence it was not possible to conduct any pollinations prior to 4 DAT.

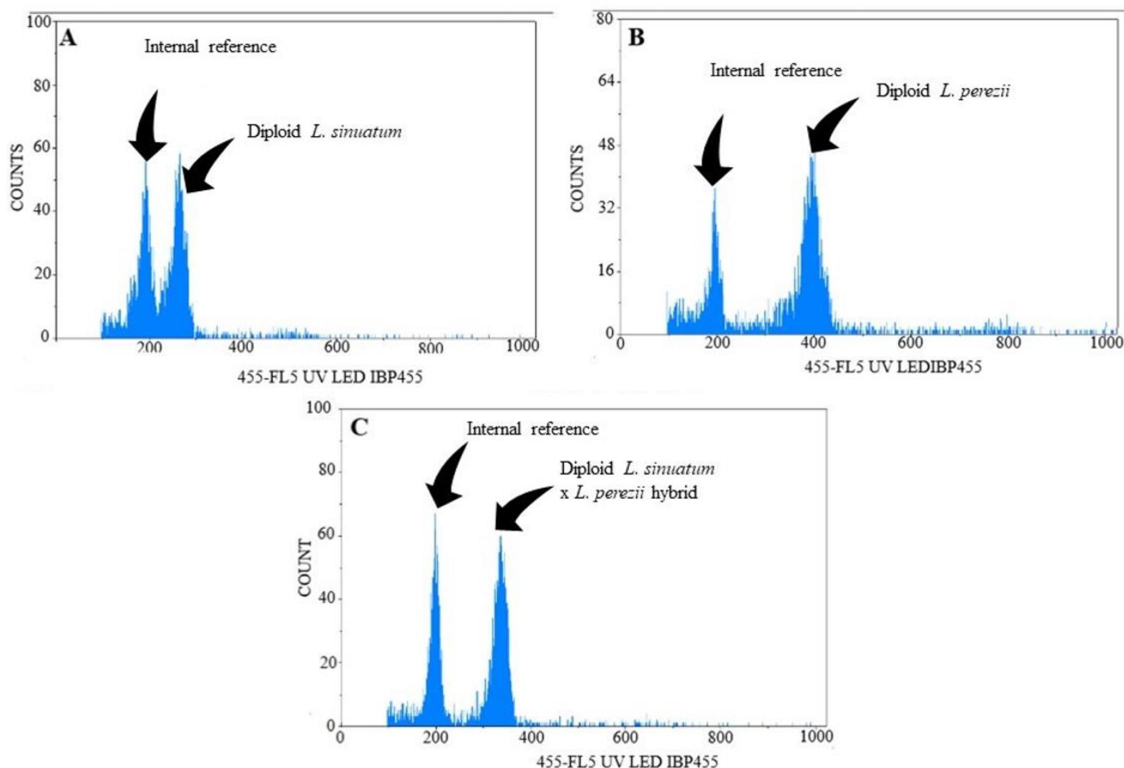

Figure A-7 Flow cytometry spectra of two representative parental genotypes and two representative seedlings. *Bellis* sp. was used as an internal reference. **A.** *L. sinuatum.* **B.** *L. perezii.* **C.** *L. sinuatum* × *L. perezii* hybrid

After N_2O-treatment, the zygote success (number of zygotes obtained divided by the number of crosses completed) varied between 0 and 44% (Y9.018.011 × Y19.001.001) with differences attributed to the seed-bearing genotype ($p<0.05$), but not DAT ($p>0.05$). The seed-bearing genotypes with higher embryo success were Y19.001.001 (7.8% ± 8%) and Y19.018.001 (6% ± 14;Table A-8).

Table A-8 Number of embryos, seedlings, and hybrids, obtained per interspecific cross (*Limonium sinuatum* × *Limonium perezii* or vice versa) and treatment (i.e., nitrous oxide (N_2O) or untreated (control))

Cross	Treatment	Number crosses completed	Number rescued embryos	Number seedlings developed	Number hybrids
L. sinuatum 13 (2018) × Y19.001.001	N_2O	453	5	5	0
Y18.011.001 × Y19.015.001	N_2O	370	4	0	0
Y19.001.001 × *L. sinuatum* 13 (2018)	N_2O	418	28	0	0
Y19.001.001 × Y19.018.001	N_2O	482	38	0	0
Y19.015.001 × Y18.011.001	N_2O	412	2	0	0
Y19.018.001 × Y19.001.001	N_2O	449	23	4	4
Y19.018.001 × Y19.001.001	Control	80	2	2	0

For the treated plants, the germination rate (number of zygotes germinated *in vitro* by number of zygotes obtained (rescued)) was 9%, with a loss of 91% of the rescued embryos in tissue culture, i.e., declining from 100 to 9 (Table A-8). In contrast, for the control crosses the two embryos rescued developed into seedlings. It was observed, but not quantified, that most of the embryos ceased their development at the heart stage (refer to Figure 1-15 for the stages of embryo development) and, in some cases, the embryo developed callus *in vitro*.

DISCUSSION

In the evaluated *L. perezii* and *L. sinuatum* genotypes, N_2O treatment for 48 h was detrimental. This was observed through no production of florets in the first four days after treatment, and the occurrence of physical abnormalities in the first florets to open. The negative effect of N_2O contrasts to the research in: begonia (*Begonia* sp.) where physiological effects of N_2O treatment only occurred after 72 h of treatment (Dewitte et al., 2010); lilium (*Lilium* sp.; Luo et al. (2016)) where pollen germination was higher after 48 h in comparison with 24 h treatment with N_2O; and tulip (*Tulipa* sp.; Okazaki et al. (2005) where the proportion of potentially unreduced pollen grains was higher after 48 h treatment with N_2O than after 24 h. However, the negative impact of a 48 h treatment has been reported in moth orchid (*Phalaenopsis amabilis*; Wongprichachan et al. (2013) and recently in other *L. sinuatum* genotypes (Siregar, 2021).

The genotypes used as parents could have affected the outcome of the N_2O treatment. The differences observed in the response of each seed-bearing genotype to the N_2O treatment, which in the experiment turns into an additional undesirable source of variation, could be the result of individual genotype differences which make them respond differently to the treatment as has been reported for *Lilium* hybrids (Akutsu et al., 2007; Luo et al., 2016). However, the effect of the genotypes could also be attributed to their natural combining ability with some genotype combinations displaying higher mating success than other, but independent of the N_2O treatment evaluated (Fasahat et al., 2016).

The absence of polyploid plants in this experiment, which also corresponded to the very first experiment done in this PhD research, would suggest N_2O does not induce production of unreduced pollen in *Limonium*. However, these results led to improvements in the experimentation which resulted in subsequent successful experiments (CHAPTER

5). The improvements included reduction of the time of treatment with N_2O from 48 h to 24 h, planned crosses starting at 12 DAT when the florets' appearance was normal. In addition, genotypes to be used in subsequent experiments were selected in advance, based on their performance in intraspecific crosses (APPENDIX A). Moreover, to have a better understanding of the N_2O effect over pollen size (i.e., plan area) and viability, a methodology for the measurement of pollen plan area was established (refer to Section 2.4.4.1).

N_2O treatment increased the hybridisation rate between *L. sinuatum* and *L. perezii*. The interspecific hybridisation rate obtained after 48 h treatment with N_2O (0.43%) was higher than the rate for untreated plants (0%; CHAPTER 5). However, the hybridisation rate after 48 h treatment was still lower than following 24 h treatment (5.5%; CHAPTER 5) reinforcing the fact that 48h treatment is likely to be too long and has a negative effect.

CONCLUSIONS

N2O for 48 h increases the interspecific hybridisation up to 0.43%. However, it negatively affects the production of florets during the first four days after the treatment and did not result in hybrid polyploids. Hybrid plants were produced but had diploid nuclear DNA contents.

APPENDIX J. Modified Brewbaker & Kwack medium for pollen germination

Table A-9 Modified Brewbaker & Kwack medium, as the most frequently reported medium for germination of binucleate pollen (Brewbaker & Kwack, 1963), was used for *in vitro* germination of trinucleate pollen of *Limonium*. Casein hydrolysate was omitted. This information was extracted from Boyd et al. (2022), corresponding to a complementary piece of work undertaking within the PhD research reported in the current document

Component	Concentration
Boric acid (ppm)	100
Sucrose (%)	10
PEG[†]-20,000 (%)	20
$CaNO_3$ [‡] (ppm)	300
KNO_3[§] (mg/L)	100
$MgSO_4$[¥] (mg/L)	200

[†] Polyethylene glycol
[‡] Calcium nitrate
[§] Potassium nitrate
[¥] Magnesium sulphate

APPENDIX K. Flow cytometry analysis for backcrosses between *L. sinuatum* × *L. perezii* hybrids and *L. sinuatum* parent (Y19.018.001)

Table A-10 Flow cytometry results for the 21 seedlings obtained after backcrossing *L. sinuatum* × *L. perezii* hybrids and their *L. sinuatum* parent (Y19.018.001) plus five control genotypes corresponding to the *L. sinuatum* parent (Y19.018.001), two diploid *L. sinuatum* × *L. perezii* hybrids (Y19.026.001 and Y19.026.003) and two tetraploid *L. sinuatum* × *L. perezii* hybrids (Y19.026.006 and Y19.026.009). *Trifolium repens* was used as an internal reference (IR) and its DAPI relative fluorescence peak was used to compare the peak of each sample and to estimate the ploidy of the sample by calculating of the ratio between each sample peak and the peak of the IR. To determine the hybrid status of the seedlings, the peak of each sample was compared with the peak produced by the known genotypes *Limonium sinuatum* (Y19.018.001) and *Limonium sinuatum* × *Limonium perezii* hybrids (Y19.026.001, Y19.026.003, Y19.026.006, Y19.026.009)

Genotype	Cross	Peak IR (*T. repens*)	Peak Sample	Ratio Peak sample/peak IR	Estimated ploidy	Ploidy	Hybrid status
Y19.018.001	Parental genotype	102	190	1.86	2	Diploid	No hybrid
Y19.026.001	Parental genotype	101	241	2.39	2	Diploid	Confirm
Y19.026.003	Parental genotype	100	236	2.36	2	Diploid	Confirm
Y19.026.006	Parental genotype	100	468	4.68	4	Tetraploid	Confirm
Y19.026.009	Parental genotype	101	474	4.69	4	Tetraploid	Confirm
Y22.002.001	Y19.006.009 × Y19.018.001	101	330	3.27	3	Triploid	Confirm
Y22.002.002	Y19.006.009 × Y19.018.001	101	321	3.18	3	Triploid	Confirm
Y22.002.003	Y19.006.009 × Y19.018.001	103	321	3.12	3	Triploid	Confirm
Y22.002.004	Y19.006.009 × Y19.018.001	106	349	3.29	3	Triploid	Confirm
Y22.002.005	Y19.006.009 × Y19.018.001	103	330	3.20	3	Triploid	Confirm
Y22.002.006	Y19.006.009 × Y19.018.001	104	331	3.18	3	Triploid	Confirm
Y22.002.007	Y19.006.009 × Y19.018.001	100	324	3.24	3	Triploid	Confirm

APPENDIX K. *Flow cytometry analysis for backcrosses between L. sinuatum × L. perezii hybrids and L. sinuatum parent (Y19.018.001)*

Genotype	**Cross**	**Peak IR (*T. repens*)**	**Peak Sample**	**Ratio Peak sample/peak IR**	**Estimated ploidy**	**Ploidy**	**Hybrid status**
Y22.002.008	Y19.006.009 × Y19.018.001	103	333	3.23	3	Triploid	Confirm
Y22.002.009	Y19.006.009 × Y19.018.001	104	330	3.17	3	Triploid	Confirm
Y22.003.001	Y19.026.006 × Y19.018.001	103	337	3.27	3	Triploid	Confirm
Y22.003.002	Y19.026.006 × Y19.018.001	97	308	3.18	3	Triploid	Confirm
Y22.003.003	Y19.026.006 × Y19.018.001	101	336	3.33	3	Triploid	Confirm
Y22.003.004	Y19.026.006 × Y19.018.001	101	318	3.15	3	Triploid	Confirm
Y22.003.005	Y19.026.006 × Y19.018.001	98	308	3.14	3	Triploid	Confirm
Y22.003.006	Y19.026.006 × Y19.018.001	101	330	3.27	3	Triploid	Confirm
NA*	Y19.018.001 × Y19.026.001	97	183	1.89	2	Diploid	No hybrid
NA*	Y19.018.001 × Y19.026.003	99	187	1.89	2	Diploid	No hybrid
NA*	Y19.018.001 × Y19.026.006	94	183	1.95	2	Diploid	No hybrid
NA*	Y19.018.001 × Y19.026.006	100	184	1.84	2	Diploid	No hybrid
Y22.004.001	Y19.018.001 × Y19.026.009	100	323	3.23	3	Triploid	Hybrid
NA*	Y19.018.001 × Y19.026.009	95	175	1.84	2	Diploid	No hybrid

*NA. No identification code was given to the genotype as it was not a hybrid

APPENDIX L. Comparison between backcrosses and outcrosses in *Limonium sinuatum*

INTRODUCTION

Hybrid incompatibility has been widely documented in multiple species including *Limonium* (Baker, 1966; Chen et al., 2016). The incompatibilities translate into reproductive barriers which could take place at different stages including: pre-pollination, post-pollination, pre-zygotic or post-zygotic (Chen et al., 2016; Fishman & Sweigart, 2018). In the present research the main reproductive barriers encountered in the *Limonium* genotypes are stigma dimorphism, different flowering times and genetic incompatibilities, which prevent/restrict fertilization (refer to Section 1.2.5.1). As a result, hybrid low viability, hybrid weakness and hybrid sterility (CHAPTER 6 & CHAPTER 7) have been observed.

For inclusion of the genotypes generated in this research into the breeding programme of *Limonium*, it was required to determine if backcrossing of those genotypes could represent a disadvantage, in comparison to outcrossing, as suspected in *Limonium* (Baker, 1966) and reported in *Hemerocallis* (Yasumoto & Yahara, 2008). Backcrossing could be detrimental in self-incompatible/obligated outcrossers, e.g., *L. sinuatum* and *L. perezii*, due to the accumulation of deleterious mutations, expression of recessive genes, or genetic linkage (Rutkoski et al., 2022; Yasumoto & Yahara, 2008). Therefore, the aim of the experiment presented here was to evaluate if backcrossing was disadvantageous in comparison with outcrossing by quantifying if less zygotes (i.e., seedlings collected 30 days after pollination) are produced from backcrosses than from outcrosses in *L. sinuatum*.

METHODOLOGY

Plant material

Five *L. sinuatum* genotypes were used in this experiment. The criteria used for genotype selection were:

1. Three genotypes should be diploid siblings from the *L. sinuatum* tetraploids obtained from crosses between Y19.016.001 and Y19.015.001 as the tetraploids would be used in future studies about pollen performance (APPENDIX I).
2. Contrasting stigma.
3. Synchronous flowering times.

The genotypes were, therefore:

- one parental genotype (Y19.015.001),
- three offspring from Y19.016.001 × Y19.015.001 (i.e., Y19.019.001, Y21.003.005 and Y21.003.008) and,
- one genotype with good performance as a parent (refer to Section APPENDIX I) but not genetically related with the other genotypes (i.e., Y19.018.001).

The plants were grown under greenhouse conditions in Palmerston North, New Zealand (40.9006 °S, 174.8860 °E), as described previously (refer to APPENDIX F). Crosses were done in September 2021, under controlled greenhouse conditions, with temperature set between 16 °C and 18 °C and natural day length equal to 11 h.

Experimental design

Crosses were done between the three offspring genotypes (Y19.019.001, Y21.003.005 and Y21.003.008) and their father (Y19.015.001) (backcrosses) or an unrelated *L. sinuatum* genotype (Y19.018.001) (outcrosses). Each genotype was used as a seed-bearing parent as well as pollen-donor parent. Ten crosses were done daily per genotype, for three days in a row.

The experimental design was a completely randomised, with one factor corresponding to the cross type: backcross or outcross. Each pollinated floret produced only one zygote; therefore the experimental unit was the pollinated floret; the 10 florets pollinated per day corresponded to sub-samples, and the crosses completed on each of the three days were the experiment replicates.

The response variables evaluated were: zygote success (number of zygotes obtained divided by the number of crosses completed), germination rate (number of zygotes germinated *in vitro* divided by number of zygotes obtained (rescued)), and cross success

(number of seedlings obtained divided by number of crosses completed). The zygote success was evaluated one month after the planned crossing, while the germination rate and cross success were evaluated seven weeks after the crossing.

Plant tissue culture

One month after the planned pollinations, the seed (i.e., zygotes) were collected from the plants and established under *in vitro* conditions following the previously established protocol (Cordoba-Sanchez et al., 2023a).

Statistical analysis

Data analysis was conducted using the software R (R Core Team, 2018) with ggplot 2 (Wickham 2016) utilised for graphic outputs. The three response variables evaluated followed a binary distribution, therefore for data analysis a generalized linear model (glm function from the stats package (R Core Team 2018)) with the binary distribution was used.

RESULTS

Zygote success was apparently lower in backcrosses in comparison with outcrosses (p>0.05), while germination rate tended to be higher in backcrosses than in outcrosses (p>0.05; Table A-11). The variable cross success with values between 5% and 8% was not different between both type of crosses (p>0.05).

Table A-11 Predicted mean value for zygote success, germination rate and cross success, for two types of crosses performed in *L. sinuatum*: backcross and outcross

Response variable	Backcross	Outcross
Zygote success	12% ± 17%	26% ± 35
Germination rate	90% ± 22%	40% ± 14%
Cross success	6% ± 9%	9% ± 17%

Differences observed in zygote success were associated with the seed-bearing genotype with values between 3% and 50% (p<0.05; Figure A-8). However, zygote success was not affected by the pollen-donor genotype presenting values between 10% and 29%

($p>0.05$). In the case of seed germination and cross success neither the seed-bearing genotypes nor pollen-donor genotype affected them ($p>0.05$).

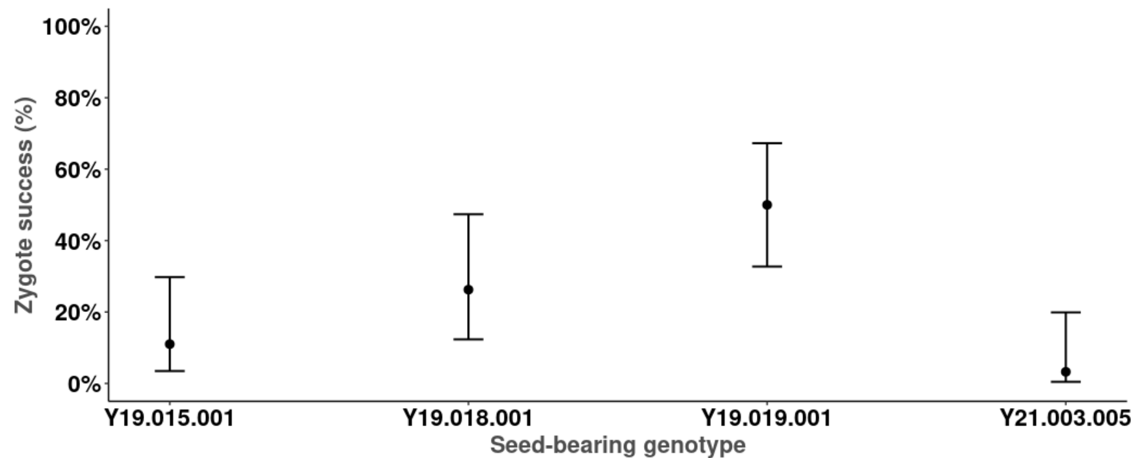

Figure A-8 Zygote success (%) obtained after backcrosses and outcrosses in *L. sinuatum* by seed-bearing parent. Dots represent the predicted mean values for pollen plan area after data modelling using a generalized linear model. Vertical lines (error bars) represent the 95% confidence interval. p = 0.05, n = 10 crosses

DISCUSSION

The low cross success observed in *L. sinuatum* was in the range reported for the species in the experiment done for evaluation of parental genotypes (APPENDIX A), and was lower that the value reported for intraspecific crosses in *L. sinuatum* (74% ± 25%) where Y19.018.001 was one of the parents (CHAPTER 5). However, the low value observed here was similar to the cross success obtained for the genotype Y19.015.001 when used a seed parent (1.3% ± 1.5%) or pollen-donor (0%); APPENDIX A). Therefore, an influence of Y19.015.001 as parent could be suspected in the seedling's behaviour in the planned crosses of this experiment.

High variability was observed in the data, even though it was not surprising given difficulties to decide which crosses should be preferred in the breeding programme when it is possible to choose between outcrossing or backcrossing. In addition, the influence over zygote success arising from the seed-bearing parent, re-confirms the importance of parental selection prior to experimentation. For future experiments in the present research, outcrossing would therefore be preferred over backcrosses whenever possible, depending on the breeding aim.

APPENDIX M. Supplementary data for crosses to evaluate their reproductive performance

Table A-12 Information about the planned crosses performed for evaluation of the reproductive performance of some genotypes of *Limonium* obtained in the current research programme including: species composition of the genotypes used as parents, zygote success (%), germination rate (%), number of seedlings derived, and E-Brida code of each seedling produced

Species of seed-bearing parent	Species of pollen-donor parent	Cross	Cross type	Zygote success (%)	Germination rate (%)	Number of seedlings available in PFR¶	E-Brida codes of the obtained seedlings	Reference
L. sinuatum (2n=2x)	*L. sinuatum* (2n=2x)	diploid × diploid	Intraspecific	70.0 ± 14.1	100 ± 0	21*	Y19.019.001 Y19.019.002 Y21.001.001 Y21.001.002 Y21.001.005 Y21.001.007 Y21.002.002 Y21.003.001 to Y21.003.007 Y21.003.009 Y21.006.002 Y21.006.003 Y21.006.005 Y21.007.002	CHAPTER 3 (Induction of Whole-genome duplication (WGD) in *Limonium perezii* using nitrous oxide (N2O)

APPENDIX M. *Supplementary data for crosses to evaluate their reproductive performance*

Species of seed-bearing parent	Species of pollen-donor parent	Cross	Cross type	Zygote success (%)	Germination rate (%)	Number of seedlings available in PFR[¶]	E-Brida codes of the obtained seedlings	Reference
L. perezii (2n=2x)	*L. perezii* (2n=2x)	diploid × diploid	Intraspecific	36.8 ± 26.6	No information	17[*]	Y21.004.001 Y21.005.001 Y21.005.002 Y21.008.001 Y21.008.005 Y21.008.006 Y21.008.008 Y21.008.013 Y21.008.014 Y21.009.002 Y21.009.003 Y21.009.006 Y21.009.007 Y21.009.008 Y21.009.010 Y21.009.012	APPENDIX A (**Error! Not a valid result for table.**) CHAPTER 3 (Induction of Whole-genome duplication (WGD) in *Limonium perezii* using nitrous oxide (N2O)
L. sinuatum (2n=4x)	*L. sinuatum* (2n=4x)	tetraploid × tetraploid	Intraspecific	25.0 ± 22.6	18.3 ± 22.3	1[†]	Y22.010.001	CHAPTER 7- Section 7.3.3 (Morphological and reproductive characterisation of tetraploid *Limonium* plants-
L. perezii (2n=4x)	*L. perezii* (2n=4x)	tetraploid × tetraploid	Intraspecific	14.3 ± 17.9	10.4 ± 19.8	3[†]	Y22.009.001 Y22.009.002 Y22.011.001	
L. sinuatum (2n=2x)	*L. perezii* (2n=2x)	diploid × diploid	Interspecific	73.0 ± 17.5[§§]	0 ± 0	0[¥]		

Species of seed-bearing parent	Species of pollen-donor parent	Cross	Cross type	Zygote success (%)	Germination rate (%)	Number of seedlings available in PFR[¶]	E-Brida codes of the obtained seedlings	Reference
L. perezii (2n=2x)	*L. sinuatum* (2n=2x)	diploid × diploid	Interspecific	8.3 ± 10	0 ± 0	0		Reproductive performance)
L. sinuatum (2n=4x)	*L. perezii* (2n=4x)	tetraploid × tetraploid	Interspecific	2.6 ± 4.5	0 ± 0	0		
L. perezii (2n=4x)	*L. sinuatum* (2n=4x)	tetraploid × tetraploid	Interspecific	3.3 ± 13	0 ± 0	0		
L. sinuatum (2n=2x)	*L. sinuatum* × *L. perezii* hybrid (2n=2x)	diploid × diploid	Homoploid-Backcross to seed-bearing genotype	5.0 ± 7.0	0 ± 0	0		CHAPTER 6-Section 6.3.2 (Production *in vitro* of tetraploid *L. sinuatum* × *L. perezii* hybrids using oryzalin-Backcrosses)
L. sinuatum × *L. perezii* hybrid (2n=2x)	*L. sinuatum* (2n=2x)	diploid × diploid	Homoploid-Backcross to seed-bearing genotype	0.8 ± 3	0 ± 0	0		
L. sinuatum (2n=2x)	*L. sinuatum* × *L. perezii* hybrid (2n=4x)	diploid × tetraploid	Heteroploid-Backcross to seed-bearing genotype	6.2 ± 8.8	3.7 ± 11.1[§]	1	Y22.004.001	

Species of seed-bearing parent	Species of pollen-donor parent	Cross	Cross type	Zygote success (%)	Germination rate (%)	Number of seedlings available in PFR¶	E-Brida codes of the obtained seedlings	Reference
L. sinuatum × *L. perezii* hybrid (2n=4x)	*L. sinuatum* (2n=2x)	tetraploid × diploid	Heteroploid-Backcross to seed-bearing genotype	24.2 ± 31	23.5 ± 36.0	15	Y22.002.001 to Y22.002.009 Y22.003.001 to Y22.003.006	CHAPTER 7-Section 7.3.3 (Morphological and reproductive characterisation of tetraploid *Limonium* plants-Reproductive performance)
L. sinuatum (2n=4x)	*L. sinuatum* × *L. perezii* hybrid (2n=4x)	tetraploid × tetraploid	Homoploid-Backcross to *L. sinuatum*	48.1 ± 22.0	37.3 ± 34.1	7	Y22.008.001 to Y22.008.007	
L. sinuatum × *L. perezii* hybrid (2n=4x)	*L. sinuatum* (2n=4x)	tetraploid × tetraploid	Homoploid-Backcross to *L. sinuatum*	7.8 ± 7.0	0 ± 0	0		
L. sinuatum (2n=2x)	*L. sinuatum* × *L. perezii* hybrid (2n=2x)	diploid × diploid	Homoploid-Backcross to *L. sinuatum*	0 ± 0	0 ± 0	0		
L. sinuatum × *L. perezii* hybrid (2n=2x)	*L. sinuatum* (2n=2x)	diploid × diploid	Homoploid-Backcross to *L. sinuatum*	0 ± 0	0 ± 0	0		

Species of seed-bearing parent	Species of pollen-donor parent	Cross	Cross type	Zygote success (%)	Germination rate (%)	Number of seedlings available in PFR[¶]	E-Brida codes of the obtained seedlings	Reference
L. perezii (2n=4x)	*L. sinuatum* × *L. perezii* hybrid (2n=4x)	tetraploid × tetraploid	Homoploid-Backcross to *L. perezii*	22.2 ± 22.2	0 ± 0	0		
L. sinuatum × *L. perezii* hybrid (2n=4x)	*L. perezii* (2n=4x)	tetraploid × tetraploid	Homoploid-Backcross to *L. perezii*	7.9 ± 11.0	0 ± 0	0		
L. perezii (2n=2x)	*L. sinuatum* × *L. perezii* hybrid (2n=2x)	diploid × diploid	Homoploid-Backcross to *L. perezii*	0 ± 0	0 ± 0	0		
L. sinuatum × *L. perezii* hybrid (2n=2x)	*L. perezii* (2n=2x)	diploid × diploid	Homoploid-Backcross to *L. perezii*	0 ± 0	0 ± 0	0		

[¶] The New Zealand Institute for Plant & Food Research Ltd

[*] Including diploids, not affected by N_2O treatment after pollination for whole genome duplication

[†] Second generation tetraploids

[¥] Interspecific hybrids (50 in total) for this cross were obtained in CHAPTER 5 in untreated plants (E-Brida codes Y22.001.001 to Y22.001.003) or after treatment with N_2O before pollination for induction of unreduced gametes (E-Brida codes Y21.010.001 to Y21.010.020; Y21.011.001 to Y21.011.023)

[§] Apomixis was confirmed in 80% of the seedlings obtained (4 out of 5 plantlets)

Species of seed-bearing parent	Species of pollen-donor parent	Cross	Cross type	Zygote success (%)	Germination rate (%)	Number of seedlings available in PFR[¶]	E-Brida codes of the obtained seedlings	Reference

[§§] Apomixis is suspected to be responsible for the unusually high zygote success value for interspecific crosses. Flow cytometry of untreated plants from the interspecific cross *L. sinuatum* × *L. perezii* obtained in CHAPTER 5 revealed 63% apomixis

APPENDIX N. Preliminary results of the use of nitrous oxide treatment for fertility restoration of abortive hybrid pollen

INTRODUCTION

Interspecific hybrids between *L. sinuatum* and *L. perezii* have gained recognition in the ornamental international market in the form of the *Limonium* siNZii™ series (Ball Horticultural do Brasil, 2023; RioRoses, 2021). However, hybrid sterility (Burchi et al., 2006; Morgan et al., 1995) still represents a challenge to sustain future breeding with this material. In the present programme of research, N_2O has improved the interspecific hybridisation rate (CHAPTER 5) and the zygote success (CHAPTER 4), but was unsuccessful in producing fertile hybrids (CHAPTER 5), raising once again the question of how could the fertility of the hybrid genotypes be restored to allow their use for breeding purposes. The first option previously successful in other *Limonium* interspecific hybrids (Morgan et al., 2001) was the induction of whole genome duplication as a result of treatment of *in vitro* shoots with oryzalin (CHAPTER 6). Despite the obvious success of the oryzalin treatment for the induction of polyploids (i.e., 18% polyploidization rate; CHAPTER 6), the key disadvantage of the technique is the necessity of having the plant material in tissue culture (i.e., *in vitro*), and the time needed for this duplication step in terms of the breeding cycle (CHAPTER 6).

The use of N_2O for treating immature *Limonium* inflorescences for induction of a ploidy increase also induces increments in: pollen size (i.e., plan area), proportion of viable pollen grains, and zygote success (CHAPTER 4 and CHAPTER 5). Additionally it induces other morphological variations of the pollen grains, such as an increase in the number of apertures and shape changes (Siregar, 2021). All the changes identified were more frequently in pollen that matures between 21 days after treatment (DAT), and up to 30 DAT, with some variations between genotypes (CHAPTER 4 and CHAPTER 5).

The rapid detection of potentially unreduced and viable pollen grains could be a useful tool for plant breeders to measure after treatment with N_2O so as to identify the optimum stage of inflorescence development for best pollen viability and higher zygote success, assuming those are most often desired to be increased. As identified previously, and

presented in other sections of the present research, changes in pollen size and/or pollen stainability, using modified Alexander's stain (Peterson et al., 2010), or even morphological changes of pollen, could be potentially used as indicators of the effect of N_2O. However, some disadvantages are still encountered with each method, and adjustments were needed as explained below:

- in the case of pollen size determination, measurement of 100 pollen grains is time demanding;
- for pollen stainability, the use of a stain (e.g., modified Alexander's or tetrazolium) aims to visualize specific compounds or cellular components, which have been demonstrated as associated with pollen viability. Alexander's stain, for instance, detects the presence of cytoplasm, with the absence of colour or a blue-green colouring being representative of the absence of cytoplasm and consequently assumption of pollen abortion. In comparison, the tetrazolium test reveals the presence of dehydrogenases (Shivanna & Rangaswamy, 1992) and, therefore, the ability for oxidative metabolism associated with pollen viability (Hauser & Morrison, 1964). However, stainability does not ensure that pollen will grow and germinate (Impe et al., 2020), as demonstrated in the present research (CHAPTER 7) with pollen stainability and pollen germination not necessarily being related for most genotypes. Besides, in the present research the viability assessment is done visually and not automated (e.g., using Fiji software (Schindelin et al., 2012) or R (R Core Team, 2018; Tello et al., 2018));
- for morphological changes of pollen, no previously published standardized methodology for their evaluation was found for *Limonium* or other crops.

The aim of this Section was to develop a rapid protocol for detection of potentially unreduced, viable pollen, following the use of N_2O on interspecific hybrid plants for restoration of pollen germinability. The hypothesis was that a rapid (one hour-long) protocol could be developed to detect potentially unreduced, viable pollen after treatment.

MATERIALS AND METHODS

Plant material and cultivation

Two interspecific *L. sinuatum* × *L. perezii* hybrids (Y21.011.009 and Y21.010.003) obtained after induction of unreduced gametes with N_2O (Refer to CHAPTER 5) were selected for this experiment, based their synchronous flowering times. One plant from each interspecific hybrid was used in the experiment. The hybrid Y21.011.009 was treated with N_2O as detailed below, while hybrid Y21.010.003 was used as the control (i.e., untreated).

The plants were grown in 1 L plastics pots filled with a commercially sourced growing medium (APPENDIX E), and were maintained under greenhouse conditions (heating at 18 °C, ventilated at 21 °C, 14 h natural light) at The New Zealand Institute for Plant & Food Research Ltd in Palmerston North, New Zealand (40.9006 °S, 174.8860 °E). The growing medium was irrigated daily to kept it at field capacity (more detail in CHAPTER 5).

Nitrous oxide treatment

All unopened florets with the calyces protruding more than 2 mm above the bract were removed from both plants, with the aim of keeping only florets with pollen in the meiotic or earlier stage (refer to Section 1.3.1.2) prior to treating Y21.011.009 with N_2O. N_2O treatment was applied for 24 h at 608 KPa (Nukui et al., 2011) using a pressure-tolerant chamber. (Figure A-6). Immediately following treatment (day after treatment zero (0 DAT)), the plant was returned to the greenhouse where the control plant (Y21.010.003) had remained.

Detection of potentially unreduced pollen

To detect of potentially unreduced pollen, three florets from different inflorescences were collected at anther dehiscence and the pollen from their five stamens pooled and fixed in an ethanol:glycerol fixation solution containing 85% ethanol and 100% glycerol in a 3:1 proportion (Hammer et al., 2015, 2011). The plan area of 20 pollen grains from each hybrid (i.e., Y21.011.009 (treated) and Y21.010.003 (untreated)) was measured every two days following the protocol described by Cordoba-Sanchez et al. (2023a)(refer to Section 2.4.4.1), starting at 20 DAT, and continued until potentially unreduced pollen was detected. To be considered as potentially unreduced pollen, the pollen grain plan area had

to be at least 1.2 times that of untreated (control) pollen, which was deemed representative of the average for those hybrids.

The experimental design used for the detection of unreduced pollen was a completely randomized design, with one factor (treatment) and two levels (i.e., N_2O-treated and untreated; Compton (1994). The 20 pollen grains measured were the replicates.

The sample size for pollen grains was calculated utilising a statistical sample size estimate (p=0.05, 99% power; Champely (2020), and any one sample comprised the pollen derived from three florets from three separate inflorescences.

The response variable, plan area, was analysed with linear models (lm function from the stats package R (R Core Team, 2018)) followed by a Least Significant Difference (LSD; Mendiburu and Yaseen (2020)) test to detect pairwise differences between means. A *p-value* equal to 0.05 was set for the analysis.

Pollen germination

Once occurrence of potentially unreduced pollen was detected, pollen was collected as flowers opened for germination for the next four days. Pollen from three florets from different inflorescences was germinated per sample. Three samples (replicates) were done daily per genotype. Pollen germination was done using the dialysis tubing method modified for *Limonium* (Zhang et al., 1997) and detailed previously in Section 6.2.4.2.3.

The experimental design was a single factor, completely randomized design, with sub-sampling (Compton, 1994). The evaluated factor was the day after treatment (DAT), with five levels: 24 DAT, 25 DAT, 26 DAT, 27 DAT and untreated (control). The replicates corresponded to the samples for germination (three) and the pollen grains observed per sample were the sub-samples. Up to 1000 pollen grains were evaluated per sample, with germination visually assessed and recorded.

The response variable pollen germination (yes/no), was analysed using a generalized linear model (glm function from the stats package (R Core Team 2018)), selecting the quasi-binomial distribution for over-dispersed data (Shoukri & Aleid, 2022). Comparison between the levels of the factor was done using a pairwise likelihood ratio test (similar to LRPAIR in Goedhart (2018)). The *p-value* was set as 0.05 for all the analyses.

RESULTS

Treatment with N_2O of young inflorescences from an interspecific *L. sinuatum* × *L. perezii* hybrid resulted in a pollen germination rate equal to 0.26% (14 out of 5368 pollen grains observed), which was statistically higher than pollen germination for untreated plants (0%; $p<0.05$).

Pollen from the treated plant with a plan area 1.2 times bigger (1465 μm^2 ± 760 μm^2) in comparison with pollen from the untreated plant used as the control (1014 μm^2 ± 231 μm^2), was detected at 25 DAT ($p<0.05$), though an increase in both the mean plan area, and occurrence of four pores on pollen grains, was noted from 24 DAT onwards (Figure A-9).

Pollen germination was subsequently tested with pollen from florets that opened between 24 DAT and 27 DAT, with pollen gemination only observed in florets that opened between 24 DAT and 26 DAT. Pollen germination ranged between 0% and 0.4%, with no differences between 24 DAT, 25 DAT and 26 DAT with a mean germination equal to 0.29% ± 0.17% ($p>0.05$). However, pollen germination decreased at 27 DAT was 0% ($p<0.05$; Figure A-10), the same as had been observed in the untreated control plant.

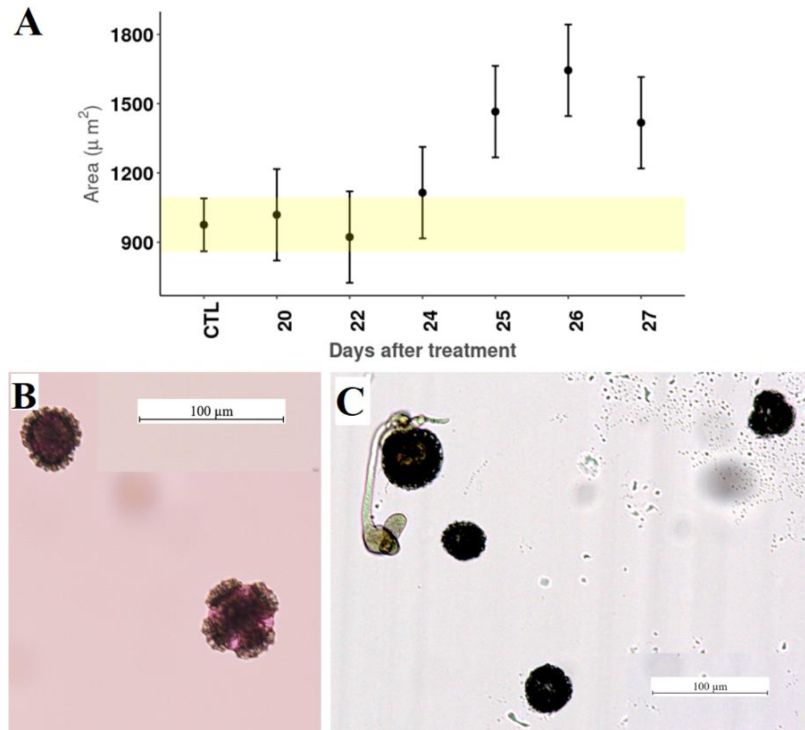

Figure A-9 Interspecific *L. sinuatum* × *L. perezii* hybrid treated with N_2O (Y21.011.009) and Y21.010.003 (untreated control (CTL)). **A.** Comparison of the pollen plan area of CTL (untreated) and treated plants. For treated plants, pollen plan area measurements were taken at different time intervals between treatment with N_2O and pollen release. Dots represent the predicted mean values for the pollen grain area after data modelling using linear models, and the vertical lines represent the 95% confidence interval. The yellow coloured bar represents the confidence interval for the plan area of the pollen grains collected from the control plant. Means with confidence intervals outside the yellow coloured bar are significantly different by the LSD test, at the 95% level of significance. **B.** Pollen collected 24 days after treatment (DAT) stained with modified Alexander's stain (Peterson et al., 2010). Four germination pores are observed in the pollen grain located at right corner. **C.** Germination of pollen grains released 24 DAT and stained with a 0.2% tetrazolium solution prepared in a 20% sucrose solution (Shivanna & Rangaswamy, 1992)

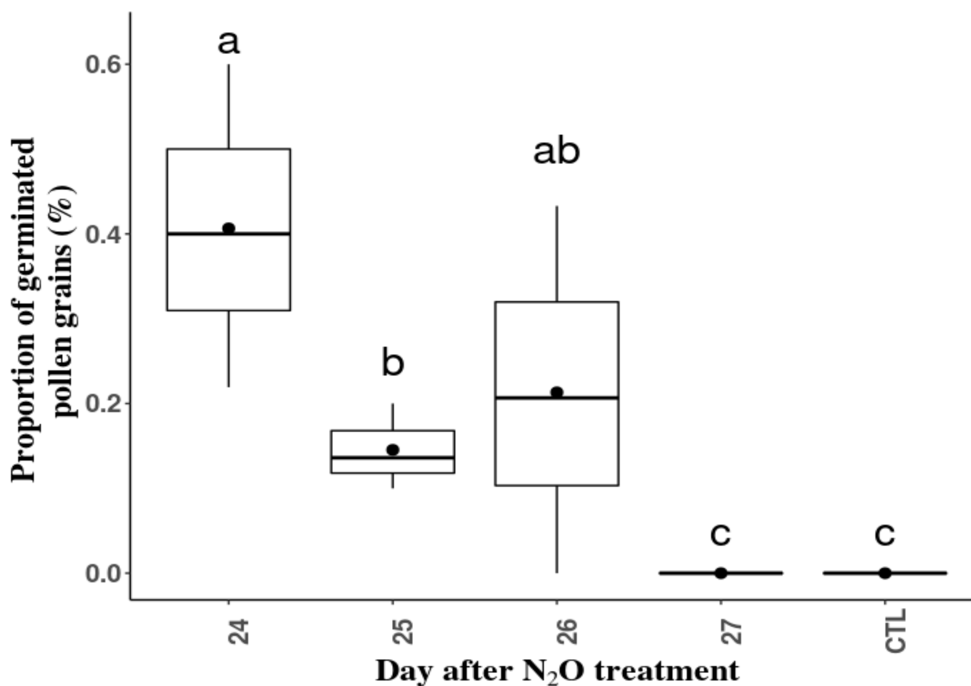

Figure A-10 Germination of pollen of an interspecific *L. sinuatum* × *L. perezii* hybrid treated with nitrous oxide (N_2O) or untreated (CTL). Horizontal lines represent median, box represents interquartile range, whiskers are minimum and maximum value and dots are the mean value. Letters in the figure denote mean groups after pairwise comparison between levels of the factors using a likelihood ratio test, with different letters indicating statistically significant differences ($p = 0.05$). n = 20 pollen grains

The evaluation of pollen size, in order to make a decision as to whether or not *in vitro* germination was required, took around 45 minutes from the time of floret collection through to data analysis of the measured pollen size. A sample size of 20 pollen grains was adequate to identify the occurrence of potentially unreduced pollen. The protocol used was based solely on changes in the plan area of pollen; however, observation of pollen grains with four germination pores was also informative and could be considered as an indication of the effect of N_2O, in combination with changes in pollen size.

DISCUSSION

The positive effect of N_2O over *in vitro* pollen germination observed in the *L. sinuatum* × *L. perezii* interspecific hybrids (Figure A-9), and potentially fertility restoration in pollen grains, has also been reported in *Tulipa* sp. (Okazaki et al., 2005) and *Lilium* (Nukui et al., 2011). The underlying mechanism involved in the increase of interspecific hybridisation in *Limonium* was not studied in this research. It is likely that the restoration

of pollen germination is associated with an increase in pollen viability and the suspected decrease in the occurrence in meiotic abnormalities or number of cells presenting them, as reported with sour cherry (*Prunus cerasus*; Akšić et al. (2016)) and maize (*Zea mays*; Defani-Scoarize et al. (1995)). Additional supporting evidence is provided by previous results on the positive effect of N_2O treatment on the proportion of viable pollen collected from partial- male sterile plants (refer CHAPTER 4), and the observed increase in pollen plan area of the hybrid pollen (Figure A-9). Previous results have also shown that 33% of stained pollen from an untreated *L. sinuatum* plant will germinate using the modified dialysis tubing method so it is expected that the levels of pollen germination observed here under-represent pollen viability.

The biological process involved in the restoration of germination could be similar to the one reported for *Lilium* (Luo et al., 2016; Nukui et al., 2011) where restitution of homologous chromosome pairing leads to normal meiosis in Pollen Mother Cells (PMC), with subsequent development of viable pollen (Barba-Gonzalez et al., 2005; Luo et al., 2016). However, it could also be a simpler mechanism associated with the odd number of chromosomes characteristic of the hybrid (Morgan et al., 1998), subsequently becoming even after treatment, and thereby facilitating the chromosome segregation during meiosis in the PMC (Oleszczuk et al., 2019; Tatum et al., 2015). Within this simpler proposed mechanism, subsequently there is reduction in the occurrence of meiotic abnormalities and an increase in the proportion of pollen with germination potential. Nevertheless, in the case of *Limonium*, cytological studies to understand the underlying mechanism of germination restoration, and evaluation of seed set, are still pending.

The protocol used for rapid evaluation, and decision making about the use of pollen, is a tool to focus the breeding efforts (e.g., planned crossing) on the DAT, confirming the occurrence of potentially unreduced pollen and, therefore, optimising the resources of time and effort. The non-occurrence of *in vitro* germination on 27 DAT is not necessarily associated to pollen inviability, but it could be related with the quite low germination rate observed (<1%). *In vitro* germination for trinucleate pollen such as produced by *Limonium* is normally low (Zhang et al., 1997), but it does not mean the pollen of *Limonium* is not able to germinate on the stigma, and fertilize an egg, under the appropriate conditions (Dafni & Firmage, 2000).

CONCLUSIONS

- Nitrous oxide promoted production of pollen that could be induced to germinate in the *L. sinuatum* × *L. perezii* interspecific hybrid Y21.011.009.

A simple and quick to use (one hour) protocol for detection of potentially unreduced-viable pollen was developed based on pollen size changes.

APPENDIX O. Germplasm diversity produced during the programme of research

Table A-13 Information about the genotypes obtained during this PhD research including parents, breeding strategy and treatment used, ploidy, and species composition

E-Brida code	Seed-bearing genotypes	Pollen-donor genotype	Breeding strategy	Treatment	Ploidy	Species composition
Y19.019.001	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Diploid	*L. sinuatum*
Y19.019.002	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Diploid	*L. sinuatum*
Y19.019.003	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.019.004	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.019.005	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.019.006	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.019.007	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.019.008	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.019.009	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.019.010	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*

Y19.019.011	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.019.012	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.019.013	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.019.014	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.019.015	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.019.017	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.019.018	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.019.019	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.019.020	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.020.001	Y19.015.001	Y19.016.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.020.002	Y19.015.001	Y19.016.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.020.003	Y19.015.001	Y19.016.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.020.004	Y19.015.001	Y19.016.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.020.013	Y19.015.001	Y19.016.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*
Y19.020.014	Y19.015.001	Y19.016.001	Ploidy manipulation after pollination (WGD)	48h-N_2O	Tetraploid	*L. sinuatum*

Y19.026.001	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	48h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y19.026.002	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	48h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y19.026.003	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	48h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y19.026.004	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	48h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y19.026.006	Y19.018.001	Y19.001.001	Ploidy manipulation after pollination (WGD)	Oryzalin-two months	Tetraploid	*L. sinuatum* × *L. perezii*
Y19.026.008	Y19.018.001	Y19.001.001	Ploidy manipulation after pollination (WGD)	Oryzalin-two months	Tetraploid	*L. sinuatum* × *L. perezii*
Y19.026.009	Y19.018.001	Y19.001.001	Ploidy manipulation after pollination (WGD)	Oryzalin-two months	Tetraploid	*L. sinuatum* × *L. perezii*
Y19.026.010	Y19.018.001	Y19.001.001	Ploidy manipulation after pollination (WGD)	Oryzalin-two months	Tetraploid	*L. sinuatum* × *L. perezii*
Y19.026.013	Y19.018.001	Y19.001.001	Ploidy manipulation after pollination (WGD)	Oryzalin-two months	Tetraploid	*L. sinuatum* × *L. perezii*
Y21.001.001	Y19.016.001	Y19.018.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. sinuatum*
Y21.001.002	Y19.016.001	Y19.018.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. sinuatum*
Y21.001.003	Y19.016.001	Y19.018.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. sinuatum*
Y21.001.004	Y19.016.001	Y19.018.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Mixoploid	*L. sinuatum*
Y21.001.005	Y19.016.001	Y19.018.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. sinuatum*
Y21.001.006	Y19.016.001	Y19.018.001		Control	Triploid	*L. sinuatum*

Y21.001.007	Y19.016.001	Y19.018.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. sinuatum*
Y21.002.001	Y19.018.001	Y19.016.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. sinuatum*
Y21.002.002	Y19.018.001	Y19.016.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. sinuatum*
Y21.002.003	Y19.018.001	Y19.016.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Mixoploid	*L. sinuatum*
Y21.003.001	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	Control	Diploid	*L. sinuatum*
Y21.003.002	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	Control	Diploid	*L. sinuatum*
Y21.003.003	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	Control	Diploid	*L. sinuatum*
Y21.003.004	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	Control	Diploid	*L. sinuatum*
Y21.003.005	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	Control	Diploid	*L. sinuatum*
Y21.003.006	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	Control	Diploid	*L. sinuatum*
Y21.003.007	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	Control	Diploid	*L. sinuatum*
Y21.003.008	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	Control	Diploid	*L. sinuatum*
Y21.003.009	Y19.016.001	Y19.015.001	Ploidy manipulation after pollination (WGD)	Control	Diploid	*L. sinuatum*
Y21.004.001	Y18.011.001	Y19.027.003	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. perezii*
Y21.004.002	Y18.011.001	Y19.027.003	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. perezii*

Y21.005.001	Y19.027.003	Y18.011.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. perezii*
Y21.005.002	Y19.027.003	Y18.011.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. perezii*
Y21.005.003	Y19.027.003	Y18.011.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. perezii*
Y21.005.004	Y19.027.003	Y18.011.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. perezii*
Y21.005.005	Y19.027.003	Y18.011.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. perezii*
Y21.005.006	Y19.027.003	Y18.011.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. perezii*
Y21.005.007	Y19.027.003	Y18.011.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Mixoploid	*L. perezii*
Y21.005.008	Y19.027.003	Y18.011.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Mixoploid	*L. perezii*
Y21.005.009	Y19.027.003	Y18.011.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Mixoploid	*L. perezii*
Y21.006.001	Y19.016.001	Y19.018.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Triploid	*L. sinuatum*
Y21.006.002	Y19.016.001	Y19.018.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum*
Y21.006.003	Y19.016.001	Y19.018.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum*
Y21.006.004	Y19.016.001	Y19.018.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Triploid	*L. sinuatum*
Y21.006.005	Y19.016.001	Y19.018.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum*
Y21.007.001	Y19.018.001	Y19.016.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Tetraploid	*L. sinuatum*

Y21.007.002	Y19.018.001	Y19.016.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum*
Y21.008.001	Y19.027.001	Y19.027.002	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. perezii*
Y21.008.002	Y19.027.001	Y19.027.002	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. perezii*
Y21.008.003	Y19.027.001	Y19.027.002	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. perezii*
Y21.008.004	Y19.027.001	Y19.027.002	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. perezii*
Y21.008.005	Y19.027.001	Y19.027.002	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. perezii*
Y21.008.006	Y19.027.001	Y19.027.002	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. perezii*
Y21.008.007	Y19.027.001	Y19.027.002	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. perezii*
Y21.008.008	Y19.027.001	Y19.027.002	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. perezii*
Y21.008.009	Y19.027.001	Y19.027.002	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. perezii*
Y21.008.010	Y19.027.001	Y19.027.002	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. perezii*
Y21.008.011	Y19.027.001	Y19.027.002	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. perezii*
Y21.008.012	Y19.027.001	Y19.027.002	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. perezii*
Y21.008.013	Y19.027.001	Y19.027.002	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. perezii*
Y21.008.014	Y19.027.001	Y19.027.002	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. perezii*

Y21.008.015	Y19.027.001	Y19.027.002	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. perezii*
Y21.009.001	Y19.027.002	Y19.027.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. perezii*
Y21.009.002	Y19.027.002	Y19.027.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. perezii*
Y21.009.003	Y19.027.002	Y19.027.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. perezii*
Y21.009.004	Y19.027.002	Y19.027.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. perezii*
Y21.009.005	Y19.027.002	Y19.027.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Pentaploid	*L. perezii*
Y21.009.006	Y19.027.002	Y19.027.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. perezii*
Y21.009.007	Y19.027.002	Y19.027.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. perezii*
Y21.009.008	Y19.027.002	Y19.027.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. perezii*
Y21.009.009	Y19.027.002	Y19.027.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. perezii*
Y21.009.010	Y19.027.002	Y19.027.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. perezii*
Y21.009.011	Y19.027.002	Y19.027.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Tetraploid	*L. perezii*
Y21.009.012	Y19.027.002	Y19.027.001	Ploidy manipulation after pollination (WGD)	24h-N_2O	Diploid	*L. perezii*
Y21.010.001	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.010.002	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*

Y21.010.003	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.010.004	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.010.005	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.010.006	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.010.007	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.010.008	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.010.009	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.010.010	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.010.011	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.010.012	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.010.013	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.010.014	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.010.015	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.010.016	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.010.017	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*

Y21.010.018	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.010.019	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.010.020	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.001	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.002	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.003	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.004	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.005	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.006	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.007	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.008	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.009	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.010	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.011	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.012	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*

Y21.011.013	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.014	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.015	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.016	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.017	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.018	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.019	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.020	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.021	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.022	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y21.011.023	Y19.018.001	Y19.001.001	Ploidy manipulation before pollination (unreduced gametes)	24h-N_2O	Diploid	*L. sinuatum* × *L. perezii*
Y22.001.001	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	Control	Diploid	*L. sinuatum* × *L. perezii*
Y22.001.002	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	Control	Diploid	*L. sinuatum* × *L. perezii*
Y22.001.003	Y19.016.001	Y19.027.005	Ploidy manipulation before pollination (unreduced gametes)	Control	Diploid	*L. sinuatum* × *L. perezii*

Y22.002.001	Y19.026.009	Y19.018.001	Backcross	Tetraploid hybrid × diploid *L. sinuatum*	Triploid	(*L. sinuatum* × *L. perezii*) × *L. sinuatum*
Y22.002.002	Y19.026.009	Y19.018.001	Backcross	Tetraploid hybrid × diploid *L. sinuatum*	Triploid	(*L. sinuatum* × *L. perezii*) × *L. sinuatum*
Y22.002.003	Y19.026.009	Y19.018.001	Backcross	Tetraploid hybrid × diploid *L. sinuatum*	Triploid	(*L. sinuatum* × *L. perezii*) × *L. sinuatum*
Y22.002.004	Y19.026.009	Y19.018.001	Backcross	Tetraploid hybrid × diploid *L. sinuatum*	Triploid	(*L. sinuatum* × *L. perezii*) × *L. sinuatum*
Y22.002.005	Y19.026.009	Y19.018.001	Backcross	Tetraploid hybrid × diploid *L. sinuatum*	Triploid	(*L. sinuatum* × *L. perezii*) × *L. sinuatum*
Y22.002.006	Y19.026.009	Y19.018.001	Backcross	Tetraploid hybrid × diploid *L. sinuatum*	Triploid	(*L. sinuatum* × *L. perezii*) × *L. sinuatum*
Y22.002.007	Y19.026.009	Y19.018.001	Backcross	Tetraploid hybrid × diploid *L. sinuatum*	Triploid	(*L. sinuatum* × *L. perezii*) × *L. sinuatum*

Y22.002.008	Y19.026.009	Y19.018.001	Backcross	Tetraploid hybrid × diploid *L. sinuatum*	Triploid	(*L. sinuatum* × *L. perezii*) × *L. sinuatum*
Y22.002.009	Y19.026.009	Y19.018.001	Backcross	Tetraploid hybrid × diploid *L. sinuatum*	Triploid	(*L. sinuatum* × *L. perezii*) × *L. sinuatum*
Y22.003.001	Y19.026.006	Y19.018.001	Backcross	Tetraploid hybrid × diploid *L. sinuatum*	Triploid	(*L. sinuatum* × *L. perezii*) × *L. sinuatum*
Y22.003.002	Y19.026.006	Y19.018.001	Backcross	Tetraploid hybrid × diploid *L. sinuatum*	Triploid	(*L. sinuatum* × *L. perezii*) × *L. sinuatum*
Y22.003.003	Y19.026.006	Y19.018.001	Backcross	Tetraploid hybrid × diploid *L. sinuatum*	Triploid	(*L. sinuatum* × *L. perezii*) × *L. sinuatum*
Y22.003.004	Y19.026.006	Y19.018.001	Backcross	Tetraploid hybrid × diploid *L. sinuatum*	Triploid	(*L. sinuatum* × *L. perezii*) × *L. sinuatum*
Y22.003.005	Y19.026.006	Y19.018.001	Backcross	Tetraploid hybrid × diploid *L. sinuatum*	Triploid	(*L. sinuatum* × *L. perezii*) × *L. sinuatum*

Y22.003.006	Y19.026.006	Y19.018.001	Backcross	Tetraploid hybrid × diploid *L. sinuatum*	Triploid	(*L. sinuatum* × *L. perezii*) × *L. sinuatum*
Y22.004.001	Y19.018.001	Y19.026.009	Backcross	diploid *L. sinuatum* × Tetraploid hybrid	Triploid	(*L. sinuatum* × *L. perezii*) × *L. sinuatum*
Y22.008.001	Y21.007.001	Y19.026.006	Backcross	Tetraploid *L. sinuatum* × Tetraploid hybrid	4.5x	*L. sinuatum* × (*L. sinuatum* × *L. perezii*)
Y22.008.002	Y21.007.001	Y19.026.006	Backcross	Tetraploid *L. sinuatum* × Tetraploid hybrid	4.5x	*L. sinuatum* × (*L. sinuatum* × *L. perezii*)
Y22.008.003	Y21.007.001	Y19.026.006	Backcross	Tetraploid *L. sinuatum* × Tetraploid hybrid	4.5x	*L. sinuatum* × (*L. sinuatum* × *L. perezii*)
Y22.008.004	Y21.007.001	Y19.026.006	Backcross	Tetraploid *L. sinuatum* × Tetraploid hybrid	4.5x	*L. sinuatum* × (*L. sinuatum* × *L. perezii*)
Y22.008.005	Y21.007.001	Y19.026.006	Backcross	Tetraploid *L. sinuatum* × Tetraploid hybrid	4.5x	*L. sinuatum* × (*L. sinuatum* × *L. perezii*)

Y22.008.006	Y21.007.001	Y19.026.006	Backcross	Tetraploid *L. sinuatum* × Tetraploid hybrid	4.5x	*L. sinuatum* × (*L. sinuatum* × *L. perezii*)
Y22.008.007	Y21.007.001	Y19.026.006	Backcross	Tetraploid *L. sinuatum* × Tetraploid hybrid	4.5x	*L. sinuatum* × (*L. sinuatum* × *L. perezii*)
Y22.009.001	Y21.009.001	Y21.008.009	Intraspecific cross with tetraploid genotypes	Tetraploid × Tetraploid	Tetraploid	*L. perezii*
Y22.009.002	Y21.009.001	Y21.008.009	Intraspecific cross with tetraploid genotypes	Tetraploid × Tetraploid	Tetraploid	*L. perezii*
Y22.010.001	Y19.019.018	Y21.007.001	Intraspecific cross with tetraploid genotypes	Tetraploid × Tetraploid	Tetraploid	*L. sinuatum*
Y22.011.001	Y21.005.004	Y21.008.010	Intraspecific cross with tetraploid genotypes	Tetraploid × Tetraploid	Tetraploid	*L. perezii*
Y23.003.001	Y19.027.003	Y23.mix2[†]	Backcross-open pollination	Diploid × triploid	undeterminable	*L. perezii × (L. sinuatum × (L. sinuatum × L. perezii))*

[†]The pollen mix includes pollen from: Y22.002.001, Y22.002.002, Y22.002.003, Y22.002.004, Y22.002.005, Y22.002.006, Y22.002.007, Y22.002.009, Y22.003.002, Y22.003.003, Y22.003.004, Y22.003.005, Y22.003.006, Y22.004.001

APPENDIX P. Information collected for the triploid block analysis

INTRODUCTION

The effect of N_2O treatment on production of unreduced gametes (pollen) was demonstrated in CHAPTER 5 and unreduced pollen has been demonstrated to be viable and capable of germination (CHAPTER 4 and APPENDIX N). After pollination, zygotes are obtained through ovule culture. This minimises the impact of endosperm failure, which could occur due to a triploid block. The offspring are expected to consist of a combination of diploid and triploid plants, and possibly tetraploid plants if unreduced male and female gametes fuse.

The proportion of stained unreduced pollen produced after treatment with N_2O varies between 6% and 41% depending on the species and genotype (refer to Section 5.5.2). It was expected that the proportion of unreduced pollen observed equated to the number of polyploid progeny. However, that was not the case. These results suggest unreduced pollen is less fit the reduced pollen (discussed in detail in Section 9.4.2) or the occurrence of the triploid block as a result of an imbalance in the endosperm number has been noted as a possibility in *Limonium* (refer to Section 1.3.7).

The following describes experimental information that was collected and compared to test whether the triploid block hypothesis was supported.

MATERIALS AND METHODS

Information collected

Progeny of the diploid genotypes Y19.016.001 (sin16) and Y19.018.001 (sin18) were selected for this analysis, as during the PhD research they have been used in intra- and inter-specific crosses (refer to CHAPTER 5 and APPENDIX I). Detailed information about the plant growing and experimental conditions can be found in the corresponding Chapter and Appendix. In brief, intraspecific crosses were done in *L. sinuatum*, followed by a 24-hour N_2O treatment (CHAPTER 5). For interspecific crosses between *L. sinuatum* and *L. perezii*, treatment involved either 24-hour or 48-hours treatment with N_2O

(CHAPTER 5 and APPENDIX I respectively). In both intra- and inter-specific crosses, untreated plants were used as control (CHAPTER 5 and APPENDIX I; Table A-14).

Table A-14 Crosses performed in the present PhD research used for analysis of the triploid block hypothesis

Cross	Cross type	N_2O treatment	Number of crosses	Reference
Y19.018.001 × Y19.001.001	Interspecific	48h	449	APPENDIX I
Y19.001.001 × Y19.018.001	Interspecific	48h	482	APPENDIX I
Y19.016.001 × Y19.018.001	Intraspecific	24h	304	CHAPTER 5
Y19.018.001 × Y19.016.001	Intraspecific	24h	310	CHAPTER 5
Y19.016.001 × Y19.027.005	Interspecific	24h	426	CHAPTER 5
Y19.018.001 × Y19.001.001	Interspecific	24h	399	CHAPTER 5
Y19.016.001 × Y19.018.001	Intraspecific	Control	45	CHAPTER 5
Y19.018.001 × Y19.016.001	Intraspecific	Control	45	CHAPTER 5
Y19.016.001 × Y19.027.005	Interspecific	Control	100	CHAPTER 5
Y19.018.001 × Y19.001.001	Interspecific	Control	80	CHAPTER 5

Expected seedlings under an EBN scenario

As explained in Section 1.3.7, the number of expected seedlings after polyploidization could be affected by an imbalance in the endosperm balance number (EBN) in these crosses. If so, the expected number and ploidy of seedlings would depend on the cross success for the cross type under analysis, and the occurrence of an EBN scenario.

Assuming, the proportion of seedlings expected under each scenario equals 25% (4 scenarios were proposed, refer to Section 1.3.7), this response variable was calculated using two equations as follows:

- Equation 1: the proportion of seedlings expected in each scenario and equal to 25% was multiplied by the cross success obtained in the corresponding control crosses, and divided by 100%, with the latter being the proportion of seedlings expected if EBN does not occur in *Limonium*.

Equation 1

$$\frac{25\%\ (proportion\ of\ seedlings\ expected\ in\ EBN\ each\ scenario) \times Cross\ success\ control\ cross}{100\%\ (proportion\ of\ seedlings\ expected\ if\ EBN\ does\ not\ occur)}$$

- Equation 2, the value obtained in Equation **1** was multiply by the number of crosses completed. Then, the obtained number is divided by 100% which represents the total percentage of crosses that could potentially result in seedlings.

Equation 2

$$\frac{Ec\ 1\ value\ \times number\ of\ crosses\ done}{100\%\ (proportion\ representing\ the\ total\ number\ of\ crosses)}$$

As a numerical example, refer Equation 3 and Equation 4 using data from Table A-12,

Equation 3

$$\frac{25\%\ \times 2.7\%}{100\%} = 0.675\%$$

Equation 4

$$\frac{0.675\% \ \times 931\ crosses}{100\%} = 17\ crosses\ expected\ to\ result\ in\ seedlings$$

Data analysis

The response variable 'number of expected seedlings' under an EBN scenario, was analysed using a chi square test, to compare the number of seedlings obtained, to the expected values in a EBN scenario. Expected values equal to zero (i.e., expected triploid seedlings) were removed for the chi square test, so as to be able to perform the analysis. The *p-value* was set at 0.05.

RESULTS

Number of expected seedlings under an EBN scenario

For plants treated for 24 h with N_2O, and used either in an intraspecific or interspecific cross, the number of diploid seedlings was between 200% and 800% higher in comparison with that expected under an EBN scenario (Table A-15). However, the number of diploids obtained after the 48 h treatment, and number of tetraploids for all the crosses and N_2O treatments, were between 44% and 100% lower than the expected under EBN scenarios. The number of diploid and tetraploid seedlings observed was significantly different from that expected (p=2.54^{e-226}).

Table A-15 Data about the seedlings obtained after induction of unreduced gametes in *Limonium* sp. by time of exposure to nitrous oxide (N_2O) (24 or 48 hours) and cross type (inter- or intra-specific). Information about the total number of crosses completed for each duration of exposure and cross type; number of diploid, triploid or tetraploid seedlings obtained; and percentage out of the number of crosses completed that they represent is also presented. In addition, the number of seedlings expected under each EBN scenario are shown

Crosses completed	Cross type	N_2O treatment	Diploid seedlings obtained (proportion of the crosses completed)	Diploid expected seedlings. Scenario 1[†]	Triploid seedlings obtained (proportion of the crosses completed)	Triploid expected seedlings. Scenarios 2[‡] &3[§]	Tetraploid seedlings obtained (proportion of the crosses completed)	Tetraploid expected seedlings. Scenario 4[¶]
931	Interspecific	48h	4 (0.43%)	6	0	0	0	6
614	Intraspecific	24h	403 (65.6%)	125	1 (0.2%)	0	1 (0.2%)	125
825	Interspecific	24h	44 (5.2%)	5	0	0	0	5
90	Intraspecific	Control	74 (82%)	90	0	0	0	0
180	Interspecific	Control	5 (2.7%)	4 (2%[¥])	0	0	0	0

[†] Scenario 1 refers to unchanged ploidy of the gametes. EBN proportion will be 2:1

[‡] Scenario 2 is when unreduced egg is fertilized by reduce pollen. EBN proportion will be 4:1. Zygotes would be lost

[§] Scenario 3 refers to reduced egg fertilized by unreduced pollen. EBN proportion is 2:2. Zygote unviable

[¶] Scenario 4 unreduced egg is fertilized by unreduced pollen. EBN proportion 4:2. Zygotes could be lost

[¥] 2% is the hybridization rate reported for crosses between *L. sinuatum* and *L. perezii* (Morgan et al., 1998)

APPENDIX Q. Synonyms for the genotype names of *Limonium* used

Table A-16 Name of the genotypes of *Limonium* used in the thesis, corresponding E-Brida code†, and synonyms used in publications derived from this PhD research

Name in the thesis	E-Brida code†	Published name	Species	Reference
Y19.015.001	Y19.015.001	sin15	*L. sinuatum*	CHAPTER 2, CHAPTER 3, CHAPTER 7, APPENDIX F, APPENDIX I
Y19.016.001	Y19.016.001	sin16	*L. sinuatum*	CHAPTER 2, CHAPTER 4, CHAPTER 5, CHAPTER 7, APPENDIX F, APPENDIX I
Y19.018.001	Y19.018.001	sin18	*L. sinuatum*	CHAPTER 2, CHAPTER 4, CHAPTER 5, APPENDIX I
Y19.001.001	Y19.001.001	per11	*L. perezii*	CHAPTER 2, CHAPTER 4, CHAPTER 5, APPENDIX I
Y19.027.003	Y19.027.003	per273	*L. perezii*	CHAPTER 2
Y19.027.005	Y19.027.005	per275	*L. perezii*	CHAPTER 4, CHAPTER 5
siNZii™ Deep Lavender	06-LPS-2002	siNZii™ Deep Lavender	*L sinuatum* × *L. perezii*	BallSB (2021)

siNZii™ Deep Lavender	06-LPS-2002	siNZii™ Lavenderish	*L sinuatum* × *L. perezii*	Ball Horticultural do Brasil (2023)

†Identification number used by The New Zealand Institute for Plant & Food Research Ltd.

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