# Initial Exploration of Unreduced Gamete (2n Gamete) Pollen Development Through Nitrous Oxide ($N_2O$) Application in *Limonium sinuatum*

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# ABSTRACT

**Key word** Meiosis, Unreduced gamete, Nitrous oxide.

Hybridisation and polyploidisation are techniques that can rapidly generate proprietary diversity in plant breeding programs. *Limonium sinuatum* (2n = 16) is diploid, with polyploid plants being of potential value for breeding and commercial applications. Although somatic polyploidisation has been reported in *Limonium*, sexual polyploidisation potentially offers scope to speed up hybridisation and breeding. Sexual polyploidisation results in increased ploidy level via formation of unreduced (2n) gametes. Unreduced gametes are produced naturally but usually at levels too low to be of practical use, and production can be dependent on genotype and environmental conditions. Practically useful levels of unreduced gametes can be artificially induced by judicious timing of treatment with nitrous oxide ($N_2O$). Flower buds at the onset of meiosis are suggested as the best stage to treat with $N_2O$. However, there is no information available on when meiosis occurs in relation to flower development, nor what the optimum conditions of treatment might be in *L. sinuatum.* The aims of this study were to identify the stage of flower development when meiosis occurs and then to test the effect of $N_2O$ treatment on 2n gamete formation before investigating the potential of 2n gametes in direct hybridisation. The results showed that meiosis across the raceme is asynchronous and occurs in very small flowers (0.8 mm in diameter) on each array of a spike in raceme. Additionally, meiotic division occurred from just after 6 AM., reaching its peak between 7and 8 AM. (sunrise at ca 6.15 AM). $N_2O$ treatments (600 Kpa) of 24 hours and 48 hours durations gave significant increases in formation of 2n gametes as indicated by a wider size range or bimodal pollen grain size distribution of pollen. Though legitimate combinations were used in crosses using 2n pollen to a diploid female plant did not result in polyploid progeny. The most likely reason for this is that a triploid block mechanism operates preventing normal embryo development.

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# TABLE OF CONTENT

# LIST OF FIGURES

# LIST OF TABLES

# CHAPTER 1

# LITERATURE REVIEW

To support the aim and objectives of this research thesis, within this first chapter there is initially a brief descriptive overview of the research focus, i.e. unreduced (2n) gamete formation in plants. A subsequent section then focuses on plant used in this thesis, i.e. plants of the *Limonium* genus, including its reproductive biology and the general objective of the current *Limonium* breeding program of which this research is part. A more detailed review is then presented of the mechanism of 2n gamete formation and the related cytology, and morphology of flowers and pollen development in other species. Finally, the mechanism of sexual polyploidisation that leads to polyploidy, is reviewed using examples from several species.

## 1.1. Introduction to central research focus

Polyploidy refers to the presence of two or more complete sets of chromosomes per cell nucleus (Acquaah, 2012; Sattler et al. 2016). The effects of a plant having extra chromosome sets may include improved yield, quality, and more resistance to biotic and abiotic stresses (Alix et al. 2017; Ríos, 2015; Salma, Kundu, & Mandal, 2017). It may also act to mask deleterious alleles (Comai, 2005; Soltis & Soltis, 2000). There is abundant evidence that spontaneous chromosome doubling can occur (Acquaah, 2012; Ríos, 2015; Sattler et al., 2016) and is heritable (Ramsey & Schemske, 1998) triggering studies to understand more about its natural occurrence, with a view to adapting it for the benefit of plant breeding. Polyploidy is important in a number of crops such as wheat, cotton, and potato etc., (Acquaah, 2012; Carputo, Frusciante, & Peloquin, 2003; Dewitte, Van Laere, & Van Huylenbroeck, 2012; Sattler et al., 2016), with breeders taking advantage of the natural occurrence of polyploidy arising from one of their parental lines and sexual hybridisation to generate improved seedlings. As explored within this thesis, polyploidy, therefore has the potential to be used further in plant breeding.

Polyploidy in nature may arise though sexual polyploidisation that begins with the formation of unreduced gametes. The unreduced gamete (2n gamete), is the product of an

abnormal meiotic cell division that results in the chromosomes not separating (Hermsen, 1984; Younis, Hwang, & Lim, 2014). This mechanism is believed to be behind the formation of most polyploids in nature and is a significant factor in the speciation of plants (Soltis & Soltis, 2009). Polyploidy through spontaneous 2n gamete formation is evident in many plant species (Carputo, Barone, & Frusciante, 2000; Ramanna, Kuipers, & Jacobsen, 2003; Ramsey, 2007; Van Laere, Dewitte, Van Huylenbroeck, & Van Bockstaele, 2009), with its rate of occurrence known to occur at a low frequency (Otto & Whitton, 2000; Wood et al., 2009). Within the context of plant breeding, the use of 2n gametes offers an advantage over polyploidy from somatic doubling of chromosomes in terms of the level of heterozygosity. Approaches used to increase the frequency of spontaneous 2n gamete formation within a breeding program can include selection of genotypes known to produce higher frequencies of 2n gametes. However, a relatively low frequency of spontaneous 2n gamete formation occurs in most species. Additionally the formation of 2n gametes may not be consistent even within a flower making it laborious to screen for the potential parent plants, and presenting a major obstacle to the selection of plants to be used in a plant breeding program (Younis et al., 2014). As explored within this thesis therefore, identification of efficient methods for stimulating 2n gamete formation at a sufficiently high frequency is needed if 2n gamete formation is to be adopted as part of a practical plant breeding program.

Approaches for achieving 2n gamete formation at high frequency are through physical treatment such as manipulation of the external environment, or chemical induction through the application of chemical agents such as colchicine, trifluralin and nitrous oxide ($N_2O$) (Akutsu et al., 2007; Crespel et al. 2015; Dewitte et al. 2010; Kato & Birchler, 2006; Okazaki et al., 2005; Okazaki, Nukui, & Ootuka, 2012; Wang et al. 2017; Wenting et al., 2019; Yang et al., 2016). These chemical treatments all result in 2n gamete formation by interfering with spindle thread formation during meiotic cell division. Use of nitrous oxide offers advantages compared to the other two chemical treatments as it is more easily used, is effective for treating target cells that are covered by layers of tissue inside the floral organs and is less hazardous both to the environment and the user. As we gain more understanding of the underlying mechanisms of how 2n gamete formation occurs in nature (Dewitte et al., 2012; Ramsey & Schemske, 1998), we are better able to apply this knowledge to artificially induce high frequencies of 2n gamete formation.

Key to the success of artificial induction of 2n gamete formation at a high frequency is identifying the best time of treatment. The highest frequency of 2n gamete formation will be achieved when the agent is applied to the right cells and targeting an early phase of meiotic cell division (Dewitte et al., 2012 ; De Storme, Van Labeke, & Geelen, 2007; Younis et al., 2014). For example, in lily and tulips, exposing flower buds targeting the pollen gamete cell to $N_2O$ for 24 hours during the period where the meiotic cells progress from prophase to metaphase I, resulted in increased frequency of 2n gamete formation (Akutsu et al., 2007; Okazaki et al., 2005; Okazaki et al., 2012). Successful treatment is commonly indicated by large pollen grains and, when used to generate a cross, polyploid progeny (Dewitte et al., 2012). Thus for the plant species in question the initial cytological study describing the phases of meiotic cell division and microsporogenesis is an important key research step for increasing the frequency of 2n gamete production. Given the duration of meiosis is known to be species and genotype dependent (Bennett, 1977), and in the absence of published literature pertaining to the research plant species in question, i.e. *Limonium sinuatum*, determining the timing of these developmental phases was a critical first step.

As noted above there is a general lack of published research on *Limonium* describing any detail of meiosis and microsporogenesis. Prior to the current investigations, the effect of $N_2O$ treatment in *Limonium* had not previously been reported. In this study *Limonium sinuatum* was selected to study 2n pollen formation via $N_2O$ treatment as a representative *Limonium* species because it is a diploid species, it grows relatively fast and can be induced to produce flowers all year around.

## 1.2. Aim of this study

The aim of this research was to use knowledge of the developmental phases of meiosis and microsporogenesis to promote 2n gamete (pollen) formation in *Limonium sinuatum*

via $N_2O$ treatment, and testing the efficacy of the resultant 2n pollen in crossing experiments, for obtaining polyploid plants.

## 1.3. The objectives of this study

To achieve the aim of this study, the research objectives were:

1. To describe the phases of microsporogenesis and pollen development in *L. sinuatum* by cytological study,
2. To identify the stage of floret development when meiotic cell division is in occurring in inflorescences of *L. sinuatum*, by cytological study and morphological flower observations,
3. To determine the time frame and duration of meiotic cell division in *L. sinuatum* by cytological observation,
4. To determine the comparative effect of two different $N_2O$ treatments on the frequency of 2n pollen formation in *L. sinuatum*,
5. To determine if 2n pollen resulting from $N_2O$ treatment is functional and can be used in crossing experiments to generate polyploid seedlings.

## 1.4. Introduction to the *Limonium* genus.

*Limonium* Mill. (Plumbaginaceae) commonly called statice, is well known as an ornamental plant commodity and is an example of a cosmopolitan genus with high diversification (Baker, 1953). The original habitat of this genus is in coastal areas of tropical and temperate zones, spread through Europe, North America and South America, Asia, Africa and Australia (Baker, 1948, 1953b). The number of species is estimated to be between 150 (Baker, 1953b) and 400 (Khan, Santpere, & Traveset, 2012), and it is still being revised (Palacios et al., 2000). There are various growth habit and growth cycles among the different species, from annuals (e.*g., L. lobatum, L. sinuatum ssp. L. beaumieranum*), to perennial herbs (*L. sinuatum ssp. Sinuatum*) and woody shrubs (*L. arborescens*) (Karis, 2004).

The growth habit and morphology of the leaf, flower and inflorescence in some *Limonium* species has been well documented (Artelari & Georgiou, 2002; Georgakopoulou, et al. 2006; Karis, 2004). In brief, the leaf can be entire, or lobe shaped in different species, and leaves are arranged in rosette formation with growth initiated from the base of the stem (Karis, 2004). As the plant reaches the generative phase it produces a number of inflorescences as a corymb panicle or spike, varying in form dependent on the species. The inflorescence comprises a dense group of individual small florets (Artelari & Georgiou, 2002). In an individual floret, there usually will be a scale leaf (i.e. bract), the long-lasting five-lobed coloured calyx, five petals, five stamens and five stigmata (Artelari & Georgiou, 2002). A wide range of calyx colours are known to exist within the *Limonium* genus, but they are predominantly from pink or violet to purple in colour, while in some species for example in *L. sinuatum* the colour is extended from red to blue, with yellow derived from *L. aureum* (Morgan & Funnell, 2018).

## 1.5. *Limonium* floral biology.

*Limonium* are reported to be either apomictic or sexual species (Baker, 1948, 1953a, 1966),), being distinguished by their ploidy, their self-incompatibility (SI) system, and the heritability characteristic during their meiotic pairing of homologous chromosomes (Baker, 1953a, 1966; Cowan, Ingrouille, & Lledó, 1998). For example, apomictic species are known to possess a higher ploidy, mostly triploid, with sterility resulting from abnormal meiosis, with an example being *L. cosyrense* (2n =3x =27). Some *Limonium* species categorised as apomictic are grouped in three subsections of section *Limonium* (Baker, 1953a).

Some *Limonium* species that are categorised as sexually derived are known to have heteromorphic sporophytic self-incompatibility (SI )(Baker, 1966). In brief, this describes a species with dimorphic stigmata (Cob/Papillate) and pollen (A, wide reticulum/B, narrow reticulum pollen types). In these species, an individual plant with papillate stigmata produces type B pollen, while an individual with a cob stigmata produces type A pollen. Successful pollination can only occur when type A pollen lands on papillate stigmata or type B pollen on cob stigmata (Baker, 1948). Thus, the success of pollination is dependent on the pairing combination between pollen and stigma. *L. sinuatum* exhibits such dimorphism.

There are various ploidy levels among *Limonium* species, as well as various basic chromosome numbers (Palop-Esteban et al., 2011). In the majority of *Limonium* species two major basic chromosome numbers have been identified; they are $X_1$= 9 ($2n = 2X_1 =18$) and $X_2 = 8$ ($2n = 2X_2 = 16$) (Castro & Rossello, 2007; Erben, 1979). Some authors have proposed to extend this to include X= 6 or 7 in some species (Darlington & Wylie, 1956). Triploid species in the genus are believed to have arisen through the formation of unreduced gametes, via hybridisation (allopolyploids) between reduced and unreduced gametes resulting in various configurations in the number of chromosome among triploid plants ( $2X_2 +X_1= 25$; $X_2+2X_1 =26$) (Palop-Esteban et al., 2011; Róis et al., 2016). Higher ploidy taxa also exist including tetraploids, pentaploids and hexaploids, though they have been found less frequently than triploids (Erben, 1979). The tetraploid, is the second most common polyploid level to be found after triploidy and it is believed that unreduced gametes are the source of these plants ($2n = 2X_1 +2X_2= 34$) (Castro & Rossello, 2007).

## 1.6. The breeding strategy in *Limonium*.

In *Limonium* breeding, hybridisation has been utilised since the early 1990s (Harada, 1992) and continues. Several hybrids that are well known in the market, such as 'Emile', 'Misty Blue', and 'Beltlaard' were derived from systematic hybridisation programs (Burchi et al., 2006). The strategy in hybridisation has mostly been through interspecific crossing, by utilising a relatively limited number of parental lines that are closely related, to produce a variety of new plant forms. Hybrids have been selected according to criteria such as floral stem branching, length of stem, size of flower, colour variation, etc. (Burge et al., 1995). In addition, agronomic features such as year round production, heat tolerance, cold tolerance, disease resistance and good postharvest life are targeted by breeders (Burge et al., 1995).

The aim of *Limonium* breeding, like in most breeding programs for ornamental crops is to introduce novelty into species already established in the market. One way to introduce novelty and diversity is to introgress traits from related species with the desired traits (Burchi et al., 2006; Burge et al., 1995; Morgan, et al., 2001; Morgan et al., 1998).

The challenges encountered with interspecific hybridisation, especially those involving relatively distant species can include pre- and post-pollination barriers such as failure of fertilisation following pollination, embryo abortion and infertility in any resulting hybrids (Morgan & Funnell, 2018). To overcome this issue, hybridisation can be accompanied with biotechnology tools such as embryo culture and ploidy manipulation to generate hybrid plants that might not otherwise be produced (Morgan & Funnell, 2018).

An example of combining biotechnology tools and conventional breeding is exemplified in the introduction of *Limonium* 'Sinzii'™, a series of cultivars bred by the New Zealand Institute for Plant and Food Research. Sinzii has been developed from crosses between *L. perezii* and *L. sinuatum*, which under normal circumstances results in no viable seed. Embryos within an enlarged ovule were grown *in vitro* to produce the new plant (Morgan et al., 1998). Subsequent somatic chromosome doubling via application of oryzalin to *in vitro* plants resulted in fertile tetraploid plants being generated. Such fertile plants have then been used in a back-crossing program, resulting in an array of novel plants with new flower colours, branching habits, and stem lengths. While this protocol has seen novel hybrids introduced to the market place, the protocol requires several steps and possibly takes a relatively longer time than needed to achieve novel plants. As explored below, the question remains as how best to speed up this process of introducing novelty into a breeding program.

In recent years introducing polyploidy via 2n gametes has been reported for many species (Lai et al., 2015; Lucidos et al., 2012; S. X. Zheng et al., 2017; Zhong et al., 2010). Doubling the chromosome number of a meiotic cell artificially is already demonstrated in several plant species yielding unreduced gametes (2n) at relatively high frequencies (Okazaki et al., 2005; Okazaki et al., 2012). Several studies have also shown the utilisation of these 2n gametes within breeding programs to produce polyploid plants in lily, tulip, poplar, and cassava (Guo, et al., 2017; Lai et al., 2015; Okazaki et al., 2005; Qu et al., 2019).

A distinct advantage of this method over somatic polyploidy is that intergenomic recombination between alien chromosomes can occur generating higher levels of heterozygosity, and thereby generating more variation in the resulting progeny (Sattler et al., 2016; Younis et al., 2014). Additionally, doubling the chromosomes of the meiotic

cell can restore fertility as exemplified in sterile begonia (Dewitte et al., 2010; Dewitte et al., 2012). In some cases triploid plants can also be obtained in the one step of fertilisation utilising 2n gametes in the crossing program (Ramsey & Schemske, 1998). Defining a straight forward methodology that would potentially be useful within a crossing program, where chromosome number is doubled, and the degree of heterozygosity in progeny is increased all as part of the normal crossing program offers considerable advantages to breeders.

Though some success with 2n gamete formation has been described in several species, no published information is evident describing this approach for polyploidy induction in *Limonium*. This therefore presents the opportunity to use *L. sinuatum* as a model plant, amongst other *Limonium* species, to study the induction of unreduced gametes.

## 1.7. The Inflorescence and floral development

The inflorescence, a floral shoot which includes the axial and the floral parts, has many functions, wherein during flower and fruit development they provide nutrients to the developing flowers and fruits prior to seed dispersal (Kirchoff & Claßen-Bockhoff, 2013). Microsporogenesis occurs in the anther of the flower with the processes of floral development and microsporogenesis to megasporogenesis being tightly linked (Koltunow et al., 1990; Scott et al., 1991). As it is possible to macroscopically identify the stage(s) of flower development that is undergoing microsporogenesis within this dynamic process, application of this knowledge requires an initial understanding of the structure of the inflorescences itself.

In a flowering plant, the inflorescence is characteristic of the species (Benlloch et al. 2007). There are various forms and structure of inflorescences that range from simple to extremely complex. It can consist only of a single main stem or as a multi-branch system, which forms that the characteristic inflorescence shape of the species (Figure.1). Generally, the more complex the structure of the inflorescence of a species, the more additional parts or modified structures may be present in the inflorescence, and vice versa (Weberling, 1992).

In general the parts of an inflorescence will comprise; a stem that holds the whole inflorescence called the peduncle; the major axis holding the flower or more branches

within the inflorescence is called the rachis. The stem/stalk of each single flower is called a pedicel, and its stalk referred to as a peduncle. Any small flower in an inflorescence may be referred to as a floret. A bract is a modified leaf formed at the node where the main stem is located within an inflorescence. The floret itself consists of the four main parts of sepal or calyx, petal, gynoecium and androecium.

Figure 1. Schematic representation of panicle inflorescence types exemplified by, a) panicle inflorescence in rice, b) panicle inflorescence in wheat; rachis (1), primary branch (2), secondary branch (3), tertiary branch (4), and rachilla (5). Figure adapted from Y. Zhang et al. (2014) and Koppolu and Schnurbusch (2019).

In the context of studying microsporogenesis relevant to 2n gamete formation, the initial step for identifying the stage of floral development within the inflorescence undergoing meiosis can best be done through first studying flower development within the inflorescence. For example, in the study of microsporogenesis in *Eucalyptus*, one approach to tracking floral developmental stages was by following the changes in diameter of the flower (Yang et al., 2016). In doing so the authors were able to successfully identify by size the developing flowers in which meiosis was occurring.

In some cases where tracking of both meiosis and floral development was undertaken, additional parameters can be added to better assist in accurately identifying the stage of floret development that is in which meiosis is occurring. For example, in rubber tree and ginseng, besides using floral diameter, colour change in the flower was shown to also be useful for identifying flowers undergoing meiosis (Silva et al., 2017; Yao et al., 2017).

From these various results, it can be concluded that the most effective parameters for observation need to be adjusted according to the species under investigation.

Within the literature there is one study that describes the process of inflorescence development for the purpose of providing basic information for androgenic culture in *Limonium*, and that was for the hybrid 'Misty Blue' (Topoonyanont et al., 2000). Through cytological and morphological observation, the authors determined the growth pattern from the beginning of the process of inflorescence initiation through to the fully expanded inflorescence. Two main growth patterns were identified during inflorescence development, comprising acropetal growth during the formation of the main stem and formation of the branches, as well as centrifugal growth arising after the formation of the spike (floret) was initiated. These differences in growth pattern showed these dynamics of flower development to be a characteristic of species that produce complex inflorescence structure (Benlloch et al., 2007). While the developmental stage of floral tissue that is appropriate for androgenic culture was determined, no detailed information was provided with regard to meiosis and microsporogenesis.

## 1.8. Microsporogenesis and the formation of 2n pollen

Microsporogenesis is a unique process in the various reproductive processes in plants (Halbritter et al., 2018). Normally, as the plant enters the generative phase of growth, the process of microsporogenesis will start when the pollen mother cell (PMC) undergoes division to form two round cells, terminating in the tetrad formation, called meiosis cell division. There are four main landmark stages that are known and described with most plant species; initiation of DNA replication (G/S2), reduction division, equatorial division, and ultimately the formation of a tetrad of meiocytes (Figure 2; Harrison, Alvey, & Henderson, 2010; Heslop-Harrison, 2013; Prusicki et al., 2019)

The process of meiosis can generally be divided into nine phases: prophase I, metaphase I, anaphase I, telophase I, interkinesis/cytokinesis, prophase II, metaphase II, anaphase II and telophase II. In prophase I commonly several sub-phases are observed; leptotene, zygotene, pachytene, diplotene and diakinesis. The important event of crossing over occurs during the progression from pachytene to diplotene, known as the process that

leads to diversity (Shi, 2014). The whole process of microsporogenesis is conserved in most species of plants, but the duration of meiosis varies among plant species (Bennett, 1971; Bennett, 1977). For example, in olive (*Olea europaea*), the process of meiosis is completed in 170 days (Fernandez & Rodríguez-García, 1988), but in *Petunia ×hybrida* is completed in less than 18 hours (Bennett, 1977).

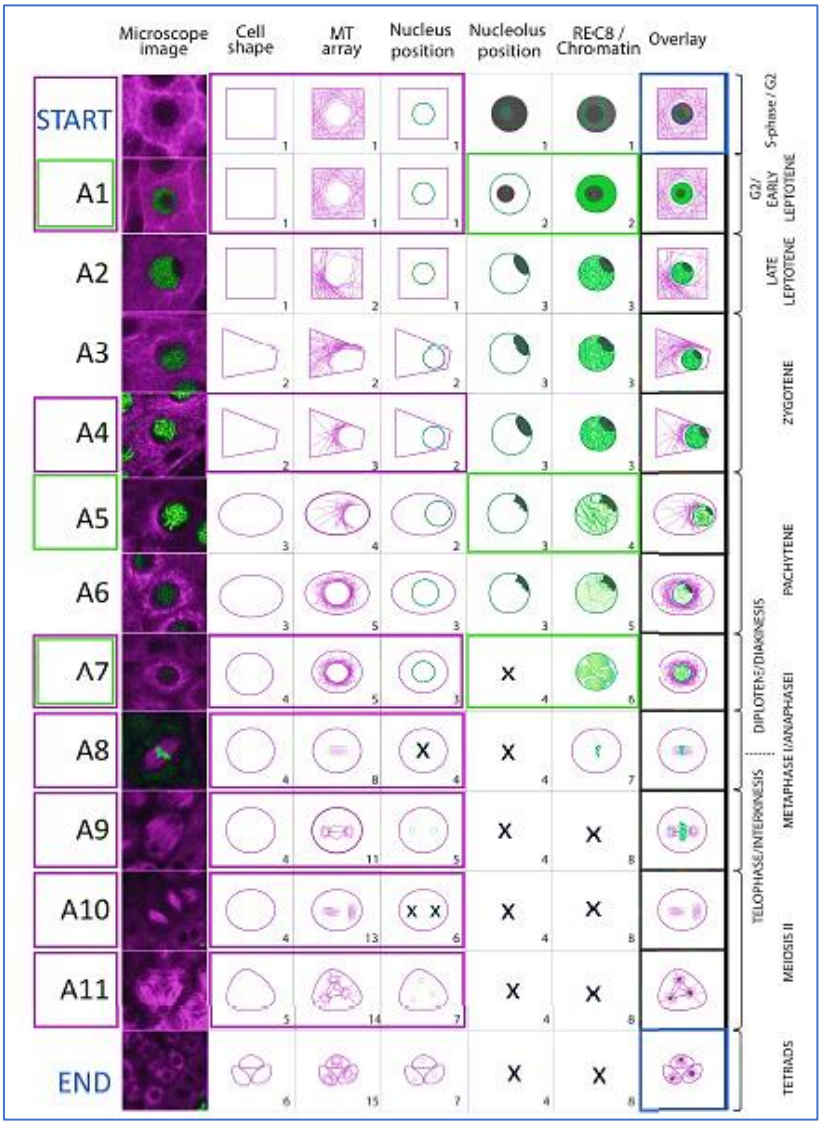


Figure 2. Schematic representation of all stages of meiotic cell division from the Pollen Mother Cell (PMC) entering early meiosis (A1) to the end of Meiosis II (A11) when tetrads are observed based on observations in Arabidopsis thaliana. Figure extracted from Prusicki et al. (2019).

Spontaneous 2n male gamete formation is known to arise due to abnormal meiosis. Cytologically, two main pathways or mechanisms are known that may lead to 2n gamete formation; namely first division restitution (FDR) and second division restitution (SDR; Figure 3). In FDR, pairing and segregation of homologous chromosomes is absent during meiosis I, or it may occur but in very low frequency (Tang & Luo, 2002; Xiao et al., 2007), while separation of sister chromatids proceeds as in normal second meiotic division (Hermsen, 1984). Whereas in SDR, the chromosome pairing and division are normal during meiosis I, but the sister chromatids fail to move towards the poles in meiosis II (Britagnolle & Thompson, 1995; Ramanna & Jacobsen, 2003). Both these abnormalities will generate a somatic chromosome number in the reproductive cell and, instead of forming the tetrad, it mostly results in dyad or tryad microspores (Younis et al., 2014).

Each mechanism of meiotic restitution has its own genetic consequences in terms of the level of heterozygosity in resulting progeny. The FDR will result a level of heterozygosity similar to the parent, except for the crossing over fragment, resulting in higher heterozygosity due to the separation of sister chromatids with the microspore containing a mixture of non-sister chromatid. The SDR results in a lower level heterozygosity compared to FDR, as it contains chromosomes of only sister chromatids (Britagnolle & Thompson, 1995; Wu et al., 2011). Besides FDR and SDR, the formation of 2n gametes can also occur due to different meiotic aberrations including, pre-meiotic doubling, anomalous chromosome pairing, exclusion of any phase during meiotic division, synaptic mutation, abnormal disoriented spindle formation, and abnormal premature cytokinesis (Dewitte et al., 2010; Douches & Quiros, 1988; Taschetto & Pagliarini, 2003).

Figure 3. The mechanism of the restitution process leading to 2n gamete formation through abnormal meiotic division in plants. FDR = First Division Restitution; SDR = Second division Restitution; PMC = Pollen Mother Cell; MPI -API-CKI = First Meiotic division; MPII-APII-CKII = Second Meiotic Division. Figure is extracted from Younis et al. (2014).

One effective approach to increase the 2n gamete frequency would therefore be through adapting the process in one of the known underlying mechanisms of 2n gamete formation. In several crop species various treatments have been used to disrupt meiotic cell division at the early stages, adapting the FDR mechanism for 2n pollen formation, and yielding

various frequencies of 2n formation (Luo et al., 2016; Wenting et al., 2019; Xin-zhong & Guo-jian, 1998; Yang et al., 2016). In tulip and lily treating the flower bud with $N_2O$ during the PMC stage while in progression to metaphase I-Prophase I, was shown to be effective to increase the frequency of 2n gamete formation by between 20% to 60% (Okazaki et al., 2005; Okazaki et al., 2012). With roses artificial induction using heat treatment at metaphase I resulted in a 24.5% increase in 2n pollen (Dewitte et al., 2012). Interestingly, treatment of Arabidopsis with nitrous oxide that was applied during second round division, adopting the SDR mechanism was also able to increase 2n gamete frequency, though no polyploid progeny was obtained from this study (De Storme et al., 2007).

Another factor that influences the success rate of increasing the frequency of 2n gametes, is synchrony or asynchrony of meiotic cell division in a species. Synchronous cell division is evident by the observation that most PMC undergo meiotic cell division at the same time, or cytologically all cells will be observed to be at the same meiotic stage. Whereas if of the asynchronous type, multiple meiotic stages will be observed among PMC. For example, in tulip and lily, development of microsporogenesis among PMC within a flower is relatively synchronous with more than 80% of cells observed to be in the same phase (Akutsu et al., 2007; Okazaki et al., 2012). Not surprisingly therefore, treatment with nitrous oxide was able to generate a 50% increase in frequency of large pollen, whereas only 20% were obtained in plants with asynchronous development such as in poplar and rubber tree (Wang et al., 2017; Yao et al., 2017; Y.-y. Zhang et al., 2019). The process of asynchronous and synchronous development of microsporogenesis is likely to be related to mechanisms of reproductive survival in a species by increasing the success of pollination (Wang et al., 2017; Wyatt, 1982).

## 1.9. Sexual polyploidisation through exploitation of 2n gametes by nitrous oxide ($N_2O$).

To achieve genetically based plant improvement in a population that has narrow variation, polyploidisation may offer an alternate solution for widening its genetic diversity (Ríos, 2015). The extra chromosomes contained in polyploid plants can provide advantages, by elevating the level of heterozygosity, by inducing fertility in sterile species, giving effect to heterosis, and masking the deleterious alleles that cause inbreeding depression (Comai, 2005; Soltis & Soltis, 2000; Younis et al., 2014). Polyploidy is commonly induced through somatic chromosome doubling, however it is known that somatic polyploidisation has a limitation in terms of the level of heterozygosity, and can produce chimeric or mixoploid plants (Morgan & Funnell, 2018; Nassar, 2004). While sexual polyploidisation via 2n gamete formation has been recognised for its ability to generate polyploidy, it also results in greater variability, no ploidy chimeras, and a high degree of heterozygosity in progeny (Dewitte et al., 2012; Younis et al., 2014), with the aditional possibility of also achieving triploid plants in one direct step (Ramsey & Schemske, 1998). Thus these advantages make sexual polyploidisation an important tool for use in crossing programs (Luo et al., 2016; Okazaki et al., 2005; Qu et al., 2019), as it offers a simple, straight forward approach, and some advantages over somatic polyploidisation for genetically improving plants.

Polyploidy via sexual polyploidisation of 2n gametes may emerge through two main crossing patterns. Firstly ‘unilateral polyploidisation’ whereby the polyploid is generated through the crosses involving the fusion of one unreduced gamete with one reduced gamete, as it might occur in normal crosses of diploid with tetraploid (Sala, Camadro, Salaberry, & Mendiburu, 1989). Secondly ‘bilateral polyploidisation’ where the male 2n gamete fertilises a female 2n gamete, as is similarly represented in crossing a tetraploid with tetraploid in normal crossing (Mendiburu & Peloquin, 1977). Both cases are utilised for polyploidy production under conditions where sufficient numbers of 2n gametes are produced to increase the chances of polyploid progeny (Ramanna & Jacobsen, 2003). Nevertheless, in some species of plants the occurrence of 2n gamete formation in nature is rather sporadic with an inconsistent frequency, and the frequency of natural occurrence is genotype dependent (Dewitte et al., 2012). Therefore, it is required to develop an

effective technique for inducing 2n pollen formation to a high frequency to increase the chances of obtaining polyploid progeny in the subsequent crossing.

Treatment with $N_2O$ is known to be effective for artificial 2n pollen induction in plants. Using other approaches to achieve 2n gamete production, such as through physical treatment (e.g. temperature) or chemicals such as colchicine or trifluralin, are also acknowledged for doubling chromosomes in many crops (Table 1). However, the heat treatment is laborious to be applied, and some of the chemical treatments are categorised as being dangerous for the applicant and detrimental for some plants. Thus, $N_2O$ comes as a useful alternative agent to doubling chromosome for several reasons. $N_2O$ is suitable for treating a target organ which is inside other tissue, e.g. anthers inside flower buds in bulbous plants such as tulip, (Akutsu et al., 2007; Okazaki et al., 2012). In addition, $N_2O$ has been shown to be a non-destructive method, because the gas easily dissipates into the atmosphere soon after the treated plant is released from the treatment, leaving no residue behind in the plant (Barba-Gonzalez, Miller, Ramanna, & Van Tuyl, 2006; A Kato & Geiger, 2002).

**Table 1. Overview of various methods used to increase the frequency of 2n gametes in various plants.**

| Induction Method | Species of plants | Reference |
|---|---|---|
| $N_2O$ | Begonia *spp.* | Angelo Dewitte et al., 2010 |
| | Lilium *spp.* | Sato, Miyoshi, & Okazaki, 2009<br>Okazaki et al., 2012 |
| | Tulipa *spp.* | Luo et al., 2016<br>Okazaki et al., 2012<br>Qu et al., 2019 |
| | *Arabidopsis* | De Storme & Geelen, 2020<br>De Storme et al., 2007 |
| Colchicine | Eucalyptus | Yang et al., 2016 |
| | Brassica | Kumar & Dwivedi, 2013 |
| | Chinese cabbage | Zhong et al., 2010 |
| | Cassava | Lai et al., 2015 |
| | Populus | Wenting et al., 2019 |
| | Strelitzia *spp.* | Zheng et al., 2016 |
| Temperature | Rubber | Yao et al., 2017<br>Zhang et al., 2019 |
| | Populus | Guo et al., 2017<br>Li, Tian, Xue, Chen, & Wang, 2019<br>Wang et al., 2017<br>Xiangyang, Zhiti, & Huibin, 1999 |
| | *Arabidopsis* | De Storme & Geelen, 2020 |
| | Roses | Crespel et al., 2015 |
| Gibberellin (GA) | *Arabidopsis* | De Storme et al., 2007 |
| Trifluralin | Begonia | Dewitte et al., 2010 |

$N_2O$ treatment is performed within a pressure chamber at 600 kPa, with the treatment time adjusted according to the specific requirement of a given species (Akutsu et al., 2007; Barba-Gonzalez et al., 2006; Angelo Dewitte et al., 2010). For example, in tulip some genotypes tolerated 48 hours treatment, but a lower number of 2n pollen were produced compared to 24 hours treatments. Whereas in lily 48 hours was optimal to induce viable 2n pollen at a high frequency (Luo et al., 2016; Okazaki et al., 2005). Application of nitrous oxide is recommended when the pollen mother cell is in progression to metaphase 1, thereby facilitating the FDR mechanism (Okazaki et al., 2005). By applying this technique a large number of 2n pollen is formed in some crops (Akutsu et al., 2007; Barba-Gonzalez et al., 2006; Okazaki et al., 2005), and polyploidy was obtained after crossing, indicating that the 2n pollen was functional (Okazaki et al., 2005). Though the underlying mode of action of $N_2O$ has not been well determined, it has been suggested that it acts similarly to other antimitotic agents like colchicine. $N_2O$ is able to disrupt or interfere with the spindle formation during meiosis division, causing the cessation of chromosome movement toward both poles during anaphase I, hence doubled chromosomes result (Kitamura, Akutsu, & Okazaki, 2009).

For $N_2O$ treatment to be used within *Limonium*, confirming its efficacy through the evaluation of the presence of 2n gametes in pollen will be important. The presence of 2n gametes can be evaluated at an early stage, i.e., at microsporogenesis through cytological observation. The occurrence of polyad formation at the end of meiotic cell division as well as monads, dyads, and tryads, is considered strongly indicative of the initial presence 2n pollen (Dewitte et al., 2012), but it is important to note that what is observed in the meiocytes at this stage is not always reflected in the real condition once mature, since in some cases they fail to grow to maturity. Thus, detection at later stages is required to confirm their presence.

Confirming the existence of 2n gametes can be done at later stages during microsporogenesis. The simplest approach to identifying the presence of 2n pollen is through observing the presence of larger sized pollen within a sample. There is a positive correlation between the DNA content and cell volume, which eventually influences pollen diameter after pollen reach the trinucleate stage (Dewitte et al, 2012). In some crops the diameter of 2n pollen was 1.5 times larger than that of normal pollen (Dewitte et al, 2012).

A mixture of sizes of pollen, including the 2n pollen, within the pollen population may be observed. For example, after $N_2O$ treatment in tulip the presence of large pollen was usually accompanied by the presence of other abnormal sizes of pollen, resulting in a bimodal pollen grain size distribution instead of a normal distribution (Okazaki et al., 2005). From recording the pollen size distribution of a sample, the frequency of 2n pollen can be estimated for the remaining pollen (Crespel et al., 2006; Ortiz, 1997; Sugiura et al., 2000), but this evaluation of size cannot definitively prove a doubled DNA content (Dewitte et al., 2012).

The most accurate way to identify the presence of 2n pollen containing double the nuclear DNA content as a measure of chromosome number, is flow cytometry analysis. Flow cytometry is used to measure the nuclear DNA content of cells, and can be used to predict the content of chromosome number precisely in pollen or in the resulting progeny (Dewitte et al., 2012; Ochatt, 2008). Analysing pollen through flow cytometry is somewhat more complex due to the need to isolate the nuclear content, with the protocol requiring digestion or rupture of the exine wall of pollen grains, and is complicated by each species having their own exine wall structure (Dewitte et al., 2009; Dewitte et al., 2012; Pan et al., 2004; Roberts, 2007). As an alternative, flow cytometry analysis can be done on the progeny resulting from crossing with putative 2n pollen (Dewitte et al., 2012).

There are examples of the successful application of $N_2O$ resulting in various frequencies of 2n gametes and polyploid progeny. Luo et al. (2016) exposed flowers buds of a sterile lily hybrid to $N_2O$ for 48 hours and observed fertile pollen with large pollen size at a 22.2% higher frequency than in the control treatment. Okazaki et al. (2005) reported that treating several varieties of tulip at meiosis, using $N_2O$ for 24 hour, resulted in large pollen with wider, and bimodal pollen size distribution, with between 17% and 85% being large pollen. In the same experiment the strategy of with or without sieving out the large pollen in the crossing increased the ratio of polyploidy obtained in the resulting progeny from 2.9% to 94.4%. Dewitte et al. (2010) showed that application of $N_2O$ in sterile begonia resulted in better pollen germination compared to treatment with trifluralin, and in a crossing experiment the frequency of triploid hybrids arising increased between 22.9% to 100%, as compared to using pollen derived from trifluralin treatments that only achieved 0.5% triploid progeny.

A polyploid (hexaploid plant) presumably arising from 2n gametes has been reported in one crossing program of *Limonium perezii* × *L. sinuatum* (Morgan & Funnell, 2018). However there has been no further exploitation of this technology reported using *Limonium*. Mastering the technique and protocol to generate 2n pollen artificially by $N_2O$ treatment would therefore be a powerful tool for a breeding program for a hybrid breeding program based on interspecies hybrids.

## 1.10. Triploid block and embryo rescue

Normally, in the crossing between two diploid angiosperm (or flowering) plants two important events occur during fertilisation; the fusion of an egg and sperm to form a diploid embryo, and fusion of the sperm nucleus with two haploid polar nuclei to form the triploid endosperm. This process is called double fertilisation and produces viable seed (the ploidy ratio of embryo : endosperm = 2 : 3; Ramsey & Schemske, 1998). When attempting an interploidy cross, it is often not possible to achieve viable seed, or if seed is formed at all, with the expected polyploid progeny not obtained (Bretagnolle & Thompson, 1995; Sato et al., 1993). This observation has been identified on many occasions either in unilateral or bilateral crosses between diploid and tetraploid plants (Ramsey & Schemske, 1998). The phenomenon related to this reproductive barrier to diploid × tetraploid and tetraploid crossing is commonly referred to as the 'triploid block' (Marks, 1966). The triploid block is usually associated with a ploidy imbalance with a malfunction in the endosperm arising due to the alteration of the ratio of embryo and endosperm and maternal, paternal ploidy ratio in the endosperm resulting from interploidy crosses (Esen & Soost, 1973; Milbocker & Sink, 1969).

They are two general concepts used to explain the cause of triploid block that mainly consider the ratio within the embryo and endosperm. According to MÜNtzing (2010), viable seed formation requires an embryo : endosperm : maternal tissue ratio of 2:3:2. An alternative idea was proposed with simplification from the later, that only the maternal : paternal ploidy ratio in the endosperm of 2:1, known as endosperm balance number (EBN), is required for proper seed growth (Johnston et al., 1980; Lin, 1984). In contrast,

if the ploidy ratio diverts from that hypothesized, the triploid block will be observed. In some plant species it is not always the case however, because in interploidy crosses a few viable seed are still able to be produced where the hypothesized ratio of maternal and paternal in endosperm of 2:1 is violated (Johnston et al., 1980). Thus some additional factor might be involved for viable seed to be formed.

A study by Ramsey and Schemske (1998) that sought to try to understand the anomaly of viable seed from interploidy crosses analysed the occurrence of autopolyploid plants. It was found that an obligate triploid block was more prominent in the crossing when the majority of unreduced gametes in pollen was involved in the crossing, while the egg is normal as it can be found in the crossing of 4n (male) × 2n (female), but not that prominent in 2n (male) × 4n (female). This also has support from the finding in citrus that the seed which was formed from intracytotype and intercytotype crosses with the embryo : endosperm ratio of 2:3, 3:5, 4:6 and 6:10 were more viable, than those with the 3:4 expected ratio resulting from fusion of 2n pollen and n eggs (Esen & Soost, 1973). It can be concluded that the formation of normal seed in interploidy crosses is driven mainly by the unreduced gamete from the egg cell formed, rather than controlled by the unreduced gamete in the pollen

Some exceptions might be evident in several species, where viable polyploid seed is formed though diverting from the genome composition in embryo and endosperm mentioned above. Firstly, a mutation in mega-sporogenesis can possibly result in formation of polyploid viable seed. For example, in the interploidy cross using a meiotic mutant in maize, a mutation in megasporogenesis can possibly generate embryo sacs containing nuclei of varying ploidies, accompanied by normal functional triploid endosperm (Lin, 1984). Thus, triploid embryos produced from it are able to grow normally. A similar mutation occurs in soybean, being the cause of polyembryony (Cutter & Bingham, 1977). Secondly, several species that have a low incidence of triploid block have been identified as having atypical endosperm characteristics (Hakansson & Eixerström, 1950; Woodell & Valentine, 1961). For example, in poplar, though mature seed have no endosperm, a large number of viable seed were set after the cross of 2x × 4x (Johnsson, 1945). Moreover, plant families such as the Asteraceae, Crassulaceae, Onagraceae, Rosaceae and Salicaceae, that produce mature seed with no endosperm, have a high incidence of polyploidy (Ramsey & Schemske, 1998).

Despite the triploid block posing a problem within a breeding program, hybrid seed from aborted embryos that might abort as the result of the triploid block, can be established through in-ovulo embryo rescue. Embryo culture is an in vitro technique that is carried out within a sterile environment and providing artificial nutrients in the medium (Reed, 2004). Morgan et al. (1998) used the in ovulo technique to recover a *Limonium* hybrids from the cross *L. perigrinum* × *L. purpuratum* and *L. perezii* × *L. sinuatum*. Similarly, through embryo rescue in tulip, a hybrid was recovered from a cross using 2n pollen that would normally produce no viable seed because of poor endosperm growth (Okazaki et al., 2005).

## 1.11. Conclusion

Naturally occurring 2n pollen formation is a recognised mechanism for plant breeding that offers a relatively straight forward method to produce polyploidy.

The only hindrance for this technique to be applicable in practical breeding is that 2n pollen usually occurs at a low frequency, and most of the time is inconsistent due to influences from a combination of genetic and environmental factors. Thus, an efficient and consistent method to produce 2n gametes should be explored to increase the frequency and reliability of formation.

For artificial application of 2n pollen generation to be successful, cytological study of microsporogenesis plays a critical role for each species if that information is lacking. To be successful in production of 2n pollen formation at high frequency the timing of the induction treatment needs to be accurate. 2n pollen can be induced artificially via treatments when the PMC are in progression from prophase I to metaphase I. The duration of meiosis is genotype dependent, thus recording as much as possible information on every stage of meiosis should be compiled before conducting the treatment application.

Nitrous oxide is one of the anti-microtubulin agents that has proven to be effective in doubling chromosomes in cells either undergoing mitotic or meiotic division. It offers some advantages in comparison to other chemical treatments, and is considered safer for both the applicant and plant, leaving no toxic residue on the plant after treatment, and the gas easily penetrating the target organ.

Several conditions for the success of $N_2O$ treatment for the formation of 2n pollen should be met, the treatment should be applied during progression of meiosis; the duration of the treatment should also be considered to be adjusted according to the need of specific requirement of a species of plants.

Following the success of $N_2O$ treatment, there are several signs that indicate the presence of 2n pollen in a population, such as the observation of polyad formation at early stages of microsporogenesis, large pollen at the later maturity stages, and the presence of polypoid seedlings in the progeny after a crossing experiment.

No previous study has been published using plants of the *Limonium* genus for 2n pollen production, and similarly crosses utilising 2n pollen have not previously been published. The *L. sinuatum* diploid species was chosen for this experiment for studying the 2n pollen formation as it has a relatively short life cycle, has all year-round flower production, and forms part of an established breeding program.

# CHAPTER 2.

# Pollen Development and Floral Morphology in *Limonium sinuatum*

## 2.1. Introduction

*Limonium sp*, known as statice or sea lavender (Figure 4) is regarded as an important horticultural commodity in the cut flower industry and is valued for its inflorescence arrangement and the attractive long lasting coloured calyces (Morgan & Funnell, 2018). The future projections for *Limonium* sales seem strong with market data from Holland from 2010 to 2015 indicating an upward trend in sales value (Morgan & Funnell, 2018). To fulfil market needs breeding programmes play important roles in supporting *Limonium* production.

Opportunities exist in plant breeding to introduce a novel cultivar by utilising lesser known species with valued. As in any other plant breeding program, a *Limonium* breeding program targets several aspects such as size, yield, novel form and increased colour ranges (Burchi et al., 2006; Burge et al., 1995). A range of approaches and techniques are available for *Limonium* breeding such as embryo rescue, chromosome doubling (for polyploidy), mutagenesis and transformation (Jeong et al., 2001; Mercuri et al., 2001; Morgan et al., 1995; Morgan et al., 1998). However, approaches that speed up progress in breeding programs are always sought. The need to work within the regulations on genetic modified organisms for example means that not all approaches are feasible particularly if the endpoint is commercial sales. Hybridisation and polyploidy breeding are not subject to this regulation and provide means to rapidly introduce novelty into a breeding programs.

Polyploid plants offer several advantages over their diploid counterparts. Polyploidy is the condition in which an organism that is typically diploid has one or more extra sets of chromosomes (Acquaah, 2012; Ríos, 2015; Sattler et al., 2016). Polyploidisation has been suggested as an important mechanism involved in the evolution of many angiosperm species (Soltis & Soltis, 2009). It is commonly considered that the extra set chromosome

in polyploid plants provides a “buffering” effect in which deleterious alleles are masked by the extra copies of the genome minimizing inbreeding depression (Sattler et al., 2016). Other effects of polyploidy on plant phenotype and plant fertility have meant that it is routinely used in many plant breeding programs. Example includes hybridising across ploidy barriers (Morgan et al., 2011), restoring fertility in infertile interspecific hybrids or simply because of the morphological traits it confers (Dewitte et al., 2010; Dewitte et al., 2012). Plant breeders are often interested in exploiting meiotic doubling (2n gametes) over mitotic doubling to generate polyploid plants in their breeding programs (Younis et al., 2014). In comparison to somatic polyploids, polyploid plants derived from 2n gametes through sexual polyploidisation offer an advantage in terms of the level of heterozygosity and heterosis in progeny due to it allowing genetic recombination before chromosome doubling take place (Dewitte et al., 2012).

A range of factors influence the frequency of unreduced gamete formation in plants have been identified. There are genetic factors with some genotypes known by breeders to generate unreduced gametes (2n gamete) at a higher frequency (Ramanna & Jacobsen, 2003; Ramanna et al, 2003; Ramsey & Schemske, 1998). The natural incidence of 2n gamete formation in a given species is typically very low (below 0.1 %), and environmental factors such as elevated temperatures or other forms of stress have been implicated as influencing the rate of formation of 2n gametes (Ramsey, 2007). The formation of 2n gametes at the typical natural low rates is not practical for breeding programs and techniques to reliably increase the frequency of formation are sought.

The artificial induction of 2n gametes has been explored in a number of crops using treatments ranging from heat to use of anti-tubulin agents, and have resulted in varying degree of success (Akutsu et al., 2007; Barba-Gonzalez et al., 2006; Crespel et al., 2015; De Storme et al., 2007; Dewitte et al., 2010; Liu et al., 2017; Yao et al., 2017). A critical aspect for success of each application is information on meiosis for the species. Success in inducing 2n pollen formation relies on appropriate timing of treatments with treatment best applied when meiosis is progressing from prophase 1 to metaphase 1 ( Dewitte et al., 2012; Younis et al., 2014). This part of the meiotic process is only observed in a narrow flower developmental range and its duration varies among species from a matter of an hour to more than a day (Bennett, 1977). Therefore an understanding of the meiotic

process is needed via cytological study of pollen development to ensure correct timing of treatment for 2n gamete formation.

Currently, there is no information available on the cytology of pollen development in *Limonium*, nor meiosis in *L. sinuatum.* Therefore, in this study the general process of pollen development along with changes in developing *L. sinuatum* flowers were investigated. The timing of particular stages and the morphological characteristics of flowers in relation to pollen development were also investigated and described.

## 2.2. Material and Methods

### 2.2.1. Plant Material

The *L. sinuatum* plants used in this study comprised 12 seed-derived, mature, potted plants that were producing multiple flower stems. Plants were grown in a plastic covered greenhouse at Plant & Food Research in Palmerston North under natural light levels during daylight hours with 24 hr supplemental lighting by High Pressure Sodium lamps at a minimum of 4.6 µmol $s^{-1}m^{-2}$ at plant height with minimum temperature of 15° C and a ventilation temperature of 24 °C.

### 2.2.2. Flower Collection and data recording.

Individual *L. sinuatum* flowers were observed within the raceme in a flower stem. The raceme consists of a compound spike with several florets of different sizes tightly enclosed in a spikelet (See Figure 4 g.) Spikes and flower buds at different stages of development were measured.

In the selected spike, each floret in the spikelet was numbered from the youngest to the oldest (mature) floret (See Figure ig.4f). Several spikes at different stages were selected with the aim of developing a morphological marker for predicting the location of florets undergoing meiosis in a spike.

In these observations, for samples over 1mm, measurements were done using a Vernier calliper, pictures were taken using a Nikon d7200 camera with a Nikon 105 mm f2.8 lens for close up pictures and a 24-70 mm f2.8 lens for large specimens.

### 2.2.3. Cytological observation

Flowers were harvested on July 2018 and fixed as described below. A whole raceme comprised of florets in different developmental stages was collected hourly from 4 AM to 1 PM, and from 7 AM to 9 AM at 15 min intervals. On the day of sample collection sunrise was at 6.15 AM.

All samples were fixed in Carnoys fixative (ethanol: acetic acid 7:3) at 4° C for 24 hours then transferred to 70 % ethanol at 4° C for longer term storage. To analyse microsporogenesis and pollen development, anthers were extracted from fixed flower buds with fine tweezers and placed onto microscope slides then stained and squashed in 2 % aceto carmine. For this the samples were prepared using a Leica MZ12 stereomicroscope at 10 x magnification. The slides containing cell spreads were covered with a cover-slip and observed using an Olympus BX51 microscope (10 X-40 X objective magnification). Pictures were captured using an Olympus SC30 camera and measurements made using Olympus software (CellSens® GS-ST-VI.7, Olympus, Germany).

## 2.3. Results

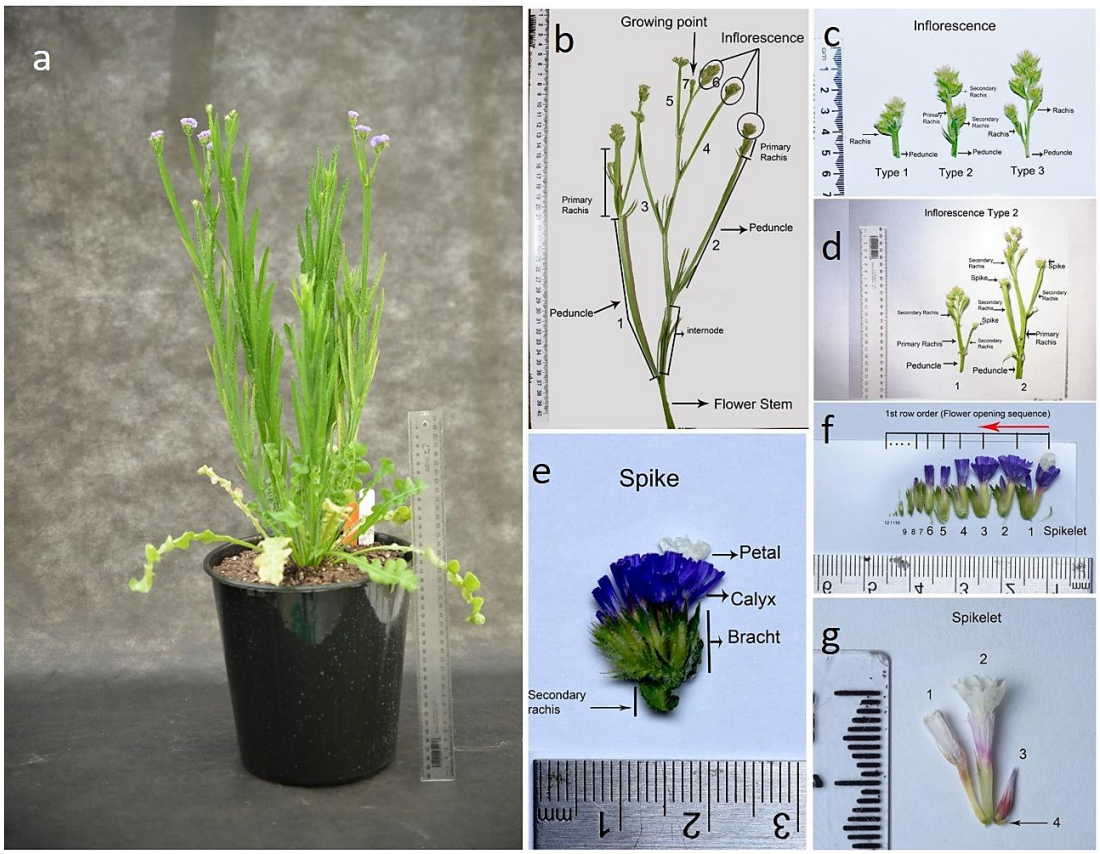


Figure 4. General features of an inflorescence of *Limonium sinuatum.* a. plant of *L. sinuatum* at an early flowering stage (5-10 flower stems) b. A flower stem or corymb of *L. sinuatum* c. three types of racemes namely type 1, type 2 and type 3 of *L. sinuatum* (each holds 6-10 spikes) d. two variants of type 2 racemes in *L. sinuatum* e. one individual spike of *L. sinuatum* (4-12 spikelets) f. arrangement of spikelets in a spike (red arrow and number indicate the sequence of flower opening) g. detail of one spikelet (bract is removed) showing it is carrying four florets.

### 2.3.1. Flowers in *Limonium sinuatum*

A single *L. sinuatum* plant can produce more than ten floral stems, panicle or corymb, arising from the plant's basal rosette (Figure 4a). Each corymb can hold more than 10 racemes (Figure 4b). A raceme is comprised of five to nine spikes. In the current research there were three types of racemes identified, namely types 1, 2 and 3 that can be distinguished by the density of arrangement of floral spikes. The type 1 raceme is distinguished by its compactness and a more dense appearance in comparison to the other types (Figure 4c). The number of spikelets within a spike varies but, in general, the

number spikelets reduced toward the distal end of the inflorescence. In a spikelet there are up to four florets enclosed by a bract (as shown in Figures 4c, 4g and 10a and 10b).

Plants with type 1 raceme were typically used for sampling in this experiment as they produce an array or sequence of flower opening that consistently begins from the very first floret located at the proximal end of a spikelet of a spike (Figure 10a and 10b).

### 2.3.2. The association between pollen and flower development stages

Based on the cytological study, the floret that is undergoing meiosis can be identified in a raceme by screening florets at different developmental stages. Five spikes of raceme type 1 with one mature floret (open) were selected then individual florets in the spike were separated and arranged in sequence. About 11 florets on each spike were arranged in order from the most mature floret to the youngest based on bud size (Figure 5a).

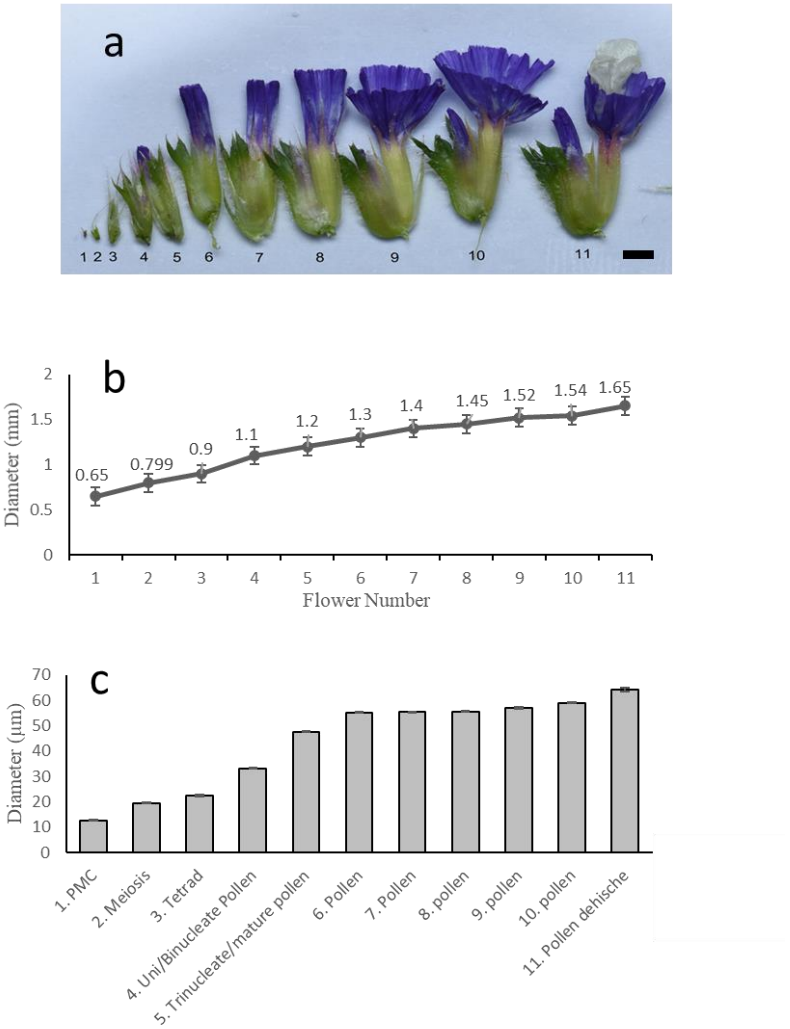


Figure 5. The sequence of pollen developmental stages in relationship to floret developmental stages in *Limonium sinuatum*. (a). An array of eleven florets from immature bud (flower 1) to mature floret at anthesis. Bar =2 mm (b). Line chart of average floret diameter (mm) at each floret stage. (c). Bar chart of average diameter of cells of developing pollen from pollen mother cells to mature pollen. The error bars are standard error.

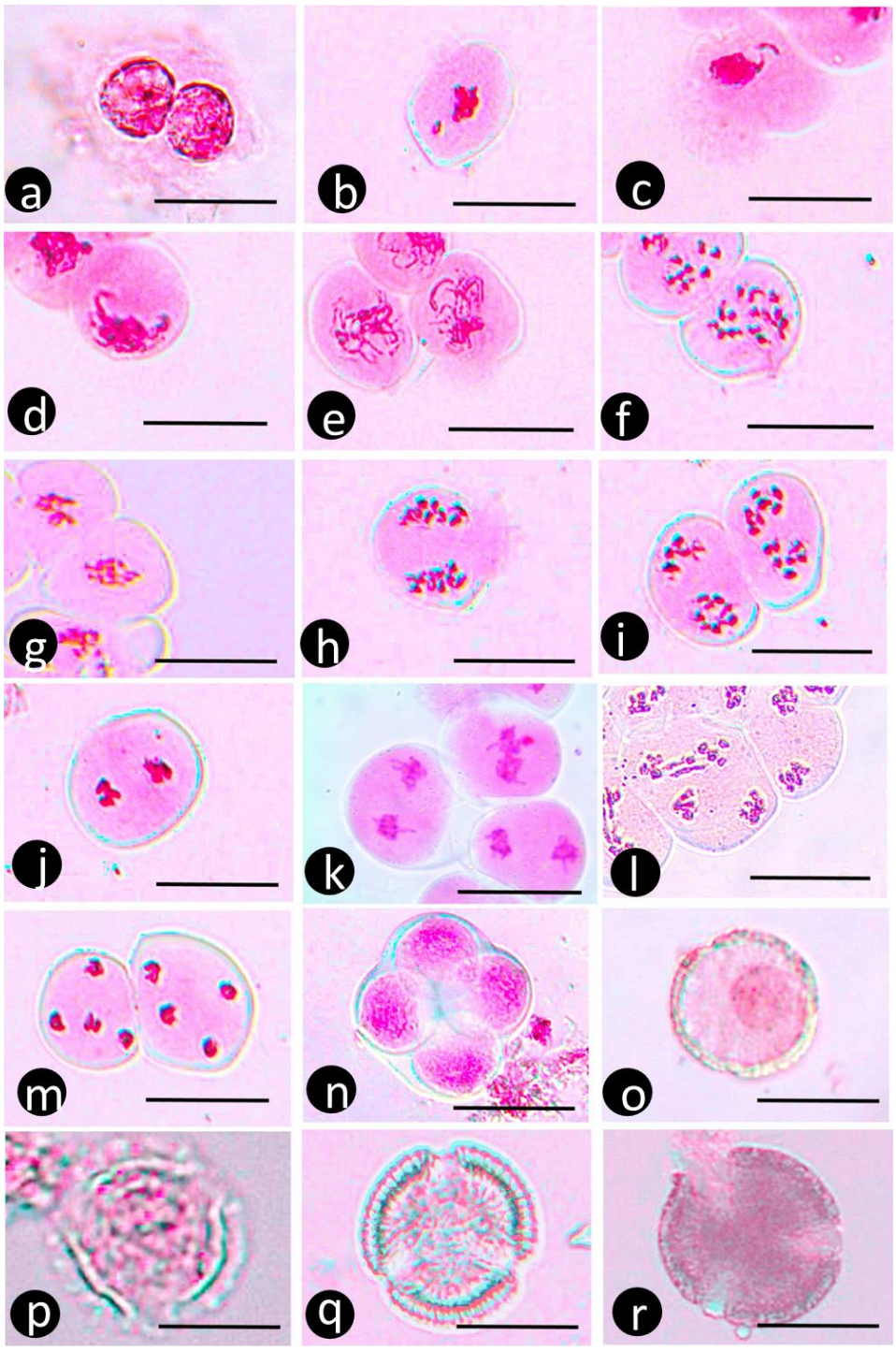


Figure 6. Microsporogenesis in *Limonium sinuatum.* a.) two pollen mother cells (PMC) b), a cell in leptotene phase, begins to commence synapses process c), two cells in zygotene phase, early synapsis d), pachytene, late synapses e), diplotene, crossing over of chromatin take place f), two cells in diakinesis phase g), three cells in metaphase I h, one cell in anaphase I i), two adjacent cell in telophase I j) prophase II k), three cells in metaphase II l), two cells in anaphase II, with spindle thread observed m), two cells in telophase II n), tetrad o) uni-nucleate microspore p), bi-nucleate microspore q), tri-nucleate pollen r), mature pollen at dehiscence.
*Bar panels a - p = 20 µm panels q & r = 40 µm*

Cytological observations using a stereomicroscope and fluorescence microscope revealed every stage of pollen development from pollen mother cells (PMC) to mature pollen shed at anthesis. The stages of microspore development and maturation are shown in Figures 5 and 6. In the most immature floret (no.1) the PMC's are 14 µm in diameter and the cells are cytoplasm rich with a central nucleus (Figure 5 and 6a).

Meiosis occurs in flower buds at position 2 (Figure 5a) and is marked by the cell size increasing almost 1.5 fold in comparison to the PMC, the cytoplasm inside the cell becoming clear and the nucleus shifting to the side of the cell (Figure 5 and 6b). The sub phases of prophase I, from leptotene to zygotene could be observed characterised by the appearance of synapsis processes and crossing over of chromatin (Figure 6b to 6e). In the next phase, chromatin was condensed resulting in the 16 pairs of homologous chromosome (bi-valent) being apparent at diakinesis (Figure 6f). From metaphase I to telophase I, the 16 chromosomes were paired up at the metaphase plate, before each eight pairs of sister chromatids segregated and moved to the opposite pole at the later stages in telophase I (Figure 6g to 6j). The spindle threads are clearly visible from metaphase II to anaphase II, pulling sister chromatids to each designated pole, before cytokinesis takes place forming the cell wall and the tetrad (Figure 6k to 6n). The tetrad stage extended to and was also seen at floret no. 3 (Figure 5a and 5c) with the process of nuclear divisions from uni-nucleate to trinucleate occurring in florets no. 4 and 5 (Figure 5a,5c,6o, 6p, 6q). The remaining stages of pollen maturation were trinucleate pollen with rich cytoplasm were observed in floret spikelets no. 6 up to no 10. When mature and ready to be released, the pollen grains have three obvious apertures (Figure 5a, 5c, 6r).

Each floret was able to produce approximately 100 to more than 300 pollen grains in its five anthers. The average pollen grain diameter was approximately 59.8 µm with the largest pollen grains at 72 µm and the smallest at 40 µm. Pollen development, from the onset of meiosis up to anther dehiscence, took more than 14 days. It was found that the floret in the spikelet undergoing meiosis was consistently located at position no. 10 counted from the most recently opened floret in a spikelet. The average diameter of the floret undergoing meiosis was approximately 0.8 mm. (Figures 5b and 5c).

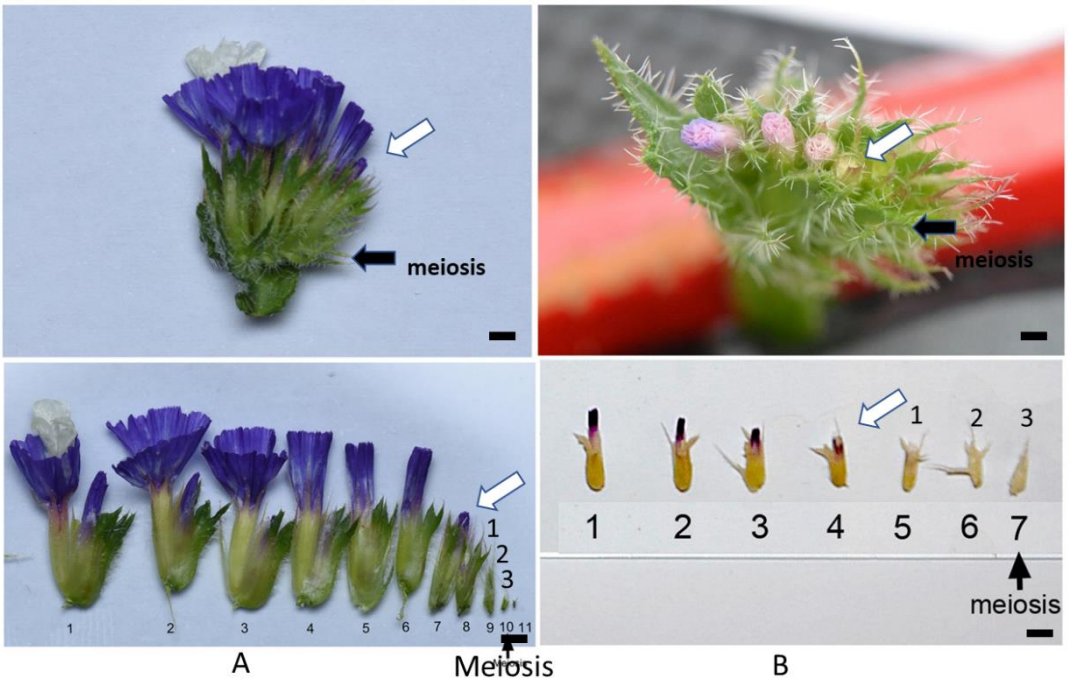


Figure 7. Appearance of morphological features of a floret as a marker to predict meiosis in *Limonium sinuatum* florets. A). Spike with one mature floret (opened) (11 florets in total, the "marker" floret is floret no.7 and meiosis is occurring in floret no.10) B), An immature spike with the "marker" floret at position no. 4 and the floret undergoing meiosis at position no.7. Bar pic =2 mm
The white arrow indicates the "floret marker" and the black arrow indicates the floret undergoing meiosis.

After the floret undergoing meiosis had been identified, further observations were aimed at finding a distinct morphological feature within a spike that could be used to identify the flower undergoing meiosis more easily (as shown in Figure 7). The floret in a spike that is undergoing meiosis is usually located three florets behind the "marker" floret. The most obvious feature of the marker floret that is visible to the naked eye is the colour of the calyx that is just beginning to protrude from the bract by (approximately1.9 mm) (Figure 7a and 7b). The marker floret can be used to identify the floret that is undergoing meiosis with a high degree of accuracy, above 90% in the field.

### 2.3.3. Flower opening sequence.

The first floret to open in a raceme is within a spike located at the proximal end of the raceme with opening proceeding acropetally toward the distal end. In contrast the florets in a spikelet within a spike follow a centripetal maturation pattern, beginning from the outer floret and proceeding inward toward the rachis (Figure 4f, Fig 10a and 10b).
Only one floret, or occasionally two florets open at a time in sequence in a spikelet of a spike within a raceme, with opening in individual florets separated by one or two days between the first floret and the next florets for the first six florets within a spikelet, but for later maturing florets the delay in opening can be 3-14 days.

### 2.3.4. The diurnal dynamics of meiosis and asynchronous meiosis progression.

#### 2.3.4.1. The diurnal dynamic of meiotic cell frequency over time.

During the period these samples were being collected, sunrise occurred at about 6.15 AM. Figure 8 shows the total number of cells undergoing meiotic cell division (PMC, Prophase1-to telophase 2, tetrad) recorded hourly from 4 AM to 1 PM. To compile the data Figure 8 every cell in a stage of meiosis observed each hour was counted and presented as a percentage of the total numbers of cells viewed. Between 4 AM and 5 AM only a small proportion of cells (around 10%) were undergoing meiosis and PMC was the dominant meiotic phase during at this time (Figure 9a). Most cells underwent meiosis from 6 AM to 9 AM with the peak number of cells undergoing meiosis observed between 7 and 8 AM (when 20 % of cells were in meiosis). The proportion of meiotic cells decreased after that and there was an approximately constant proportion toward the end of the observation time at 1 pm (Figure 8).

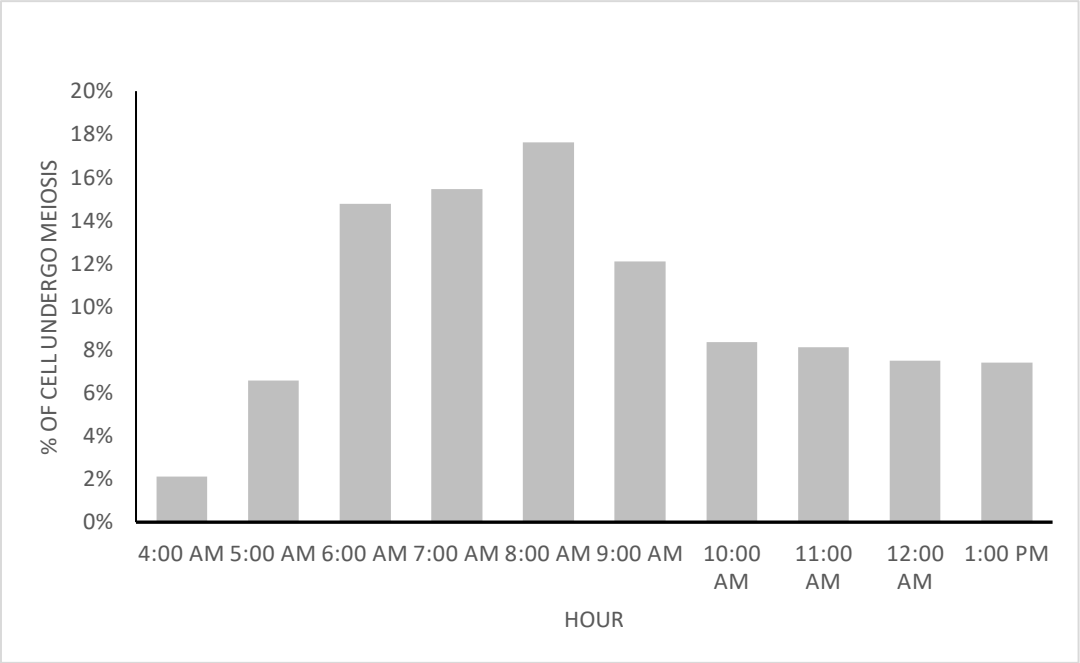


Figure 8. The percentage of cells in *Limonium sinuatum* florets undergoing meiosis observed per hour from 4 AM to 1 PM. Sunrise was at 6.15 AM.

The passage of cells through meiosis was also observed. The numbers of cells at each meiotic stage was counted each hour and then at the peak of meiotic activity (from 7 to 9 AM) this was recorded at 15 min intervals to investigate the dynamic in much more detail (Figure 9a and 9b).

The first cycle of meiotic stages, from when PMC started actively entering into prophase I up to tetrad formation were completed in a relatively short time (from 6 to 9 AM). A first cycle of meiosis began at 6 AM with the observation that the PMC were entering into the early phases of meiosis and ended with the total disappearance of PMC and the emergence of tetrad formation at 8 am (as shown in Figure 9a). Once the first cycle of meiosis was completed, a second cycle started with the re-appearance of a high number of PMC-prophase 1 cells at 9 AM. This cycle was completed at 11 PM, a shorter time of two hours. A third cycle was observed to begin at 12 PM and was still underway at the last observation time at 1 PM.

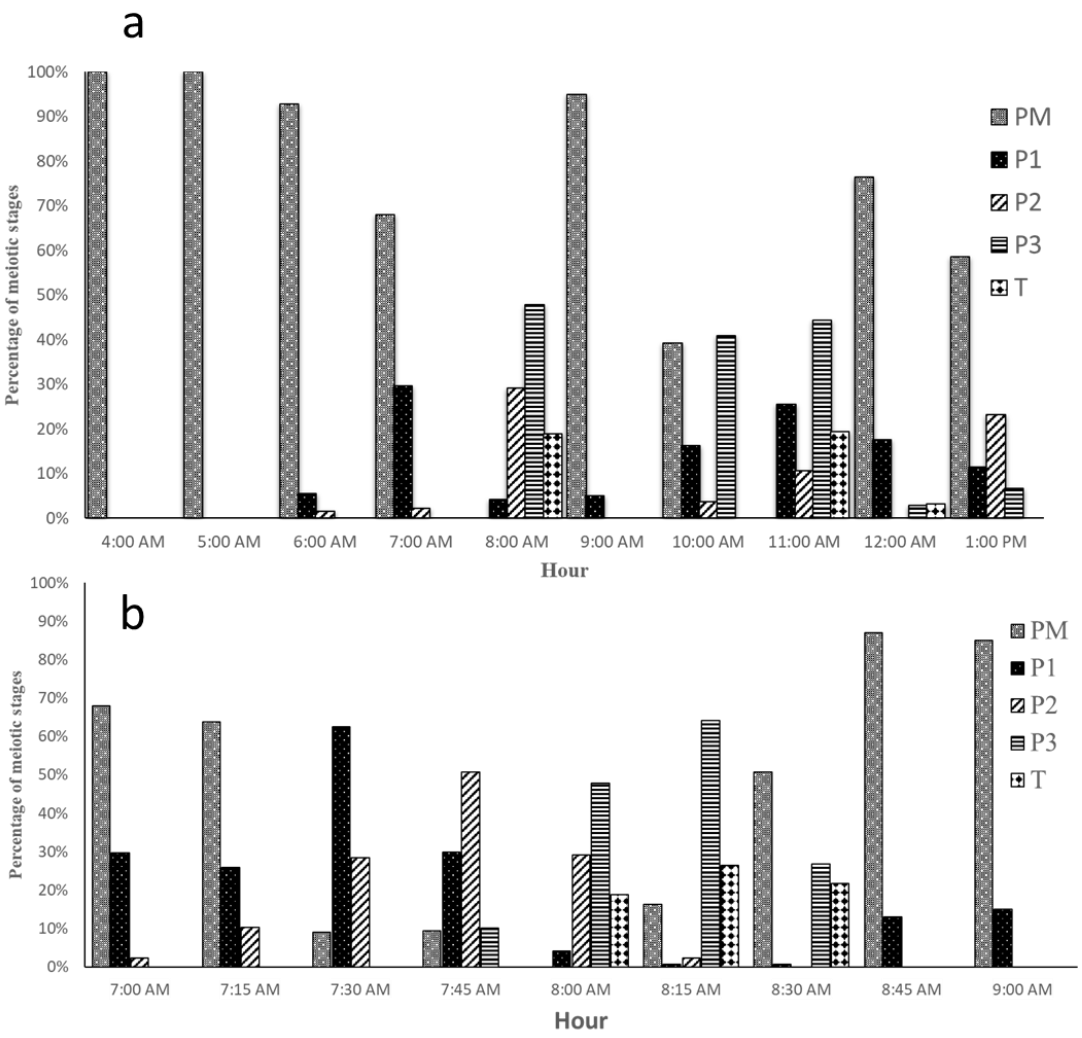


Figure 9. Dynamics of meiosis in *Limonium sinuatum.* a) The proportion of all cells in different meiosis phases recorded hourly from 4 am to 1 pm b) The proportion of cells at different meiosis phases recorded in 15 min intervals from 7 am to 9 am.
PM =premeiotic interphase-leptotene-zygotene; P1 = pachytene to diakinesis; P2: metaphase I to telophase I; P3 = prophase II to telophase II; T = tetrad.

The most rapid progression of meiotic cell division occurred from 7 to 9 am and was marked by the emergence of most meiotic phases from leptotene to the formation of tetrads (Figures 9, 10a and 10b). Prophase I to metaphase I were the most commonly observed meiotic phases between 7.15 am and 7.45 am (Figure 9b).

#### 2.3.4.2. Asynchrony of meiotic cell division in a floret and among florets within the spikes of an raceme

For the purpose of this observation, all samples were collected at 07.45 AM. The first observation was that in each individual floret cells were in multiple meiotic phases but there were commonly two or three dominant meiotic phases, indicated by a large percentage of cells in the phases counted (Table 2).

**Table 2. Stages of microsporogenesis stages in a floret of *Limonium sinuatum* at different spikes in an raceme sample collected a 07.45 AM (the schematic position of each floret is provided in Figure 10)**

| Floret | Microsporogenesis stages (%) | | | | | Total cells examined |
|---|---|---|---|---|---|---|
| | PM | P1 | P2 | P3 | T | |
| Spike 1 (floret no. 7) | 16.06 | 13.14 | 31.39 | 39.42 | - | 137 |
| Spike 2 (floret no. 6) | 8.90 | 20.42 | 40.84 | 29.84 | - | 191 |
| Spike 3 (floret no.5) | 15.89 | 62.91 | 10.60 | 10.60 | - | 151 |
| Spike 4 (floret no.4) | 18.29 | 39.02 | 41.68 | - | - | 82 |
| Spike 5 (floret no.3) | 4.26 | 17.02 | 76.60 | 2.13 | - | 141 |
| Spike 6 (floret no.2) | - | 7.69 | 92.13 | - | - | 91 |
| Spike 7 (floret no.1) | - | 43.75 | 56.25 | - | - | 80 |
| Spike 8 | - | - | - | - | - | - |

Note PM =Premeiotic interphase-leptotene-zygotene; P1 = Pachytene to Diakinesis; P2 : metaphase I to telophase I; P3 = prophase II to telophase II; T = tetrad

On the other hand, synchrony of meiotic cell division was also observed among florets within a spike in an inflorescence. Among the florets in an inflorescence there was greater asynchrony between florets located at the proximal end and florets at the distal end of the inflorescence with these florets observed to be undergoing a very different range of multiple meiotic phases (Table 2 and Figure 10).

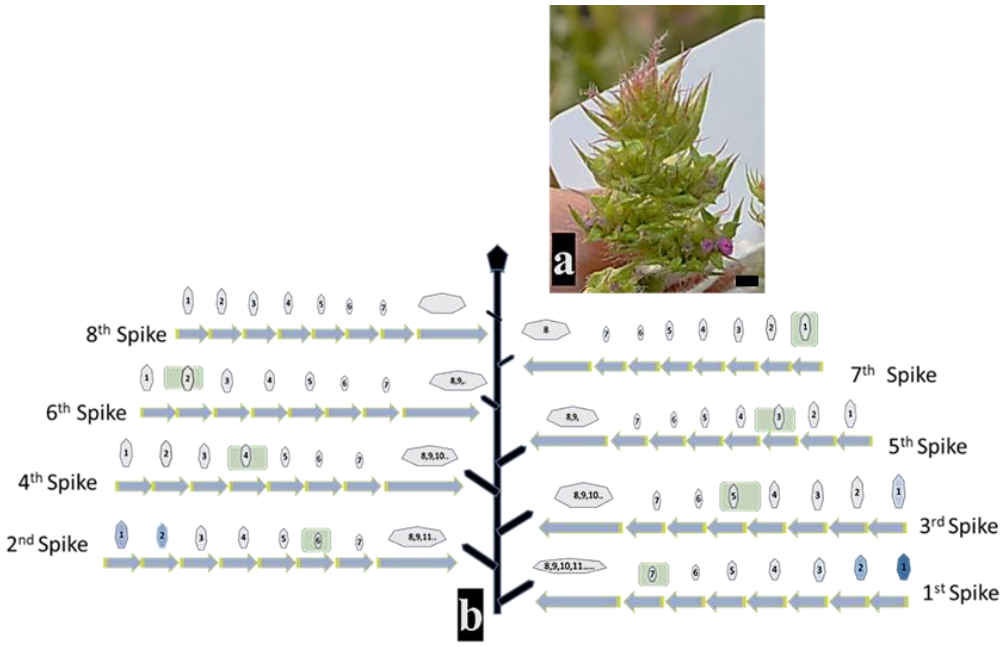


Figure 10. The schematic presentation of individual florets within a single spike of a raceme of *Limonium sinuatum*. (a). An example of a type 1 spike raceme. (b). A map of every floret undergoing meiosis within a raceme (occurs in only one floret per spike in one observation mark in green color). Bar pic = 5 mm

( ) = indicate floret in meiosis; Arrow = direction of floret opening on the spikelet; number = indicate floret position; florets represented by darker colours are more developed.

## 2.4. Discussion.

In its natural habitat *L. sinuatum* (2n= 16) is considered as perennial species producing flower stems all year around. The erect flower stems of approximately 40-60 cm are comprised of densely packed small florets forming a unique arrangement of spikes supported by a winged stem (Artelari & Georgiou, 2002), which is a distinctive feature of *L. sinuatum*. In line with observations of the flowering morphology of *L. sinuatum* in the natural habitat, the *L. sinuatum* used in this experiment mostly share similar characteristics. However, there is additional knowledge that can be included here. Three general type of raceme spikes types were identified instead of the only irregular pattern reported by Artelari & Georgiou (2002. The raceme types can be distinguished from each other based on the compactness and arrangement of the florets, particularly for the type 1 that is quite visibly distinct as it has a triangular shape with florets arranged more densely in comparison to the other two types of raceme (please refer to Figure 4c).

Meiosis is generally classified into nine phases: prophase I (leptotene, zygotene, pachytene, diplotene and diakinesis), metaphase I, anaphase I, telophase I, interkinesis, prophase II, metaphase II, anaphase II and telophase II (Heslop-Harrison, 2013; John, 2005; Prusicki et al., 2019). In *L. sinuatum*, cytological observation of meiotic division revealed all of the meiotic phases except interkinesis. Interkinesis, occurring between telophase I and prophase II is characterised by the appearance of the channelling furrow within a cell and ends with formation plate of a plate-like structure that separated the cell into two equal parts, is also known as the resting period (Heslop-Harrison, 2013). There are two general types of species in plant kingdom with regard to cytokinesis, those that undergo successive and those that undergo simultaneous cytokinesis (Heslop-Harrison, 2013). Species with simultaneous cytokinesis usually skip interkinesis and at the later stages form a tetrahedral instead of tetragonal tetrad formation (Davis, 1967). From observations described here, *L. sinuatum* can be categorised into this group as the tetrad is arranged tetrahedrally and interkinesis was not observed, this is observed in other dicots for example it is reported in *Desmodium* sp (Davis, 1967).

In this study the relationship between flower development and the whole process of microsporogenesis and megasporogensis in *L. sinuatum* was described in detail. The progression of each microsporogenesis to megasporogenesis phase was consistent with changes in flower size and flower developmental stages, for all parameters such as such as bract and calyx development, and diameter of floret. Thus, the observed transformation of shape and size of floret is in line with the progression of pollen development over time, indicated by a tight correlation between them in *L. sinuatum*. A strong relationship between pollen development and floral bud length also has been reported in other crops (Kim et al., 2016; Wang et al., 2011; Yang & Kang, 2015). In many plant species, the developmental events of microsporogenesis and pollen formation occur in a precise chronological order that correlates well with the floral bud size or corresponds to flower developmental stages (Koltunow, Truettner, Cox, Wallroth, & Goldberg, 1990; Scott, Hodge, Paul, & Draper, 1991; Yao et al., 2017).

A necessary step prior to the artificial induction of 2n pollen is treating flowers that are undergoing meiosis. In many studies in other crops, flower bud diameter can be regarded as a predictor of the timing of meiosis with a high degree of accuracy prior to applying

treatments for the purpose of inducing 2n pollen (Okazaki et al., 2012; Sato et al., 2009). Size can be easily used for crops with larger flowers such as tulip or *Lilium* (Luo et al., 2016; Okazaki et al., 2005; Okazaki et al., 2012). By contrast in *Limonium*, it was difficult to identify meiotic florets in the field if only using floret diameter as the size of floret at meiosis was so small (less than 0.8 mm). Fortunately, it was noted that there is a feature of flower development could be used to identify meiotic florets and this was the floret marker used here (the floret in which the coloured calyx is just visible as shown in Figure 7.) and was used to identify the floret undergoing meiosis with high accuracy (up to 90 % in the field). The use of a distinct flower development stage as a marker alongside flower diameter has also been applied in other crops to identify meiotic flowers, particularly for species with complex compound flowers such as eucalyptus, cassava, rubber tree (Lai et al., 2015; Yang et al., 2016; Yao et al., 2017).

Another significant finding is a typical characteristic of meiosis of *L. sinuatum*. First of all, asynchrony of meiosis was evident, characterised by the observation of cells undergoing different meiotic phases at the same time (Figure 6 and Table 1). The asynchrony is more profound during the active period of meiotic cell division 1-3 hours after sun rise as indicated by cells in more than three meiotic phases instead of the usual one or two phases. Secondly, *L. sinuatum* has a relatively rapid meiotic cell division, which in one cycle of meiotic cell division is completed in approximately 2-4 hours. In comparison to the short duration of meiotic cell division in *L. sinuatum*, it is 18 hours in petunia hybrids and in olive tree approximately 170 hours (Bennett, 1977; Fernandez & Rodríguez-García, 1988), thus *L. sinuatum* can be considered a species that has a short cycle in meiotic cell division. It is believed that there is an association between species with a short meiotic cell division in one cycle and the asynchrony of meiosis within florets and within the inflorescence observed here. This type of association is known in Arabidopsis, and in other species with dense arrangements of small flowers typical of grass and forage plants, for instant in red clover (Bullitta et al., 1995; Prusicki et al., 2019; Sato et al., 1993; Taylor et al., 1976). Species with a longer time span in one meiotic cycle tend to undergo meiosis in a more synchronised manner, e.g., in larger flowered species such as in tulip and lily (Luo et al., 2016; Okazaki et al., 2005). This variation in asynchrony and duration of meiosis are believed to be an adaptation to increase the success of pollination (Wyatt, 1982; Yang & Kang, 2015). It is important to note that the cytological studies mentioned above and in this experiment were destructive, thus the

type of asynchrony and the duration of meiosis might be different than described if a live imaging system for tracking meiosis such as used in a recent Arabidopsis study (Prusicki et al., 2019) was used here.

In the studies of microsporogenesis in *Eucalyptus*, the estimation of particular timing of any meiotic stages was done by examining the total number of cells in all stages of meiosis and identifying the dominant meiotic stages at particular time (Yang & Kang, 2015). By this approach, the approximate dominant stages of meiosis are able to be identified. It is known that the best stage for applying treatment for 2n pollen formation is during the progression from prophase 1 to metaphase 1 (Dewitte et al., 2012; Younis et al., 2014). In this experiment these meiotic phases predominantly started from around 7 to 7.45 AM characterised by the observation of a relatively high ratio of cells undergoing prophase 1 to metaphase 1.This occurred approximately one hour after the sun had risen and was coincident with the PMC actively entering into early meiosis phases. Prophase I (Leptotene to diplotene) is the only dominant phase observed during this period (approximately occurs from 6 to 7 AM, following sun rise at 6.15 AM).
It is known that the temperature is a main factor that regulates the duration of meiosis in plants (Bennett, 1977), but little is known of the effects of photoperiod affecting meiosis. In mitotic cell division, light intensity is regarded as an important factor, together with temperature regulating cell division in plants ( Babil et al., 2010; Bouget et al., 2007).

## 2.5 Conclusion

This study of microsporogenesis provides new information for pollen development and meiosis in *L. sinuatum*. Through the morphological analysis of floret development it was shown there is a close link between the progression of pollen development and floret developmental stages. The linkage between pollen development and flower development stages and the finding that meiosis only occurs in a specific floret called the meiotic floret provided a convenient and accurate way of identifying the meiotic floret using a marker floret, a floret that has distinct visible features. The intense focus on the study of meiosis cell division yielded important information of the timing and duration of when meiotic cells undergoing cell division progress from prophase 1 to metaphase 1. The study also described some degrees of asynchrony in meiotic cell division in *L. sinuatum*. All of this information will serve as basic information for the timing of $N_2O$ treatment application.

It is possible that the duration and time of meiosis in other species of the *Limonium* genus will be different. And if using different *Limonium* species, further investigation will be needed

# CHAPTER 3.

# Nitrous oxide treatment for 2n pollen production in *Limonium sinuatum.*

## 3.1. Introduction

*Limonium* (Miller) is a cosmopolitan genus with variation in ploidy between species, with triploid status being the most predominant (Erben, 1979; Lledó et al., 2005; Palop-Esteban et al., 2011). As in other genera the occurrence of polyploidy is believed to arise through involvement of 2n gametes during hybridisation (i.e. allopolyploids) (Ramanna & Jacobsen, 2003; Ramsey & Schemske, 1998). Triploid plants may display agronomically valuable features but are typically sterile (Husband 2004; Wang, Cheng, Zhi, & Xu, 2016), meaning that usually they cannot be used in further breeding due to the abnormal meiosis or, if they produce viable seed it is at a very low frequency not feasible for breeding purposes, or through apomixis. To increase the incidence of polyploidy in particular for triploid breeding, a possible approach is to artificially induce the meiotic chromosome doubling.

Nitrous oxide ($N_2O$) is a gas that is able to inhibit microtubule polymerisation, and thereby serves as an alternative to colchicine treatment to achieve polyploidisation (Dewitte et al., 2012; Ríos, 2015; Sattler et al., 2016). Experiments with several crops using $N_2O$ have achieved a high rate of success in doubling the chromosome number, not only in mitotic cells (e.g. the zygote but also in meiotic cells for 2n pollen formation to generate polyploidy) (Akutsu et al., 2007; Chou et al., 2013; Kato & Geiger, 2002; Okazaki et al., 2005). Interestingly, $N_2O$ treatment is also known method to overcome sterility, for instance exposure of sterile lily and begonia plants to $N_2O$ restored fertility and the viable pollen obtained could be used in breeding (Barba-Gonzalez, et al., 2006; Dewitte et al., 2010a). In comparison to colchicine, use of $N_2O$ treatment offers the advantage that special chemical disposal is not required after treatment, as the gas is easily released into the atmosphere ( Kato & Geiger, 2002) and it is more suitable for treating

organs inside tissue such as bulbs, or physically small flowers as occurs in *Limonium* species.

Polyploidy via the formation 2n pollen is known to be artificially induced through $N_2O$ application (Dewitte et al., 2012; Sattler et al., 2016). There are two important aspects that determine the success of the $N_2O$ application for 2n pollen production. First of all, identifying the correct flower stages to treat. For achieving the optimum frequency of 2n pollen in the $N_2O$ application it is recommended to treat the plants when cells are undergoing meiosis, particularly at the early stages (Dewitte et al., 2010b; Ríos, 2015; Sattler et al., 2016). In studies of 2n pollen induction in lily and tulip, the highest frequency of 2n pollen formation was when flower buds were treated when cells were in meiosis progressing from prophase I to metaphase I (Okazaki et al., 2005; Okazaki, Nukui, & Ootuka, 2012). The determination of the initial information on meiosis should be obtained through cytological study (Sala et al., 1989; Silva et al., 2017). Another important factor is the duration of treatment. Longer durations that work in some plants, fail to yield 2n pollen in other species (Dewitte et al., 2012).

Many plants will survive 24 hours $N_2O$ treatment (Akutsu et al., 2007; Barba-Gonzalez et al., 2006; Chou et al., 2013; Dewitte et al., 2010a; Kato & Birchler, 2006; Kato & Geiger, 2002; Lucidos et al., 2012) and in some plants continuous exposure for 48 hours resulted in high yields of 2n pollen (e.g., lily, but not in tulip) (Luo et al., 2016; Okazaki et al., 2005). Following successful treatment by applying the correct timing and duration of $N_2O$ exposure, large pollen grains will be present within the pollen population indicating the presence of 2n pollen (Bretagnolle & Thompson, 1995; Dewitte et al., 2012).

There is no prior publication of any attempt being made of $N_2O$ treatment in *L. sinuatum* for the formation of 2n pollen. Therefore in this study, the effect of exposure of $N_2O$ on *L. sinuatum* was investigated in two different durations (24 and 48 hours).

## 3.2. Materials and Methods

### 3.2.1. Plant Material

The six diploid (2n= 2x =16) plants of *L. sinuatum* used in this study were pot growing mature plants at early flowering (4-6 months old). The plants were grown and maintained in a plastic film covered greenhouse at Plant & Food Research in Palmerston North, New Zealand. Plants were grown under natural light levels during daylight hours with 24 hr supplemental lighting by High Pressure Sodium lamps at a minimum of 4.6 µmol $s^{-1}m^{-2}$ at plant height. The greenhouse was heated from a minimum temperature of 15° C and vented from 24 °C.

### 3.2.2. Induction of 2n Pollen by N2O treatment

Three plants were assigned to the 24-hour treatment, and the other three were assigned to a 48 hour treatment with $N_2O$. Using information from previous experiments, the "floret marker" was used to identify meiotic stage marker florets before the $N_2O$ treatment. The marker florets were labelled by marking their calyces using a waterproof marker before the plants were put into the $N_2O$ chamber. Three plants at a time were placed inside the chamber, 1.5 hr after dawn (7.30 AM on 2nd & 6th October 2018). The plants were treated in the $N_2O$ chamber at 600 kPa for 24 hour or 48 hour and were removed exactly after 24 or 48h of $N_2O$ treatment and immediately transferred back to the greenhouse.

### 3.2.3. Cytological observation.

In order to observe the early effects of the treatment on tetrad formation, 10 marked florets at a developmental stage at which they were expected to have formed microspores were randomly collected 30 minutes after transfer to the green house. The florets were transferred into a freshly made solution of Carnoys fixative (as described below) to fix them prior to storage.

In order to observe the effect of $N_2O$ treatment on pollen grain development, the meiotic florets, along with adjacent florets in the spikes were harvested 15 days after treatment.

All collected samples were fixed in freshly made Carnoys fixative (ethanol: acetic acid 7:3) and held at 4° C for 24 hours. After 24h the samples were transferred to 70 % ethanol for longer term storage and held at 4°C until microscopy was carried out. For microscopy, anthers were dissected from the fixed florets, squashed, and stained in 2 % aceto carmine, the large remaining debris were removed leaving only the pollen grains. The size of the samples meant that they were prepared under a Leica MZ12 stereomicroscope. After preparation the cover slips were sealed to the slides. The prepared cell spreads and pollen were observed using an Olympus BX51 microscope (10 X-40 X objective magnification). Images were captured using an Olympus SC30 and all measurements were many using Olympus software (CellSens® GS-ST-VI.7, Olympus, Germany). All data and statistics were processed in Microsoft Excel.

**Size distribution of pollen grains**

The diameter of pollen grains arising from control plants was measured in all seven first florets within a spikelet, of a spike that were arranged in order from the youngest (estimated flower opening was 10 days away) to a well-developed floret (estimate flower opening was in two days).

## 3.3. Results

### 3.3.1. Cytological observation in control plant

#### 3.3.1.1. Tetrad formation in control plants

Cytological observation of florets from plants within the control treatment illustrated that at the tetrad phase the tetrad microspore is arranged tetrahedrally and enclosed by a layer of callose. Each microspore was equal in size and appeared to be elliptical in shape (Figure 14a). Formation of a tetrad was the most frequently observed, with tryads present at a relatively low frequency (Figure 12).

#### 3.3.1.2. Size distribution of pollen grains in control plants

The average diameter of pollen grains from each floret varied slightly, with the largest pollen observed in the more mature florets at 59.98 ± 0.70 µm, and the smallest in the youngest florets at 33.25 ±0.17 µm (Figure 11a).

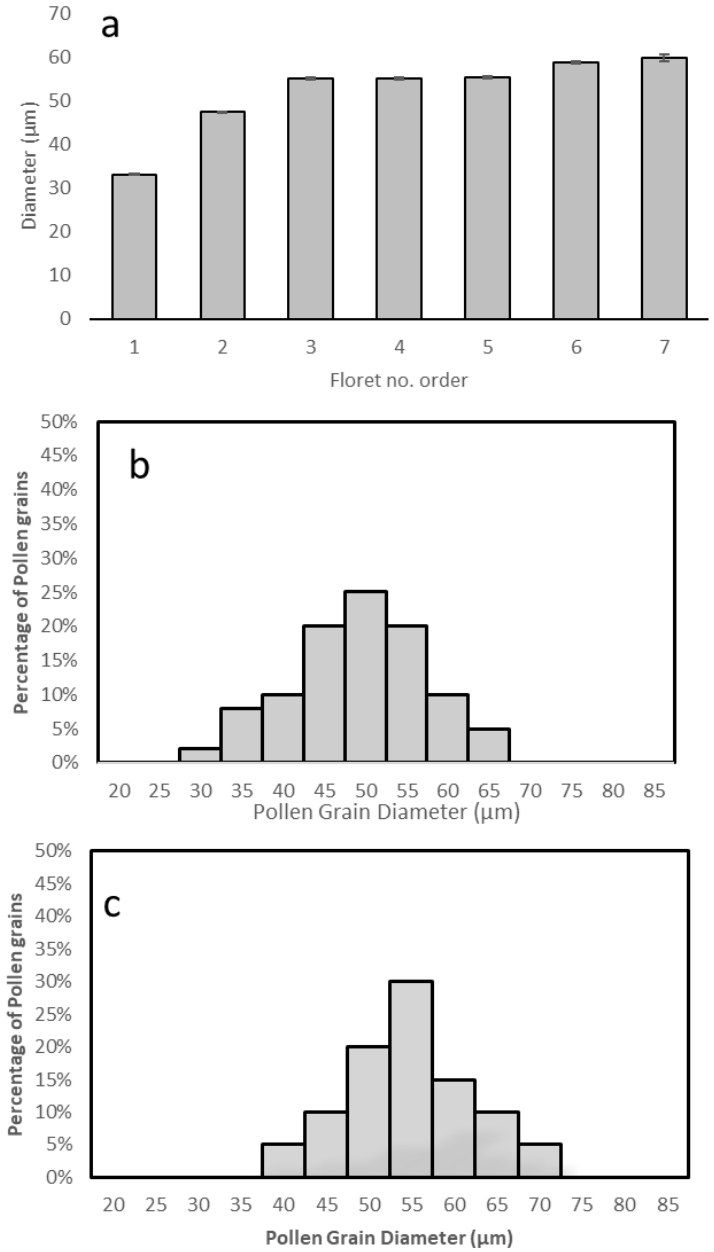


Figure 11. The average pollen diameter of pollen collected from florets at different development stages, and their pollen size distribution in *Limonium sinuatum* flowers. a) average diameter of each floret ordered in an array from one to seven (youngest floret to most mature floret). b) distribution of pollen grain diameters in the control plant youngest floret (floret no.1) c) distribution of pollen grain diameters in control plant oldest floret (floret no.7).

A more detailed analysis of distribution of pollen size diameter for the most developed floret and the least developed (i.e. youngest) floret, resulted in a normal distribution of pollen size with only one peak. For the most developed floret, 45% of the pollen ranged

from 56 to 65 µm in diameter (Figure 11c). In contrast, within the least developed floret, 40% of the pollen measured 51 to 60 µm in diameter (Figure 11 b).

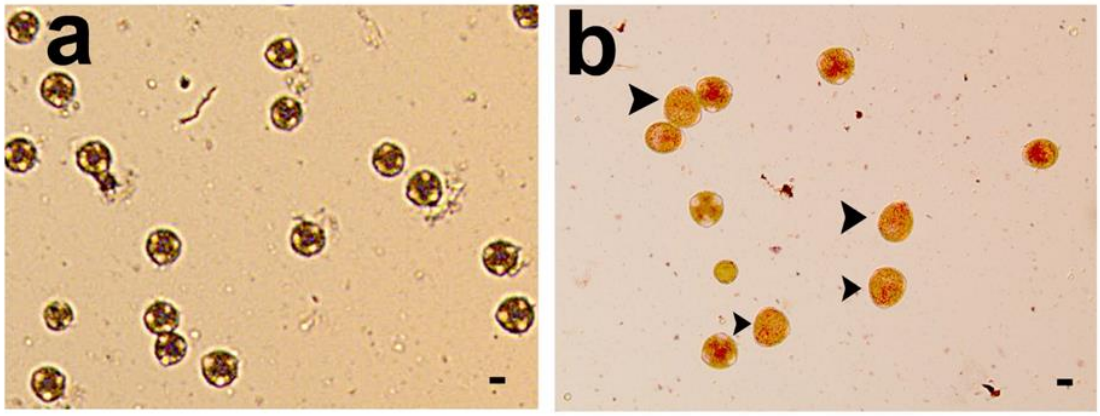


Figure 12. Pollen grains of *Limonium sinuatum.* A) pollen grains from control plant; B. large pollen grains obtained following$N_2O$ treatment for 24 hours (arrowed). Bar pic. = 50 µm

### 3.3.2. The Effect of $N_2O$ Treatment on Tetrad and Pollen Grain Sizes

#### 3.3.2.1. Tetrad formation by $N_2O$ treatment

The $N_2O$ treatment for 24 hour and 48 hour on buds of florets undergoing meiosis resulted in various types of polyad. For the plants that were treated for 24 hours in addition to the tetrad, pentad structures with five microspores were the most common type formed at 16%, followed by dyads and hexads, at 6% and 1%, respectively. When buds were treated for 48 hours a wider range of polyad types was noted, via the inclusion of monads, albeit at a low frequency of 2 % (Figure 13).

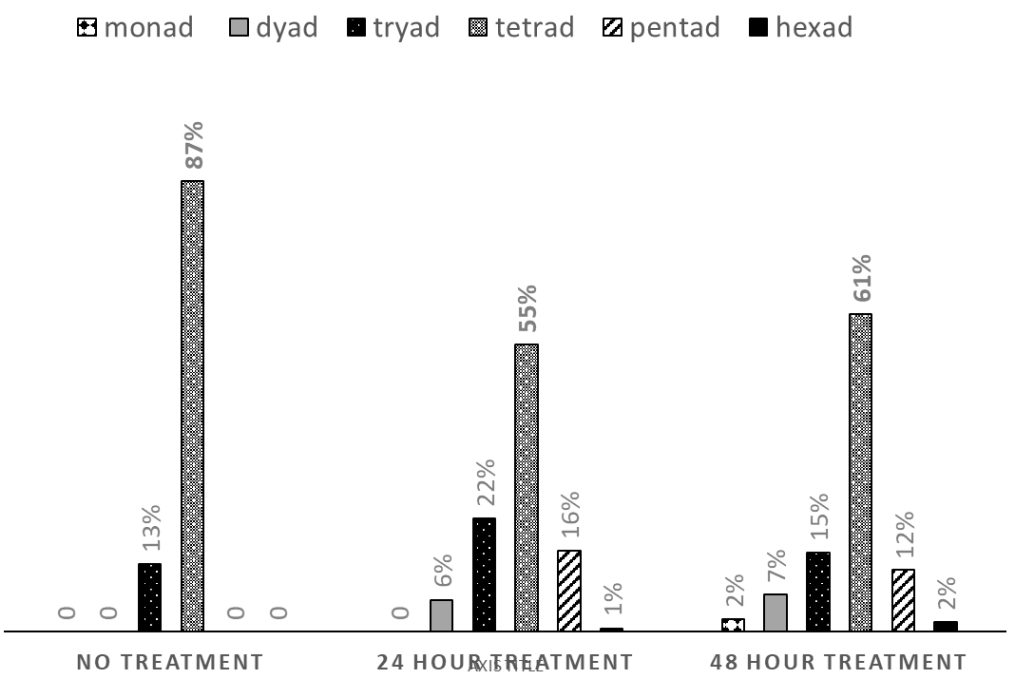


Figure 13. Ratio of polyad tetrad formation in *Limonium sinuatum* following different exposures to $N_2O$ at 600 kPa.

Each individual microspore type varied in shape, and ranged from spherical to an irregular ellipse, and the majority were not equal in size (Figures 14b-f).

Although tetrads were observed from the treated plant, they were distinct in their form compared to the tetrahedral tetrad found in the control plant. The typical characteristic of this off-type tetrad was that each microspore varied in size and shape (Figure 14e).

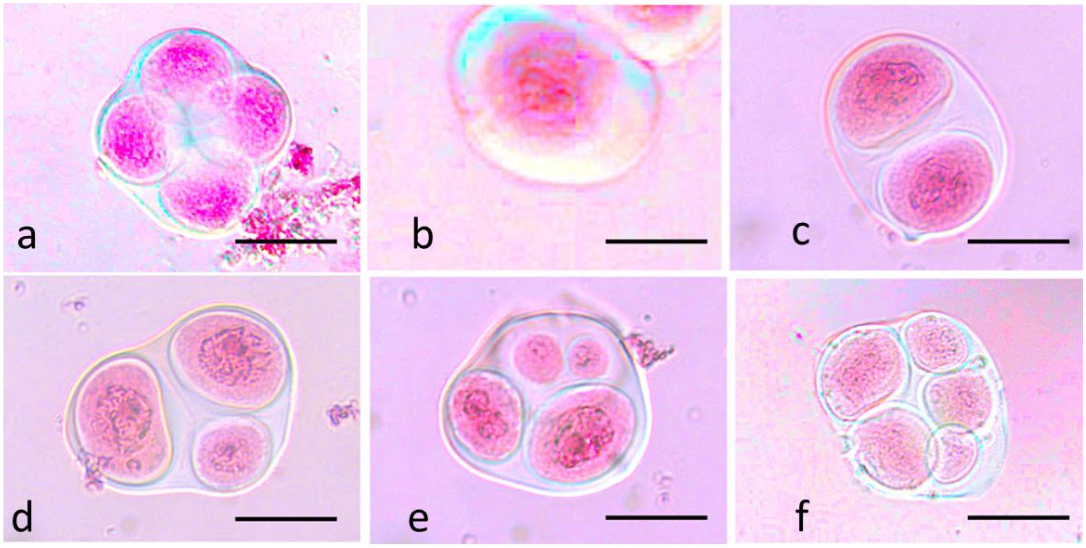


Figure 14. Formation of polyads in *Limonium sinuatum* following nitrous oxide treatment for 24 and 48 hours a) Tetrad in control plant b) Monad c) Dyad d). Triad e). Tetrad f). Pentad
*Bar pic. = 20 µm ;*

### 3.3.2.2. Pollen size distribution

The 48-hour $N_2O$ treatment had an obvious detrimental effect on the development of all the florets on the treated plant, including those florets which would have been actively in meiosis. Following the 48-hour treatment, 15 days after treatment, the growth of the florets was noted as having ceased, wherein they had become stunted and abnormal in their physical appearance. At this stage the florets were obviously wrinkled and withered, with changes in calyx colour, initially to pale white and subsequently brownish on the outer bract (Figure 15a).

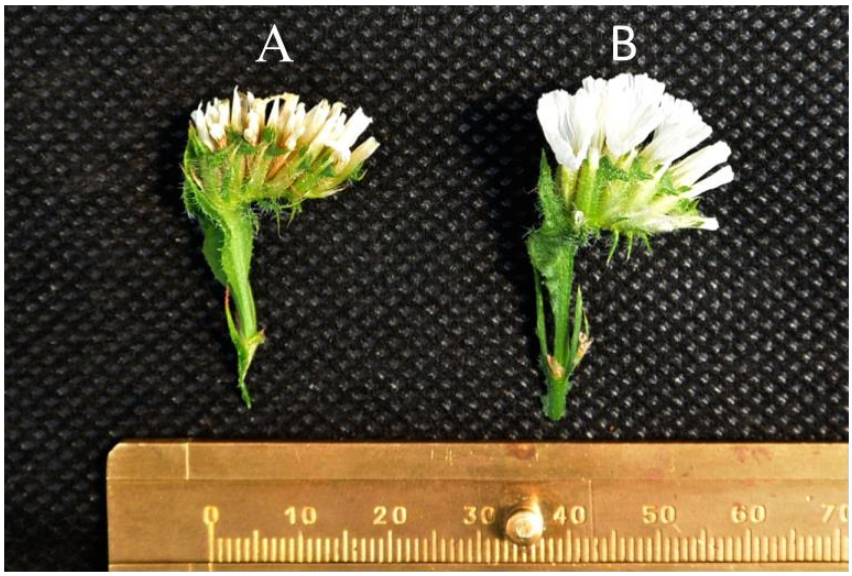


Figure 15. Flowers of *Limonium sinuatum* following treatment with $N_2O$ A) 15 days after 48 hour $N_2O$ treatment B) normal untreated flower.

The petals of the flower were also damaged by $N_2O$ treatment for 48 hours with the effect seen six days after treatment. Dissection of the flower showed stunted and necrotic petal tissue. As the anthers are positioned under the petal in most florets examined the 48h treatment also had also negatively affected anther growth. No pollen could be harvested from florets that received the 48-hour treatment. Thus, no data were obtained in pollen grain sizes of $N_2O$ treatment for 48 hours.

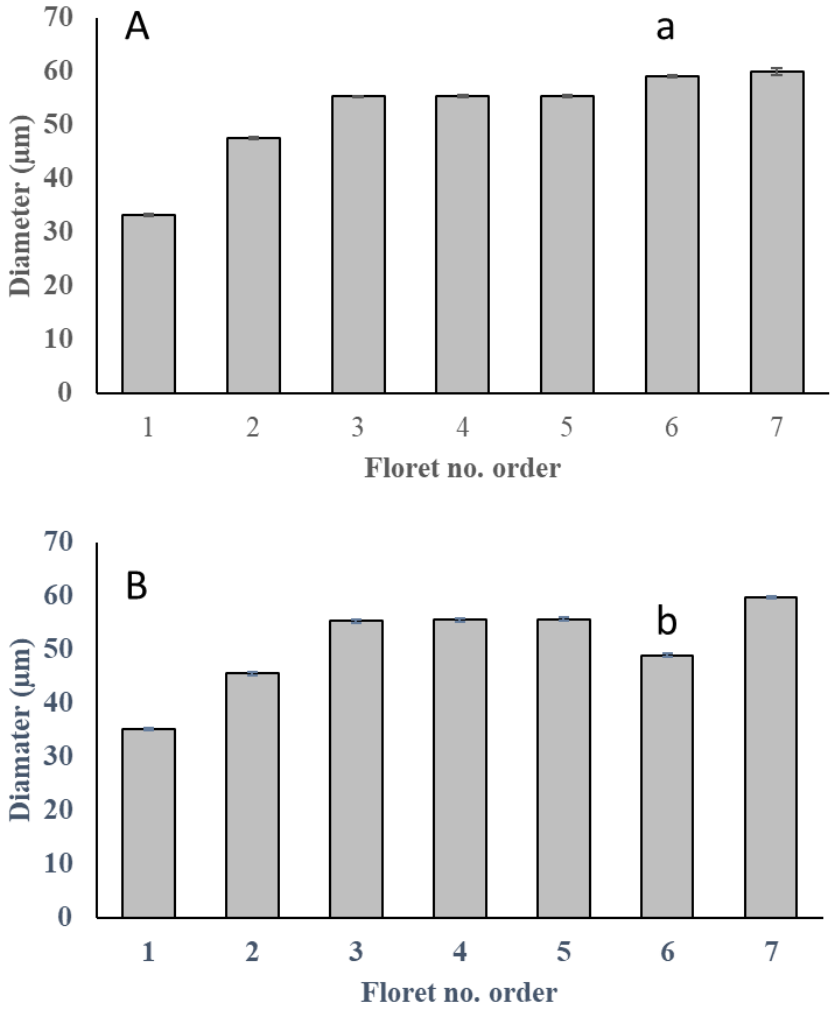


Figure 16. Average diameter of pollen grains of *Limonium sinuatum* florets from an inflorescence arranged from undeveloped (1) to mature (7) florets. A). average pollen grain diameter in each floret of Control plant. B). average diameter of pollen grain in each floret 15 days after $N_2O$ treatment for 24 hours.
Note: different letter indicate statistically significant different at floret no. 6, T.Test $p < 0.05$

The 24-hour $N_2O$ treatment only effected the floret undergoing meiosis at the time of exposure within the treatment chamber. The $N_2O$ treatment for 24 hours at the targeted stage of floret development resulted in a 14% reduction in the average pollen grain diameter of the floret undergoing meiosis at the time of treatment as compared to the average pollen size on control plants (Figures 16a and 16b).

In florets that were undergoing meiosis at the time of $N_2O$ treatment a detailed analysis of the distribution of pollen grain diameter revealed either a bi-modal distribution or a wide size distribution of pollen grain diameters (as shown in Figures 17b and 17c). In the

bi-modal distribution the one peak occurred at 70 µm and the second at 45 µm. In the florets with the wide distribution there was a peak at 40 µm with the largest and the smallest pollen diameters at 25 µm and 80 µm respectively.

On the other hand, the $N_2O$ did not affect florets adjacent to the targeted florets, i.e. those either before or after meiosis. This can be confirmed from the pollen size distribution model that showed no difference in the size of pollen grains between the treated and control plants, as seen in Figure 17a.

The characteristics of pollen resulting from the $N_2O$ treatment were that they were larger in diameter, with an elliptical shape, with the additional appearance of having one or two small apertures in the exine layer and, in some cases, no aperture at all (Figures 12b and 12C). The features of most mature pollen from the control plants include an elliptical shape surrounded by a layer of exine with three obvious apertures typical of type A pollen as seen in Figure 12a.

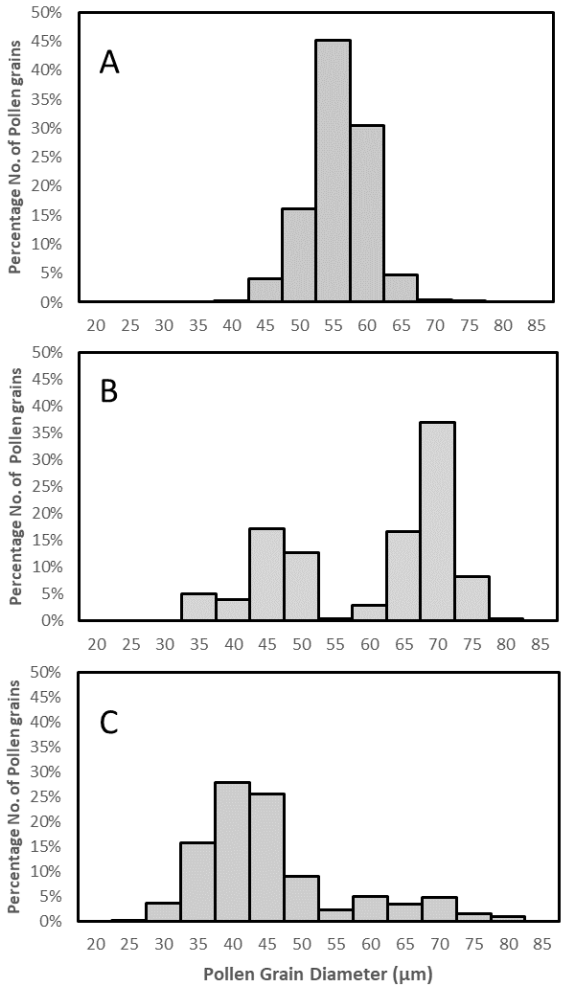


Figure 17. Distribution of pollen grain sizes in *Limonium sinuatum* following 24 hours treatment with $N_2O$. A) Normal distribution in control plant B) Bi-modal distribution in floret from $N_2O$ treatment for 24 hours C) Bi-modal distribution with wider and flatter second peak in floret from N2O treatment for 24 hours.

## 3.4. Discussion

In the present study data from the control plant revealed basic information on the characteristics of the normal pollen. Firstly, the pollen grain size distribution from control plant, showed a histogram characterised with only one peak that was observed in all flowers in any developmental stage from when the developing pollen reaches the early trinucleate phase to near dehiscence. This finding corresponds to that reported in other crops such as tulip, lily, cassava and rubber tree where the control plant would exhibit normally a grain pollen size distribution with one peak, but there is some variation in the average diameter of pollen grains (Lai et al., 2015; Luo et al., 2016; Okazaki et al., 2005;

C. Wang et al., 2011; Yao et al., 2017). Differences in average pollen grain size within and between species is common and suggests that pollen diameter is genotype dependent and characteristic of a given species (Bedinger, 1992; Crespel et al., 2006; Halbritter et al., 2018). Secondly, an elliptical shape was observed in *L. sinuatum* pollen from control plants with three obvious apertures seen in mature pollen. Such a shape is also found in pollen of other crops (Gómez et al., 2015; Kang & Wang, 2010; Lersten, 2004 ; Yang et al., 2016). Acquiring the data on normal pollen is important for detecting 2n gametes in the pollen population (Bullitta et al., 1995; Dewitte et al., 2012; Okazaki et al., 2005).

The $N_2O$ treatment did not affect pollen from any florets except those treated at meiosis. The remaining florets all produced the normal pollen grain size distribution as observed in untreated control plants. There was no evidence of the presence of large pollen. Kitamura et al. (2009) in their experiment in lily found that the $N_2O$ was only effective when developing pollen was treated during meiotic cell division. As in the attempts to induce 2n pollen in tulip and rubber tree, treatment outside of meiotic cell division failed or if it occurred, it was only at a very low frequency (Okazaki et al., 2005; Yao et al., 2017). Basically, the $N_2O$ has properties like colchicine in halting spindle formation or breaking the constitution of the spindle thread so the affected cells retain the chromosomes at the centre; thus the movement of chromosome to the opposite side of the cell doesn't occur resulting in doubled chromosomes in one cell while the other cell remains empty (Dewitte et al., 2012; Kitamura et al., 2009). Therefore the effect of $N_2O$ might be observed in any kind of cell that undergoing cell division by interfering with spindle thread formation as in cells undergoing both meiosis (Akutsu et al., 2007; Barba-Gonzalez et al., 2006; Luo et al., 2016; Nukui et al., 2011), and mitosis as seen in zygotes of maize and *Phalaenopsis* (Chou et al., 2013; A Kato & Geiger, 2002). It was expected there would be also an effect of $N_2O$ on other flowers besides the meiotic flower, as two mitotic events occur during the development of pollen between the uni-nucleate to tri-nucleate stages with cell divisions to produce two generative cells and one vegetative cell (Halbritter et al., 2018; Heslop-Harrison, 2013) but no other flowers were observed to produce large pollen grains. It appeared that 2n gamete formation was not possible in mitosis 1 and 2 during pollen development from the uninucleate to trinucleate stages, but no further investigation was undertaken to confirm this.

The efficacy of 24-hour $N_2O$ treatment for 2n pollen formation can be only seen in a floret where cells are undergoing meiotic cell division. In comparison to the control plants, the affected pollen population had a bi-modal pollen grain size distribution with two peaks or a wider pollen grain size distribution with one major peak, indicating the presence of a more varied range of sizes in the pollen population. The presence of different pollen grain sizes and shapes (spherical or round without obvious apertures) including from extremely small sizes to larger sized pollen is known to be indicative of the partial production of 2n pollen following $N_2O$ treatment (Dewitte et al., 2012; Okazaki et al., 2005). This result is identical to the findings of $N_2O$ treatments in tulip, *Ipomoea* and day lily (Okazaki et al., 2005; Orjeda et al., 1990; Santen et al., 1991), showing a small portion of 2n pollen with mixed sized pollen was common following a successful treatment. The occurrence of these extreme sizes was most likely due to abnormal chromosome numbers in some cells as a result of the treatment (Ramanna & Jacobsen, 2003; Ramanna et al., 2003). Supporting evidence was seen in this experiment with the early observation of small cells containing minute nuclei observed among larger microspores, as shown in Figure 4d and 4e. The presence of abnormal numbers of chromosomes in a gamete is believed to be a source of aneuploidy (Iizuka & Ikeda, 1968).

To determine the success rate of the $N_2O$ treatment, estimation of the frequency of 2n gametes is necessary. There are two possible ways to estimate the frequency of 2n gametes proposed here. Firstly, the estimation of frequency of 2n gamete can be done by directly comparing the pollen grain size distribution in treated plants with control plants. The estimation of frequency of 2n gamete using this approach will not be difficult if the pollen sizes between treated and untreated flowers do not overlap (Bullitta et al., 1995; Okazaki et al., 2005). By doing so, using the control plant pollen grain size distribution in Figure 17 a. pollen larger than 65 µm is considered as 2n pollen.Thus the estimated frequency of 2n pollen formation the the flowers in Figures 17 b and 17 c is from 9% to more than 50%.

On the other hand, the estimation of frequency can also be done by assuming that 2n pollen will have two times more volume than n pollen. This approach provides a way to estimate 2npollen frequency when the pollen grain szie distribution in untreated plants is unknown. Large pollen grains with a population are frequently attributed as 2n pollen,

and accompanied by a positive correlation between DNA content and cell volume and, therefore pollen diameter (Dewitte et al., 2012). Theoretically, according to Okazaki et al. (2005) multiplying, the average pollen diameter of control plant by factor of 1.26 value that derived from assumption that if the volume ratio of n and 2n pollen grain was 1:2, then the diameter ration of n and 2n was 1: $\sqrt[3]{2}$ (ca,. 1.26), Since the average diameter of mature pollen from control plants of *L. sinuatum* was around 59.98µm, the expected diameter of 2n pollen, based on this formula, would be ~ 75 µm (59.98 µm X 1.26). Using this value as a standard estimator for the presence of 2n gametes, all pollen with diameter above 75um was categorised as 2n pollen. From the data in the two type bi-modal pollen grain size distributions of treated plants in Figures 17b and 17c, the estimated frequency of the 2n pollen was approximately 2% and 9 %, respectively..

Even though there are differences in the frequency between the both analyses, it does appear that $N_2O$ treatment for 24 hours increased the frequency of 2n pollen formation.

In line with this experiment, an increase of 2n gamete frequency of up to 20 % was reported in tulip, with increased frequencies of 2n gametes also found in other crops such as begonia and lily following $N_2O$ treatment (Dewitte et al., 2010a; Lucidos et al., 2012; Okazaki et al., 2012). However, it is important to note that the presence of large pollen might not reflect doubled chromosomes. Dewitte et al. (2012) recommended confirming the presence of doubled chromosomes through flow cytometry analysis of pollen or progeny which is not done yet in this experiment.

Nitrous oxide treatment is known for its efficacy for increasing the frequency of 2n gametes in several crops at correct timing (Prophase1-Methaphase 1) (Akutsu et al., 2007; Barba-Gonzalez et al., 2006; Lucidos et al., 2012; Okazaki et al., 2005; Okazaki et al., 2012; Younis et al., 2014) and in this case has resulted in 2n pollen production in *L. sinuatum*. In comparison to the general natural occurrence of 2n gametes, estimated to occur at approximately 0.1-2% (Ramsey, 2007) and the frequency of 2n pollen generated in some hybrids (about 28%) (Ramsey & Schemske, 1998) it appears that the rate 2n pollen formation from $N_2O$ treatment in *L. sinuatum* (from 9 % to up to 50%) is moderate to high. The partial production of 2n pollen in this experiment is thought most likely to be related to the asynchrony and rapid progression of meiosis in *L. sinuatum* (as described in Chapter 2). The $N_2O$ treatment in *L. sinuatum*, for 24 hours in this experiment was started at 7.30 AM (the best description of meiosis condition at this time can be seen in

figure 9b from 7.30 AM to 7.45 AM in chapter 2) where a large proportion of the pollen mother cells of the marked floret were undergoing meiosis from Prophase 1 to Metaphase 1, and that the $N_2O$ treatment has a strong tendency to affect cells at those stages . Of the remaining cells, a smaller proportion were still in early stages of prophase 1 and some were already progressing toward the end of telophase II. This indicates that meiosis was halfway toward the complete first cycle of meiosis, and it was suspected the cells within those stages seemed to be getting a partial affect of $N_2O$ treatment resulting in pollen grains with varied sizes. This sort of dynamic situation is also seen in experiments with rubber tree to induce 2n gametes, where the asynchrony was quite strong, thus an unexpected frequency of 2n gametes was observed after heat treatment (Yao et al., 2017).

In *Lilium* and tulip experiment where the progression of meiosis is relatively less asynchronous, the relatively high frequency of the 2n gamete formation (up to 85%) can regularly be achieved (Luo et al., 2016 ; Okazaki et al., 2005)). Therefore, the best possible approach to optimise the production of 2n pollen is the $N_2O$ application when most cell are better synchronised. I *Limonium sinuatum* this is observed between 6 to 7 AM where cells were progressing toward the early metaphase 1 (figure 9a in chapter 2) while rapid progression and strong asynchrony started to occur after 7 AM, after metaphase 1. Therefore, treatment of $N_2O$ starting between 6 to 7 AM would be an best to optimise the production of 2n pollen in a future experiment. .

## 3.5. Conclusion

This study proved that $N_2O$ treatment can be used for the production of 2n pollen in *L. sinuatum.* The treatment with $N_2O$ for 24 hours during meiotic cell division is an effective method to generate 2n pollen, with only cells undergoing meiotic cell division at the time of treatment being affected. This treatment resulted in an increased frequency of 2n pollen in *L. sinuatum* up to more than 9 %. The presence of 2n pollen following the $N_2O$ treatment for24 hour can be detected from as early as microspore tetrad stage 30 minutes after the plant has been removed from $N_2O$ treatment chamber. The putative 2n pollen revealed unique characteristics, they are large, almost 1.25 times larger diameter in comparison to the n pollen. The $N_2O$ treatment for 24 hours also generated a wide variation in pollen grain size, including very small and very large pollen grain and with a bi-modal pollen gran size distribution. The $N_2O$ treatment is a reliable method for 2n pollen formation, which can give a further benefits in plant improvement programs. The

additional approach to confirm the 2n large pollen chromosome number by flow cytometry analysis will be useful to improve the accuracy of detecting 2n pollen in future experiments.

# CHAPTER 4.

# Cross breeding of *L. sinuatum* utilising pollen derived from the $N_2O$ treatment for 24 hours

## 4.1. Introduction

Generating polyploid plants via sexual polyploidisation through 2n pollen can be used to generate superior plants (Younis et al., 2014). The role of 2n pollen to generate polyploidy cannot be underestimated because in many cases a polyploid plant is able to be generated through hybridisation by utilising induced 2n pollen (Dewitte et al., 2012; Younis et al., 2014). Interspecific crosses in *Limonium* utilising closely related species resulted in several prominent cultivars such as 'Misty Blue', 'Beltlaard', and 'Emile' (Morgan & Funnell, 2018). When relatively distant species are used in breeding program, issues can arise with low seed in the hybrid progeny due to infertility and ploidy incompatibility (Morgan & Funnell, 2018). In some instances, through the utilisation of 2n gametes including 2n pollen sterility and low production of viable seed can be solved in one step as exemplified in studies with lily, begonia and tulip (Dewitte et al., 2010; Dewitte et al., 2012; Okazaki et al., 2012). Therefore, optimising possible implementation of 2n pollen production in *Limonium* breeding programs, is expected to offer new opportunities to speed breeding programs.

The basic knowledge in parental plant pairing necessary for achieving a high success in crossing for viable seed in *Limonium* is understood. *Limonium* has heteromorphic breeding (Baker, 1966). *L. sinuatum* (2n =16) is categorised as a sexual species having dimorphic system with two types of pollen and two types of stigma (type A/B pollen and cob or papillate stigma) (Baker, 1948, 1953b). Plants with cob stigmata always produce type A pollen and plants with papillate stigmata produce type B pollen (Baker, 1948, 1953a). The successful cross combination requires that type A pollen land on papillate stigmata and type B pollen land on cob stigmata (Baker, 1966). In this case *L. sinuatum* is obligate outcross species, It is not necessary to emasculate *L. sinuatum* florets prior to cross pollination.

In this study, the pollen derived from the 24 h $N_2O$ treatment were used in compatible crosses expected to give viable seed. Florets containing the putative 2n pollen were

crossed one way to a female diploid parent of *L. sinuatum*. The ploidy of the progeny from this cross and those from a (untreated) control cross of *L. sinuatum* were examined using flow cytometry.

## 4.2. Materials and methods

### 4.2.1. Plant Materials

Eighteen diploid *L. sinuatum* (2n=16) flowering plants (4-6 months old) were used in this crossing experiment with six of them assigned as male parents used for 2n pollen induction by $N_2O$ treatment from Chapter 3 (three plants had type A pollen and three plants had type B pollen) and twelve were female parents (6 plants with cob stigma and 6 plants with papillate stigma). Plants were grown and maintained in a plastic house in the Plant & Food Research facility in Palmerston North with a minimum temperature of 15° C and a ventilation temperature of 24 °C.

### 4.2.2. Induction of 2n Pollen by N2O treatment for 24 hours

Six diploid *L. sinuatum* plants (three plants with type A pollen and three plants with type B pollen) were selected for $N_2O$ treatment for 24 hours. Before the plants were put into the chamber, all the meiotic stage florets on each plant were marked on their calyces by waterproof marker. Three plants at a time were placed inside the $N_2O$ chamber, 1.5 hour after sunrise. The plants were treated with $N_2O$ at 600 kPa for 24 hours. After treatment the plants were removed from the chamber and held in the green house until the anthers in the meiotic florets dehisced. From earlier results in this thesis this took approximately 16 days from treatment.

### 4.2.3. Crossing Experiment

There were two groups of plants used, the control cross and the crossing experiment group. For the control cross, six diploid *L. sinuatum* were used (three plants assigned as male parents, two plants with type A pollen and one with type B pollen) and three others as female parents with two plants with cob stigmata and one with papillate stigmata). Pollinations were carried out soon after anther dehiscence. The plants assigned as female

parents, for example with cob stigmata were pollinated by type B pollen from the assigned male parent plants and vice versa. For pollination dehiscent anthers were collected from the freshly opened florets and pollen transferred by rubbing the anther onto the stigma of female parent. Emasculations were unnecessary before carrying out the pollinations.

In the crossing experiment utilising putative 2n pollen from $N_2O$ treatment, pollinations were carried out soon after dehiscence of the anthers of the indicated meiotic florets from the $N_2O$ treatment. Before pollination florets of all female parent plants used in crosses were emasculated to ensure that only pollen from flowers treated at meiosis landed on the stigma surface. The pollinations were conducted in the same way as in the control cross except that the number of pollinations that could be carried out using pollen from the flowers treated at meiosis was limited, with one floret containing putative 2n pollen used to pollinate a maximum of four to five female florets.

All seed were collected, 30 days after pollination and sown in a soil-less (bark; pumice 50 : 50) medium then grown in a fog tent within the greenhouse for four weeks. Seedlings were then transferred to an open mist bench for continued growth after another four weeks.
The weight, width and length of all seeds were measured before sowing and planting.

### 4.2.4. Determination of ploidy level within seedling population.

The analysis of ploidy in the seedlings was performed by flow cytometry. Young leaves were collected from all seedlings from the control cross, from the crosses involving 2n pollen and from the parent plants. The protocol for obtaining the nuclear DNA content followed the method described by Morgan et al (1995) with samples of the same *Bellis* plant used as an internal standard.

## 4.3. Results

The control cross resulted in a large number of viable seed. Mature viable seed, indicated by the brown colour of its seed coat was able to be harvested at 30 days after

pollination. There was one seed per ovary in all successfully pollinated florets, with the proportion of the florets producing viable seed from the crosses being 76 %. The shape of the seed derived from the control cross was uniformly torpedo (Figure 19 a) with an average weight of 4.67 ±0.07 gram (Figure 18). Out of 20 seed sown, 16 were successfully germinated and all 16 seedlings were grown into normal plants (80% germination success rate).

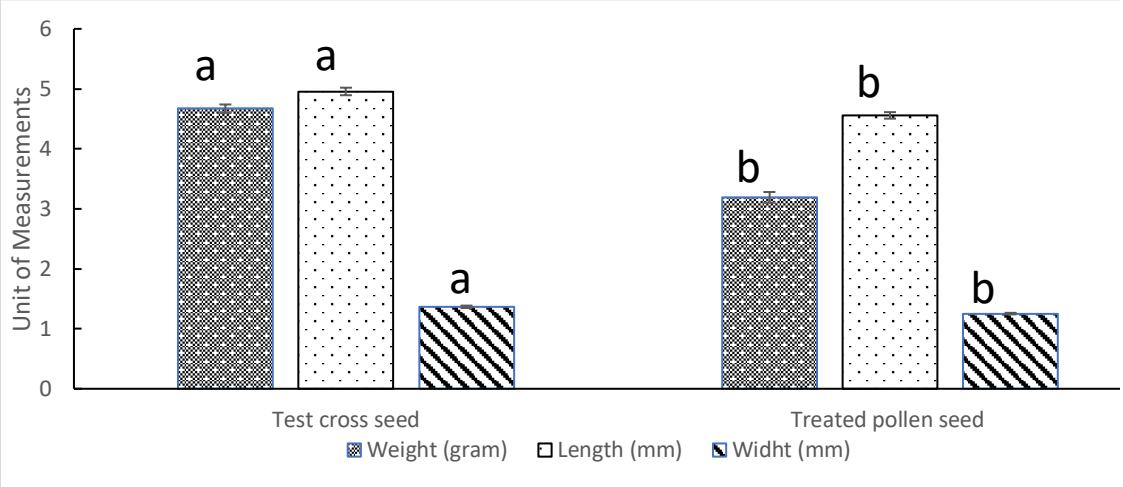


Figure 18. Bar chart of seed weight, length and width between seeds derived from pollination using pollen from *Limonium sinuatum* flowers treated with $N_2O$ at 600 kPa for 24h at meiosis. Different letters indicate significant differences using T-Test at 0.05.

The crosses using pollen from flowers treated at meiosis resulted in a low frequency of viable seed. Out of 500 crosses only 114 florets produced viable seed (22.8 % pollination success rate). One observation of an immature seed from a pollinated floret at 10 days after pollination (under microscope), showed an undeveloped ovule with some necrotic tissue providing evidence of abnormal seed development (Figure 19d).

At seed harvest at 30 days after pollination, it was observed that 14 seed out of 114 appeared to have an abnormal shape and varied in size (Figures 19 b and 19c). The average seed weight from this cross was 30 % lighter in comparison to seed from the control cross (Figure 18). Following sowing and planting, 88 out of 114 seed germinated and grew normally (77% germination rate).


Figure 19. Seed of *Limonium sinuatum* derived from crossing experiments. a.) five viable seeds from a control cross with all seed of an identical torpedo-like shape b) seed derived from a cross in which the pollen was sourced from flowers that had been treated with N2O (24h and 6atm) at meiosis to induce 2n pollen formation were small but with a torpedo-like shape. c). two seeds with irregular form of ellipsoid shape derived from a cross involving pollen from N2O treated flowers. d.) undeveloped ovule from a cross using 2n pollen at 10 days after pollination e.) Ovule derived from a control cross at 10 days after pollination

a-c Bar = 1mm (d-e) Bar = 2 mm.

Flow cytometry analysis of progeny and parent showed similar histograms from crossing involving pollen from treated and untreated flowers and their parents are shown in Figure 20.

All progeny and parents from the control cross resulted in a histogram where the sample peak was about 100 channels. The progeny derived from the cross that involved pollen from treated flowers also had histograms with peaks at about 100 channels. No polyploid plants were detected (the peak for tetraploid plants was predicted at about 200 channels).

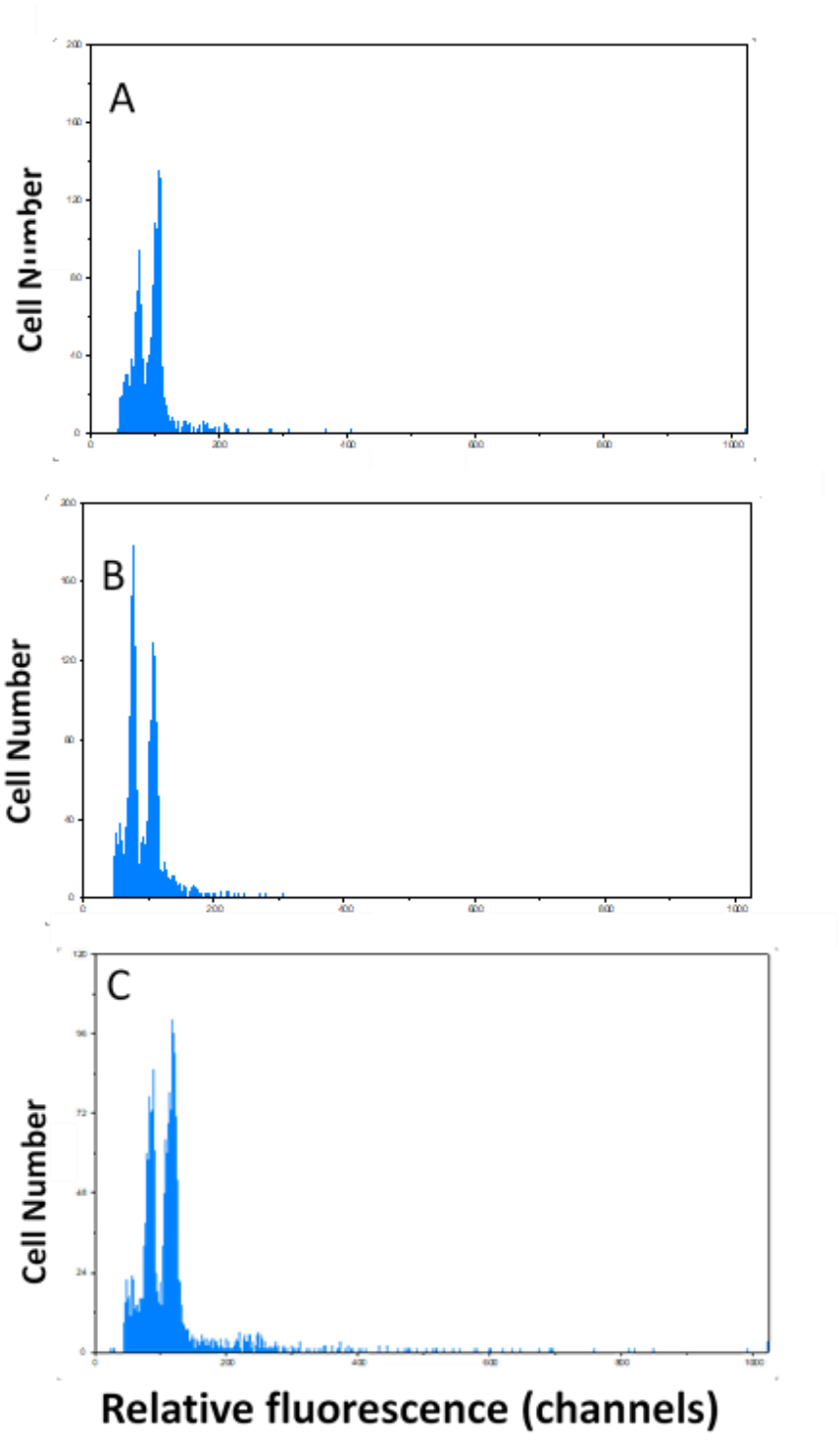


Figure 20. Histograms from flow cytometry analysis of *Limonium sinuatum.* (A) Histogram of the *L. sinuatum* parent. (B) Histogram of *L. sinuatum* seedling from controlled of two diploid plants (C). Histogram of *L. sinuatum* seedling derived from a cross pollen from flower treated at meiosis and with pollen shown to have a larger average diameter.

All histograms shows two peaks. Peak 1 is the internal reference, *Bellis*. The second peak is the *Limonium* sample.

## 4.4. Discussion

In this experiment, there were no polyploid seedlings obtained from crosses utilising the pollen from $N_2O$ treated flowers. The result in this experiment was in contrast with previous findings of the effects of $N_2O$ treatment in crops such as in tulip and lily (Luo et al., 2016; Okazaki et al., 2005). Therefore, the possible reason for the cause of this constraint and a possible approach to overcome the issue is also proposed.

The main factor that might explain the failure in obtaining polyploid progeny is the triploid block mechanism. The triploid block is a term used to explain the difficulties in producing viable triploid seeds commonly observed in interploidy (e.g. diploid x tetraploid) hybridisation which results in failure of endosperm development and ultimately seed development (Marks, 1966)). According to Okazaki and Nishimura (1998) the triploid block will only result in a high portion of diploid plants in progenies irrespective of the parent line used as the polyploid, as exemplified in 2x X 3x crosses in tulips.

The triploid block may range from weak to strong as determined from the analysis of progeny (Ramsey & Schemske, 1998). An obligate triploid block operates when there is no direct polyploidy detected in progeny (Ramsey & Schemske, 1998). In this experiment, it is assumed that an obligate or strong triploid block operates in *L. sinuatum* due to the fact that no direct polyploid progeny were obtained. However, it is expected that within the amount of undeveloped or aborted seed (77.2 %), high in comparison to the control cross (24 %), some might have contained a polyploid embryo. This is based on an indication of some ovule growth that may in turn indicate some early embryo development (Figure 19 d) though no further investigation was undertaken to investigate this in more detail. If this is the case, then the opportunity existed to recover polyploid progeny.

Practically, to recover polyploidy plant from of abortive seed, embryo rescue technique would be conducted. Embryo rescue is an in vitro culture method to recover plants from abortive embryos that is conducted in an aseptic environment with the embryos planted in an artificial medium. Embryo rescue is a common technique to increase the frequency of production of hybrid plants particularly from crosses between distantly related species

(Reed, 2004). Morgan et al. (1995) found that embryo rescue is an effective approach in *Limonium* to recover hybrids from seed that would normally abort. Embryo rescue has been applied in *Limonium* to recover embryo in hybrids between *L. perigrinum* X *L. purpuratum* and *L. perezii* and *L. sinuatum* (Morgan et al., 1995; Morgan et al. 1998). Similarly in tulips, embryo rescue was used to recover polyploid plants that would normally abort from the cross utilising the putative 2n pollen, with triploid, tetraploid and few aneuploid plants recovered (Okazaki et al., 2005; Okazaki et al., 2012).

In addition, prior to embryo rescue being implemented, the improvement of pollination techniques may favour a high proportion of polyploidy plant. The issue related to the pollination technique in this experiment is the mixed pollen which consisted of some portion of 2n pollen that were present following the $N_2O$ treatment for 24 hours. The proportions of large and small pollen grains means that chances for only large pollen to fertilise the egg are reduced. An approach to overcome this problem was demonstrated in tulip by sieving the pollen to screen for large pollen prior to pollination. This technique increases the probability of only 2n pollen landing on the stigma surface and fertilising the egg resulting in polyploid progeny in tulip (Okazaki et al., 2005).

## 4.5. Conclusion

The use of 2n pollen in the crossing experiment resulted in no polyploid plants. Despite this result there is evidence that 2n pollen was successful in pollination. The finding that a high number of pollinations failed to produce viable seed in pollination using 2n pollen, compared to the control pollination, suggested that some portion of embryos from the failed pollinations resulted from 2n pollen fertilising the egg cell. Moreover, there is no polyploid plant from germinated seed though viable seed was harvested in pollination from this experiment, indicating that the pollen that successfully proceeded into double fertilisation and hence viable seed is n pollen instead of 2n pollen. It is likely a mechanism operated that prevents the normal formation of seed in such pollination called the triploid block. One of strategy recommended for overcoming this problem is through embryo rescue that has been previously applied in *Limonium*.

# CHAPTER 5

# Overall Conclusions and Recommendations

## 5.1. Conclusion

The exploration of diploid (2n) pollen formation through the application of $N_2O$ treatment in *L. sinuatum* has been examined. In the scope of this thesis work, studies involved a sequence of steps including a preliminary study on meiosis through to a study of pollen development and finally study of the effect of N2O treatment on production of 2n pollen and its use in crossing.

In investigations of meiosis (presented in detail in chapter 2) a cytological study in combination with a study of flower development provided basic information on the sequence of pollen development in *L. sinuatum.* The flower developmental stage analysis was relevant for later use in timing of N2O application.

The complete sequence of pollen development stages were documented from the formation of the pollen mother cell (PMC) to shedding of mature tri-nucleate pollen from dehiscent anthers. Pollen development was tightly linked with the flower developmental stages and it takes approximately 18 days to complete from the PMC entering meiosis to anther dehiscence and pollen shedding, or from the time the floret is at the bud size where meiotic cell division occurs to fully developed florets. This time frame strictly only applies for the very first six to seven florets to develop in an array within the spike in the arrangement of an inflorescence.

All meiotic cell division activity takes place in a floret in bud of approximately 0.8 mm in diameter, this is called the meiotic floret. The meiotic floret is consistently located three florets behind the floret marker, a term used to describe the floret used to identify the meiotic floret on intact inflorescences in the field. The distinctive visible feature of this floret marker is that its calyx is just starting to show colour because the length of the calyx has just begun to exceed the length of the enclosing bract (Chapter 2, Figure 7).

Once the meiotic floret is reliably identified, the meiotic cell division can be investigated in more detail. Meiotic cell division activity begins to increase approximately 15 minutes after sun rise (ca 6.15 am sun rise), and enters the dominant phases from prophase I to metaphase 1 start at 7.15 am to 7.45 am (approximately 1 to 1.5 hour after sun rise).

Asynchrony of meiosis was detected in *L. sinuatum*, among cells in a floret and among florets within a raceme. The asynchrony was characterised by the presence of two or more meiotic stages at the same time in a single floret. This asynchrony became apparent from immediately after the sun had risen mostly as only two different stages, and it became more obvious during the rapid meiosis progression, one hour after sun rise when four different stages were observed. These stages ranged from the cells starting to enter metaphase I to stages at the end of the meiotic cell division such as tetrad microspore formation. Moreover, asynchrony in meiotic cell division was also detected among florets and was more profound among florets in different locations within the raceme. For example, in one observation a floret located at the proximal end of the raceme had meiotic cells undergoing prophase1 to metaphase 1, while florets at the distal end were undergoing prophase II to tetrad formation.

The $N_2O$ treatment for 24 hours in *L. sinuatum* has great potential and is effective in increasing the frequency of 2n pollen formation. The presence of 2n pollen can be inferred from cytological observations at the end of meiotic division (at tetrad microspore stage), by the observed formation of various polyads. Apart from the tetrad and tryad type, dyads, pentads and hexads are the most common. Tryads and tetrads constitute up to 75 %, dyads and pentads combined constitute up to 20 %, and hexads occur at less than 2 %. Interestingly, some tetrad types derived from the treatment showed characteristics that differed from the normal tetrad as shown by the unequal and irregular shapes of microspores.

When observed at pollen maturity 15 days after $N_2O$ treatment, various sizes of pollen grains within the pollen population were seen only in the florets undergoing meiosis at the time of treatment. The size distribution of pollen grains following treatment at meiosis formed two types of histogram, either a bi-modal pollen size distribution with two distinct peaks (at 35 μm and 75 μm) or a bi- modal distribution with what appeared to be a flattened second peak, similar to size distribution of normal pollen but with extreme

pollen grain sizes (from 30 μm to 80 μm). All other florets in the treated spike had a normal pollen size distribution similar to that in control plants. This result indicated that $N_2O$ treatment only affected the cells undergoing cell meiotic division. While the average diameter of pollen in the control plant is approximately 59.98± 0.70 μm, the smallest pollen size is approximately 40 μm and the largest approximately 70 μm, this pollen size distribution histogram comprised only one peak.

From this result, it can be estimated that the frequency of 2n pollen which was expected to be approximately 1.25 times larger in diameter than the normal pollen grains (the 2n pollen was assessed as pollen with a diameter larger than approximately 65 μm). The frequency of pollen that has this size in this experiment was about 9 % to more than 50% of the total pollen population.

The treatment with $N_2O$ for 48 hours resulted in cytological changes observed at the tetrad microspore stages, with observation of monad polyads in addition to the dyad to pentad type, however no pollen resulted. The 48 hours treatment was so detrimental to flower development, that floret growth aborted and pollen was not produced.

A crossing experiment in which the 2n pollen derived from the 24-hour $N_2O$ treatment was crossed to a diploid female parent of diploid plant (2n=16) *L. sinuatum* was undertaken. In the control cross, using diploid plants as male and female parents, the pollination resulted in approximately 76 % of pollinated flowers producing viable seed of the normal torpedo shape typical of the species. In the treated cross using pollen from treated flowers, out of 500 crosses only 114 floret produced viable seed a pollination success rate of 22.8 % which was much lower than the control cross. The shape of the seed resulting from the treated cross was variable, with some seed having irregular shape and form, and being smaller than seed from the control cross. There were however no polyploid plants in the progeny from the treated cross identified using flow cytometry. There is the likelihood that a triploid block mechanism operated in preventing maturation of seed with polyploid embryos however this was not investigated further in these experiments.

The 2n pollen as an agent of polyploidisation is known to offer a promising benefit in the breeding program in several crops. In this current experiment the treatments of $N_2O$ is

effective to increase the frequency of 2n pollen in *L.sinuatum*. The cytological study of pollen development then provides basic information prior to N2O treatment, in particular the knowledge of the timing of the specific events in meiotic cell division. The most obvious sign that indicate the present of 2n pollen in the pollen population following the $N_2O$ treatment is large pollen grains within the pollen population. The presence of 2n pollen also can be detected as early as the microspore tetrad stage, by the observation of polyad. Though no polyploid plants were obtained from this experiment the further use of 2n pollen in direct hybridisation in *L. sinuatum* has potential to produce polyploidy to speed the breeding of *Limonium*, in situations where the polyploid plant can be recovered from the abortive seed. Opportunity still exists for overcoming the issues in future experiments to obtain polyploids as explained in the recommendation section

## 5.2. Recommendation

The following provides several recommendations for factors that should be taken into account to improve the result obtained in this preliminary study in future experiments.

Firstly, the cytological approach utilising the destructive method may not have been the best method with which to follow the rapid progression in meiotic cell division seen in *L. sinuatum*. This is a possibility that some important details of meiosis were missed using the protocol described here. To improve the cytological study a live cell imaging system for studying the cytological events is recommended. It is anticipated this will allow discovery of more detail of the asynchrony in meiotic cell division. The additional detailed information, might help better target the $N_2O$ treatment application in future experiments.

Secondly, in the crossing experiment, there are two suggestions proposed as to why only diploid but no polyploid progeny was obtained. The crossing resulted in a large percentage of pollinations with no seed when using pollen from treated flowers. This might indicate a complete triploid block mechanism is involved, in which case embryo rescue will increase the likelihood of recovering polyploid hybrids in the seedling population.

Furthermore, following $N_2O$ treatment pollen consisted of not only 2n pollen, but also n pollen and other mixed pollen. In this case, to avoid mixed pollen in pollination, it is recommended to sieve the larger pollen grains with a diameter more than 75 μm (this size is used considering that it does not overlap in size between the control plant and treated plant) before crossing to increase the chances of only 2n pollen fertilizing the ovule and diminishing the portion of diploid seed.

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