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Pollination in Plants

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Notice

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Meet the editor

Phatlane William Mokwala received his BSc, BSc HONS, and MSc degrees from the University of Limpopo, South Africa, then called the University of the North. He received his PhD degree from the University of Pretoria, South Africa. He is currently working as a senior lecturer in Plant Physiology and Plant Biotechnology at the University of Limpopo. He went through the ranks from a Senior Laboratory Assistant, a Tutor, and a Lecturer to the current Senior Lecturer position in Plant Physiology and Plant Biotechnology. His research interests focus on the biotechnology of soybean (*Glycine max*) and reproductive biology of watermelon (*Citrullus lanatus*).

Contents

Preface

Pollination is an important precedence to reproduction in flowering plants. The productivity of plants in terms of fruit and seed set depends on the efficiency of pollination. As a result, pollination is equally essential for the productivity of plants in crop production systems and ecosystems. In nature, this process has been perfected over the ages with plant evolving mechanisms to ensure pollen deposition on the stigma. Through coevolution, plants have developed mechanisms of attracting pollinators and rewarding them for the performance of pollination.

Changing agro-ecological conditions—such as land degradation, deforestation, monoculture agricultural production systems, and global warming—result in low productivity of plants. This makes it necessary that pollination systems within these changing conditions be studied and appropriate measures taken to maintain productivity in the agro-ecological systems.

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Chapter 1

Introductory Chapter: Pollination

Phetole Mangena and Phatlane William Mokwala

Additional information is available at the end of the chapter

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1. Introduction

Pollination is the transfer of pollen grains from the anther, which is the male part of the flower, to the stigma, which is on the female part. This process normally precedes fertilization. It is an important process in the reproduction of plants without which sexual reproduction will not take place. It is a process that has been coordinated and perfected over the ages as plants coevolved with animals, where the animals act as pollinators or pollination agents. The plants and animals coexist in same habitats [1].

The coevolution between plants and animals in respect of pollination led to the development of pollination syndromes. In pollination syndromes, specific pollination agents pollinate specific plants or flowers. This is, however, not a water tight arrangement as there are polyphilic flowers which attract and are pollinated by different types of pollination agents and polytrophic pollinators which are attracted and pollinated by different types of flowers. Pollination syndromes are a symbiotic relationship between the plants and the agents or animals in which both benefit. The agents get nutrients from the flowers and the plants benefit from improved reproduction (**Figure 1**). The latter is very important in crop production.

There are two types of pollination, namely, cross- and self-pollination. In cross-pollination, pollen grains are transferred from the anther to the sigma of a different plant. Cross-pollination when followed by fertilization leads to the production offspring with heterosis or hybrid vigor. Self-pollination, on the other hand, is the transfer of pollen grains from an anther to a stigma of the same flower or plant. This often results in inbreeding depression where undesirable recessive traits are expressed in the offspring. Self-pollination occurs in species where pollination agents are scarce and in closed flowers where the pollination agents have limited access to the sexual structures of the flower.

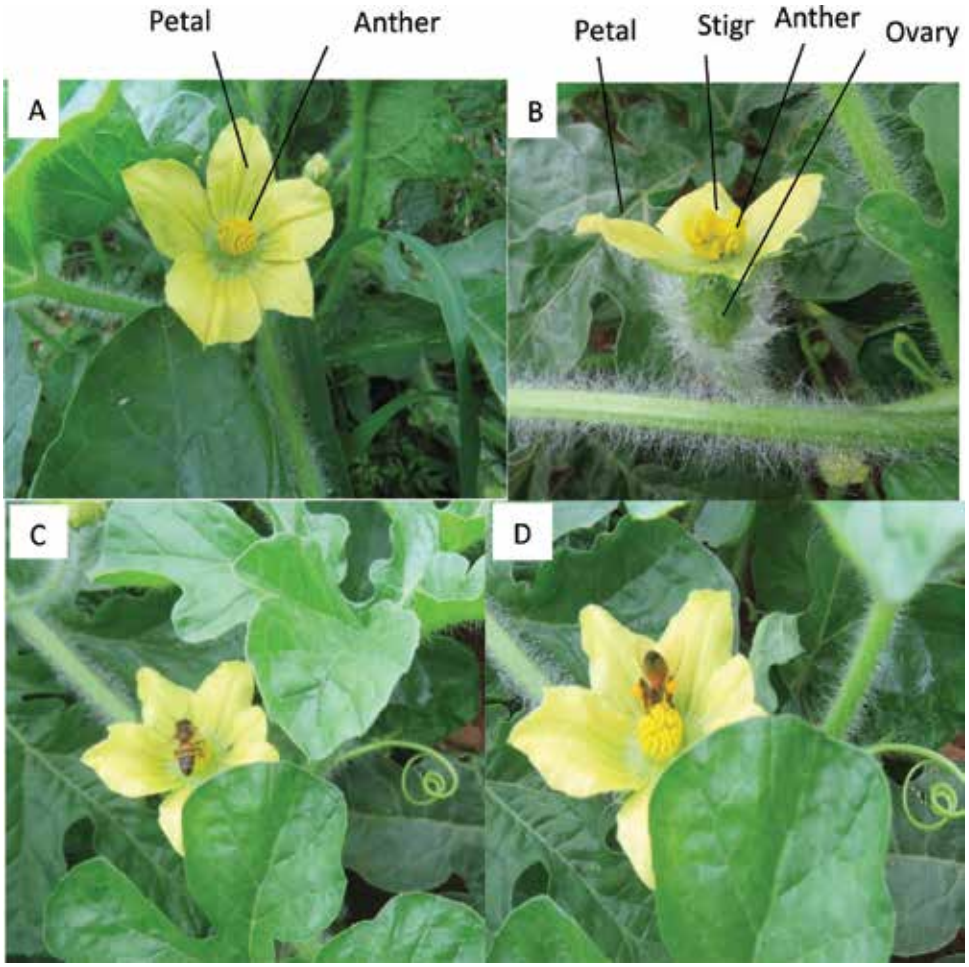

Figure 1. Flowers of the watermelon (*Citrullus lanatus* Thunb.); A is the male flower and B is the bisexual flower; the species is andromonoecious producing male and bisexual flowers on the same plant; in C, a honeybee is foraging for pollen grains on a male flower and in D, it is foraging for nectar with pollen grains visible on its legs.

2. Pollination syndromes

Within pollination syndromes, plants and animals exploit each other for their own advantage. Animals exploit the relationship to guarantee their reproduction and survival. They forage on the flowers for carbohydrates (in nectar) and proteins (in pollen grains), which are the rewards. On the other hand, the plant guarantees its pollination and reproduction by providing the rewards and offering other attractive features like color and scent [2].

Scent plays a major role in cases where the pollination agents are deceived. The deception involves mainly reproduction hues like pheromones and reproduction substrates or sites [3]. Different plants produce flowers of different colors. The colors reflect light of different wave

lengths. Because animals can perceive color of specific wavelength, each type of animal will be attracted by a flower of different color [4]. A combination of floral structure, reward, color and scent from the plant and color perception and nutrient preference on the side of the animal will result in a specific pollination syndrome. The following pollination syndromes are recognized: cantharophily (beetles), melittophily (bees), myophily (by flies), psychophily (butterflies and moths), chiropterophily (bats), and ornithophily (birds). In these pollination syndromes, the pollen grains are attached on some parts of the animals and become brushed onto the stigma. On the other hand, some pollen grains are transferred by abiotic factors (wind and water) from anther to stigma.

2.1. Cantharophily

The flowers in beetle pollinated plants are unspecialized. They are dull in color, scented, and produce lots of pollen grains. The beetles feed on the pollen grains and other flower parts. The scent is emitted from different parts of the flower [5].

2.2. Chiropterophily

Flowers pollinated by bats are large, bowl-shaped, dark to green in color, produce large amounts of nectar and pollen, and smell like rotten fruit. Bats pollinating the flowers are frugivores and nectarivores [6].

2.3. Melittophily

Bees are known to forage flowers for pollen and nectar for their hives. Such flowers are blue, yellow in color or reflect UV light and produce lots of nectar and pollen grains. Carneiro et al. [7] reported of oil gathering bees on flowers containing elaiophores or oil secreting bodies.

2.4. Myophily

Flies are attracted by the mimicry of carrion and feces, a phenomenon called sapromyiophily [8].

2.5. Ornithophily

Flowers pollinated by birds are red in color, tubular in shape, scented, and nectariferous (Medan). The birds are attracted by the red color and scent while they obtain nectar as reward. Bird pollinators are mainly hummingbirds and sunbirds [9]. These are hoverers which flip their winds while sucking. Others are perchers which sit on branches while sucking nectar.

2.6. Other pollination syndromes

In wind pollinated flowers (anemophily), the pollen grains are very fine released on dangling anthers. The stigmas are sticky and hang out of the flower like beard. This increases the chances of the pollen grains landing on the stigma. This type of pollen transfer occurs mainly

in the grasses and some acacias. Water aided pollination occurs mainly in submerged water plants. Both water and wind pollinated flowers do not produce nectar and are not brightly colored. It is suggested that anemophily evolved from entomophily (pollination by insects) is a result of limitation of insect availability [10].

3. Processes that ensure cross-pollination

Nature devised a means of ensuring that cross-pollination succeeds and self-pollination is prevented. Some plants are dioecious. In dioecious plants, male plants produce female flowers and female ones produce female flowers. This is different from monoecious plants where plants produce both male (staminate) and female (pistillate) or bisexual flowers. Clearly, self-pollination cannot take place in dioecious plants since one plant produces either male or female flowers. Prevention of self-pollination in monoecious plants includes mechanisms like dichogamy, heterostyly, and self-incompatibility. Dichogamy is a condition in which either the anthers mature or release pollen grains before the stigmas are receptive (protandry) or the stigmas become receptive before the anthers mature (protogyny) [11]. In self-incompatible species, the pollen tube fails to reach the embryo sac. Either the pollen grain fails to germinate on the stigma or the pollen tube is hydrolyzed in the style after germination. In some cases, self-incompatibility is due to morphological characteristics. Some plant species produce long-styled flowers (pin morphs) and short-styled flowers (thrum morphs). Pin morphs can only fertilize thrum morphs and vice versa [12].

4. Systems in which self-pollination occurs

Self-pollination occurs in species with cleistogamous flowers and in plants in which pollinators are scarce. In cleistogamous flowers, the sexual structures (androecium and gynoecium) are enclosed by the petals which form a keel. Pollination agents cannot access the stamens and pistils within the keel. Self-pollination occurs also in monoecious plants.

5. Pollination in agricultural production systems

Pollinators play a major role in crop production. A balance between plants and pollination agents in ecosystems was maintained through the ages though disturbed now and then by natural disasters like wild fires and diseases. However, with the advent of monoculture and expanding agricultural land, the balance is constantly disturbed. Pollinators became supplemented with managed bee hives [13, 14]. On the other hand, an increase in managed honey bee hives has a negative impact on natural pollinators like bumble bees [14]. It is suggested that the introduction of honey bees needs to be managed in combination with pollinator habitat and pesticide use in a system called integrated crop pollination [15].

6. Pollination in changing climatic conditions

Global warming will have an effect on both plants and pollinators. Bumble bees were found to be less sensitive to temperature change than managed honey bees [9]. According to [16], expected climate change will negatively affect the geographical distribution of five native bees in Brazil which will potentially decrees tomato production by the year 2100.

7. Conclusion

Pollinators are necessary for ecosystem services and crop production productivity. Changes in ecosystems due to global warming as well as agricultural production systems will need to be studied and managed in order to keep ecosystem productivity and crop production sustainable and to feed an increasing world population.

Conflict of interest

The author has no conflict of interest in the publication of this article.

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Chapter 2

The Regulation of Sperm Cells Delivery to the Embryo Sac

Ryushiro Dora Kasahara

Additional information is available at the end of the chapter

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Abstract

Pollination, or the first contact between male and female gametophytes, is one of the most important steps in plant reproduction. After pollination, the pollen grains, male gametophytes, are hydrated and then germinate pollen tubes. The pollen tube initially penetrates and grows through the intercellular spaces of the stigma and then grows through the transmitting tract to the placenta connected to an ovule. The pollen tube grows along the surface of the ovule's funiculus, through the micropyle, and into the female gametophyte. After the pollen tube enters the female gametophyte, it ruptures and releases two sperm cells with its contents. The two sperm cells then move toward and fuse with the egg cell and central cell to produce embryo and endosperm, respectively. Multiple sperm cells typically strive to "win the race" and fertilize an egg cell during animal fertilization; however, in flowering plants, each ovule harboring an egg cell generally encounters only one of many pollen tubes conveying plant sperm cells. This chapter mainly addresses reproductive strategies of plants following pollination from the pollen tube extension and the guidance of two sperm cells to the female gametophyte for fertilization in the ovule.

Keywords: plant fertilization, pollen tube guidance, MYB98, LUREs, fertilization recovery system, POEM

1. Male and female gametophytes

Discussing the journey of the pollen tube first requires an introduction to the smallest fertilization units, namely, the male and female gametophytes (**Figure 1**). The male gametophyte (pollen) comprises two sperm cells and one vegetative cell and is found in the stamen of a flower. The two sperm cells fertilize the egg and central cells inside the female gametophyte

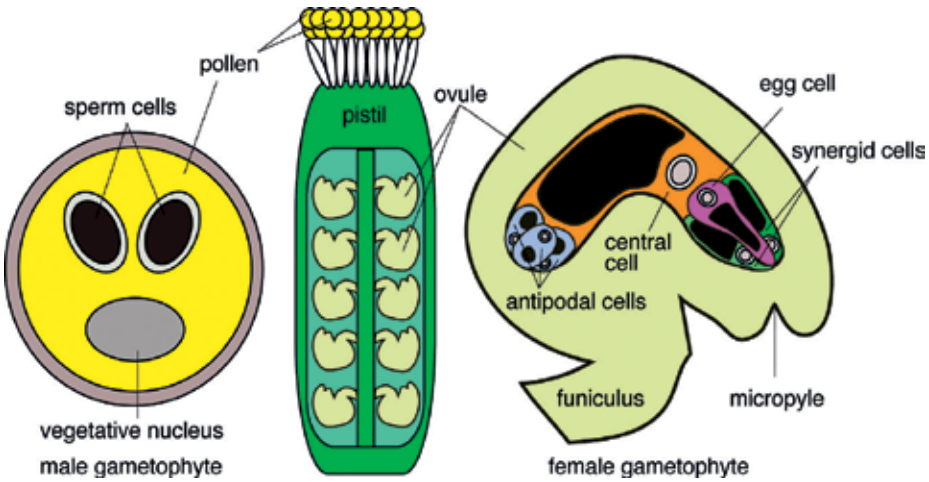

Figure 1. Male gametophyte and female gametophyte. The male gametophyte, also called the pollen grain or microgametophyte, develops within the anther and consists of two sperm cells encased within a vegetative cell (left). The mature female gametophyte (right) inside the pistil (center). The egg and central cells are polarized such that the nuclei of both cells lie very close to each other. This feature is important for double fertilization because these two nuclei are the targets of the two sperm nuclei. After double fertilization, the egg cell forms embryo and the central cell forms endosperm. The synergid cells have at least two functions associated with the fertilization process. First, the synergid cells secrete pollen tube attractants. In addition, the pollen tube enters the synergid cell, suggesting that the synergid cells are important for pollen tube reception. The black areas represent vacuoles of the cells in the female gametophyte.

via a guided pollen tube journey that is described later. The female gametophyte, which is embedded in an ovule within the pistil, contains seven cells of four different types: an egg cell, a central cell, two synergid cells, and three antipodal cells. The egg and central cells are polarized such that their nuclei lie in very close proximity, a feature facilitating double fertilization of these two sperm nuclei targets [1–3]. The synergid cells are extremely essential for the attraction of pollen tubes, as discussed below [4–8].

2. From the stigma to the funiculus

Once a pollen grain reaches the stigma at the top of a carpel, the pollen tubes elongate toward the funiculus to form a bridge-like structure to an ovule, as shown in **Figure 2**. This pollen tube growth through the stigma to the funiculus is controlled via multiple signals from both sporophytic and gametophytic maternal tissues in the carpels. The roles of the female tissues in pollen tube guidance have been focused upon.

Light and transmission electron microscopy studies of *Arabidopsis* have led to several observations regarding pollen tube growth in the female tissues of carpels [9–12]. Although the morphologic features of the pollen tube journey are well understood, the underlying regulatory molecular mechanisms remain unclear. Accordingly, previous studies used sporophytic mutants to elucidate the relationship between pollen tube growth and ovule/female

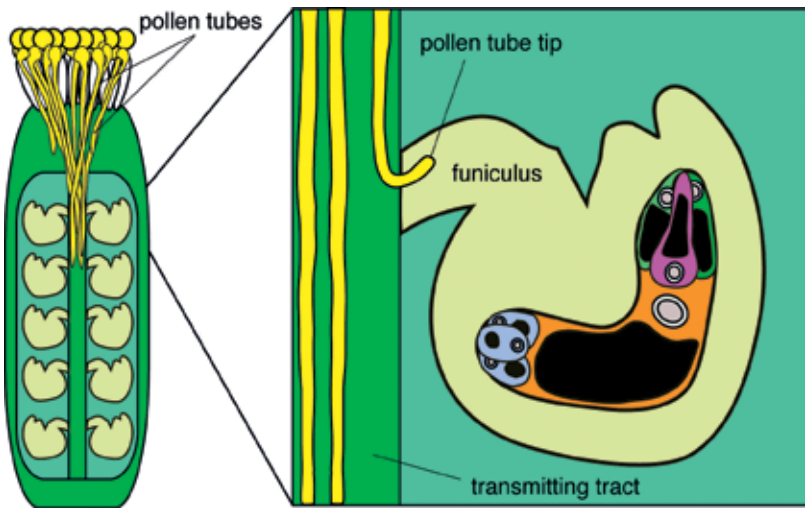

Figure 2. Pollen tube guidance from the stigma to the funiculus. Soon after pollination, the male gametophyte becomes hydrated and then germinates a pollen tube. The pollen tube initially penetrates and grows through the intercellular spaces between the papillar cells of the stigma and then grows through the transmitting tract of the carpel's style and ovary. The pollen tube then emerges from the transmitting tract and grows along the surface of the placenta toward an ovule.

gametophyte development. Particularly, these homozygous mutants mostly produce defective ovules. Although wild-type pollen tubes grow normally during initial phases from pollen hydration to tube emergence from the transmitting tract, they fail to grow toward the mutant ovules, which lack female gametophytes. In other words, although the female gametophyte does not influence early pollen tube growth, it appears to be required for subsequent pollen tube guidance to the ovule [11, 13–15]. These observations suggest that a molecular approach is essential for understanding pollen tube growth from the stigma to the funiculus.

3. From the funiculus to the female gametophyte

The pollen tube is subsequently guided from the funiculus to the female gametophyte. Although the molecular mechanisms underlying this step have been relatively well elucidated, as shown in **Figure 3**, a complete understanding requires a discussion of synergid cell biology (**Figure 1**). Synergid cells within the female gametophyte are essential for reproduction. After the pollen tube grows from the stigma to the funiculus, it enters the female gametophyte by growing into one of the two synergid cells, which typically undergo cell death before or upon pollen tube arrival. Soon after arrival, the pollen tube ceases to grow and subsequently ruptures to release its sperm cells into the receptive synergid's cytoplasm, thus triggering the completion of degeneration. Finally, one sperm cell each migrates to the egg cell and central cell to complete double fertilization of the female gametophyte [3, 16–19].

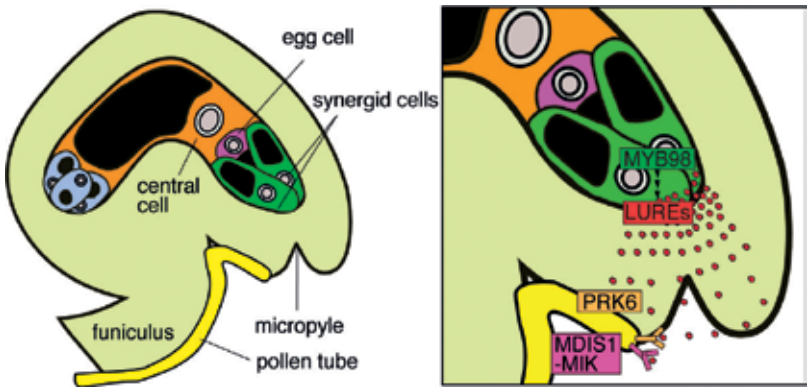

Figure 3. Pollen tube guidance from the funiculus to the micropyle. Synergid cells are required for pollen tube guidance. Several studies using *Arabidopsis thaliana* mutants have reported that pollen tubes fail to grow onto ovules containing abnormal female gametophytes, indicating that the embryo sac provides a guidance cue for the pollen tube. AtLURE1 peptides are attractants that guide pollen tubes to the ovular micropyle. These AtLURE1 peptides are particularly expressed in synergid cells and secreted toward the funicular surface through the micropyle. A transcription factor MYB98 is required for AtLURE1 since myb98 mutants do not express AtLURE1 peptides. PRK6 and MDIS1-MIK receptors are AtLURE1 pollen tube attractant counterparts.

Synergid cells are required for pollen tube guidance. Several studies using *Arabidopsis thaliana* mutants have reported that pollen tubes fail to grow onto ovules containing abnormal female gametophytes, indicating that the embryo sac provides a guidance cue for the pollen tube [11, 20, 21]. Higashiyama *et al*. used laser ablation in an *in vitro Torenia* pollen germination system to demonstrate that synergid cells, but not other female gametophyte cells, produce a pollen tube attractant [4]. Early in 2005, the requirement of a small protein, maize EA1, for pollen tube guidance was reported; however, maize EA1 has no homolog in *Arabidopsis* or other dicots and is unlikely to be a universal attractant [5].

MYB98, which is exclusively expressed in synergid cells (**Figure 4**), provides the first molecular evidence of pollen tube guidance in *Arabidopsis* [6]. Laser ablation studies have demonstrated that synergid cells secrete attractants that guide the pollen tube to the female gametophyte [4], suggesting that defects in pollen tube guidance should be observed in the *myb98* mutant. Accordingly, Kasahara *et al*. observed the pollen tubes of *myb98* mutant pistils pollinated with wild-type pollen. In the wild-type plant, the pollen tube grew along the funiculus of the ovule and through the micropyle to the female gametophyte; however, the wild-type pollen tubes grew abnormally on ovules containing *myb98* female gametophytes, specifically, the pollen tubes grew from the placenta to the funiculus but failed to grow into the micropyle (**Figure 4**).

MYB98 is expressed during the very early stage of synergid cellularization during female gametocyte development, consistent with the *myb98* mutant phenotype, in which pollen tube guidance and filiform apparatus structure are affected. However, several observations indicate that other synergid cell development aspects, including cell specification, remain normal in *myb98* mutants. Female gametophyte development and overall synergid cell morphology appear to be unaffected in *myb98* mutants. Additionally, the *myb98* mutation does not appear to affect the steps of fertilization process subsequent to pollen tube guidance, including the control of pollen tube growth cessation, pollen tube rupture, and sperm cell migration. These

data suggest that MYB98 functions as a transcription factor within the synergid cell gene regulatory network, where it particularly controls the expression of downstream genes required for pollen tube guidance and filiform apparatus formation.

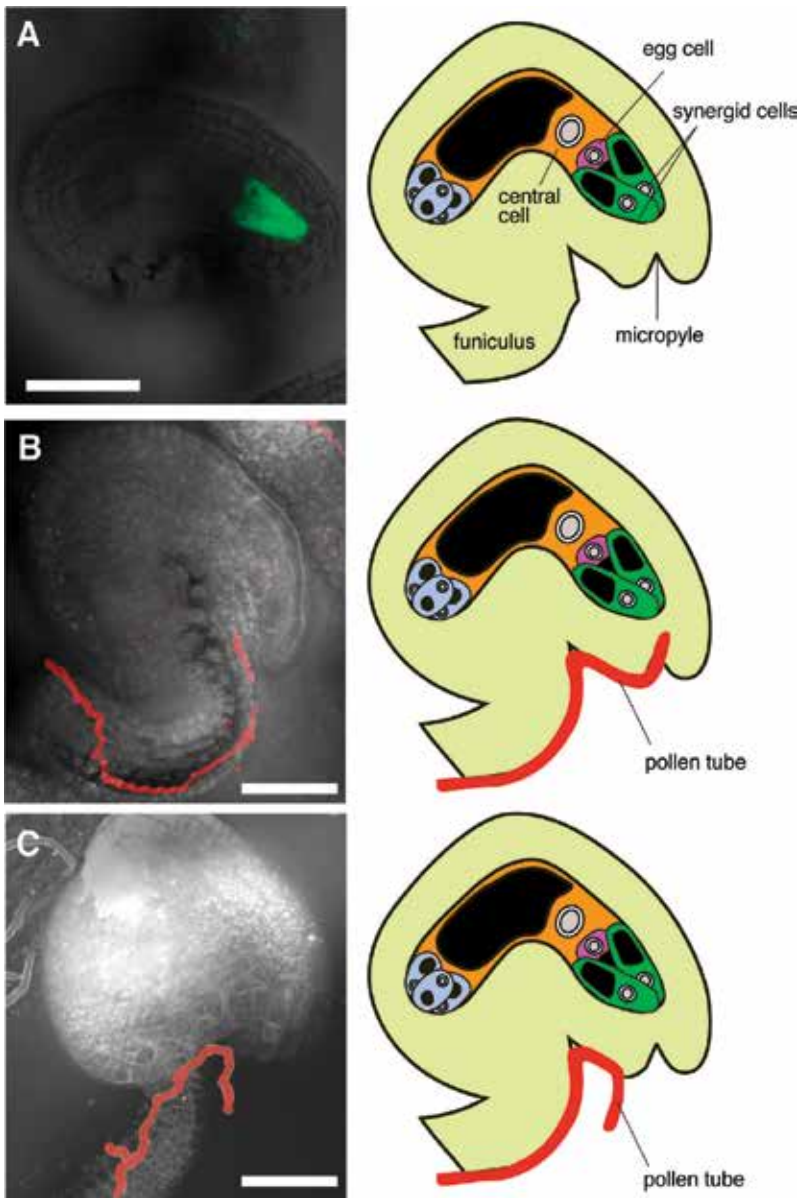

Figure 4. pMYB98::GFP expression and *myb98* phenotype. (A) MYB98 is expressed predominantly in the synergid cells (*pMYB98::GFP* photograph captured by Liyang Xie, HBMC, FAFU). *myb98* female gametophytes are defective in pollen tube guidance. (B) In the wild type, the pollen tube grew along the ovule's funiculus, through the ovule's micropyle, and into the female gametophyte. (C) By contrast, wild-type pollen tubes grew abnormally on *myb98* ovules. Pollen tubes grew from the placenta to the funiculus but then failed to grow into the micropyle. These data suggest that MYB98 functions as a transcription factor within the synergid cells to regulate the expression of genes required for pollen tube guidance. Bars = 30 μm.

The female gametophyte pollen tube attractants LURE1 and LURE2 have also been identified in *Torenia* [7]. LUREs are cysteine-rich proteins (CRPs) within the defensin-like (DEFL) family. *LURE* genes are expressed in synergid cells, which secrete the encoded proteins into the filiform apparatus. Accordingly, LURE downregulation reduces pollen tube attraction, and recombinant mature proteins attract pollen tubes *in vitro* and in a species-specific manner [7]. As discussed above, *myb98* mutation affects the filiform apparatus within synergid cells. However, MYB98 is also required for the expression of at least 83 genes encoding CRPs similar to LURE1 and LURE2 [22, 23]. Many of these CRPs exhibit localization and diffusion patterns similar to those of ZmEA1 [5, 24]; particularly, the CRPs are secreted into the filiform apparatus and subsequently diffuse into the micropylar region [22]. In 2012, Takeuchi and Higashiyama [8] finally identified a recently evolved DEFL gene cluster in *Arabidopsis* and demonstrated that these DEFL [cysteine-rich peptide (CRP810_1)] peptides, or AtLURE1 peptides, are attractants that guide pollen tubes to the ovular micropyle. These AtLURE1 peptides are particularly expressed in synergid cells and secreted toward the funicular surface through the micropyle. Genetic analyses have revealed that gametophytic mutants defective in micropylar guidance *myb98* [6], *magatama3* [21], and *central cell guidance* [25] do not express AtLURE1 peptides and that recombinant AtLURE1 peptides were found to preferentially attract *A. thaliana* pollen tubes vs. *A. lyrata* pollen tubes, indicating that these peptides act as species-preferential attractants in micropylar guidance [8]. Several female-secreted peptides have been identified as species-specific attractants directly controlling pollen tube growth direction. However, the method by which the pollen tubes precisely and promptly respond to guidance signals from their own species remains unknown. In 2016, two research groups reported AtLURE1 pollen tube attractant counterparts [26, 27]. Takeuchi and Higashiyama [26] reported that the tip-localized pollen-specific receptor-like kinase 6 (PRK6), featuring an extracellular leucine-rich repeat domain, serves as an essential sensor of LURE1 [8] in *Arabidopsis* under semi-*in vivo* conditions and is important for ovule targeting in the pistil. PRK6 interacts with pollen-expressed ROPGEFs (Rho of plant guanine nucleotide-exchange factors), which facilitates pollen tube growth by activating the Rho GTPase ROP1 [28, 29]. Particularly, PRK6 acts as a key membrane receptor for external AtLURE1 attractants and recruits core tip-growth machinery, including ROP signaling proteins. Furthermore, Wang *et al*. [27] identified that a cell-surface receptor heteromer, MDIS1-MIK, perceives the female attractant AtLURE1 on the pollen tube of *Arabidopsis*. MDIS1, MIK1, and MIK2 are plasma-membrane-localized receptor-like kinases containing extracellular leucine-rich repeats and an intracellular kinase domain. AtLURE1 particularly binds the extracellular domains of MDIS1, MIK1, and MIK2, whereas *mdis1* and *mik1 mik2* mutant pollen tubes respond less sensitively to AtLURE1.

4. Discharge of sperm cells from the pollen tube tip to fertilization

Immediately after growth cessation, the pollen tube ruptures at or near its tip, leading to release of the pollen tube's contents, including the two sperm cells. In *Arabidopsis* and *Torenia*, rupture occurs within 1 min after entry of the pollen tube into the female gametophyte [16, 17]. Regarding the molecular mechanisms underlying this step, two proteins localized in the sperm cells have been reported (**Figure 5**): GCS1 [30] and GEX2 [31]. Mori *et al*. [30] identified

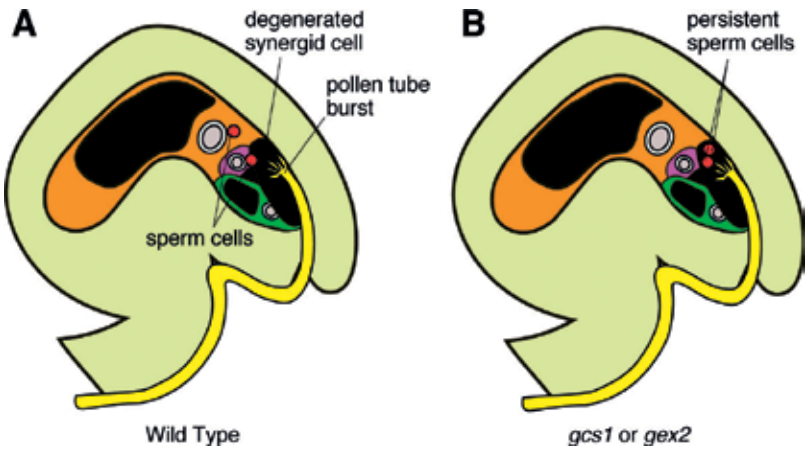

Figure 5. Discharge of sperm cells from the pollen tube tip to double fertilization. (A) Upon reaching an ovule, the pollen tube grows along the surface of the ovule's funiculus, through the micropyle, and into the female gametophyte. The pollen tube enters the female gametophyte by growing through the synergid cells. The pollen tube then comes in contact with the synergid cells and ceases growth. One of the synergid cells then undergoes cell death. Finally, soon after synergid degeneration is initiated, the pollen tube ruptures and releases two sperm cells into the degenerating synergid cytoplasm. The two sperm cells then move toward and fuse with the egg cell and central cell to complete double fertilization. (B) The male gametes of *gcs1* mutant fail to fuse with the egg or central cell. GCS1 accumulates during late gametogenesis and localizes on the plasma membranes of generative cells. The male gametes of *gex2* mutant also fail to fuse with the egg or central cell but the frequency of failure is lower than the *gcs1* mutant.

a protein, GCS1 (generative cell specific 1), using generative cells isolated from *Lilium longiflorum* pollen. Homologs of GCS1, possessing a carboxy-terminal transmembrane domain, are present in various species, including non-angiosperms. Immunological assays have indicated that GCS1 accumulates during late gametogenesis and localizes on the plasma membranes of generative cells. Notably, *Arabidopsis gcs1* mutants exhibit male sterility because the male gametes fail to fuse with the egg or central cell (**Figure 5**). Mori *et al*. [31] identified another important male factor, GEX2 (gamete expressed 2), which encodes a sperm-expressed protein of unknown function that localizes to the sperm membrane and contains extracellular immunoglobulin-like domains, similar to the gamete interaction factors in algae and mammals. Using a novel *in vivo* assay, Mori *et al*. demonstrated the requirement of GEX2 for gamete attachment, as double fertilization is compromised in its absence.

5. Fertilization recovery system

In angiosperms, double fertilization within the ovule occurs with the entry of two sperm cells, which are usually delivered by a single pollen tube. In 1904, Wylie [32] observed the insertion of two pollen tubes in an *Elodea canadensis* ovule and concluded, "It often happens that two pollen tubes pass into one ovule; in such cases both synergids disappear." Since this discovery, the reception of two pollen tubes in an embryo sac, although rare, has been reported in at least 12 species [33]. Similarly, the reception of two pollen tubes has been reported in several *Arabidopsis* mutants, including the *gcs1* mutant [30]. Although this phenomenon is interest-

ing, it has long been considered anomalous. However, Kasahara *et al*. [34] investigated the mechanisms underlying this phenomenon in higher plants upon frequently observing ovules that accepted two pollen tubes in the fertilization-defective *hap2-1* (allelic to *gcs1*) mutant [35], as shown in **Figure 6**. As the *hap2-1* mutant pollen tubes were marked by the pollen tube-specific reporter gene *LAT52:GUS* [36], Kasahara *et al*. traced the tube behaviors *in vivo* by staining for GUS activity, followed by aniline blue staining, to trace the behaviors of the first and second pollen tubes. Accordingly, most ovules contained one pollen tube at 10 hours

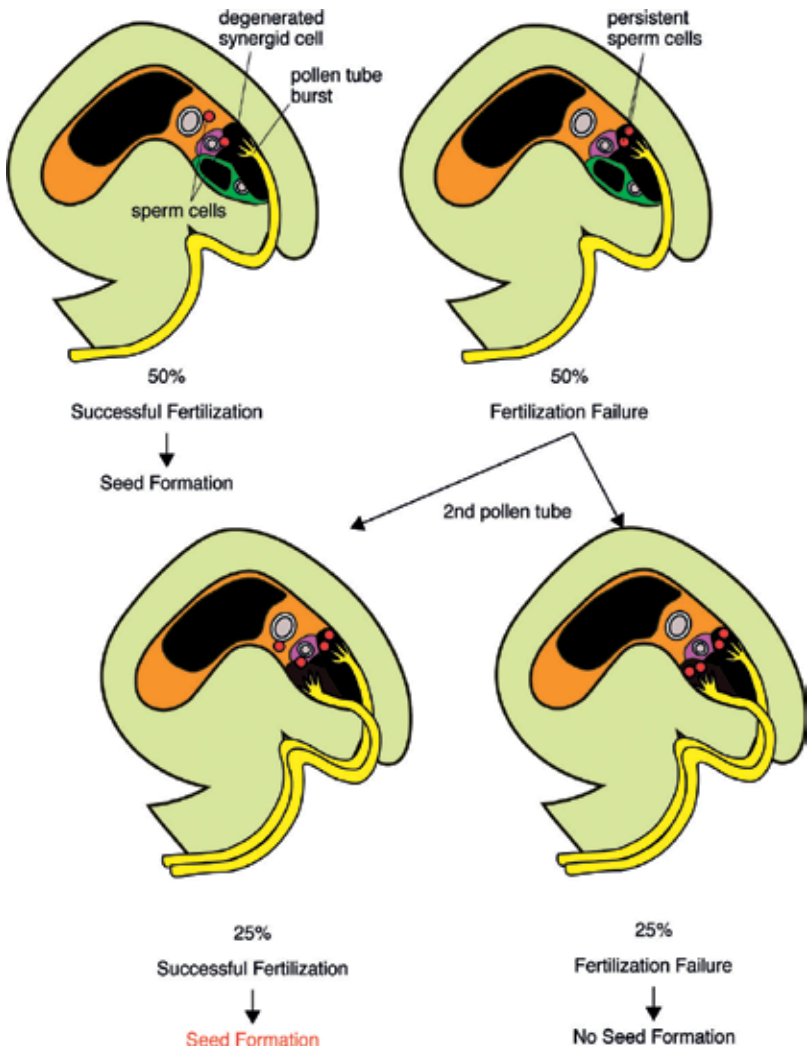

Figure 6. Fertilization recovery system. Upon insertion of a single pollen tube into an ovule, the pollen tube bursts and releases two sperm cells. When the sperm cells complete fertilization, the ovule blocks the entry of the other pollen tubes and develops into a seed by forming an embryo and endosperm. When fertilization fails, the ovule attracts a second pollen tube to rescue fertilization. The rescued ovule develops into a seed, resulting in increased fertility. In the case of failure of fertilization by the second pollen tube, the ovule does not attract a third pollen tube, possibly due to depletion of the pollen tube attractant from synergid cells, since both synergid cells collapse after entry of two pollen tubes.

after pollination (HAP), indicating that the reception of a second pollen tube is independent of sperm cell fertility until several hours after the arrival of the first pollen tube. This delay may represent a blocking system by which ovules avoid polysiphonogamy [34, 37]. However, after 10 HAP, ovules that failed to be fertilized by the first *hap2-1* pollen tube began to attract a second tube. In this case, the persistent synergid cell, which would degenerate upon successful fertilization, continued to attract pollen tubes, leading to a second pollen tube acceptance rate of ~80% among failed ovules by 28 HAP. Although no particular role has been proposed for synergid cell persistence after the arrival of the first pollen tube, Kasahara *et al*. demonstrated that the second synergid cell could retain its function and thus attract and accept a second tube to rescue fertilization. This might explain the presence of two synergid cells in many higher plants (**Figure 6**).

Previously, several research groups [35, 38, 39] studied why several sperm cell–defective mutants exhibited an enhanced fertility phenotype (60–70% fertility); particularly, the frequency ratio of double pollen tube reception was almost completely consistent with the frequency of enhanced fertility (**Figure 6**). Additionally, the GUS staining experiment revealed that by 10 HAP, ~50% ovules had accepted a mutant allele, indicating that the mutant and wild-type pollen tubes were similarly competent to enter the embryo sac and release their contents. von Besser *et al*. [35] suggested that *hap2-1* sperm cells affect pollen tube guidance. However, our data led us to conclude that sperm cells are passive pollen tube cargo and do not influence pollen tube guidance in *hap2-1* mutants, consistent with the observation that sperm cell–defective mutants (i.e., no transmission via the male germline) exhibit only 30–35% sterility, instead of the expected 50%. Very recently, Zhang *et al*. [40] demonstrated that in the absence of two bHLH transcription factors, *Arabidopsis* produces an abnormal, sperm cell-free pollen exhibiting behavior similar to its wild-type counterpart, thus indicating that sperm cells are dispensable for normal pollen tube development. This result reinforced our concept of sperm cells as passive cargo, with no control over pollen tube growth and behavior.

According to previous report by Kasahara *et al*. [37], all hand pollination experiments were performed using large numbers of pollen grains. Particularly, *Arabidopsis* pistils, usually containing 50–60 ovules, were pollinated with approximately 20, 40, 80, 120, and 700 grains. Two days after pollination, the insertion of few second pollen tubes into ovules were observed among the pistils pollinated with 20 and 40 grains, indicating that under restricted conditions (ovules > pollen tubes), the pollen tubes selectively inserted into ovules that had not previously accepted any pollen tube. Conversely, when a wild-type pistil was pollinated with 80 (ovules ≤ pollen tubes) and 120 (ovules < pollen tubes) grains, approximately 12 and 25% of the ovules accepted second pollen tubes, respectively, suggesting that ovules accept second pollen tubes while under saturated conditions (ovules < pollen tubes) [37]. In other words, excess pollen is required to saturate the fertilization recovery system, and approximately 80% (not 100%) of the failed ovules can accept a second pollen tube to complete recovery, consistent with a previous report [34] that a substantial period of ~28 h is required for ovules to complete the fertilization recovery system. This delay in secondary guidance may be attributable to the functional synergid cell numbers; a previously penetrated ovule contains only one persistent synergid cell (the other would have been disrupted by a burst pollen tube) to provide guidance. Higashiyama *et al*. [4] reported that an ovule containing two synergid cells

attracts more pollen tubes than does an ovule with one synergid cell, suggesting that the latter produces insufficient levels of attractant. This may explain why only approximately 80% ovules with one synergid cell will attract a second pollen tube.

6. Pollen tube-dependent ovule enlargement morphology (POEM)

In angiosperms, the pollen tube releases its contents (including sperm cells) into the embryo sac upon insertion into the ovule, thus completing double fertilization. Recently, Kasahara *et al*. [41, 42] reported that the expansion and initiation of seed coat formation occurred even in ovules wherein fertilization failed after pollen tube insertion. This phenomenon was designated as pollen tube–dependent ovule enlargement morphology (POEM), which occurs only when the ovule accepts the pollen tube content (PTC). POEM was the first report addressing the paternal functions of PTC in facilitating the ovule's maternal development without fertilization in plants.

In animals, once semen is discharged into the uterus, the seminal plasma carries the sperm to the egg [43, 44], whereas in plants, PTC, which transports sperm cells to the ovules, has an analogous function. In mice, fertilization requires seminal vesicle secretory protein 2, which localizes only in the seminal plasma [45]. As seminal plasma is essential for fertilization in animals, Kasahara *et al*. proposed that PTC should also be important for fertilization in plants. To understand the function of PTC, a *gcs1* mutant [30] was used, which fails to accomplish fertilization despite releasing PTC to evaluate transcriptional variations after PTC release into the embryo sac and compared the transcriptomes between two ovule RNA types: one after normal fertilization and the other after PTC release without fertilization. At 12 and 24 HAP, the observation of similar expression profiles for both RNAs was unexpected because early events after pollen tube insertion were thought to be fertilization-dependent. However, these events were instead found to depend on PTC. Notably, between 24 and 48 HAP, multiple genes associated with cell expansion, cell division, and seed coat formation were upregulated regardless of fertilization, suggesting that PTC can affect ovule shape. Hence, the ovule phenotype was investigated. Interestingly, ovules that accepted PTC expanded without fertilization because of cell expansion and division and the production of a partial seed coat, consistent with the results of the transcriptome analysis. Using data from the successful transcriptome analysis, Kasahara *et al*. identified that the novel plant phenomenon POEM occurs only when the ovule accepts PTC, irrespective of fertilization (**Figure 7**) [41].

In angiosperms, pollination is the first step toward fertilization. Once the pollen reaches the stigma, the grains elongate to form pollen tubes and move toward the synergid cells observed within the female gametophyte. Fertilization occurs when the pollen tubes pierce the female gametophyte; this action terminates pollen tube growth and induces bursting, resulting in the deposition of the two sperm cells inside the female gametophyte. The phenomenon represents a new reproductive phase between pollen tube guidance and fertilization because PTC release itself could induce POEM. Roszak and Köhler [46] demonstrated failure of seed coat synthesis in *agl62* mutant ovules (Kang *et al*. [47]), which exhibited early endosperm cellularization, resulting in abnormal endosperm formation. Roszak and Köhler suggested that central cell

fertilization and normal endosperm formation was required for the initiation of the seed coat formation. Contrarily, our observation of vanillin staining in *agl62* mutant ovules indicated that the central cell fertilization is not required for the initiation of the seed coat formation (**Figure 7**).

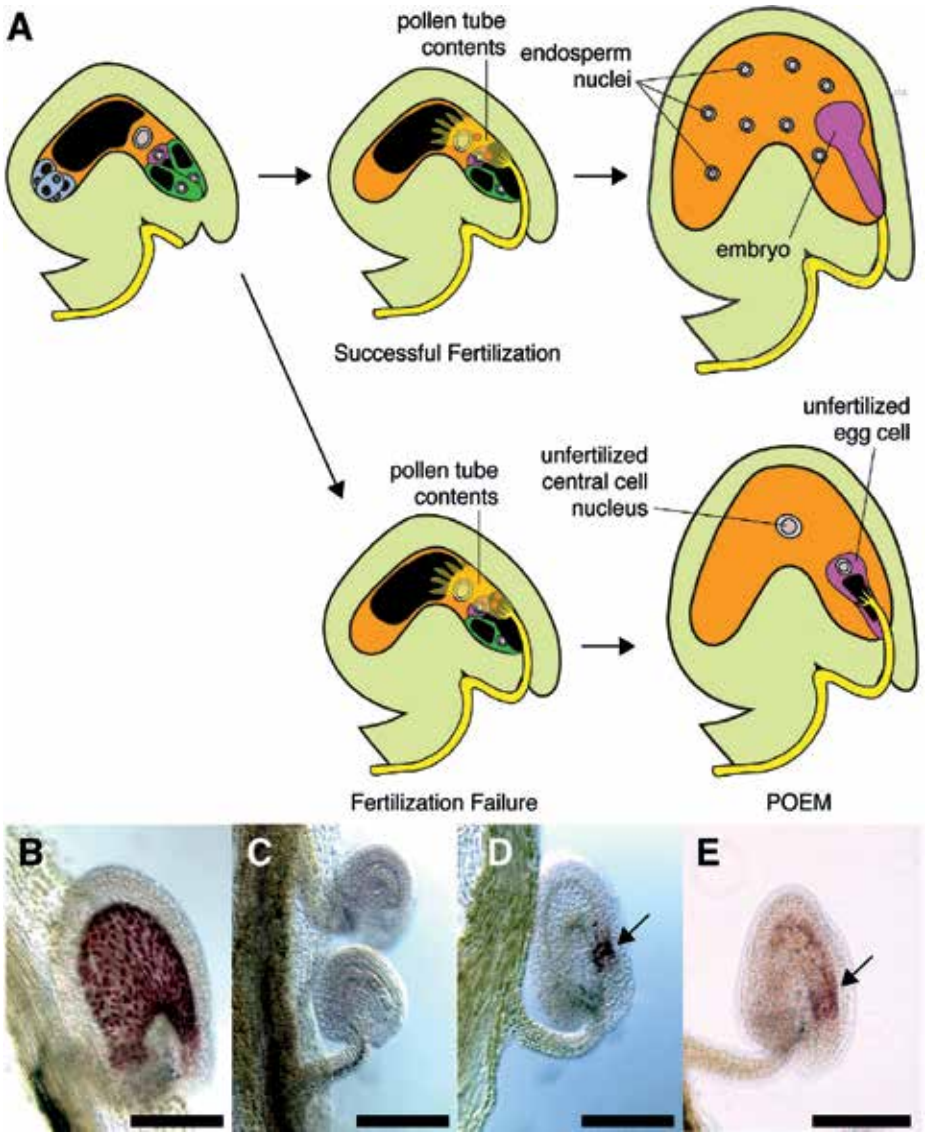

Figure 7. Pollen tube–dependent ovule enlarged morphology (POEM). (A) After the pollen tube is inserted to the female gametophyte, the pollen tube bursts and releases its contents (yellow region) with two sperm cells. Double fertilization is accomplished by these sperm cells fertilizing egg cell and central cell, respectively. *gcs1* mutant sperm cells fail to fertilize and the ovule does not produce a seed. It had long been suggested that the ovule will just die if the ovule fails to fertilize. However, if the pollen tube contents are supplied to the ovule, the ovule will be enlarged and initiate seed coat formation without fertilization. (B) A wild-type seed stained by vanillin at 3DAP. Whole seed coat region is stained. (C) Ovules without pollen tubes. Seed coat is not stained by vanillin. (D) A wild-type ovule crossed by *gcs1*/*gcs1* pollen. The ovule is partially stained. (E) An *agl62* mutant ovule. The ovule is partially stained even though it has abnormal endosperm. Arrows indicate the stained part by vanillin. Bars = 100 μm.

PTC was previously found to initiate central cell/endosperm nuclei division without fertilization when it was released to an autonomous endosperm mutant, *mea* [48, 49]. The finding that segmentation can be induced in a fertilization-independent manner by physical stimuli, leading to the development of some eggs into normal tadpoles, was first reported in 1910 [50]. In plants, Kasahara *et al*. [41] showed that PTC could increase central cell/endosperm nuclei division in the absence of fertilization, suggesting a function parallel to the observed fertilization-independent division of animal germ cells in response to external stimuli. By inducing endosperm nuclear division, PTC facilitates apomixes [51] in important crops when the POEM phenomenon is combined with autonomous endosperm and embryo mutants. In plants, seed formation without fertilization, or apomixis, is agriculturally valuable because important genetic traits can be easily fixed in apomictic crops, which then propagate without interference from unfavorable environmental conditions. POEM could therefore be categorized as "pseudogamy," which is defined as any reproductive process requiring pollination but no inheritance from the male gametophyte [52]. Although Focke [53] first defined pseudogamy as a part of apomixis, the underlying cellular or molecular mechanisms have remained obscure. Given the conceptual similarities, POEM may therefore be a key in understanding pseudogamy, particularly concerning pollen and PTC stimuli.

7. Summary

This chapter discusses the journey of the pollen tube from the stigma to fertilization as well as the POEM phenomenon. Because very few factors related to pollen tube guidance from the stigma to the funiculus of the ovule have been elucidated, additional insights into this step are eagerly awaited. However, the molecular mechanisms underlying pollen tube guidance from the funiculus to the female gametophyte are well known in *Arabidopsis* because the pollen tube attractants AtLURE1 peptides had previously been identified downstream of the master synergid cell regulator MYB98. During the final step after pollen tube bursting, only two proteins, GCS1 and GEX2, have been identified as direct male-related key fertilization factors in the pollen tube. Accordingly, further molecular evidences are required to understand the final step for plant fertilization. Finally, very few factors related to new plant phenomena, fertilization recovery system, and POEM have been identified. New insights into the underlying molecular mechanisms are anticipated.

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Chapter 3

Pollination Ecology of the *Manicaria saccifera* (ARECACEAE): A Rare Case of Pollinator Exclusion

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Luis Alberto Núñez-Avellaneda

Additional information is available at the end of the chapter

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Abstract

We studied the reproductive biology and pollination ecology of the palm cabecinegro (*Manicaria saccifera*) in very wet tropical forest, in the Chocó, Pacific region of Colombia. We present data about the phenology, floral morphology, floral biology, reproductive system, and pollination. *M. saccifera* is monoecious, self-incompatible, lacks apomixis and has dichogamy in the form of protogyny. Flowering occurs all year round with a peak between April and May. A single individual may produce up to five inflorescences in its reproductive period. Each inflorescence has unisexual flowers grouped in dyads and triads; anthesis is diurnal and the flowers may be receptive for 72 h. Flowers are visited by 10 species of insects. The inflorescences in the female-phase do not offer reward and insects are attracted by olfactory mimicry; in the male-phase flowers reward visitors with pollen and a place to oviposit. The most efficient pollinator is *Mystrosp cercus* (Nitidulidae), the only visitor arriving in abundance during the female-phase. Other insects do not enter the flower because the peduncular bract and the petals act as barriers, blocking the entrance of insects greater than 2 mm. Having one exclusive pollinator which in turn depends on the palm for its survival is an example of extreme specialization and mutual dependence.

Keywords: Arecaceae, beetle pollination, *Mystrops cercus*, neotropical palm, phenology

1. Introduction

Palms are one of the most important plant families because they are a food source for wildlife [1]; they provide a variety of products that rural peoples use for construction, food, medicine and handicraft purposes [2, 3]; they are a source of raw materials for a great variety of products that communities exploit commercially at small or large scales, always in an extractive

way [3, 4], and finally, palms are culturally important because many species are essential to the cultural development of local peoples [5].

Due to this importance, studies on their reproductive biology have increased in the last decades [6]. Initially, most studies focused on cultivated palms or on widely used species [1, 7]; however, recent works have focused on a wider range of palms [8–10].

Research on the reproductive biology of this plant family has increased our knowledge on the great variety of pollination strategies and mechanisms found in plants and on the strong dependence and intimate association that most palm species share with the insects that pollinate them, mainly species of coleoptera belonging to the families Nitidulidae and Curculionidae [9–11]. Due to palms economic, ecologic and cultural importance, studies on palms reproductive biology are essential for their sustainable use, conservation and future domestication [10].

Manicaria saccifera is a widely distributed species [12], of ecological importance [1, 13] and great economic potential [14, 15]. It ranks among the most utilized and economically valuable palms for the Afro-American and indigenous communities of the Chocó biogeographic region: [16] reported 36 different uses for this species, evidencing its versatility as a non-timber forest product. The most significant uses of *M. saccifera* in the Pacific region of Colombia include: the use of the peduncular bract as a source of fiber for crafts and textiles [15–17]; weaving the leaf veins for basketry [15]; the use of unexpanded leaves for brooms; the leaves for thatch houses; the immature fruits filled with a liquid resembling coconut water are consumed while working in the forest. Additionally, fruits are sold in local markets for medicinal purposes [16]. In Venezuela the Warao Indians use *M. saccifera* for construction, sailing, food, medicine, and for crafting bags and hats [18, 19].

M. saccifera is a little studied palm in terms of its reproductive biology [20], who mentions the possibilities of auto-pollination and the great quantity of larvae inside the flowers. On the other hand, [21] reported several visiting insects, with *Mystrops cercus* and *Mystrops erviki* (Nitidulidae: Coleoptera) being frequent visitors. The most important aspect to highlight in this species is that, contrary to most species in the family, anthesis and all remaining reproductive mechanisms occur "hidden" within the interior of the peduncular bract, without opening or exposing the male and female flowers. This aspect makes an understanding of the species reproductive strategy even the more intriguing.

Our work constitutes the first significant contribution detailing the principal aspects of the reproductive biology and pollination ecology of one of the most important palms for the inhabitants of the Pacific region of Colombia and a key species of tropical pluvial rainforests. In this work we evaluate the periods of flower and fruit production, the reproductive system, floral biology, flower visitors and we quantify the role each visitor has in the pollination of the palm.

2. Materials and methods

2.1. Study area

Our study was conducted in the Quibdó municipality, Pacific coast of Colombia, in an area adjacent to the road leading from Quibdó to the Atrato municipality, at 5° 39′ N, 76° 38′ W

(**Figure 1**), and 90 m of elevation. With a mean annual temperature of 28° C, a relative humidity of 89% and a mean annual precipitation of 5000–7000 mm, this area is classified under very wet tropical forests in the Holdrige life zone system [22].

2.2. Study species

Manicaria saccifera Gaertn., known as "cabecinegro" in the Colombian Pacific (Galeano and Bernal [13]) is a monoecious palm that grows in swampy inundated areas, preferably near the edge of rivers and creeks. Its individuals are medium sized, solitary or cespitose, with large regularly pinnatisect or simple leaves and with a dentate margin. The inflorescences are interfoliar and solitary [13]. It is distributed in Central America, in the Pacific littoral of Colombia southwards to northern Ecuador, and in the Orinoco and Amazon basin of Colombia, Brazil and Venezuela [12].

2.3. Inflorescence morphology

A morphological description of the inflorescence was performed based on 20 inflorescences of *M. saccifera*. We (i) recorded the height at which the inflorescences are found, (ii) measured the length of the inflorescence, rachillae and flowers; (iii) counted the number of rachillae, of female and male flowers per rachillae and of female and male flowers per inflorescence; and (iv) determined the position of the flowers in the rachillae, the number and shape of the

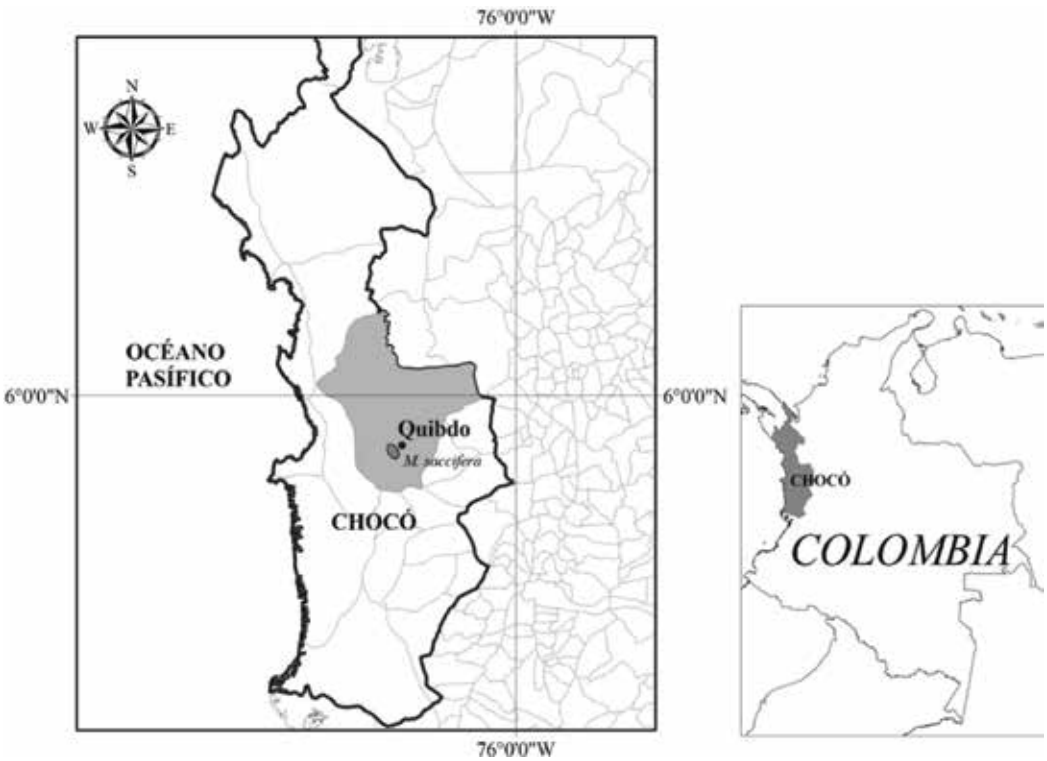

Figure 1. Study zone.

stigmas and the number of anthers. To estimate the number of flowers per inflorescence, we multiplied the total number of flowers of each rachillae by the mean number of rachillae counted in 10 inflorescences.

2.4. Reproductive phenology

We recorded the reproductive phenology of 48 individuals of *M. saccifera* during a 12-month period, marking individuals along a pre-defined trail in the forest. Each week we revisited the study area during 3 days to register in each individual if flowering and fruiting occurred. We calculated the monthly percentage of flowering and determined the flowering synchrony. Following [23] we defined flowering events as being: (i) asynchronous, when less than 20% of the individuals are in flower; (ii) low synchrony, between 21 and 60%; and (iii) high synchrony when over 60% of individuals flower at the same time.

2.5. Floral biology

To view the reproductive structures and carry out our observations, a longitudinal slit was opened on the peduncular bract and was later covered with paper tape. Direct observation of the flowering buds and opened flowers were made at intervals of 6, 12, and 24 h in 10 inflorescences of 10 individuals. We registered the (i) hour of anthesis of the flowers, (ii) the daily rhythm of anthesis of the flowering buds, (iii) the presence and longevity of pollen, and (iv) the stigmatic receptivity. We used three methods to evaluate the stigmatic receptivity: (1) direct observation of the stigmas, noting changes in morphology, color and presence of exudates; (2) signs of peroxidase activity, using hydrogen peroxide tests [24]; (3) colorimetry tests, applying the Perex-Test solution by Merck [25]. Additionally, we registered the increase in temperature within the inflorescence using digital thermometers made by Cox Technologies Inc., with a range of temperature between −35 and 210°C. We performed measurements in five closed inflorescences of five individuals, introducing the thermometer's sensor to register the internal temperature of the inflorescence, which was compared with ambient temperature.

2.6. Reproductive system

We determined the reproductive system of *M. saccifera* by performing controlled pollinations on five inflorescences from different individuals using four different treatments: (i) Autopollination: we isolated inflorescences to avoid the entry of pollen; (ii) Open pollination: natural pollination without our intervention; (iii) Apomixis: without pollination; (iv) Allogamy: controlled pollinations using pollen from different individuals of *M. saccifera.* In all cases, the flowers were isolated with synthetic mesh at least 30 days. After each treatment we checked whether fruits were developing and recorded the percentage of fruits formed. The degree of genetic compatibility was evaluated using the self-incompatibility index proposed by [26].

2.7. Floral visitors and pollinators

The composition of floral visitors was studied in 10 inflorescences from 10 individuals. When the rate of visits was highest, inflorescences were covered with bags and shaken so insects would fall

inside the bags. This procedure was repeated three times each day during all the flowering phase. For each floral visitor we documented its (i) abundance, (ii) behavior and (iii) role in pollination. To document the behavior of floral visitors we performed observations on the female- and male-phase of each palm, noting: the hour of arrival and departure to the flower, the activity within the flower, the utilized resources, and the permanence in the flower, and the contact with the stigmas in the female-phase. Following [10] we calculated the pollinator importance value (PIV) and the pollinator relative importance value (PRI). The variables used to calculate these indices were: the relative abundance of insects in the female flowers (AB), pollen-transport capacity (PTC), fidelity (F), constancy (C), and pollen-transport efficiency (PTE). For details on each variable refer to [10]. To determine the CTP and ETP, we collected five insects of each species, preserved them in 70% alcohol and took them to the laboratory to conduct the pollen load analyses following the methods in [9]. To evaluate fidelity we compared the pollinators of *M. saccifera* with pollinators of the palms *Attalea allenii, Attalea cuatrecasana, Oenocarpus bataua, Oenocarpus minor, Socratea exhorriza* and *Wettinia quinaria*, all of which are palms that grow in the study area together with *M. saccifera.*

3. Results

3.1. Inflorescence morphology

The inflorescences of *M. saccifera* are interfoliar and located at a mean of 1.89 ± 1.1 (SD, n = 20) meters above the ground (**Figure 2A**). An individual during the reproductive phase may produce 1–5 inflorescences (n = 45), which are found in different developing stages and which flower alternately. Each inflorescence can measure up to 1.50 m, including the peduncle, which measures 46 cm and the prophyll (35 cm). Each inflorescence is covered by a fibrous peduncular bract, which is closed without suture, is brown and has the shape of a long hood. Throughout all development phases of the inflorescence, the peduncular bract acts as a mesh or as a selective barrier to insect visitors (**Figure 2B** and **C**). The bract suffers changes in morphology and coloration at its exterior surface and thickening due to the development and growth of the fruits in the inner part. Inflorescence present a mean of 35 ± 19.5 (SD, n = 10) rachillae. The flowers in inflorescences are distributed in (i) triads, with a female flower in the center and two male flowers at the sides in the basal part; (ii) toward the apex as dyads, with two male flowers (**Figure 3**). This distribution of flowers distinguishes the subfamily Arecoideae from the other palm subfamilies. Generally, the last rachillae to develop contain only male flowers.

Each rachillae contains a mean of 2.88 ± 0.84 (SD, n = 504) female flowers and a mean of 218 ± 132 (SD, n = 504) male flowers so that each inflorescence can harbor 199 ± 32 (SD, n = 10) female flowers and 15,085 ± 532 (SD, n = 10) male flowers.

The flowers are of rigid texture, yellowish in color, and the female flowers are greater, measuring 0.7 ± 0.3 cm (SD, n = 16) in length, and with a laminar stigma in the shape of a pyramid (**Figure 2E**); each male flower measures 0.8 ± 0.3 cm (SD, n = 16) in length and has numerous stamens (mean of 35) (**Figure 2D**).

The fruits are spherical, 5 cm in diameter and covered with woody pyramidal or pointed projections; occasionally the fruits have the shape of two or three united spheres forming a

Figure 2. Morphology of *Manicaria saccifera*. (A) Habit. (B) and (C) inflorescence covered by the peduncular bract. (D) Flowers of *M. saccifera* in triads. (E) Receptive female flower. (F) Open male flower.

Figure 3. Distribution of the female- and male-flowers at the rachillae of *Manicaria saccifera.*

triangular structure. The seed is spherical, very hard, and is covered by a brownish or light purplish kernel of brittle texture (**Figure 2G**).

3.2. Reproductive phenology

M. saccifera flowered throughout the year (**Figure 4**), with a peak in inflorescence production in the month of May, when nearly 61% of individuals had one or two inflorescences; the remaining individuals flowered during the months of June until August. There was no relationship between flowering and precipitation, flowering being constant in months of low rainfall (February) and highest rainfall (November). The individuals of the studied population showed low synchrony in flowering, with 27% of the individuals flowering simultaneously. The fruiting period was relatively constant throughout the year (**Figure 4**).

3.3. Floral biology

The events that take place during floral biology can be summarized as: (1) flower buds emerge; (2) buds of pistillate flowers develop completely; (3) anthesis begins; in female flowers it occurs simultaneously whereas it is alternate in male flowers and lasts up to 2 days. During anthesis there is an increase in temperature above ambient temperature in female and male inflorescences of 4.0 and 4.7°C, respectively (both n = 3); (4) maximum stigmatic receptivity, evidenced by the white color of the stigmas covered by a hyaline-brilliant substance; (5) pollination, with subsequent loss of stigmatic receptivity evidenced

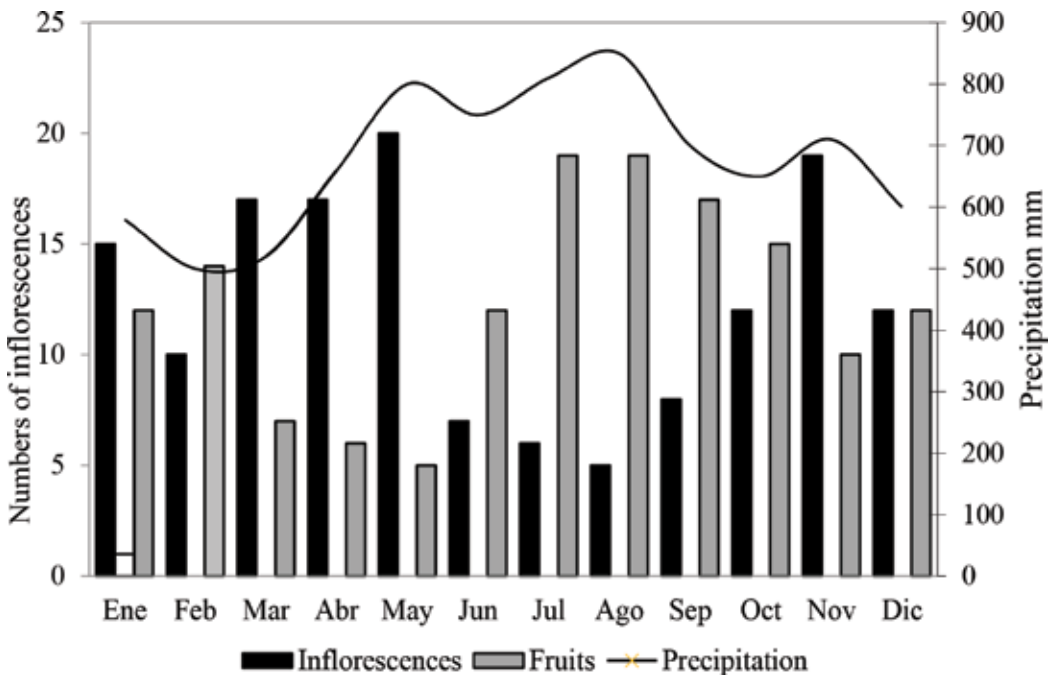

Figure 4. Monthly proportion of flowering and fruiting individuals of *Manicaria saccifera* compared with annual precipitation.

by a change of color from white to brown to black; (6) fruit formation, with maturation lasting approximately 15 months.

3.4. Reproductive system

Our controlled pollination treatments revealed that *M. saccifera* is strictly xenogamous. Thus, neither of the apomixis or auto-pollination treatments led to fruit formation. In contrast, with the open pollination and allogamy treatments the percentage of fruits (**Table 1**).

3.5. Floral visitors

The inflorescences of *M. saccifera* were visited by eight species of insects (**Table 2**), with a mean abundance of 716 ± 60 visitors per inflorescence (n = 10). *Mystrosp cercus* was the most abundant floral visitor and the only insect able to cross the two barriers imposed by the fibrous peduncular bract and the petals of the female flowers. It had the highest pollinator relative importance value, representing 99.9% of the pollination in *M. saccifera*. In contrast, the remaining species were occasional visitors with low abundances and were only present in the male-phase of the inflorescences. Among these frequent species were *Amazoncharis* sp.1 and *Xanthogypus* sp.1 (Staphylinidae).

Treatments	Nº palms/ N°flowers	Nº fruits/% set fruits
Apomixis(A)	5/410	0/0
Open pollination (OP)	5/410	225/62
Auto-pollination (AP)	5/410	0/0
Allogamy (AL)	5/410	220/53

Table 1. Percentage of fruits formed in ***Manicaria saccifera*** after four controlled pollination tests.

Floral visitors	AB	CTP	ETP	C	F	IVIP	IRIP
Mystrops cercus	560	1564	569	1	1	498,352,960	99.9
Mystrops erviki	12	156	234	0.25	1	109,512	0.021
Trigona fulviventris	23	669	245	0.05	0.5	94245.3	0.018
Trigona ferricauda	12	456	123	0.05	0.5	16826.4	0.003
Derelomini sp.1	12	123	12	0.25	0.33	1461.24	0.0002
Xanthopygus	34	167	32	1	0.33	59959.68	0.012
Atheta sp.1	12	23	12	1	0.33	1092.96	0.0002
Amazoncharis sp.1	123	12	12	1	0.33	5844.96	0.001
Total						498,641,903	100

AB: abundance in female phase, PTC: pollen-transport capacity in female phase, PTE: pollen-transport efficiency, C: constancy in the phase female, F: fidelity, PIV: pollinator importance value and PRI: pollinator relative importance value

Table 2. Role of visitor in the pollination of ***Manicaria saccifera***.

4. Discussion

Results of the reproductive system indicate that self-pollination is unlikely because *M. saccifera* is a monoecious palm with unisexual flowers and anthesis time of the male and female phase do not match due to the type of protogynous dicogamia present. No fruit formation occurs via apomixis (**Table 1**), and the probability of geitonogamy is low due to the non-coincidence of two inflorescences in anthesis in the same individual; however, if the two inflorescences in anthesis manage to coincide, fertilization is prevented by the self-incompatibility found. And because the anthers and stigma remain covered avoiding pollen dispersion by wind anemophilia is unlikely to occur. Consequently, the non-presence of apomixis, the non-occurrence of self-pollination, the degree of self-incompatibility found and the fact that no wind pollination occurs, determine that *M. saccifera* should be considered a xenogamous palm, dependent on insects for pollination.

Cross pollination apparently works well in *M. saccifera* as open-pollination and allogamy testing showed 62 and 53% of fruit formation, respectively (**Table 1**), such efficiency indicates the importance of pollinators as carriers of pollen between individuals in the population, given the obligatory xenogamy of the palm. Cross-pollination is efficient despite that the pollination mechanism that occurs in *M. saccifera* is atypical to what usually happens in palms, in which visitors insects have full access to the flowers. In *M. saccifera* the peduncular bract keeps hidden and isolated male and female flowers, and only *Mystrops cercus* can cross the peduncular bract when the stigmas are receptive, the rest of the insects that are attracted at this time cannot enter; they do it in male phase when receptivity has passed and focus their activity in male phase.

The fact that the peduncular bract does not open preventing access to a group of insects, mainly larger than 2 mm, becomes a selective filter that limits free access of insects into the inflorescence at a critical moment in the reproduction of the palm. Large insects like bees (Apidae) or with larger sizes cannot cross the peduncular bract, while small insects or smaller than 2 mm, are the only ones who can access the flowers when they are receptive.

The peduncular bract acts as a barrier or selective filter that restricts access of large insects to female flowers at the most important time for fertilization, but is not the only one: those insects that may cross the first barrier are immediately faced with a second barrier and therefore a second filter, this time generated by the petals of the female flowers which do not open completely either and only two small slots of 1–2 mm are the space between the petals that insects may use to enter the flower, access the stigma and deposit the needed pollen to fertilize each flower of the inflorescence. Consequently, the bract that covers the inflorescence and the petals of the female flowers become two barriers that act as filters for selecting by size the type of pollinator in *M. saccifera* and only *Mystrops cercus* is the insect that passes through the filters and access the stigma that are hidden for the rest of the visitors.

Keep flowers hidden at the time of anthesis is a rare phenomenon in palms, presented only in other species of the genus *Pholydostachys* (personal observation), which have a fibrous small peduncular bract similar to *M. saccifera* and insects must pass through at the time of anthesis in a yet unknown mechanism. The closest thing to the selective filter imposed by the

peduncular bract occurs in some palms species of the genus *Attalea* in which as at the time of anthesis the peduncle bract leaves only a small slit that acts momentarily as a filter, but over time the bract exposes most of the flowers and insect access is complete [9].

In angiosperms the mechanism of pollination where flowers are not exposed and the androecium and gynoecium are hidden at the time of fertilization is rare but still occurs in several plant families and this type of pollination is called cleistogamy [27]. Cleistogamous plants are usually hermaphroditic, self-compatible and the release of pollen and stigmatic receptivity occurs at the same time therefore self-pollination and autogamy are predominant [28], freeing themselves from dependence on pollinators. Although *M. saccifera* reproductive structures remain hidden, cleistogamy is unlikely because the palm has unisexual flowers, with temporal phase separation and high values of self-incompatibility, therefore highly dependent on insects to fertilize the flowers.

The selective filter imposed by the peduncular bract of *M. saccifera* really influences the access of floral visitors as evidenced by the fact that the diversity of insects found within the inflorescence was low (only eight species). Comparing the rate of visitors to palms with characteristics similar to *M. saccifera* as size, rewards offered, type and location in the forest, these are visited by great diversity of visitors; for example, in *Oenocarpus batau*a [10] reported 81 species; *Phytelephas macrocarpa* is visited by 45 [29], as *P. seemanii*; and *Astrocaryum mexicanum* is visited on average by 35 species [30].

Therefore, the mechanism of isolating the reproductive structures and to have selective barriers can bring advantages and disadvantages for *M. saccifera*. Among the disadvantages, access by insects is limited and therefore the options of species that can act as pollinators is reduced, which may limit pollen flow with consequent pollination problem [31]. It has often been suggested that plants that display their flowers can attract more visitors and potential pollinators than those with few exposed flowers [32] and thus the pollination probabilities increase. Moreover, the filters presented in *M. saccifera* can bring advantage in the fact that there is a real selection of insects that prevents the entry of those who have little part in pollination, and thus an antagonist interaction with the palm, generating actions that directly or indirectly affect the reproductive success of the species. This phenomenon of insects that are not involved in pollination of palms is very common and widely reported for other species, where only a small number of visitors is actively involved in pollination and most visitors focus their activities exclusively on male stage or male flowers [9, 10, 33–35].

The low diversity of visitors generated by the selective filters in *M. saccifera* is balanced by an intimate and exclusive association of the palm with its main pollinator *Mystrops cercus*, which has easy access to the inflorescence through the selective filters and deposits pollen with efficiency values reaching 99% of the pollen transported and used for fertilization of the flowers of the palm (**Table 2**), ensuring fertilization of flowers and thus a constant fruit production. The pollination mechanism present in *M. saccifera* with *M. cercus* as main pollinator is summarized in a general model of pollination (**Figure 5**).

Because *Mystrops cercus* depends *M. saccifera* flowers as an ideal environment for feeding with pollen, protection (isolated flowers) and an ideal microenvironment to develop part of their life cycle due to the thermogenesis of the flowers, such association entails the

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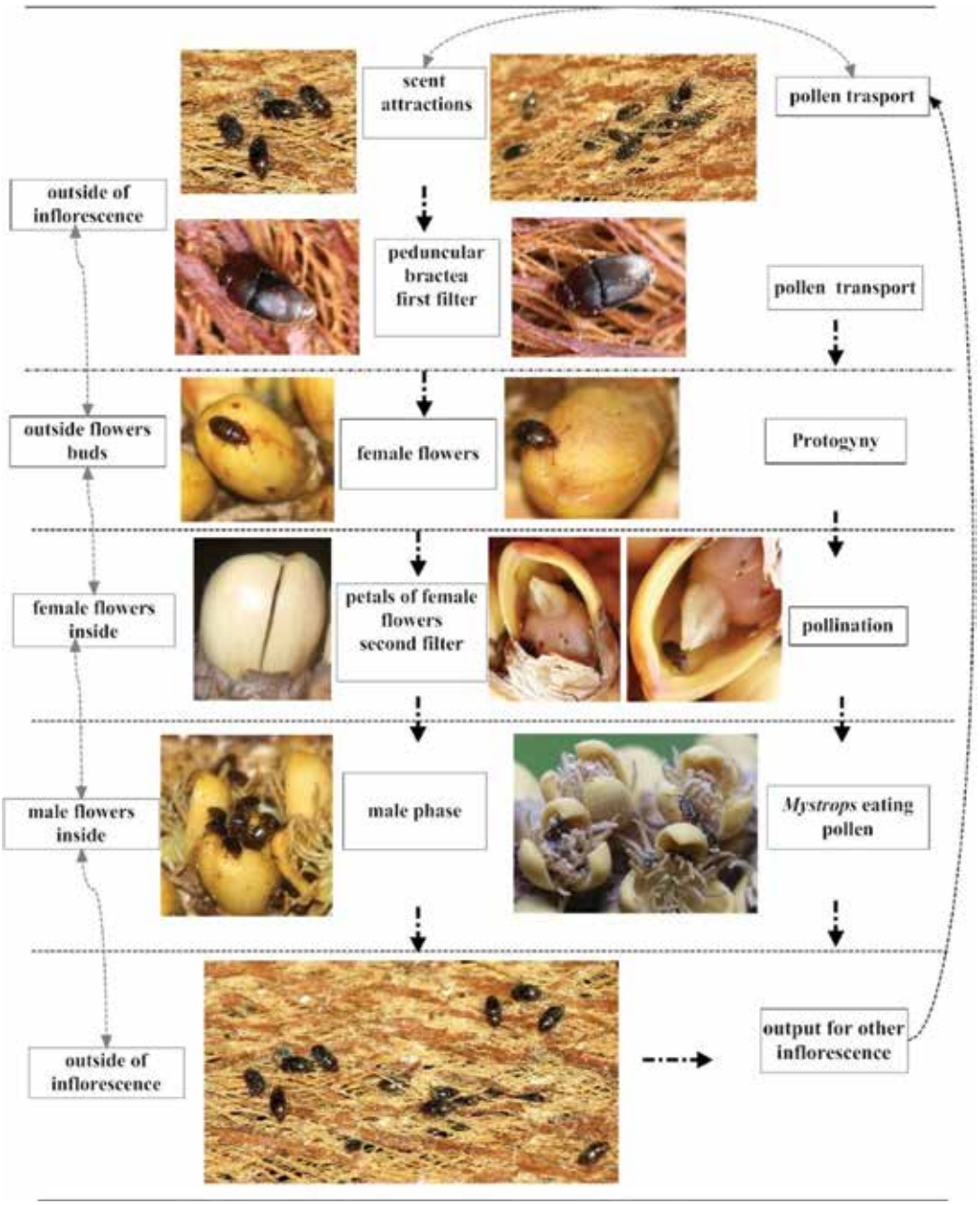

Figure 5. Pollination of *Manicaria saccifera* by *Mystrops cercus*.

establishment of a relationship of mutual dependence or obligatory mutualism between the palm and its pollinator. In that mutualism, pollinators need the palm they host for food, find a mate, make their life cycle; while for the palm the benefit of having a close relationship with the pollinator ensuring their loyalty, perseverance and efficiency in pollen transfer necessary to achieve reproduction.

Obligate mutualisms *Mystrops* - *Palmae* are more and more recognized, sometimes in one-to-one relations as in *Attalea allenii* [9, 36, 37]; or a *Mystrops* species associated with several species of the same genus of palms as with *Mystrops rotundula* and *Mystrops pulcra*, which pollinate seven species of the genus *Ceroxylon* [36]. The mutual dependency between *Mystrops cercus* and *M. saccifera* ensures reproductive success of the palm and the permanence of pollinators through the coordination of several mechanisms of association including attraction, maintenance and fertilization of flowers by their primary pollinator *Mystrops cercus*, this leads us to suggest that a high degree of specialization exists between *Mystrops cercus* and *Manicaria saccifera*, which has also been reported in other palm species pollinated by *Mystrops* species [1, 7–9, 21, 36, 38]. Likewise, two additional evidences can support the degree of specialization found and suggested in this paper: specificity and distribution of interaction.

Regarding specificity [36] conducted a comparative study of *Mystrops* species in at least 80 species of palms including *Mystrops* species visiting and pollinating flowers of palms found in the Chocó (*Attalea allenii*, *Attalea cuatrecasana*, *Oenocarpus bataua*, *O. minor*, *Socratea exhorriza*, *Wettinia quinaria* and *M. saccifera*), and found that species of pollinators are not shared: each palm has its own association with a particular *Mystrops* species. In terms of distribution, we have found *Mystrops cercus* in five additional locations to our study area.

Participation of the genus *Mystrops* in the pollination of palms has been amply demonstrated, whether acting as principal pollinators, secondary or co-pollinators [9, 30, 34]. However, to the extent that detailed reproduction studies of tropical palms increase, species of the genus *Mystrop*s are showing greater relevance and importance as pollinators of palms: one or more species of *Mystrops* are the most important pollinators, and in some cases, are the solely responsible for the movement of pollen in a particular palm species, e.g., *Mystrops* sp. nov. 1 in *Attalea allenii* and *Mystrops sp*. nov. 2 in *Wettinia quinaria* [9], *Mystrop*s sp. in *Mauritia flexuosa* [37], *Mystrops* sp.15 in *Wettinia praemorsa*, and *Mystrops* sp. nov. 22 in *Cryosophila kalbreyeri* (Núñez [36]) *Mystrops* in *Wettinia kalbreyeri* [39].

In synthesis, *M. saccifera* presents a specialized pollination system with morphological barriers that blocks access of floral visitors when the stigmas are receptive, and is closely associated with *Mystrops cercus*, a kind of small beetle that, in an exclusive way, visits and pollinates the flowers of this important palm with morphological constraints. The most striking examples of highly specific mutualism relationships and close interdependence between plants and pollinating insects are given in cases where the flower morphology limits the visitor access to reproductive or floral rewards structures and consequently the possibilities of pollination are minimal, *M. saccifera* is a clear example.

We recommend further studies, mainly focused on evaluating features like the changes in time and if this mutual dependence is maintained throughout the disjunctive distribution of the palm.

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Chapter 4

Challenges in Cocoa Pollination: The Case of Côte d'Ivoire

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Additional information is available at the end of the chapter

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Abstract

Cocoa (*Theobroma cacao* L.) is mainly pollinated by ceratopogonid midges (*Forcipomyia* spp.). However, other insect species will also pollinate cocoa flowers when these midges are scarce. In Côte d'Ivoire, inadequate pest control practices (insecticide spraying, mostly against the mirids *Distantiella theobromae* and *Sahlbergella singularis*) and landscape degradation as a result of deforestation and cocoa monoculture, have decreased overall pollinator population levels and, as a result, pollination services to cocoa trees. The current low average Ivorian cocoa yield of 538 kg per ha (in 2016) is the result of global agricultural mismanagement (deteriorated soils, lack of fertilizers, inadequate or absent pest control, absence of shade trees and intercrops). However, there is also an evidence of a pollination gap that could cause low cocoa yield. More research is needed to understand: (i) which agro-ecological efforts to enhance cocoa pollination can improve yield, and (ii) which strategies are effective in enhancing cocoa pollination. In this chapter, we briefly describe the cocoa sector. Next, the cocoa flower and pollinator biology and phenology are presented, followed by an overview of current environmental and management constraints to cocoa pollination in the context of Côte d'Ivoire, the largest cocoa producer in the world. We conclude with exploring possibilities to enhance pollination in the Ivorian small-scale cocoa sector.

Keywords: cocoa, pollination, *Theobroma cacao*, fructification, cherelle wilt, *Forcipomyia*, phenology, pesticides, IPM, Côte d'Ivoire

1. Introduction

Non-bee insect pollinators play a significant role in global crop production [1]. Cocoa (*Theobroma cacao* L.) is one of the 13 most important commercial crops in the world. It entirely

depends on insects for pollination and successful production [2]. In cocoa, pollination is almost exclusively performed by ceratopogonid midges (Order Diptera) from genus *Forcipomyia* [3, 4]. In 2016, global cocoa production was 4.472 million tons of dry beans, of which 2.655 million tons (59%) were produced in West Africa and 1.472 million tons (33%) in Côte d'Ivoire [5]. In 2016, global average cocoa yield was 438 kg dry beans per ha and per year (480 kg per ha in West Africa), whereas it was shown in research stations that cocoa bean yield could attain up to 2000 kg per ha and per year [6].

Cocoa yield remains under the latter potential level due to: (i) an inadequate cropping system mainly consisting of full-sun monocultures without shade, leading to soil erosion, nutrient depletion, water shortages, weed growth, and increased pest and disease outbreaks [7–11]; and (ii) inadequate pest and disease management [12, 13]; both leading to (iii) below-optimum pollinator population levels. Earlier research showed that increasing pollination, either manually [14] or indirectly by improving breeding opportunities for pollinating midges [14–16], had a significant impact on cocoa yield compared with normal agricultural practices control plots.

Over the past 50 years, cocoa demand has consistently increased annually by some 2.5% [17]. Demand continues to rise, particularly as a result of newly emerging chocolate markets such as China and India [18]. However, cocoa production levels have decreased by 3–5% over the past five years (compared to 2012 levels), leading to unstable prices because of market shortages. Cocoa production could expand through increasing global cocoa acreage (as it has always been done in the past). However, this is not a sustainable solution as it is mostly achieved at the cost of deforestation in tropical areas [19]. The other, more sustainable, approach is increasing productivity per ha. The latter strategy not only increases overall cocoa production without further deforestation, but can also increase income of cocoa farmers who nowadays often leave the cocoa production sector because of its low profitability [9].

In this chapter, we explore how cocoa farmers—besides by improving soil conditions, adequate pruning and integrated pest and disease management—can increase cocoa yield by increasing pollination intensity of their cocoa trees. We will first present the global cocoa sector and then focus on Côte d'Ivoire, the leading cocoa-producing country in the world. Next, we give background information on the biology and phenology of both cocoa flowers and pollinating midges (*Forcipomyia* spp.), followed by a discussion of pollinator-reducing factors (environmental and managerial) in Côte d'Ivoire. We conclude by discussing some options for relieving the constraints on cocoa pollination.

2. The cocoa crop sector

2.1. Global production and economic value

Although global yearly cocoa production quantities are below those of other tropical commodity crops such as sugar cane, rice, soybean, oil palm, cassava or banana, it is a unique crop because more than 90% of its production comes from small-scale farmers (each with a

cultivation area not larger than 3 ha) [5, 17]. As such, cocoa provides a livelihood to around 4.5 million farming families. Globally, 14 million people work in cocoa production [17]. In 2013, the total chocolate confectionary retail consumption had a value of 109,992 million USD [20]. Cocoa is produced on around 10 million ha, which is just 0.7% of the total global arable land, but 7% of the global permanent crop area. As a result, cocoa cultivation, and particularly cocoa agroforestry systems, play an important role in carbon sequestration and consequently have important climate mitigation potential [21].

2.2. The cocoa sector in Côte d'Ivoire

Côte d'Ivoire comprises the main cocoa-producing region in the world. In 2016, the country provided one third (1.472 million tons) of global cocoa supplies on 2.851 million ha of land. Average cocoa yield in Côte d'Ivoire was thus 516 kg/ha of dry cocoa beans, which is slightly below the global average yield of 538 kg/ha for that year [5]. In Côte d'Ivoire, cocoa is exclusively produced by around 1,000,000 small-scale farmers, each cultivating on around 2–3 ha [22]. These smallholders operate in a difficult context. Between 2000 and 2011, Côte d'Ivoire was generally considered a failed state with frequent occurrences of violent conflicts, where cocoa tax revenues were often used to fuel the conflicts [23, 24]. Moreover, the cocoa production in Côte d'Ivoire has often been linked to child slavery on plantations [23]. As in most cocoa-producing regions, fluctuating prices (between 1500 and 3500 USD per ton in the period 2011–2018, see http://www.nasdaq.com/markets/cocoa.aspx) affect Ivorian cocoa smallholders because in a situation with volatile prices, it is difficult to make informed choices on the "right" crop investments [25].

3. Cocoa pollination

Pollination intensity and fruit set largely determine cocoa yield [26]. If natural pollination is limiting cocoa yield, then enhancing pollinator population levels should result in increased fruit set and consequently yield.

3.1. Biology and phenology of cocoa flowers and fruit set

3.1.1. Biology

Cocoa flowers are hermaphrodite. They are produced on the trees' trunks and branches (cauliflory). After 2–3 years, so-called flower cushions, i.e., thickened flower-producing leaf axils, are formed. Every cushion bears up to 50 flowers per flowering season. There are two flowering seasons per year, which thus yields 100 flowers per year. The pentamerous flower is about 15 mm in diameter. A petal consists of a pouch—which conceals the anthers—and a wide tip. The function of the latter tip is unknown, but it does not specifically attract pollinators [27]. A particular aspect of cocoa flowers is the outer whorl of purple staminodes around the style. Right after anthesis, these staminodes align parallel to the style (**Figure 1**). Pollinators move around on the inner side of the staminodes, thereby rubbing their pollen grain-carrying bodies against the style. On older flowers, staminodes are somewhat withered and flexed away

Figure 1. Closed and open flowers as well as fruits (pods) on the trunk of *Theobroma cacao*. Flowers are produced in clusters directly on the trunk and older branches (this is known as cauliflory) and are small, 1–2 cm in diameter, with pink calyx. The floral formula is ✶ K5 C5 A(5° + 5) G(5) [31]. While many of the world's flowers are pollinated by bees (hymenoptera) or butterflies/moths (Lepidoptera), cocoa flowers are pollinated by tiny flies, *Forcipomyia* midges in the family Ceratopogonidae [16, 94]. The tree flowers profusely, but few flowers set particularly in the dry season. When the tree is under water stress, all flowers are dropped within about 5 days. Successful pollination requires the deposition of at least 35 suitable pollen grains on the receptive parts of the flower, and is dependent on the season [95]. (Photos by Guy Smagghe in cocoa plantation at Tiassalé, Côte d'Ivoire, 15/01/2018).

from the style, which obstructs pollen deposition on the style [28]. The ovary consists of 40–70 ovules with axile placentation [29]. At least 20 ovules need to be successfully fertilized for a pod to develop and mature. Maximum pollination is achieved when pollination intensity, i.e., the number of pollen grains deposited on the style, exceeds 115 [30]. Usually, a mature pod contains between 30 and 40 beans [31, 32]. Flower morphological characteristics (size, color, and shape) can differ greatly among varieties. However, even the most noticeable differences (e.g., white vs. red sepals) have no effect on pollination [33].

3.1.2. Bud development and maturation

Flower bud development from meristem to receptive flower takes at least 20 days and can take up to 30 days [31, 32, 34]. In India, it was shown [32] that flower bud development is faster in months with higher mean temperatures (e.g. June with a mean air temperature of 28°C) compared to colder months (e.g. November with mean air temperature of 25°C). Prolonged dry (<125 mm per month) or cold (mean monthly air temperature < 23°C) periods inhibit flowering [35]. Flowering is optimal during rainy days with high relative humidity and moderate temperatures (100 mm per month, 70% RH, and 27°C). High solar radiation incidence is linked with increased flower abscission [32]. Pollen grains are only able to germinate on a receptive stigma [36, 37]. The receptive period is at about 2–3 days after anthesis. Unsuccessful pollination leads to flower abscission. Reported flower abscission rates vary from 63% on the main trunk and 81% on the fan branches to over 90% for all flowers [27, 32, 35].

Anthesis starts at around 2–4 pm. The latter becomes evident through splitting of the five sepals [27]. The process of sepal splitting continues overnight and finishes at around 4–6 am. Complete anthesis (flower fully open) is quickly followed by pollen release from the anthers (also between 4 and 6 am). Higher air temperature, as well as low air humidity, facilitates

anther dehiscence [32]. However, pollen release is maximum between 8 am and 2 pm [10]. Styles and stigmas mature later than anthers, and have maximum receptivity around 12 am–2 pm. Maximum stigma and style receptivity does not concur with maximum anther dehiscence, thus limiting the possibility of self-pollination. The period during which the stigma is receptive to pollen and consequently during which successful pollination is possible, only lasts one day. Non-fertilized flowers will abscise the next day. About 1–5% of all flowers develop into a pod [31, 32].

3.1.3. Cherelle wilt

Even after cocoa flowers are successfully pollinated and led to fruit set, not all young fruits (cherelles) will grow to mature cocoa fruits. Up to 80% of cherelles will shrivel, turn black, and become rapidly colonized by pathogens, while the pod remains on the tree. This so-called cherelle wilt is a physiological mechanism whereby the fruits are naturally thinned to balance nutrient allocation in the tree. Cherelles can wilt up to day 100 after fruit set [38]. Poor soils and impeded photosynthesis result in increased cherelle wilting [39, 40]. Leguminous shade trees, which supply nitrogen to the soil, can therefore lower cherelle wilt [7]. Wilting in an early stage saves energy that can be invested in the development of the remaining fruits [30, 31]. Apart from resource limitation, inadequate pollination (insufficient pollen grains deposited on the stigma surface) and incompatible pollen may also cause cherelle wilting [41].

3.1.4. Pod maturation

There are 130–160 days between fertilization and pod harvest [32]. The cocoa fruit is an indehiscent drupe. During the first 40 days after fertilization, pod growth is slow. Afterward, growth accelerates. The first division of the zygote only takes place between day 40 and 50. Pod and ovule growth decrease from day 85 onwards, when embryos start to develop. On day 140, the embryo has completed its development and pod ripening starts [38].

3.1.5. Self-incompatibility

Most cocoa trees are self-incompatible. Self-pollination on a self-incompatible variety will not result in successful fertilization; as such, cross-pollination is then the only way for successful fertilization [42]. Self-incompatible trees are mostly cross-compatible; i.e., they are able to successfully fertilize flowers on other trees, including trees of the same variety. Incompatibility takes place at the stage of gamete fusion: incompatible gametes are unable to fuse. The underlying mechanism is of a genetic nature [32, 43]. Following unsuccessful fertilization due to incompatibility, the flower drops off after 2–3 days. Even within a single variety, not all trees are necessarily either self-incompatible or self-compatible. However, the proportion of self-incompatible trees of a certain variety is determined by the specific variety. Self-compatible varieties that are cross-incompatible can restrict bean yield. In commercial plantations, it is therefore recommended to always plant different varieties [31]. Self-compatible hybrids produce larger fruits with a higher dry bean yield [44].

Viable pollen is able to germinate (producing a pollen tube) when it reaches the stigma. Pollen viability lies between 80 and 90%, hence does not limit fertilization [32, 45].

3.1.6. Flower phenology

The number of flowers per tree varies throughout the season and is a function of climatic factors, such as photoperiod and temperature regime [46], whereas it is also cultivar dependent [31]. Furthermore, it seems that fruit production in the previous year determines flower production in the following year. Years of high pod production alternate with years with a low level of flowering [47, 48]. In most tropical countries, flowering occurs year-round. Flowering peaks are often preceded by increased temperature and rainfall, and occur at the onset of the rainy season, after which flower numbers gradually decline [45]. In West Africa, the major rainy season commences in April and climaxes in June, a period that is characterized by intense flowering (flowers on branches and trunks) [6]. In the minor rainy season (September–November), flowering intensity is lower (flowers on branches only). Few flowers are observed during the dry season (December–March) [47]. When pods are developing and this sink for assimilates is increasing, new flower production diminishes [40].

3.2. Biology and phenology of cocoa pollinators

3.2.1. Overview of cocoa pollinating species

Early studies have ruled out wind as a pollinating agent—pollen grains form chunks, due to their viscosity and become too heavy to travel on their own [49]. However, in South America, experiments have been conducted to increase pollination by artificially increasing air currents in the field with motorized knapsack sprayers, thus stimulating wind pollination. This technique, however, only proved to be effective (doubling of cocoa bean yield) on self-compatible varieties [50].

Cocoa is almost exclusively pollinated by insects. The most important pollinators are midges from the family Ceratopogonidae. In reference [26], the author claims based on a review of five papers that female specimens are the main pollinators, although in reference [28], four times more males than females were collected in cocoa flowers. Ceratopogonids are biting midges of 1–4 mm length [51]. Males also pollinate, but to a lesser extent. It is not clear why females visit cocoa flowers more frequently than males [28, 52, 53]. Females presumably visit cocoa flowers to feed on the protein-rich pollen grains, necessary for egg maturation.

Besides ceratopogonids, other small dipteran insects such as Cecidomyiidae (gall midges), Chironomidae (non-biting midges), Drosophilidae (fruit flies), Psychodidae (moth flies), and Sphaeroceridae (small dung flies) have been documented to visit cocoa flowers. Other insects, such as aphids, coccids and cicadellids (Hemiptera), thrips (Thysanoptera), and ants (Hymenoptera), also occasionally visit cocoa flowers. However, their contribution to pollination is most probably very low. Up to date, pollen grains have not been detected by microscopic observation on insects other than *Forcipomyia* spp. In some cases, observations suggest that cecidomyiids (in Cameroon) and drosophilids (in Ghana) may contribute to some extent to pollination [26].

Only Diptera, and particularly genus *Forcipomyia* (Fam. Ceratopogonidae), are morphologically able to pollinate cocoa. *Forcipomyia* holds the largest number of cocoa pollinators. Within

that genus, the most frequently reported pollinators belong to the subgenera *Euprojoannisia* (before: *Proforcipomyia* and *Euforcipomyia*), *Thyridomyia,* and *Forcipomyia* [26].

It is well-documented that ceratopogonids breed in humid, decaying organic material such as cocoa leaf litter, decomposing cocoa pod husks, banana pseudostems, and bromeliads [4, 54]. Besides being moist, these breeding substrates are cooler than the ambient environment and provide dark conditions which all benefit ceratopogonid breeding [31].

In the 1970s, cage experiments [28, 52, 53] were performed to characterize the pollination capacity of different ceratopogonid species. However, results of these experiments have little value as they were performed under unrealistic conditions (exposure of a high number of flowers to a single midge and use of small cages, both causing pollination levels that the same midges would not achieve in nature). The only valid method to determine whether a species is a pollinator is through field observation [26]. It has been shown that artificial circumstances bias lab experiment results considerably; for example, successful pollination by *Tyora tessmanni* was shown under lab conditions, but could not be confirmed under field conditions, where the putative pollinator was abundantly present [55].

There is weak evidence for the indirect influence of the ant *Azteca chartifex spiriti* Forel (in Brazil) on cocoa pollination, as it has been shown to attract ceratopogonid midges [56]. There is some evidence of the pollination potential of stingless bees *Tetragona jaty* (Smith), *T. testaceicornis* Lep., *T. coryina* Ckll., *T. pallida* Latr.; *Nannotrigona testaceicornis punctata* (Smith); *Paratrigona lineata subnuda* Moure, and *Plebeia mosquita* (Smith). However, cocoa pollination by the latter species is merely coincidental. Sweat bees (*Lasioglossum* spp.) have also been suggested as possible cocoa pollinators [57, 58].

3.2.2. Biology and phenology of Forcipomyia spp.

Forcipomyia eggs hatch 3 days after deposition. Twelve days later, larvae transform into pupae. Pupation lasts 3 days. Adults live 1–12 days (under laboratory conditions) [59, 60]. A complete life cycle thus covers about 28 days [31].

Female ceratopogonids, in search for sugary nectar, start pollinating cocoa flowers early in the morning (5–8 am) and also actively visit flowers in the afternoon (4–6 pm) [3, 52]. Ceratopogonids carry cocoa pollen grains on their thoracic hairs. Weather conditions affect their flower visiting activities: rain and clouds decrease their activity whereas sunny weather increases it [3]. Some trees receive more attention from pollinators than others, resulting in a greater fruit set in some trees as compared to others. The interest for particular trees shifts with time. Why this happens, is not clear. Female ceratopogonids commonly visit cocoa and other flowers everywhere in the world [4, 61].

Ceratopogonid midge flights might cover long distances, but it is not known how far exactly [26]. Distance traveled during one foraging event, and consequently during which pollination is performed, can reach up to 50 m. However, midges mostly deposit pollen from a certain cocoa tree on flower stigmas of neighboring cocoa trees [31, 62]. It has been shown that there are 5–7 times more *Forcipomyia* specimens above the cocoa canopy than below the

canopy [26]. Since wind speed above the canopy is higher than below, it can be expected that wind could play an important role in horizontal cocoa pollinator distribution over the cocoa field.

Besides feeding on flower nectar, adult ceratopogonids also suck the blood of other insects and mammals. In general, pollinating activity is very limited in time during the lifetime of these pollinators.

Ceratopogonid pollinator populations can be abundant and exceed one million individuals per ha [3]. Moist environments favor ceratopogonid midge abundance. In fact, there is a positive correlation between soil moisture and ceratopogonid population levels [26]. Stable moist conditions are indispensable for successful development of eggs and larvae [63]. It is suggested that the West African harmattan (dry, hot wind from the north) results in withered breeding places, rendering them unsuitable for insect breeding [26]. Pollinator populations thus increase with each rainy period, to decrease again with the onset of a drier period [31].

3.3. Pollination gap in cocoa

The yield gap in cocoa (i.e., the difference between yield at optimal, experimentally determined growing conditions and the current cocoa farm yield) is caused by multiple factors including disease, pest and weed pressure as well as inadequate phytosanitary practices, lack of improved varieties, low soil fertility, etc. [64]. However, there is increasing evidence that the present yield gap is also linked with inadequate pollination. This so-called pollination gap was already observed in the late 1970s when it was found that during the dry season, the number of ceratopogonid pollinators, as well the relative number of pollinated flowers were lower than in the wet season [3, 4, 26]. Because rotten, moist organic material is an ideal breeding substrate for ceratopogonid midges, attempts have been made to increase reproduction opportunities for these midges by adding such organic material in cocoa plantations. In an experiment in Ghana, banana pseudostems, cocoa pod husks and leaf litter were added as pollinator breeding substrates next to cocoa trees. It was found that midge population increased to 500% of the control tree levels whereas fruit set in treated trees was four times higher than in control trees. Cherelle wilt also increased in treated trees but was lower than increased fruit set rates so that the final number of mature fruits was twice as high for all substrate-treated trees compared to the control trees [65]. A more direct proof of the pollination gap was found when cocoa trees in Sulewesi (Indonesia) were artificially pollinated. Optimum dry bean yield was achieved when 40% of flowers were hand-pollinated [14]. The latter treatment increased dry bean yield by 350 kg per ha as compared to a pollination intensity of 10%, which concurs with natural pollination intensities observed over the past 20 years [30, 66]. In North Queensland (Australia), it was recently shown that adding cocoa pod husks as a pollinator breeding substrate considerably increased fruit set (110 times more cherelles) and yield (60 times more fresh fruit production). However, hand pollination in fields where breeding substrate had been added did not result in extra yield, indicating that breeding substrates had already increased pollination intensities to optimum levels [16].

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4. Constraints to cocoa pollination in Côte d'Ivoire

4.1. Deteriorating pollinator environment

4.1.1. Cocoa monoculture

Cocoa is a shade-tolerant tree. Traditionally, cocoa is grown in shaded, agroforestry systems where it is intercropped with forest trees that were spared when the forest was cleared for cocoa cultivation. However, it was shown that—provided soil nutrition levels are adequate—cocoa production with shade trees is lower when compared to full-sun production [31, 67–69]. As a consequence, agroforestry systems have globally been replaced by monoculture systems with low shade provision [70]. Over the past few decades, cocoa cultivation has intensified not only by removing shade trees but also by extensive application of fertilizers and pesticides. As a result, the insect assemblage of cocoa cultivation systems has changed considerably. When compared to agroforestry systems or natural forests, insect biodiversity has decreased in present-day cocoa plantations, often at the expense of predators, leading to increased pest outbreaks and pollinators [71–75].

4.1.2. Landscape degradation

In Côte d'Ivoire, the cocoa sector is largely responsible for landscape degradation [19]. Over the past few decades, cocoa was typically cultivated on freshly cleared land where its production rapidly expanded, after which the land was abandoned 10–15 years later due to declining yields. Since the 1970s, such continuous so-called boom-and-bust cycles, as well as cocoa expansion from the southeast to the southwest of Côte d'Ivoire, have led to massive deforestation in the country [9, 22, 76]. In the 1960s, total tropical primary forest cover amounted to around 8.14 million ha. In the 1980s, that area had dropped to 2.6 million ha, whereas in the 2000s, primary forest cover was just over 1.35 million ha, meaning that since its independence, Côte d'Ivoire has lost 80% of its forest cover [77].

Almost all cocoa plantations in Côte d'Ivoire have less than 50% of shade, meaning that the majority of trees are fully exposed to sunlight, leading to biodiversity loss and soil deterioration, often resulting in reduced addition of organic matter to the cocoa plantation soils [78]. It has been extensively shown that *Forcipomyia* spp., which are the predominant pollinating midges, require moist and decaying organic material to breed [15, 28, 52, 79]. Also, the vicinity of natural forest and moist refuges promote diversity of *Forcipomyia* spp. and cocoa pollinators in general [52, 80]. It is therefore fair to assume that in Côte d'Ivoire, massive landscape degradation has led to decreased breeding opportunities and consequently to lower population levels of cocoa pollinating midges.

4.2. Pesticide use in the Ivorian cocoa sector

4.2.1. Target pests and insecticide products used

The major cocoa pest problem in West Africa is caused by mirids (Order: Hemiptera, Fam. Miridae). *Sahlbergella singularis* and *Distantiella theobromae* suck the sap from cocoa pods

and young shoots, causing commercial cocoa losses of up to 30% [81, 82]. In West Africa in general, more than 75% (in some areas 100%) of cocoa farmers use chemicals to control mirid infestation [83]. Nowadays, most frequently used insecticides in cocoa cultivation are the pyrethroids bifenthrin, cypermethrin, deltamethrin and lambda-cyhalothrin, and the neonicotinoids acetamiprid, imidacloprid and thiacloprid [84]. In Côte d'Ivoire, almost all farmers who use insecticides, apply commercial products containing a systemic neonicotinoid insecticide, usually in combination with a contact pyrethroid insecticide two times per year (July–August and January–February). The pyrethroid would thereby kill the mirid adults as well as the nymphal instars, whereas the systemic neonicotinoid would ensure that mirids that hatch after insecticide applications are also killed (personal communication with local pesticide dealers) (**Table 1**). However, the precise impact of these specific insecticides on cocoa pollinators in Côte d'Ivoire is unclear and should be further investigated.

4.2.2. Impact of pesticides on cocoa pollinators

Broad-spectrum insecticides (such as β-hexachlorocyclohexane and dichlorodiphenyltrichloroethane), which were historically widely applied in cocoa crop production, did not affect pollinator population levels [26]. It is suggested that breeding sites are protected from

Pesticide brand	Neonicotinoid	Conc. (g/L)	Pyrethroid	Conc. (g/L)
Thiodalm Super	Acetamiprid	20	Bifenthrin	20
Callifan Super BD	Acetamiprid	20	Bifenthrin	20
Gourou Super 45 EC	Acetamiprid	25	Cypermethrin	25
Onex Super 40 EC	Acetamiprid	20	Cypermethrin	20
Caomine 40 EC	Acetamiprid	20	Cypermethrin	20
Blinde 20 EC	Acetamiprid	10	Lambda-Cyhalothrin	10
Gawa 30 SC BTE	Imidacloprid	30	—	—
Thiosulfan 60 EC	Imidacloprid	60	—	—
Caostar 60 EC	Imidacloprid	60	—	—
Gawa Pro 80 SC	Imidacloprid	60	Bifenthrin	20
Koumabana	Imidacloprid	30	Bifenthrin	20
Grosudine Super 50	Imidacloprid	30	Bifenthrin	20
Tropinex Ultra	Imidacloprid	30	Lambda-Cyhalothrin	20
Actara 240 SC BTE	Thiamethoxam	240	—	—
Azudine 50 SC	Thiamethoxam	30	Deltamethrin	20
Boradyne 45 ZC	Thiamethoxam	30	Lambda-Cyhalothrin	15

Table 1. Insecticides, designed for application in cocoa cultivation, randomly collected by the authors from pesticide shops in Abidjan, Côte d'Ivoire in October 2016.

insecticide sprayings by leaves and other organic material. However, residual effects of insecticides might affect cocoa pollinators [3, 54]. A study in West Africa on the effect of large-scale insecticide treatments (against mirids; Fam. Miridae) on both pollinator population levels and cocoa pod production showed that there is only a short-term negative impact of insecticide treatments on pollinator population levels [31]. Also in West Africa, it was shown that fogging instead of spraying insecticides is less harmful for pollinators, as fogging only negatively influences the population level for 2 days compared to 8 days with spraying [85]. Alternative approaches are to (i) reduce insecticide dosages during the period that pollinator population levels are low, and (ii) use narrow-spectrum insecticides.

5. Recommendations

5.1. Integrated pest management (IPM) options in the Ivorian cocoa sector

Despite the currently widely applied spraying programs, mirid infestation remains the most severe cocoa production limitation factor [81]. Although the precise impact of pyrethroids and neonicotinoids on cocoa pollinators in Côte d'Ivoire is unknown, it cannot be excluded that apart from these regular pests, pollinators are also affected by these products. Therefore, novel and more integrated pest management (IPM) approaches should be tested against mirids. The latter approaches might include: (i) further development and testing of mirid pheromones [86]; (ii) increasing shade levels by planting shade trees to avoid so-called "mirid pockets" (i.e., mirids particularly occurring in non-shade areas of the plantations) [81]; and (iii) enhancing ant populations as they are most probably natural mirid predators [87].

5.2. Enhancing cocoa pollinator environment

In cocoa plantations, pollinator population levels can be increased by augmenting the amount of natural pollinator breeding sites or by adding artificial breeding substrates. Since it is known that *Forcipomyia* spp. breed in moist and rotting organic material, introducing such material in the cocoa field will most likely enhance pollinator breeding and subsequently their population levels. Banana pseudostems are preferred as a pollinator breeding substrate over cocoa husks, because the latter are a possible source of black pod disease [88]. Intercropping with fruit trees will not only provide shade, but (provided that not all fruit is harvested), will also introduce rotting fruit in the plantations as potential pollinator breeding sites. As shown in **Figure 2**, in Côte d'Ivoire, we currently investigate, together with the cocoa farmers of the local cooperatives, the effect on pollination levels of squared pits (0.5 × 0.5 m and 0.3 m deep) that are spaced in 10 × 10 m squares and in which organic material such as fresh empty pod husks, cut banana pseudostems, and fruits from intercropped trees such as *Citrus* spp. will be deposited to enhance pollinator breeding.

5.3. Pollinator mass breeding and mass release

Mass breeding and subsequent mass release of *Forcipomyia* spp. at times when cocoa flowering peaks, might also have a significant effect on effective cocoa flower pollination. The idea

Figure 2. Midges of *Forcipomyia squamipennis* in the family Ceratopogonidae are believed to be the most important pollinators of cocoa globally, based on field observations and laboratory rearings [53, 96]. Indeed, early on, scientists figured out that most *Theobroma cacao* trees are not able to self-pollinate, but for years, they could not figure out what moved cocoa pollen between trees. It turned out that cocoa flowers are pollinated by midges not much bigger than tiny specks of airborne dust. Midge populations are greatest in the rainy season. Adult midges spend the day in shady spots such as between the buttress roots of large trees, in crevices in logs, in hollow stumps or in piles of husk debris. They emerge at variable times of the day to swarm near their hiding locations, and disperse in the early morning and late afternoon. Most midges do not move further than about 6 m. The females lay batches of eggs on damp piles of plant debris, on moist decomposing wood, cocoa husks and other plant debris, in batches of 40–90 eggs. Eggs hatch after 2–3 days and the larvae pass through four instar stages before pupating at about 12 days; the pupal stage lasts 2–3 days. The adults survive for about a week and there are thought to be about 12 midge generations per year. Adult females require liquid plant food for survival and oviposition, although ovary maturation is independent of adult food intake or mating. In a joint project between Ghent University and Barry-Callebaut, and in collaboration with local cocoa smallholders and their cooperatives, we introduced squared pits of 50 × 50 cm and 30 cm deep, filled with organic material such as cut banana pseudostems, fresh empty pod husks and fruits from intercropped trees such as *Citrus* spp., at a density of 1 pit per 100 m^2 (spaced at 10 × 10 m as based on presumed midge flight radius of the midges) to enhance the establishment of the cocoa pollinating midge populations in the field (main picture is a photo by Guy Smagghe in cocoa plantation at Tiassalé, Côte d'Ivoire, 15/01/2018; inset photo is of a mating pair of *Forcipomyia* midges by Christophe Quintin, https://www.flickr.com/photos/34878947@N04/).

is based on similar practices commonly applied in the horticultural sector where bumblebees (*Bombus terrestris*) are commercially bred and subsequently released in tomato (*Solanum lycopersicon*) greenhouses for tomato flower pollination [89]. As compared to the earlier used vibrating sticks to induce pollen release from tomato flowers, bumblebees increase tomato fruit set by 45%. Another example is the black soldier fly (*Hermetia illucens*) (Order: Diptera, Fam: Stratiomyidae) that is used to enhance composting of food waste and reduction of organic manure volumes, and which can be mass bred prior to release on organic material [90]. To our knowledge, no mass breeding attempts for *Forcipomyia* spp. have been undertaken up to date. The hematophagous nature of *Forcipomyia* midges can be a constraint to their mass breeding success [91]. Laboratory experiments showed that *F. townsvillensis* eggs will not develop without complete blood meals [92]. Research is needed to test the most appropriate midge rearing conditions (temperature, humidity and feeding).

Forcipomyia spp. mostly pollinate flowers neighboring the ones where they have collected pollen [93]. We assume therefore that they do not swarm further than 10 m from their breeding sites. Under that assumption, mass release should be performed at least each 20 × 20 m in cocoa plantations (25 releases per ha). Given the wide diversity of *Forcipomyia* spp. that have been

identified as cocoa flower visitors and the fact that some are restricted to either Africa, Central America, or South America (only one cocoa flower pollinator, *F. fuliginosa* was observed in all regions), it can be assumed that specific pollinating midges are restricted to certain areas [4]. It is therefore recommended that *Forcipomyia* spp. mass breeding for use in a certain cocoa area would start with locally sampled *Forcipomyia* midges, as exotic midges might disturb local biotic equilibria. Obviously, as a precondition to adoption of commercial mass breeding of pollinating midges by resource-poor smallholders in Côte d'Ivoire, the technology should be cost-effective.

6. Conclusion

Since cocoa production essentially depends on insect pollination, any threat to pollinators will have a negative impact on cocoa production. There is evidence that currently, cocoa pollination is below the optimum level and that enhancing pollinator populations in cocoa fields could increase cocoa production [14–16]. It is clear that cocoa pollinators are threatened by the currently predominant cocoa production system, which consists of full-sun cultivation on often deforested land with degraded soils and chemical pest control. Pest control, shade tree planting, and landscape management all influence cocoa pollinator presence, making pollination management very complex.

Many research questions on cocoa pollination remain. They include: (i) quantification of the pollination gap (only in [14] attempts have been made, but just by comparing hand-pollination treatments with unpollinated controls); (ii) evolution of the pollination gap throughout the year (e.g., in West Africa, the gap might be narrower during the dry season when flowering is less abundant); (iii) the relation between pollination and cherelle wilt (can cherelle wilt be decreased by improving pollination efficiency?); (iv) success rates of artificial pollination (a difficult task requiring a lot of agility and experience); (v) influence of insecticide applications on pollinator and other insect population levels; (vi) role of landscape and cocoa cropping systems (agroforestry, intercropping, soil mulching) on pollinator species composition and abundance; (vii) pollen load and pollination efficiency of cocoa flower visitors other than *Forcipomyia* spp.; (viii) evaluation of pollinator roles in self-compatible as compared to self-incompatible cocoa trees; (ix) promotion of self-pollinating self-compatible trees; and (x) effectiveness of enhancing ant populations to improve cocoa pollination.

As final conclusion, we believe that the answers to these research questions will undoubtedly lead to decreasing the current cocoa yield gap, which is the only sustainable solution to increasing global cocoa supplies.

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Conflict of interest

The authors have no conflict of interest.

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Chapter 5

Artificial Pollination in Kiwifruit and Olive Trees

Tacconi Gianni and Michelotti Vania

Additional information is available at the end of the chapter

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Abstract

In the last 10 years, kiwifruit vine artificial pollination became a widespread practice useful to increase fruit quality. Kiwifruit size is directly proportional to the number of seeds, i.e., to the number of fertilized ovaries. However, artificial pollination efficiency depends on many parameters such as pollen quality (germinability, humidity, and conservation), pollination system (dry or liquid), coadjuvants, and flowering stage. Those parameters were well defined in *Actinidia* in recent studies, however, they remain quite undefined for other anemophilous pollinated trees such as olive tree, hazelnut, pistachio, and palm. In these plants, the flowers are very small and extremely numerous, so the pollination was difficult to study. In addition, there are incompatibility factors (genetic and physic), long lap time from pollination to fertilization, and alternate bearing, lower economic gain for these fruits, low agronomic input, and low innovation level in the field. All these aspects had reduced the application of pollination technique for these cultivations. The experiences developed in kiwifruit lead to define a new model crop fruit set that could be applied to anemophilous pollinated plants such as olive tree, where the fruit set are lower than 2%. The first experiences have shown a great potential and have encouraged the development of this technique.

Keywords: kiwifruit, olive, pollination, equipment, quality, flowering stage, germinability, humidity

1. Introduction

Pollination of crop plants is often the major requirement in achieving sufficient crop set [1, 2]. Insufficient pollination has been found to be one of the important causative factors of low yield and low quality in many fruit tree species [3]. Supplementary pollination is a valid support to increase productivity in crop species such as strawberry [4, 5], olive [6], kiwifruit [7, 8], almond [9, 10], pistachio [11, 12], hazelnut [13], macadamia [14, 15] and date palm [16, 17]. Artificial pollination

leads also to increase final set, weight, kernel recovery, and, in many cases, fruit quality in terms of nutritional characteristics and shelf life [4]. Moreover, in olive tree, a greater pollination and fruiting cause a slower ripening of the drupes, and consequently harvest times are more suitable to the improvement of olive and oil quality. In many cases, natural pollination (both wind and bee) is often unsatisfactory or not constant in the years (**Figure 1**), because it can be affected by climatic factors, wrong synchronization of male and female flowering, and low attraction for bee since the absence of nectar in the flowers of wind-pollinated (anemophily) plants.

Kiwifruit artificial pollination was first studied by Dr. Hopping in 70 years [7, 18, 19] in New Zealand and in Italy, in collaboration with Dr. Cacioppo and Dr. Galimberti in Latina, in 1987 (**Figure 2**).

Kiwifruit (*Actinidia chinensis* var. *deliciosa* and *A. chinensis* var. *chinensis*) is a dioecious plant, and, in order to have good pollination, in orchard, there are female and male plants in 6:1 ratio. The pollination is mainly anemophilous (wind-pollinated), and the fruits size depends on the number of seeds: a 100 g fruit has more than 1000 seeds, and it is estimated that about 10fold pollen grains are necessary to reach this seed number [21, 22]. Also, an increase in male:female ratio to 1:1 (**Figure 3**) was not enough, in many cases, to optimize the pollination.

Moreover, in many specialized orchards, there are installed anti-hail net or plastic tunnel to protect the plants from climate injuries or from the bacterial disease *Pseudomonas syringae* pv. *actinidiae* [23]. These installations reduce the ventilation and indeed pollen movement. Furthermore, in yellow flesh kiwifruit (but also in green ones), often male plants were not planted in the orchard in order to have a higher yield (male occupy 16% of the surface) and an easier management of the plants (treatments for plant protection due the higher disease susceptibility of male, pruning, fertilization, and irrigation). In the cases where male plants are absent in the orchard, pollen are kept from specialized male orchards or buy on market (following plant protection rules to avoid diseases contaminations).

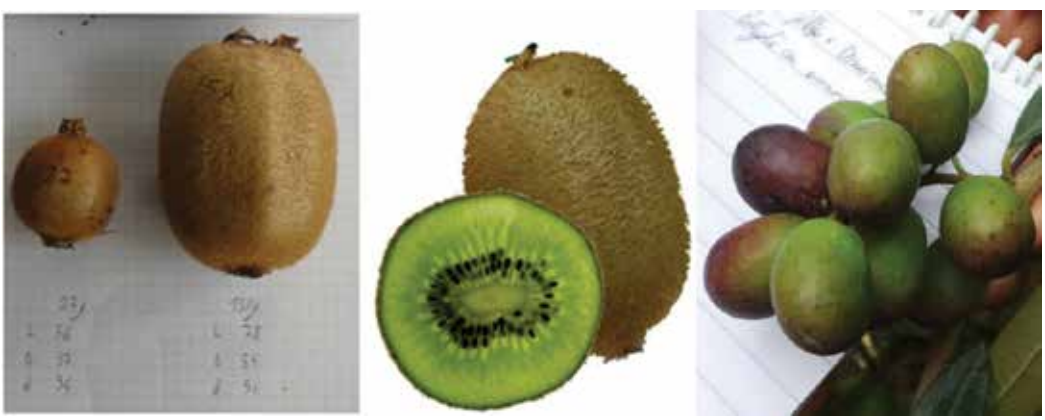

Figure 1. Kiwifruit with opposite size due to the pollination efficiency (left), perfect pollination with many pollinated ovules (center), and abundant pollination in olive (right).

Figure 2. M. E. Hopping who developed, in New Zealand, artificial pollination in kiwifruit (left). Hopping's group of researchers during the experiment of spray pollination in Latina in 1987 (right) [20].

Figure 3. *A. deliciosa* cv. Hayward in T-bar orchard in Verona with the male permanent leader in the middle. Despite this configuration, ideated by Tacconi Lorenzo in 1987, the pollination efficiency is low and is necessary in collecting and distributing pollen to obtain fruits with good size.

Kiwifruit artificial pollination is nowadays a consolidate technique to increase kiwifruit quality and size [8, 24]. However, pollination not always reacts with maximum efficiency: the results could change in different years and depend on the pollen harvesting system, pollen storage technique, pollination system (dry or wet), added substance to dilute pollen (dry or liquid) or

to help the germination, pollination equipment, moment of pollination, floral stage of application, and economic impact of the operation (cost and gain). The analysis of these aspects could be applied to other crops and could be summarized in a flowchart where physiological aspects and human practices/decisions are integrated (**Figure 4**). Given the optimal pollen quality and optimal agronomical management (irrigation, fertilization, pruning), the results could vary in relation to the choice of the floral stage of intervention in relation to the type of pollination. In the reported studies, many parameters were analyzed alone and in interaction in different environments in Italy and for many years: pollen quality, pollination system, and flowering stage.

High-quality pollen is basic for good results: germinability, germination energy, and humidity were evaluated under different conditions of pollen harvesting, conservation at different temperatures and time of exposition at different temperatures, and manipulation before and during pollination in different pollination systems (dry and liquid).

The interaction of the pollination systems and the flowering stage were also evaluated.

Many aspects are in common with olive (*Olea europaea* L.) and can be applied to its pollination. Olive fruit set are very low, less than 2% of flowers, which in Northern Italy means about 10 kg of fruit per plant [25]. The main problems are self-incompatibility, scarce pollen from wild, wrong pollinator cultivars in the orchard, pollen quality and quantity, lack of coincidence of blooming period, and adverse climate conditions.

This observation leads to define kiwifruit pollination as a new model for crop fruit pollination that could be applied in other wind pollination (anemophily) trees such as olive tree, hazelnut, and pistachio that were studied but without a practical application (**Figure 4**) [6, 12, 13].

This chapter does not want be a review on biology of the pollination in kiwifruit and olive but an update of the research applied in the field supported by scientific data. Here, publicized works but also original researches data are reported.

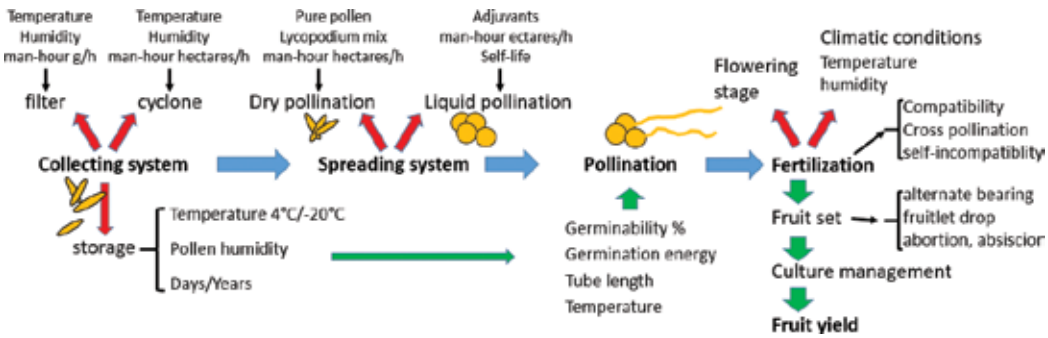

Figure 4. Flowchart of the main phases ranging from pollen collection to pollination and critical points analyzed in this chapter. Physiological aspects, climatic conditions, and human practices are integrated.

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2. Pollen quality for kiwifruit pollination

The first parameter that is considered to define the pollen quality is the germinability. Pollen could germinate but stop early in the tube growth: must it be considered right for pollination or not? Many grains germinate, but in different ways due to their different germination energies (germinability related to the time or germination tube length), and it is evident recording pollen germination under microscope (**Figure 5** and related movie). Other parameters must be considered in order to evaluate pollen quality, as humidity and germination energy [26].

These parameters were evaluated under different conditions of pollen harvesting, conservation at different temperatures and different times, exposition at different temperatures, and manipulation before and during pollination. For example, stresses against pollen during harvest and manipulation result in decrease of germination energy more than decrease of germinability.

2.1. Materials and methods

The experiment was performed on *Actinidia deliciosa* cv. Hayward (female) and cv. Tumuri (male) in the Tacconi Lorenzo's farm in Verona (North Italy), on a plantation built in 1982, (T-bar system, 4.5 × 3 m) having a permanent corded male suspended in the middle of the inter-row (**Figure 3**). The pollen samples were collected in two seasons (2008 and 2009) having opposite conditions of high relative humidity (RH) and low temperatures and low RH and high temperatures, respectively. Pollen samples were collected with two different systems (**Figure 6**): filter separator (Aspir@ Polline TR Biotac, Verona, Italy, www.biotac.it) and cyclone separator (AspiraPollineMini2 Biotac, Verona, Italy). The pollens were extracted from the machine and placed at 4°C every 45 min.

The germination temperature test was made with fresh Tumuri pollen (collected with Aspir@ Polline TR Biotac) on standard substrate (sucrose 85 g/l, boric acid 0.5 g/l) by taking a photo of the same field of view under microscope every minute for 14 h. The temperatures considered were 18, 24, and 30°C. To test the in vitro germination conditions, the germination of two

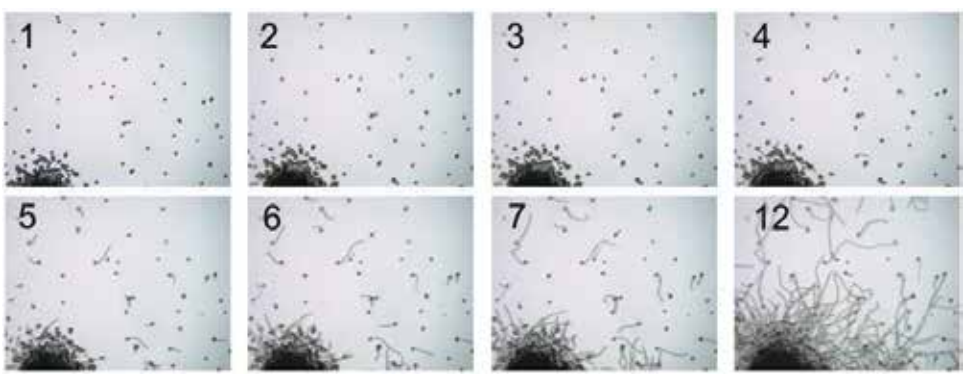

Figure 5. Time lapse during pollen germination at 20°C: the number indicates hours.

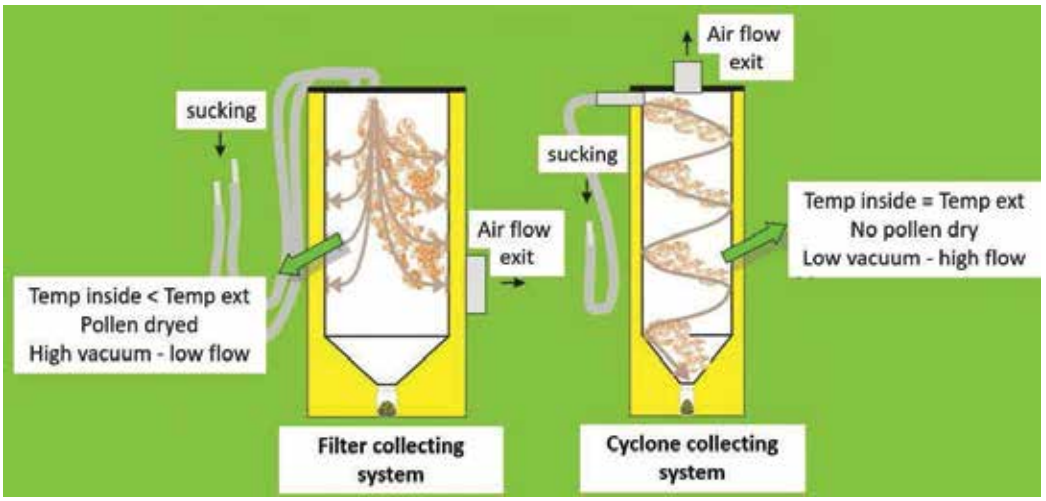

Figure 6. Schematic representation of the operating principle of the two pollen separation systems and related critical points.

pollen samples (collected with Aspir@Polline TR and AspiraPollineMini2, Biotac) on different agar growth media was compared (**Table 1**). The germination was observed at intervals of about 1 h for 15 h under the microscope (Olympus BX51 microscope at 200 magnifications with Olympus DP50 camera). The germination was made at a constant temperature of 20°C in a growth chamber (Sanyo Gallenkamp PLC, Loughborough, UK) with RH 100% and with cold light. The germination was calculated as percentage of germinated pollen counting about 100 pollen grains in three different optical field; tube length was evaluated using UTHSCSA ImageTool software and reported as fold grain diameter (D, about 30 micron).

2.2. Results and discussion

2.2.1. Germination: effect of temperature and growth media

Germination latency period and tube length are inversely proportional to the temperature of germination (**Figure 7**). During pollen application in field lower temperature is useful due the

N.	growth media	concentr.	filter system					cyclone system				
			germ. start	2 h		12 h		germ. start	2		12 h	
			minute	%	tube lenght	%	tube lenght	minute	%	tube lenght	%	tube lenght
1	water		>15 h	0	0	0	0	>15 h	0	0	0	0
2	sucrose + boric ac.	85+0.1 g/l	120	22	1.1	88	14	120	8	0.4	90	8.5
3	Biotac sol.	20 ml/l	60	60	1.4	90	8.5	60	20	1.1	92	9.5
4	Arabic gum	0.05 g/l	>15 h	0	0	0	0	>15 h	0	0	0	0
5	Biotac sol. + arabic gum	(as above)	>15 h	0	0	0	0	>15 h	0	0	0	0
6	PollenAid	20 ml/l	40	85	3.5	95	12	40	85	3	88	10
7	Biotac sol.	40 ml/l	60	48	2.4	94	12	60	58	2.2	88	10

Table 1. Percentage of germination and germination energy of pollen collected with different systems and germinated on various media.

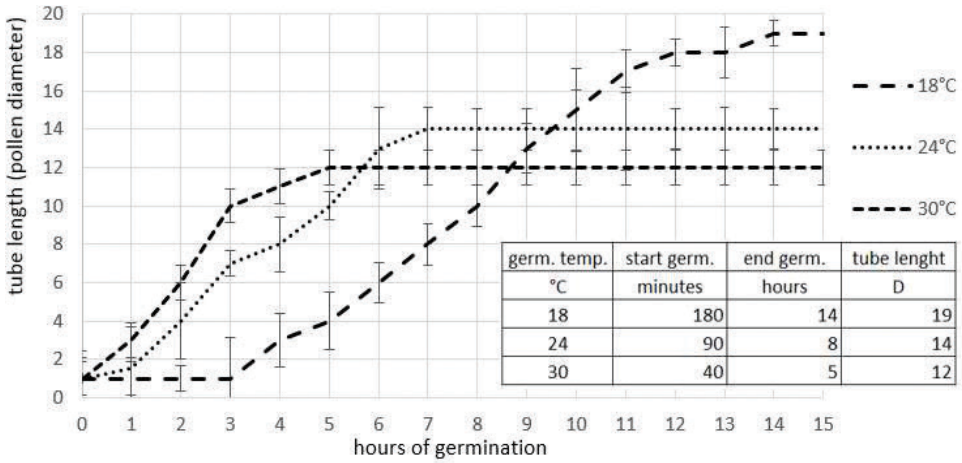

Figure 7. Dynamic of pollen germination at different temperatures and tube elongation.

observation that the pollen tube length is higher if the germination appends at about 18–24°C, whereas at higher temperature (30°C), the germination stops early and tube length is lower. Moreover, the suspension of pollen in water must be sprayed before the germination starts, in practical within 40 min, to avoid pollen damage.

The different media used in vitro can give useful indications for pollen suspensions in the case of liquid pollination and for analysis. The different media showed a different percentage of germination and germination energy (given by start time of germination and final lengths of pollen tubes; **Table 1**).

The analyses carried out show how the result evaluation of germination could vary according to the growth media used and depending on the moment in which the observation is made. Furthermore, a media that is too nutritious (i.e., n. 6 and 7) could overestimate the real germination that would occur in vivo in field condition, whereas a less stimulating substrate (i.e., n. 2) would be more useful as it highlights any weakness (less germination energy). PollenAid and Biotac solution could be useful in liquid pollination because they encourage germination [26].

2.2.2. Pollen harvest systems, pollen humidity, and pollen viability

Different pollination machines are available in the market, and these fall in two categories basing on the separation system: filter and cyclone (centrifugation). The comparison of these systems in two different climatic conditions during pollen collection, in particular relative air humidity (RH), reveals some differences in the pollen quality. Regarding cyclone system, pollen RH increases with air RH increasing (RH), whereas in the case of filter system, pollen RH is about 10% independently to the air RH (**Figure 8**). This difference is due to the lower pressure inside the filter system and, therefore, lower temperature in comparison to external one, such that water vapor in the air is condensed and extracted.

The humidity of the pollen is important for pollen long-term storage. One advantage of artificial pollination is the possibility to store pollen at −18°C for years maintaining its viability.

Year	2003		2004	
Collecting system	Filter	Cyclone	Filter	Cyclone
air T°C during collecting	23		29	
average air T°C	17.6		22.9	
average air RH%	85.6		63.9	
minimum air RH%	55.3		25.9	
T°C insiden filter	18	23	21	29
Pollen RH%	9.8	20.5	10.2	16.2

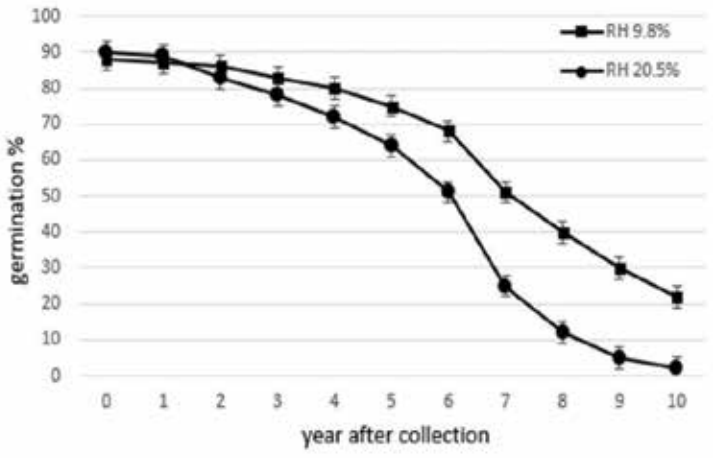

Figure 8. Pollen humidity related to collecting systems and environmental conditions (left) and effect of pollen humidity on germination during years of storage at −18°C (right).

For this purpose, pollen RH must be about 10–12%; in other ways its germinability decreases in direct proportionality with RH and years (**Figure 8**). For practical usage, it could be considered that pollen can be stored about 3 years if its humidity is low or after drying with silica gel.

2.3. Conclusion

The highest pollen quality was obtained when the pollen was picked up from the collecting machine frequently during the day (about every hour), to avoid any stresses, and stored at 4°C for no more than 7 days. Pollen can be stored at −18°C up to 3 years, better with low humidity or pre-dried to 10–12% with silica gel at 4°C. A recent method to estimate pollen viability was developed and is based on physical analysis of the single cells by impedance flow cytometry [27], and it could be interesting to compare the two methods especially during pollen storage.

3. The interaction between pollination systems and flowering stage in *Actinidia*

High-quality pollen is essential for good pollination, but pollination efficiency depends also on the equipments used and the time of application: dry pollination with pure pollen or diluted with lycopodium, liquid pollination in water suspension with adjuvants, handing application, or with mechanized tools [8]. In this paragraph, the interaction between pollination systems and the flowering stage will be elucidated, in order to understand which is the best flowering stage in relation to the pollination system adopted.

3.1. Materials and methods

All the experiments were performed on *Actinidia deliciosa* cv. Hayward in field condition with three repetitions per treatment, among 5 years (2009–2013) in three different environments: Cuneo (NO Italy), Verona (NE Italy), and Latina (Central Italy).

The comparing of pollination-systems (**Figures 9** and **10**) was conducted in collaboration with Agrion (Cuneo, www.agrion.it); the pollination was carried out with 90% of flowers at the stage of petal fall (with white pistils) with 600 g of pollen per hectare with a single-step distribution. The experimental design was a randomized block in standard orchards (female:male rate 1:6) with T-bar (Verona and Cuneo) and pergola (in Latina) trellis systems. The liquid distribution was 12 g/l of pollen in deionized water and 5 ml/l of activator PollenAid (Kiwi Pollen, New Zealand) for a total of 50 l/ha of water suspension. The machines used in the pollination system's comparative test were reported in **Figure 9**.

The role of *Lycopodium* was evaluated in 2013 in Verona by comparing two systems of dry pollination with and without *Lycopodium* added. *Lycopodium* was added to pollen in dry pollination as inert in some machines like Speedy. Experimental design consisted of three theses (two rows each): pollination with the Soffi@Polline system with pure pollen, pollination with SoffiaPolline with pollen:*Lycopodium* mixture (55%:45%), and pollination with Speedy with pollen:*Lycopodium* mixture (55%:45%).

To understand the relation between flowering stage and the type of pollination, dry or liquid, just before pollination the flowers were labeled according to their flowering stage (**Figure 11**). The signed stages were, according to BBCH scale [28] are the following: closed flower (55–59), white petals (60–64), ocher petals (65–66), early petal fall (67), and petal fall (68) with most of the pistils white and stigmas viscous, just before pistils dry and ovary increasing (69). To understand the success of the pollination, about 100 fruits for three biological replicates were weighed at harvesting time (end of October). This experiment was repeated for 4 years (2010–2013) in Verona, Cuneo, and Latina using Soffi@PollineZ for dry pollination and "ElettroEASY" (or similar diaphragm pump) for liquid pollination.

3.2. Result and discussion

3.2.1. Comparison of equipments for pollination

Usually, the best pollination method considered is the manual method of pon-pon but because of its considerable employment of labor is rarely used in commercial orchards. As shown in **Figure 10**, it was overcome by Soffi@PollineZ pollinator, probably because with pon-pon some flowers were not touched, whereas the pollen powder blown reaches all the canopy. Analogously, with Speedy some flowers were not pollinated and, in addition the role of *Lycopodium*, will be analyzed in another experiment. Good results were also obtained with liquid pollination applied 2 days before the other when the 90% of flowers at the stage of petal fall. In other terms, it seems that liquid pollination could be better before petal fall. The following experiments will elucidate this aspect [8].

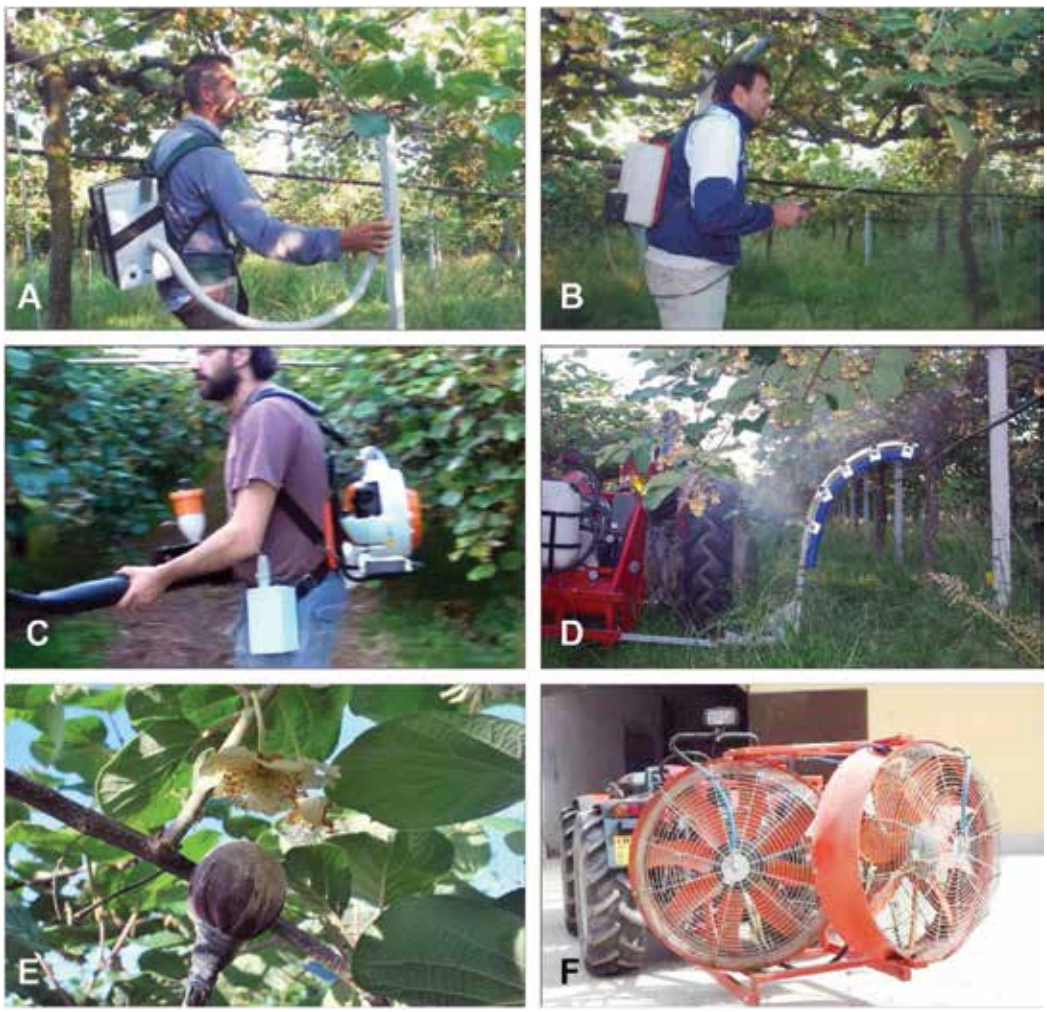

Figure 9. The commercial available equipments used in the pollination system's comparative test and their working capacity. (A) "speedy" (Dall'Agata, Forlì, Italy) is a battery dry distributor for pollen: *Lycopodium* (45–55%) mix (5–7 h/ha); (B) "ElettroEASY" (Volpi, Mantova, Italy) is a battery diaphragm pump for liquid pollination (4 h/ha); (C) "Soffi@ PollineZ" (Biotac, Verona, Italy) is an engine blower with dry distributor for pure pollen for dry pollination (1 h/ha); (D) "Spruzz@Polline TR" (Gerbaudo, Cuneo, Italy) is a sprayer with fogger-type nozzles attached to the tractor, for liquid pollination (2 h/ha); (E) "pon-pon" (homemade ball covered with velluto) for flower-to-flower manual dry pollination) (25 h/ha); and (F) "Ventole" (Romani, Verona, Italy) consists of two fans attached to the tractor for air and pollen shuffling (0.5 h/ha).

3.2.2. Role of Lycopodium in pollination

The low pollination rate observed using the pollen-*Lycopodium* mix Speedy machine (**Figure 9A**) is due to the drying effect of the *Lycopodium* on pistils and does not depend on the machine: the addition of *Lycopodium* to the Soffi@Polline gave the same results.

The fruit size obtained in the thesis pollinated with pollen-*Lycopodium* mixture was lower than the thesis pollinated with pure pollen: average weight 96 g with the addition of lycopodium,

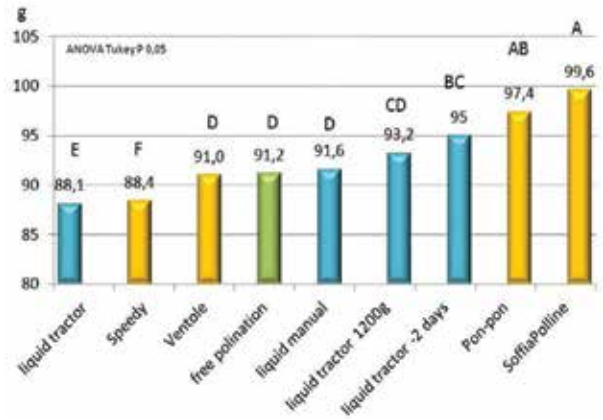

Figure 10. Average weight of the fruit pollinated with different equipments. Blue color is for liquid pollination systems: Spruzz@Polline TR 2x means double pollen dose, and Spruzz@Polline TR-2 means that the application was made 2 days before the other pollination. Yellow color is for dry pollination system: Green color is for natural pollination (control). Different letters indicate statistically significant differences (ANOVA Tukey P 0.05).

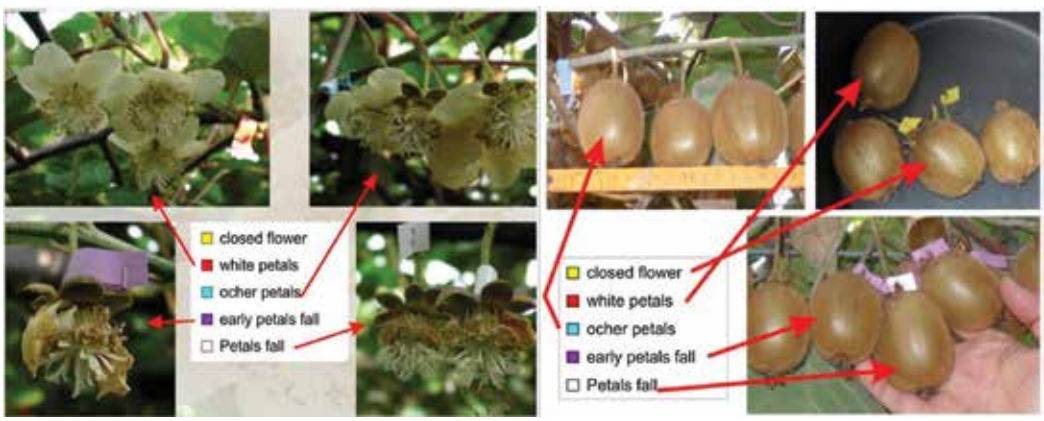

Figure 11. Fruits marked at the time of harvesting with the ribbon attached during pollination in order to go back the original flowering stage during pollination.

106 g with pure pollen, and 75 g free pollinated fruit (data not shown). That result indicates that the presence of this inert may adversely affect fertilization, regardless of the distribution system.

3.2.3. Flowering stage

After the first evidence where liquid pollination appears more efficient before petal fall, the interaction between flowering stage and pollination system was investigated. *Actinidia* flowering is scalar, and the same flower is viable for about 4 days, in normal climatic condition, after that the pistil degenerates and starts the fruit set (**Figure 11** and related movie). Regarding liquid pollination, the best results were at full bloom and at early petal fall (**Figures 11** and **12A**), whereas for dry pollination, the best results were reached at petal fall (**Figures 1** and **12B**) before pistil

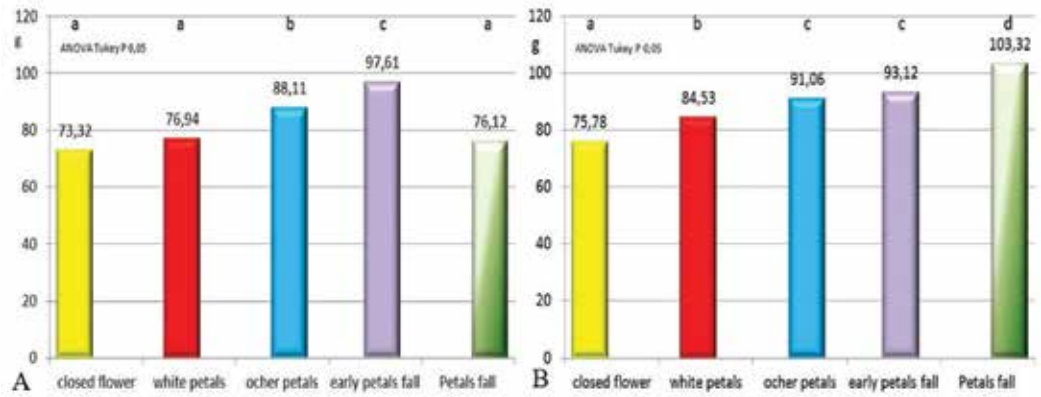

Figure 12. Average weights of the fruits pollinated by spray pollination system (A) and by dry pollination system (B). This experiment was repeated for 4 years in Verona, Cuneo, and Latina using Soffi@PollineZ for dry pollination and a sprayer diaphragm pump for liquid pollination. Different letters indicate statistically significant differences (ANOVA Tukey P 0.05).

senescence [8]. The pollination efficiency is evident at the harvest but could be useful approximately within 30 days after pollination (see related movie). In this period there are endosperm cellularizations that define the final fruit size and are important to proceed with the thinning of the bad pollinated fruit to avoid loose of energy and favorite the growing of the best pollinated fruit.

The flowering stage is easily described observing the petals, but it reflects more important aspect of the flower and in particular the pistil exudate, essential for pollen adhesion, germination, and the ovary receptivity. The pistil's exudate production increases during flower life and, in cv. Hayward, is maximum at the petal fall stage (**Figure 13**). For yellow flash kiwifruit, it is less evident, and the flower has a lower self-life compared with Hayward and evolves within 1–2 days to late flowering stages. In this case, it is not possible to wait that all flowers reach the petal fall stage

Figure 13. Easy test for the evaluation of pistil exudate production: the maximum dry pollen receptivity is at early morning with flowers at the petal fall stage when almost all ovules are receptive (left). Longitudinal section of the fruit showing lack of seeds on the tip due to not fertilized ovules because of an early pollination (right).

and the artificial pollination must be done every 1–2 days, depending on the climate conditions. It is notable also that, due to physical properties, the pistil's exudate increases the pollen attached if it is powder, whereas decreases pollen adhesion if it is conveyed with water. Moreover, in dry pollination the fruit size is higher with respect to liquid pollination (**Figure 12**). This observation indicates indeed the receptivity of the ovules in the flower that is maximum just before pistil senescence (change from white to brown color) after petal fall. Often, early pollination leads to ovary-growing and pistil senescence even if not all ovules were fertilized, thus precluding the possibility of a complete pollination of the fruit. This phenomenon is visible observing the longitudinal section of the fruit (**Figure 13**) because, excluding phenomena of water stress, the ovule's maturation is not simultaneous and starts from the petiole side to the tip side of the flower.

New histological analysis is under way in order to study the relationship between flower stage and ovary maturation. The process from pollen adhesion to fertilization could be observed in vivo by staining the pollen with aniline blue under UV light (**Figures 14** and **15**).

In *Actinidia*, fertilization appends within only 6 h after pollination (**Figure 15**), and this aspect facilitates the study of the relation between the moment of pollination and the flower stage. The *Actinidia* floral biology could be useful as model of wind-pollinated trees in field condition.

3.3. Conclusion

Kiwifruit artificial pollination, in conventional orchard, increases the production up to 30% (**Figures 10** and **12**) due to bigger fruit size. Pollen is collected from male plants, and to maintain its viability is necessary to avoid high temperature and high humidity. In practice it is picked up from the collecting machine every 45 min and stored at 4°C for ready usage (up to 7 days) or for long storage at −18°C (3 years if its humidity is lower than 12%) (**Figure 8**). Both liquid and dry pollinations are effective if done at the right flowering stage: liquid pollination not later than early petal fall stage, dry pollination, with pure pollen, at petal fall stage (in cv. Hayward) when the pistils exudate is maximum (**Figures 11**–**13**), at early morning with high air humidity. In order to pollinate early and late flowering flowers, the pollination must be done in two steps or more in particular in yellow flash kiwifruit. In any cases, dry pollination seems to be most suitable because it is applied when the number of mature ovaries in the flower is maximum.

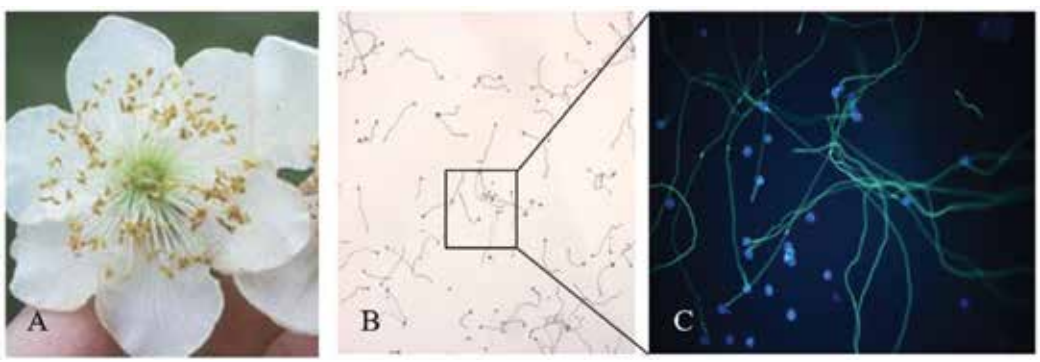

Figure 14. (A) Male flower, (B) pollen during germination under optical microscope (40×), and (C) magnification of germinated pollen stained with aniline blue under UV light (100×).

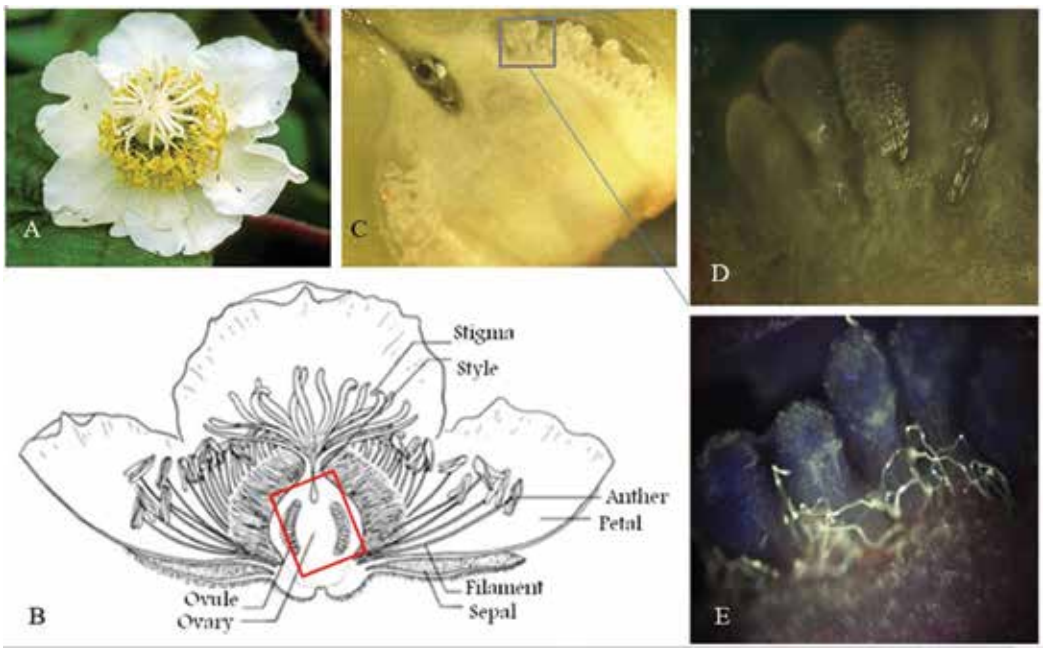

Figure 15. (A) Female flower, (B) schematic section and flower organs, (C) section of flower under optical microscope (40×), (D) magnification of ovules (100×), and (E) the same section stained with aniline blue under UV light 6 h after pollination.

4. Artificial pollination in olive tree

Olive trees (*Olea europaea* L.) bear both hermaphrodite and staminate flowers [29, 30] in the form of panicles [31]. Hermaphrodite flowers generally have two stamens and a bilocular ovary with a short style and stigma. Artificial pollination seems particularly suited also to olive tree because of a wind-pollinated crop, hermaphrodite but with many flowers specialized for pollen production, and in many cases self-incompatible [32]. In staminate flowers, the pistil is either rudimentary or absent. The flowers are not entomophilous pollinated, in fact they produce large quantity of pollen and don't have nectaries [33, 34]. The problems linked to pollination/fertilization olive cultivation are numerous: the blooming period of male and female trees does not overlap, and pistillate flowers are usually unable to receive pollen grain [35]; adverse climatic conditions during fruit set; compatibility relationships among cultivars; pollinizers could be bad oriented and/or in a non-satisfactory ratio with the cultivar of interest; and even if pollen is abundant, it could have low viability [35] and can be scarcely retained by the stigma surface. Moreover, depending on the cultivar, the environmental conditions, the specific tree and shoot, and ovary abortion could occur many weeks after pollination [36]. Shedding of staminate flowers begins just after full bloom [37] and partially overlaps the abscission of unfertilized flowers, triggered by pollination and fertilization of adjacent flowers. It takes place in the days after petal drop [38]. Most fertilized ovary abscission, occurring after 2 weeks and until about 6 weeks after full bloom, is affected by substrate

competition among growing fruits and other sinks [39]. After petal fall, about 25% of the ovaries are retained, but only a small percentage of fruits reach maturity. It was estimated that a good commercial yield could be reacted if at least 1% of the total number of flowers set fruits and remaining until harvest [31].

4.1. Materials and methods

Many steps of pollination were optimized, and many parameters were evaluated during the experimentations. Artificial pollination was tested by taking advantage of previous expertise developed in artificial pollination of *Actinidia*, using Aspir@PollineMini2 (**Figure 16**) to suck pollen and Soffi@PollineZ (**Figures 9C** and **16**) (Biotac, Verona, Italy). Pollen was stored at 4°C for short-term usage and at low temperature (−20 and −80°C) for long-term usage. The influence of the time of distribution was evaluated using a completely randomized block design, with four replicates on cv. Leccino. Pollen germinability was evaluated as described for *Actinidia*. The pollination experiment design will be aimed at understanding: the influence of the pollinizer on productivity of fruit, the influence of artificial pollination on alternate bearing, the optimization of distribution in relation to the flowering stage, and the influence of the amount

Figure 16. Mr. D'Isola during pollen sucking from olive tree varieties compatible with Leccino (left) and during pollination (right).

of pollen spread (standard application was 2 g per plant). To get a detailed experimentation, many parameters must be taken into account. During pollen collection the data recorded were air temperature and RH, amount of pollen per hour, cultivars collected, germinability, and pollen RH. Regarding pollination, the data were number of flowers per panicle, number of panicle per twig, position of the twig on the branch, position of the branch in the canopy, and cardinal orientation. In particular, two levels in the canopy and one shoot in each cardinal point per tree were considered. These parameters were recorded just before flowering and during all flowering time every day and once a week after the end of flowering for 6 weeks to take in account abortion and fruitlet abscission. The pollination was made one time in the early morning with moderate air temperature and higher RH, at the middle of flowering when 95% of flowers were open and many corollas were fallen down but with white pistils. After pollination other parameters were recorded: shoot length in order to evaluate the vegetative and reproductive competition and number of growing ovaries and fruits twice the time in the season. In order to evaluate the influence of the fruit, the number on the fruit quality, at harvesting was measured: harvesting time, total fruit collected per tree, fruit diameter, average weight, and oil yield. All these parameters were recorded also for some pollinizer plants in order to understand the influence of pollen collection in the hypothesis of a yield reduction due to pollen subtraction or not. Open-pollinated plants were used as control in all the experiments.

4.2. Results and discussion

Many data are available in literature about olive flower and fruit set biology; anyway, most of the knowledge are not adopted in the field. The experimentations started on 2014 thanks to the resourcefulness of an olive grower, Gianfranco D'Isola, on Lake of Garda (Brescia, North Italy). His 23 plants had never yielded anything being all Leccino, a self-incompatible cultivar without other pollinators nearby: the compatible cv. Frantoio was dead because of frost winter. The idea of artificial pollination came from kiwifruit pollination technique. The principle is simple: taking pollen from compatible varieties and, at the time of flowering, "blow" it on the target plants (**Figure 16**). Also, a modest improvement in the percentage of fruit setting leads to significant production increase [25]. Using the equipment employed in kiwifruit, the results were surprising: an average yield of 48 kg of olives per plant (**Figure 17**), an exceptional value compared to 10 kg, and the average production of North Italy [25, 40].

Pollen was collected from olive cv. Pendolino, Moraiolo, and Casaliva and stored at 4°C for a few days until the time of pollination. Pollination was carried out with pure pollen in the early morning by delivering a total of about 2 g of pollen per plant in two steps within 2 days.

The experiment was successfully repeated in 2016 (**Figure 17**) [25], whereas in 2017 the extremely high temperature during olive flowering (the subsequently in weeks) and the absence of rain until the end of the season have provoked a copious fruit abscission.

These experiments have led to a larger trials on Lake of Garda cultivation conducted by AiPol (www.aipol.bs.it) in collaboration with CREA and new trials in other regions [40] and in other countries like in Japan by Associazione Italia Giappone and Biotac (www.biotac.it). Because these experiments aim to a practical application for farmers, the field trials were done in conventional orchards with pollinizers present in order to evaluate the effective gains given by the pollination technique in the real situations.

Figure 17. Result of olive tree pollination on self-sterile cv. Leccino in 2014 (left) and 2016 (right).

Despite the pollen collected during the day when air RH is low, olive pollen has a higher RH than kiwifruit pollen, and the incubation at 4°C for 24 h with silica gel before storing or spreading is suitable. Pollen germinability ranges from 35 to 68% and RH from 15 to 26% just after collection. After dehydration for 12 h at 4°C with silica gel, the RH decreases to 12–15%. During storing, the germinability decreases about 1.8% per day at 4°C and 3.4% per year during 3 years of observation when stored at −18°C. The collected pollen quantity seems not to be a problem thanks to the abundant production of olive tree in the "charging" season [35, 41] which are not economically sustainable during "off" season [40]. Anyway, pollen can be easily stored for several years in domestic fridge with a very low viability decrement. During harvesting period it was noted that the pollen collected at the beginning of the flowering is very low (few grams per hour), while after full blooming, when 20–30% of the corollas drop down, the amount reaches 100 g/h in many cultivars (also in "olivastro") and also 200 g/h in Ascolana.

In these experiments, the fruit set improvement ranges from 10 to 30% more than the control free pollinated. Moreover, the pollinizer plant where pollen was collected, often self-compatible, showed a fruit set about 10% higher than the control (**Table 2**). It has been hypothesized that the movement of the braches and the panicles made during pollen suction increases pollen dispersion inside the tree.

	buds with flower	buds with shoot	buds not developed	Total	average shoot lenght	fruit set	average flower per panicle	fruit harvested	average fruit wheigth	average fruit diamete	average plant yield
	n°	n°	n°	n°	cm	%	n°	kg	g	mm	kg
pollinated	775	261	538	1574	10.45 a	15.05 a	11.3 a	798	5.9 a	22.1 a	39.9 a
pollinizer	533	373	481	1387	12.24 b	11.76 b	11.1 a	597	5.6 b	20.3 b	29.9 b
control	694	265	527	1486	12.78 b	8.68 c	11.2 a	184	5.6 b	20.7 b	9.2 c

Table 2. Example of data collected by AiPol on 2016 in sale Marasino (Brescia, IT) in a field trial of 20 plants of cv. Leccino with five cv. Casaliva as pollinizer. Different letters indicate statistically significant differences (ANOVA Tukey P 0.05).

4.3. Conclusions

Regarding the practical application, these preliminary results confirm the possibility to improve olive yield by pollination and aim to understand the better moment for pollination in relation to the flowering stage during flowering. The pollination technique can also be used to balance the vegetative-reproductive balance of the plant and mitigate the alternation of production. Of course, the pollination must be inserted in a context of adequate agronomic management in order to support the greater production. In this sense, plants with a great fruit set must be "prepared" to support a larger production: fertilization and irrigation must adequately satisfy the major removal of the plant, pruning must balance vegetative and reproductive aspects and must be done every year, and, during the summer, plant protection must be applied in order not to vain all the improvements. Using the methods described (**Figure 4**), it is possible to set up experiments that allow to investigate the pollination process in more detail in order to understand, also at the molecular level (**Figures 14** and **15**), the fertilization, the fruitlet drop, and the alternate bearing. These experiments will help also to elucidate the real cultivar cross compatibility: conflicting reports about the classification of pollen compatibility exist. Often, these classifications came from field observation with contradictory results obtained in different locations and years [33, 34, 36, 42], and one of the main constraints is the overlapping of flowering period. The possibility to collect and store pollen for many years allows to test the compatibility of varieties with very different flowering times: it is possible also pollinate an early flowering variety with the pollen taken from a late flowering ones. Moreover, it is possible to establish a pollen bank for the farmers and for research purposes and identify some varieties with the higher compatibility producing the higher amount of pollen and perhaps a universal pollinizer. It could be interesting to develop also a super-intensive orchard where mechanized pollen collecting will be possible, analogously to the mechanical fruit harvesting, whereas the pollen spreading could be easily mechanized with blower for the tractor (already used in kiwifruit as reported in www.biotac.it) or with drones. Unlike happen in kiwifruit, in olive tree the pollination technique is at the begin but with very interesting perspectives.

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Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 6

Pollen Germination *in vitro*

Jayaprakash P

Additional information is available at the end of the chapter

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Abstract

Pollen germination *in vitro* is a reliable method to test the pollen viability. It also addresses many basic questions in sexual reproduction and particularly useful in wide hybridization. Many pollen germination medium ranging from simple sugars to complex one having vitamins, growth regulators, etc. in addition to various minerals have been standardized to germinate pollen artificially. The different media, successful pollen germination methods, procedures from pollen germination studies with wheat, rye, brinjal, pigeonpea and its wild relatives are discussed.

Keywords: *in vitro* pollen germination, grasses, PGM, pollen germination method

1. Introduction

Pollen germination in the stigmatic tissue of *Portulaca* was first observed as early as 1824 by Amici and later he observed the germinating pollen tube entering ovule. Pollen acts as a vehicle for the transfer of male gametes to embryo of female plant. Pollen viability is an important factor in successful hybridization which may last from few minutes mostly in self-pollinated crops to many hours or days in cross pollinated crops. Assessing pollen viability is very crucial in artificial pollination especially involving different species or genera. Among diverse techniques used to assess pollen viability, *in vitro* pollen germination is the most reliable method. Pollen has been germinated in variety of media which differs from species to species and even for different varieties of a crop [1, 2]. Linskens [3] used simple sucrose/boric acid media and later many complex medium have been reported with addition of polyethylene glycol, various amino acids, etc. [4–6]. Among the many PGMs the one developed by Brewbaker and Kwack [7] has been widely used with some alterations.

A brief discussion was made in this chapter to develop a pollen germination protocol for a crop/species.

2. Different pollen germination media and methods

2.1. Pollen germination medium

The composition of some of the successful media used pollen germination are given below

Brewbaker and Kwack medium [7]

- 10% sucrose
- 100 mg l^{-1} boric acid
- 300 mg l^{-1} calcium nitrate
- 200 mg l^{-1} magnesium sulfate
- 100 mg l^{-1} potassium nitrate

Roberts medium [8]

- 20% sucrose
- 10 mg l^{-1} boric acid
- 362 mg l^{-1} calcium chloride
- 100 mg l^{-1} potassium nitrate
- Tris 60–130 mg l^{-1}

PEG medium [4]

- 0.1 to 1.1 M PEG 400,
- 100 mg l^{-1} Boric acid

EACA medium [1]

- 37.5% sucrose
- 15% PEG 4000
- 250 mg l^{-1} boric acid
- 300 mg l^{-1} calcium nitrate
- 100 mg l^{-1} potassium nitrate
- 200 mg l^{-1} magnesium sulfate
- E amino caproic acid (EACA)-0, 100, 250, 500, 750 or 1000 mg l^{-1}
- 1% agar

Raffinose medium [9].

- 0.75 M raffinose,

- 100 mg l^{-1} boric acid,
- 300 mg l^{-1} calcium chloride

Peptone PGM of Wheat [2] (**Figure 1**)

- 19% Maltose
- 13% PEG 6000
- 50 mg l^{-1} boric acid
- 30 mg l^{-1} calcium nitrate
- 80–100 mg l^{-1} peptone
- BK salts
- 1% agar

Tryptone PGM of rye [10]

- 19% Maltose
- 13% PEG 6000
- 50 mg l^{-1} boric acid
- 30 mg l^{-1} calcium nitrate
- BK salts
- 1% agar

Agarose medium [11]:

- 0.5% agarose
- 18% sucrose
- 0.01% boric acid
- 1 mM $CaCl_2$
- 1 mM $Ca(NO_3)_2$
- 1 mM KCl
- 0.03% casein enzymatic hydrolysate
- 0.01% myoinositol
- 0.01% ferric ammonium citrate
- 0.25 mM spermidine

A basic medium contains a sugar, calcium nitrate and boric acid to which poly ethylene glycol, vitamins, amino acids, growth regulators etc. are added to make a complete pollen germination medium. The pH and temperatures are also important factors.

2.2. Pollen germination methods

Pollen is collected from freshly opened flowers and enough pollen is dusted over medium and culture by any one of the following methods:

- **Cavity slide technique (Rangaswamy and Shivanna** [12, 13]**)** : It is used for liquid pollen germination medium(PGM). A drop of medium is placed in the cavity, pollen is dusted over and it is covered with a dust free cover slip with its periphery sealed with Vaseline. It creates a required relative humidity inside. The slide is placed inversely over a pair of glass

rods in a humid chamber. A Petri dish is used as humid chamber where a moist filter paper is placed inside the lid.

- **Agarified medium in Petri dishes** [14]**:** Pollen was extracted from fresh buds (with bud break) and was spread evenly on a drop of medium in a Petri dish and covered with Petri plate lined with moist filter paper. The plats were incubated in a BOD incubator with a temperature of 18°C/20.5°C depending on the pollen sample
- **PGM droplet technique** [15]**:** In a Petri dish a droplet of pollen germination medium was placed using a glass rod. Thus drops of different media could be placed within few centimeter distance between them For example nine media can be placed in 3 × 3 fashion (**Figure 2**).

1-Medium A	2-Medium B	3-Medium C
4-Medium D	5-Medium E	6-Medium F
7-Medium G	8-Medium H	9-Medium I

Further in order to have a better visualization, a drop of stain was placed carefully over the medium droplet before observation. The extra stain was removed with a piece of dried filter paper. (Acetocarmine, and components of Alexander stain *viz*., Malachite green, Orange G, Aniline Blue and acid fuchsine may be used).

- **The cellulose membrane/agarose culture apparatus** [11]: Rectangular agarose pad was prepared over a microscopic slide with PGM containing 0.5% agarose. Appropriate size of cellulose membrane was cut and layered over agarose pad. The pollen is placed over the membrane and cultured in a moist chamber.

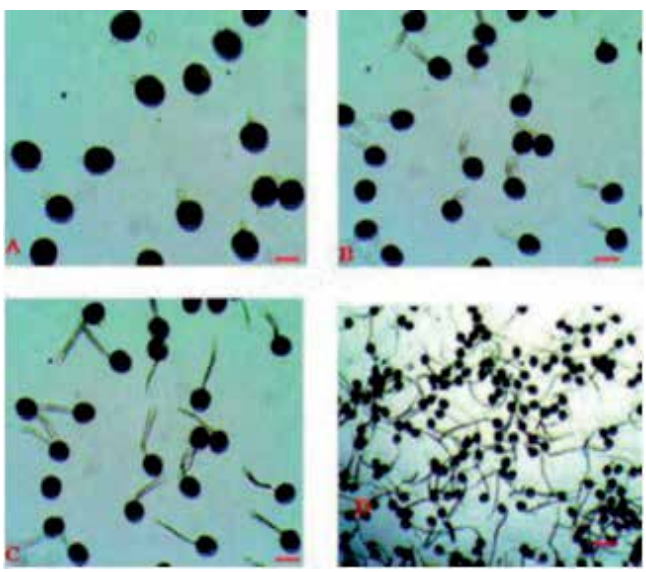

Figure 1. Wheat *in vitro* pollen germination (a) initiation of pollen germination (b, c, d) pollen germination after 5, 10, and 15 min of incubation.

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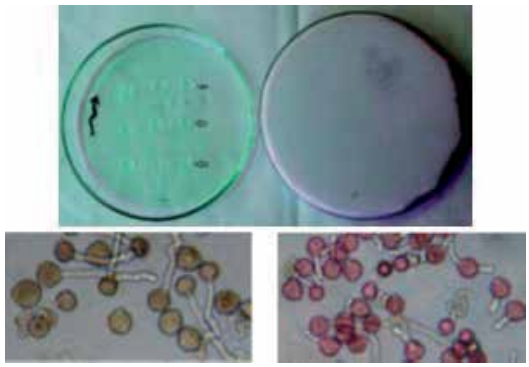

Figure 2. PGM droplet technique. (a) Incubation chamber showing 3 × 3 combination of media; (b) unstained pollen tubes; (c) pollen tube with acid fuchsine.

In our experiments initially liquid medium was used but profuse pollen bursting was seen due to quick hydration. To overcome this problem later agarified media were used by adding 0.5 to 1% agar to pollen germination medium. PGM droplet technique is a further refined technique which may be used effectively to fix the initial levels of inorganic salts level for factorial experiments. Each season, at least 200–300 fresh media were prepared and tested. This allowed large number of media to be tested in a single day, thus avoiding preparation of fresh medium each time and furthermore, more than two genotypes or species could be tested simultaneously.

3. Effect of different components of pollen germination medium (PGM) on pollen germination

Sugars: The sugar in the medium acts as an osmotic regulant which regulates the diffusion rate of water from the medium into pollen grains [16] and sucrose is generally used in PGM. The failure of pollen to germinate and its bursting may indicate lack of sugars within pollen grains and critical dependence on external supply. Sucrose has given satisfactory pollen germination for pigeonpea, wild pigeonpeas and wild brinjals whereas it was a poor osmatic regulant for pollen of wheat, rye and brinjal. Maltose was used as carbon source for these species.

Boron and calcium nitrate: The mineral requirements are also different for any two species. Pollen grains are believed to be deficient in boron, which is normally compensated by high levels of boron present in stigma and style. Boron combines with sugar to form a sugar-borate complex which facilitates translocation of sugar molecules. Boron is reported to be toxic to plants even at as low as 5.1 ppm [17]. However, pollen seems to tolerate very high concentration of boron. Visser [18] showed that certain species of crop plants require as much as 1200 ppm boric acid for optimal germination and tube growth. Boron deficiency leads to pollen tube bursting as its required in the pollen wall structure [19]. Calcium is involved in cationic balance and is essential for tube elongation [7]. Pollen germination involves many ions with Ca^{2+} as the key player and extracellular calcium proved essential for pollen tip growth [20]. It was observed that the grass pollen (wheat and rye) requires very low levels of these minerals (30–50 mg l^{-1}) as compared to the dicots (pigeonpea and brinjal) (300–400 mg l^{-1}).

Polyethylene glycol (PEG): It is known to be a non-penetrating osmotic agent that decreases water potential of culture medium [21]. In pollen grains, PEG is considered to regulate the permeability of plasma membrane and to give stability to the pollen tube membrane [6] and to give stability to the pollen tube membrane. PEG of different molecular weight has been in pollen cultures of different species and in all cases it has a promontory effect [4, 6, 22]. Generally 10–15% of PEG was added to PGM. PEG 4000 was preferred for pigeonpea and brinjal whereas wheat and rye pollen germination satisfactorily with addition of PEG 6000.

Peptone: Inclusion of peptone arrested pollen tip bursting in wheat at concentration 80–120 mg l^{-1}which was used in PGM for the first time [2]. Peptone has been used in plant tissue culture for various effects such as induction of shoot regeneration in avocado [23], promotion of hairy root formation in ginseng [24] etc. Addition of peptone, contained pollen tube burst to a great extent, it was less than 20% as compared to >85% in the medium which is devoid of peptone however, the level of peptone varied with genotypes.

ε-Amino caproic acid (EACA): It is an amino acid derivative, a saturated six carbon fatty acid ($C_6H_{12}O_2$, $CH_3(CH_2)_4$-COOH) which occurs in milk fats. This immunosuppressor has been used to overcome self-incompatibility and incongruity in *Phaseolus* [25] and *Vigna* [26, 27]. It has been used as a component of PGM of pigeonpea for the first time [1] and subsequently in the medium of wheat and rye [2, 10].

Tryptone: It was good growth-stimulating nitrogen sources used in the cultivation of *Trichoderma hamatum* and *T. harzianum* [28]. It was found to be the best organic nitrogen source for kefiran production by *Lactobacillus kefiranofaciens* (Dainiel et al., 2015), asparaginase production from *Enterobacter cloacae* [29], production of biosurfactant by *Bacillus subtilis* [30]. In rye pollen germination, tryptone at 50–75 mg concentration gave satisfactory and reproducible level of pollen germination [2].

Besides many organic supplements were added to the PGM to enhance smooth pollen tube growth such as casein hydrolysate, vitamins etc.

4. A protocol to standardize *in vitro* pollen germination medium

To standardize pollen germination medium for any crop initially a set of media are used. Based on the response, one of the media is picked up and modified. Here a procedure to develop a PGM for pigeonpea and its wild species is discussed. Initially two sets of key media(PGM) were used Set I consisted of Brewbaker and Kwack (BK) medium [7] with 1% agar at different levels of sucrose *viz*., 10, 20, 30 and 40% (A–D). With the addition of polyethylene glycol (PEG) 4000 at 15% concentration to each of A, B, C and D; the media E, F, G and H, respectively, were obtained. These were designated as Set II.

Composition of Brewbaker and Kwack's (BK) medium

Sucrose	10%
Boric acid	100 mg l^{-1}
Calcium nitrate	300 mg l^{-1}
Potassium nitrate	100 mg l^{-1}
Magnesium sulfate	200 mg l^{-1}

Key media

Set I	**Set II**
A. 10% sucrose + BK salts + 1% agar	E. Medium A + 15% PEG 4000
B. 20% sucrose + BK salts + 1% agar	F. Medium B + 15% PEG 4000
C. 30% sucrose + BK salts + 1% agar	G. Medium B + 15% PEG 4000
D. 40% sucrose + BK salts + 1% agar	H. Medium B + 15% PEG 4000

Based on the germination/initiation of germination and bursting of pollen, one of the key media was first selected (**Figure 3**). The complete medium for each species was then standardized by altering the concentration of sucrose, boric acid and/or calcium nitrate one by one to obtain maximum pollen germination and good pollen tube growth. First sucrose concentration and temperature were varied to prevent bursting of pollen before germination. Secondly, varying concentrations of boric acid was tried *viz*., 50, 100, … 300 ppm keeping 300 ppm of calcium nitrate (as in BK medium) and other components unchanged. Lastly, the optimum concentration of calcium nitrate was determined (100, 200, … 600 ppm). The observations were recoded for three best concentrations of each boric acid and calcium nitrate.

4.1. Standardization of *in vitro* pollen germination medium for *C. platycarpus*

In the medium (B) containing 20% sucrose, 1% agar and standard Brewbaker and Kwack medium's salts, pollen of *C. platycarpus* showed 99.15% germination and tube length over 172 μm in 1 h duration at 20.5°C. Lowering the concentration of sucrose to 10% (medium A) and leaving other constituents unchanged gave 61.60% germination with a mean tube length of over 83.88 μm. Though the medium (C) containing 30% sucrose gave 94.25% germination, the tube length was reduced considerably (33 μm), also pollen tube burst at the tips.

The best medium which gave 99.15% germination for *in vitro* pollen germination and pollen tube growth of *C. platycarpus* was

Sucrose	20%
Boric acid	100 mg l^{-1}
Calcium nitrate	300 mg l^{-1}
Magnesium sulfate	200 mg l^{-1}
Potassium nitrate	100 mg l^{-1}
Agar	1%
Temperature	20.5 ± 2°C

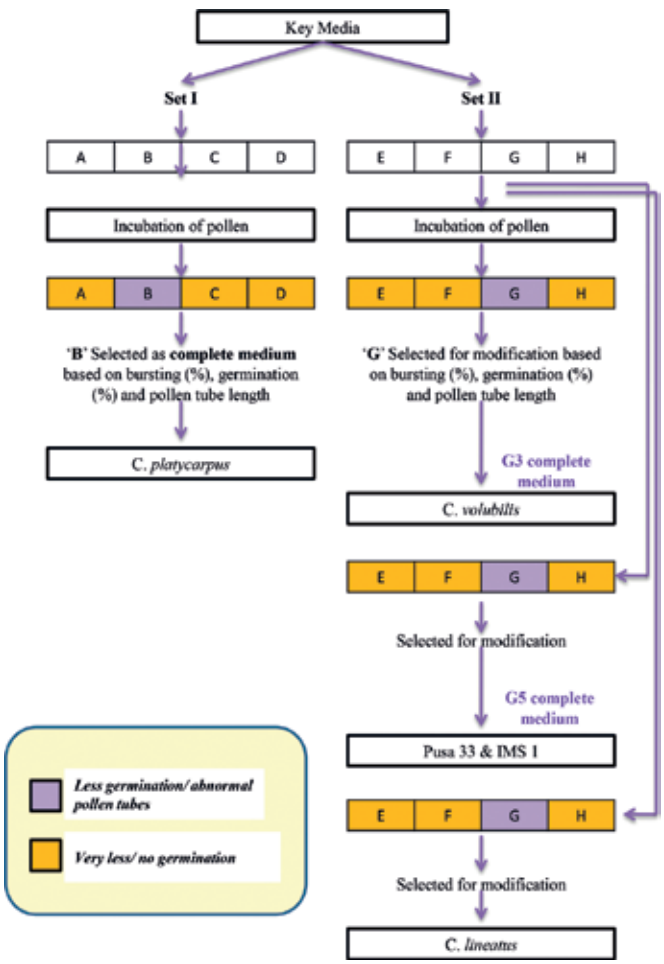

Figure 3. Procedure for standardization of *in vitro* pollen germination medium of *Cajanus* spp.

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4.2. Standardization of *in vitro* pollen germination medium for *C. volubilis*

On key media G (from Set II) the pollen of *C. volubilis* showed initiation of germination (budding) but almost all pollen grain showed bursting before or after budding. To contain bursting of pollen, sucrose level was increased to 35% and also the temperature was reduced to 18°C.

In order to improve germination, first boric acid concentration was altered keeping $Ca(NO_3)_2$ at 300 ppm. At 250 ppm, maximum pollen germination was obtained (approximately 63%) and increasing or decreasing boric acid concentration over 250 ppm did not improve germination.

Then, the concentration of calcium nitrate was modified keeping boric acid concentration at 250 ppm to further improve the germination of *C. volubilis* pollen. At 100 ppm $Ca(NO_3)_2$, pollen of *C. volubilis* showed 94.6% germination and pollen tube growth of 27.47 μm. *C. volubilis* showed 94.26% germination and pollen tube growth of 27.47 μm. Increasing the $Ca(NO_3)_2$ concentration to 200 ppm showed. The complete pollen germination medium for *C. volubilis* has the following constituents:

Sucrose	35%
Calcium nitrate	100 mg l^{-1}
Potassium nitrate	100 mg l^{-1}
Magnesium sulfate	200 mg l^{-1}
Polyethylene glycol	15%
Agar	1%
Temperature	18 ± 1°C

This gives 94.26% pollen germination.

4.3. Standardization of *in vitro* pollen germination media for *C. cajan* vars. Pusa 33 and IMS-1

In the medium D (Set I) only initiation of germination was observed whereas no germination was observed in media A, B and C. On varying the concentrations of boric acid and calcium nitrate, it was observed that the medium with 40% sucrose, 250 ppm H_3BO_3, 300 ppm $Ca(NO_3)_2$, 1% agar and BK salts gave 43.80% germination with abnormal pollen tubes.

From Set II, medium G showed over 45% germination (**Figure 2**) but there was a lot of bursting of pollen grains; medium F also showed little germination. To control the bursting of pollen grains sucrose level was raised to 37.5% and further, different combinations of boric acid and calcium nitrate were tried. Even though over 87% germination was seen, almost all the pollen grains showed bursting after germination. Among the permutations

and combinations of media tried by changing the concentration of boric acid and calcium nitrate, six combinations showed better germination (70–80%) but accompanied with bursting of pollen tubes.

4.3.1. Pretreatments

In order to standardize optimal media for pollen germination different methods of pre-conditioning the pollen were attempted. Heat treatment of pollen at 40°C for 30 min or at 60°C for 20 min did not improve germination of *C. cajan* pollen. Pre-hydration for 30 min in Petri dish lined with moist filter paper also did not improve germination. The stamen of pigeonpea is diadelphous (five short and five long stamens). Pollen extracted separately from short and long stamens showed approximately 70% germination. Pollen extracted from single anthers also showed about 70% germination. Thus, germination percentage did not vary between different anthers. Out of six combinations of media tried, the PGM with 250 ppm boric acid and 300 ppm calcium nitrate showed maximum normal pollen tubes.

Another pre-treatment of pollen was tried by incubating young buds (12 h or 36 h before anthesis) at 20.5°C in

(a) Petri dish lined with moist filter paper for 36 h.

(b) Agarified medium containing 37.5% sucrose in (a) for 36 h.

(c) Agarified medium containing 37.5% sucrose +15% PEG in (a) for 36 h

(d) Pollen germination media for 12 h

(e) Pollen germination media for 36 h.

Then upon anther dehiscence, pollen was collected and germination was tested on six different media. The pollen from treatments (a), (b) and (c) showed good germination on medium G_5 but burst soon after.

For treatments (d) and (e) media G_1–G_6 were used both for pre-treatments and germination tests. The pollen from the treatment (d) showed much variation for germination and bursting. Pollen extracted from the treatment (e) showed improved germination for *C. cajan* var. Pusa 33 and IMS-1. Among the media tested (G_1 to G_6) the medium (G_5) with 250 ppm H_3BO_3 and 300 ppm $Ca(NO_3)_2$ showed over 92% germination for both varieties. Besides media G_3, G_4 and G_6 also showed over 90% germination but there was pollen tube bursting. The result indicated pre-treatment of young buds (36 h before anthesis) in PGM (G_5) for 36 h and germinating pollen in the same PGM gave over 92% germination $Ca(NO_3)_2$ some other media (G_3, G_4 and G_6) also showed over 90% pollen germination but there was bursting of pollen tubes at tips and variation in pollen tube length.

Thus, the complete media for both Pusa-33 and IMS-1 should have the following composition:

Sucrose	37.5%
Boric acid	250 mg l^{-1}
Calcium nitrate	300 mg l^{-1}
Potassium nitrate	100 mg l^{-1}
Magnesium sulfate	300 mg l^{-1}
PEG 4000	15%
Agar	1%
Temperature	20.5 ± 20°C

To the above medium EACA at a concentration of 250–750 mg l^{-1} was added to arrest pollen tip bursting.

The results of studies on *in vitro* pollen germination of *Cajanus cajan* and its wild species are summarized in **Table 1**.

Based on the initial tests on *C. lineatus* it was found that modification of medium "G" could give optimum germination but due to non-availability of pollen, PGM could not be optimized.

Optimal media composition	***C. platycarpus***	***C. volubilis***	***C. cajan* vars. Pusa-33 and IMS-1**
Sucrose (%)	20	35	27.5
Boric acid (ppm)	100	250	250
Calcium nitrate (ppm)	300	100	300
Potassium nitrate	100	100	100
Magnesium sulfate	200	200	200
PEG 4000 (%)	—	15	15
Agar (%)	1	1	1
Incubation period (h)	1	3	3
Incubation temperature (°C)	20.5	18	20.5
% Pollen germination	99.15	94.26	92.52
Pollen tube length (μm)	172.56 ± 0.315	27.47 ± 0.933	16.34 ± 0.264

Table 1. Results of ***in vitro*** pollen germination of ***C. cajan*** and its two wild relatives.

4.4. Varietal response to PGMs

It was noticed that there are genotypic differences for pollen germination. In pigeonpea, among the genotypes tested, IDTSP51 alone did not require EACA, but for others the requirement varied(250–750 mg l^{-1}). Similar response was seen with wheat pollen germination. The medium supporting more than 90% pollen germination were selected for each genotype though some of them showed pollen tube bursting which indicated the need for fine tuning of the medium. The genotypes HW 971 and HW 741 responded well in M19 giving >95% pollen germination and pollen tube length of >400 μm. This is adjudged as the best medium since it did not require supplementing either EACA or peptone. It is noted that some genotypes (HD 2833, HW 971, HW 1095 etc.) require EACA alone in PGM whose response were at par with medium having EACA + peptone. Some varieties such as MACS 6195, HW 1095 etc. showed better responses (>92% germination) in PGM with a combination of EACA (500 or 750 mg) and peptone water (100 mg). These varieties responded poorly when peptone water was increased beyond 100 mg which reduced the pollen tube length (**Table 2**).

Variety	Pollen germination medium	Pollen germination (%)	Pollen tube length (μm) (mean ± S.E)	Range for pollen tube length (μm)
HD2833	*M19 + 500 E	97.9	418.8 ± 15.39	
	M19 + 750E	94.32 ± 3.67	667.7142 ± 32.18	
	M19 + 500 E + 100 P	97.51 ± 2.98	553.75 ± 23.14	334.37–713.00
	M19 + 750 E + 100 P	95.38 ± 3.09	334.37 ± 18.39	
	M19 + 500 E + 120 P	97.12 ± 1.87	680.00 ± 24.255	
	M19 + 750 E + 120 P	97.22 ± 1.74	713.0 ± 21.55	
HW2044	M19 + 500 E	98.02 ± 1.03	265.60 ± 9.88	265.60–467.50
	M19 + 750E	98.01 ± 0.076	467.5 ± 9.55	
HW5207	M19 + 750E	98.0 ± 0.043	313.529 ± 12.45	246–350.62
	M19 + 500 E + 120 P	98 ± 0.054	350.625 ± 14.91	
MACS 6145	M19	98.11 ± 0.87	360.58 ± 12.05	360.58–603.13
	M19 + 750 E + 100 P	97.21 ± 1.54	603.13 ± 22.48	
	M19 + 750 E + 120 P	97 2.63 ± 2.11	546.25 ± 26.89	
HW 971	M19 + 500 E	98 .13 ± 1.42	462.50 ± 18.76	462.50–551.25
	M19 + 500 E + 100 P	98.0 ± 0.6	551.25 ± 25.85	
HW 741	M19	98.23 ± 0.042	403.75 ± 21.13	241.87–403.75
	M19 + 500 E	97.23 ± 1.99	241.87 ± 8.55	
HW1085	M19 + 500 E	98.18 ± 1.11	244.375 ± 11.44	
HW1095	M19 + 500 E	98.01 ± 0.43	268.75 ± 11.83	190–371.87

Variety	Pollen germination medium	Pollen germination (%)	Pollen tube length (μm) (mean ± S.E)	Range for pollen tube length (μm)
	M19 + 750 E + 100 P	92.91 ± 4.53	371.87 ± 16.36	
	M19 + 500E + 120 P	90.82 ± 4.76	278.125 ± 18.13	
	M19 + 750 E + 120 P	96.37 ± 3.75	190 ± 3.65	

P = peptone; E = EACA.*M19 = 19% Maltose + 13% PEG 6000 + 50 mg l^{-1} boric acid + 30 mg l^{-1} calcium nitrate + BK salts + 1% agar.

Table 2. Response of wheat genotypes in pollen germination medium.

Among the varieties tested, two of them *viz*., HD 2833 and HW 1095 showed more that 94% in all the media tested. The genotype HD 2833 showed the maximum pollen tube length of 713 μm in PGM with 750 mg l^{-1} EACA and 120 mg l^{-1} peptone and the pollen of HW1095 achieved a mean pollen tube length of 190 μm in the same medium The variety HD 2833 showed a mean pollen tube length in the range of 334.37 to 713 μm followed by MACS6145 with 360.58 to 603.13 μm and HW671 with 462.50 to 551.25 μm for mean pollen length. Similar kind of genotypic differences for pollen germination requirement have also been reported [1].

5. Conclusion

A simple or a complex medium may be developed to suit the germination requirement of pollen (binucleate or trinucleate pollen). The protocol suggested would give a better guidance in development of PGM. The author has developed pollen germination medium (PGM) for many crops *viz.* pigeonpea and its wild species, wheat, rye and brinjal by using the base constituents of PGM of Brewbaker and Kwack [7]. These media were supplemented with polyethylene glycol, e-amino caproic acid, peptone etc. besides the addition of BK salts. Grasses pollen so far considered as recalcitrant can also be germinated in the artificial medium e.g. wheat and rye. The grass pollen is released at high moisture content (30–40%) as compared to 1–5% in the case of orthodox species. This trait makes it unsuitable for *in vitro* germination. With combination of maltose, PEG and tryptone/peptone contained the initial pollen bursting and resulted in successful pollen germination of wheat and rye. The grasses pollen was so far classified as recalcitrant [31]. It is observed that monocots require low salt content than the dicots in the pollen germination medium. Also wild species require minimal medium than the domesticate ones.

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Chapter 7

Comparison of Pollination Graphs

James H. Lee, David M. Chan and Rodney J. Dyer

Additional information is available at the end of the chapter

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Abstract

From the agent-based, correlated random walk model presented, we observe the effects of varying the maximum turning angle, $\delta_{\max}$, tree density, ω, and pollen carryover, κ_{max}, on the distribution of pollen within a tree population by examining pollination graphs. Varying maximum turning angle and pollen carryover alters the dispersal of pollen, which affects many measures of connectivity of the pollination graph. Among these measures the clustering coefficient of fathers is largest when $\delta_{\max}$ is between 60 and 90°. The greatest effect of varying ω is not on the clustering coefficient of fathers, but on the other measures of genetic diversity. In particular when comparing simulations with randomly placed trees with that of actual tree placement of *C. florida* at the VCU Rice Center, it is clear that having specific tree locations is crucial in determining the properties of a pollination graph.

Keywords: pollination network, correlated random walk, agent-based model, pollen carryover, tree density

1. Introduction

While the movement of genes from one generation to the next ensures the cohesiveness of plant species through time and space [1–3], the extent to which individual sites and populations are functionally connected is mitigated by both biotic and abiotic factors [4]. For wind dispersed pollen, features such as the direction and speed of the wind and physical properties of individual pollen grains [5] play prominent roles in how genes are carried across the landscape. In addition to intrinsic factors, site-specific features, such as the structural

complexity of the landscape and co-occurring species [6], also influence connectivity to an extent that it is easy to discern.

Genes that are dispersed via active agents—animal mediated pollination—add increasing layers of involvement for at least two reasons. First, the way in which an animal disperser identifies, perceives, and interacts with features in the environment directly impact realized genetic connectivity. Over the last decade, enough work has been focused on this topic to denote a new subdiscipline of population genetic research, dubbed landscape genetics [7, 8], has been is devoted to developing methods for this task. From the perspective of the plant population, the characteristics of the intervening landscape determine the overall *porosity* of the landscape. Second, while the determination of which subset of features is important as it specifies the matrix through which pollinators move, the consequences to plant population genetic structure from alternative modes of pollinator movement is largely unexplored—even for the same plant species, alternative pollinating species leave discernibly distinct genetic structure [4, 9].

In this manuscript, we examine how the way in which pollinating individuals move across the landscape may influences population genetic structure. Here we develop an agent-based model (ABM) to simulate pollinator movement across a spatially explicit landscape. Individual pollinators are tracked as they pick up and disperse pollen among a set of individual plants. We adopt an underlying model of a correlated random walk (CRW) [10], where the direction and rate of movement is both temporally autocorrelated, though constrained. Within this framework, we explore the extent to which the spatial arrangement of trees interacts with variation in model parameters in producing variation in pollination statistics. We then apply this model to a data set from an natural population of the understory tree, *Cornus florida* L. (Cornaceae) [11]. This data set consists of both spatial and genetic information upon which previous landscape genetic studies have been conducted [11].

2. Background and methods

The agent based model developed herein uses two different categories of actors; trees act as the source and destination of pollen, and pollinating agents move individual pollen grains across the landscape. While the *trees* are spatially fixed on the landscape, the movement characteristics of the pollinating agents determine the ability of the plant population to maintain population genetic structure and determine relative reproductive output for individual trees. The movement of pollinating agents across the landscape is defined as a correlated random walk parameterized by inertia and speed. Across model runs, the aggregate movements of pollinating agents define a *de facto* pollination network whose characteristics are used to infer the robustness of the overall mating network and provide insights into population genetic stability. Across replicate runs, we extract parameters describing pollinator-movement dynamics parameters (average and maximum dispersal distances), pollen network robustness (pollen donor connectance and spatial clustering), and future population genetic structure (pollen donor density and diversity).

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2.1. Field characteristics

The field size for model runs is set as a square 100×100 units grid. The density of the trees simulated on this landscape, ω, is determined as

$$\omega = \frac{\tau}{100 \times 100} = \frac{\tau}{10,000} = 0.0001\tau, \tag{1}$$

where τ is the number of trees. In our simulation runs, we used tree densities, measured in trees per square unit, of $\omega \in \{0.0250, 0.0500, 0.0750, 0.1000, 0.1500\}$.

Previous simulation and empirical work has shown that density of pollen donors can have significant impacts on the genetic structure and diversity of offspring [6, 12], and as such should be a parameter across which we evaluate the other features of this model. The simulation field has rigid boundaries, and is considered impermeable. As such pollinators cannot leave the field nor are new pollinators allowed to enter the field during a model run. When an pollinator comes into contact with the edge of the field, its subsequent heading is set such that it 'bounces' off of the barrier at the opposite angle from which it approached.

Simulations were also run for a field size of $\frac{3100}{\sqrt{541}} \times \frac{1100}{\sqrt{541}} \approx 133.2794 \times 47.2927$ units. This was used based on data for trees sampled from the experimental natural population at the Virginia Commonwealth University, Rice Rivers Center (http://ricerivers.vcu.edu). This population was used as it has been the focus of previous work on pollen-mediated gene flow [11]. The tree density that results from this data set was $\omega = 0.071552$ trees per square unit, which was also simulated as a uniformly distributed scenario along with others previously mentioned.

2.2. Tree characteristics

For tracking purposes, each tree, T, is numbered such that $1 \leq T \leq \tau$. Let $\mathbf{\Upsilon}^{(T)} = \left(y_1^{(T)},\ y_2^{(T)}\right)$ be the location of tree T, which is static. For the initial model runs, trees are randomly placed using a uniform random distribution within the allotted field size. For both simplicity and comparison to temperate tree species, we assume that all trees are self-incompatible and that all successful pollination and fertilization produces non-inbred individuals. This means that all pollination distances will be strictly greater than zero since at most one tree can occupy any location on the landscape. We also applied the model to a spatial arrangement of trees mimicking a natural population for which we have already conducted extensive empirical studies of insect-mediated pollination and gene flow. To create the pollination graph, data was collected to determine the number of seeds fertilized on each tree, which pollen donor trees are sired those seeds, and the frequency each tree fathered seeds on other trees.

For the second part of the study, coordinates of the trees at the VCU Rice Center were provided by the Dyer Laboratory [13]. These coordinates were used to create pollination graphs to compare with the random location pollination graphs to gauge the extent to which spatial heterogeneity influences broad trends in pollen connectivity.

2.3. Pollinator movement

Both natural and managed landscapes contain a broad range of species that are commonly distributed with a high degree of spatial heterogeneity. For tree species, reproductive structures may be nestled among several other taxa both below and above the target species in a mixed forest canopy. Under these conditions, a movement model based upon correlated random walk is preferred over alternatives such as Levy walks due to the complexity of the intervening landscape and the lack of long thoroughfares in the forest. Correlated Random Walk (CRW) models have been widely used to describe foraging behavior across a range of animal taxa [14–18].

In our simulations, we begin with an allotment of 1000 pollinators starting at random location with a random direction of travel on the simulated landscape. At each discrete time step, each pollinating agents will obtain a new heading based upon its previous heading with a specified random deviance. The individual will then move in this new direction 1 distance unit. This process continued for n_{max} time steps.

If a pollinator is within one unit distance of a tree, it will visit flowers on the tree. Each flower on a tree can be pollinated with equal probability. Pollinators visit one tree at a time. If multiple trees are within 1 unit the closest one is chosen. When visiting a tree, the pollinator may both gather pollen and deposit pollen from other trees. Due to the short length of the simulation we assume there are a sufficient number of flowers to gather pollen from and deposit pollen to on each tree.

Let β be the total number of pollinators in a simulation, and let $\mathbf{X}_n^{(i)} = \left(x_{1,n}^{(i)},\ x_{2,n}^{(i)}\right)$ be the location of the i^{th} pollinator, $1 \le i \le \beta$, at time step n, $0 \le n \le n_{\max}$. For all simulations, we assume $\beta = 1000$ and $n_{max} = 600$.

The initial position of each pollinator, $\mathbf{X}_0^{(i)}$ is uniformly distributed throughout the field. Each pollinator's initial heading, $-180^\circ \le \theta_1^{(i)} \le 180^\circ$ is chosen from a uniform random distribution. At each subsequent time step, the pollinator's new heading $\theta_{n+1}^{(i)}$ is dependent upon its current heading $\theta_n^{(i)}$ and a random number $\delta_{n+1}^{(i)}$. That is

$$\theta_{n+1}^{(i)} = \theta_n^{(i)} + \delta_{n+1}^{(i)} \tag{2}$$

where $\delta_{n+1}^{(i)} \in (-\delta_{\max},\ \delta_{\max})$ for each $n = 1,\ \ldots,\ n_{\max}$. Similarly, the initial step size of each pollinator, $r_1^{(i)} = 1$ at time $n = 1$. Each subsequent step size, $r_{n+1}^{(i)} \in (0,\ r_{\max})$ uniformly distributed for each $n = 1,\ \ldots,\ n_{\max}$. In Cartesian coordinates, the position of the i^{th} pollinator at each subsequent time step will be

$$\mathbf{X}_{n+1}^{(i)}\left(\mathbf{X}_n^{(i)};\ r_{n+1}^{(i)},\ \theta_{n+1}^{(i)}\right) = \left(x_{1,n}^{(i)} + r_{n+1}^{(i)}\cos\left(\theta_{n+1}^{(i)}\right),\ x_{2,n}^{(i)} + r_{n+1}^{(i)}\sin\left(\theta_{n+1}^{(i)}\right)\right) \tag{3}$$

for each $n = 1,\ \ldots,\ n_{\max}$. In our simulations we used values of $\delta_{\max} \in \{0^\circ, 15^\circ, 30^\circ, 45^\circ, 60^\circ, 75^\circ, 90^\circ, 120^\circ, 150^\circ, 180^\circ\}$.

Sample paths based on different values of δ_{max} are shown in **Figure 1**. As δ_{max} increases the paths do not generally travel away as far from the starting point and loop around more often. A path with $\delta_{max} = 0$ would be along a straight line, which is not shown here.

2.4. Pollination

If a pollinator visits a flower, it will collect pollen from that individual. Pollen will be deposited with a probability of P_κ, where κ is the number of previously visited flowers, to account for pollen carryover. Carryover probability $P_\kappa = 0$ if $\kappa > \kappa_{\max}$ where $\kappa_{\max}$ is the maximum pollen carryover. In the simulations the pollination carryovers used were $\kappa_{\max} \in \{1, 3, 5, 7, \infty\}$.

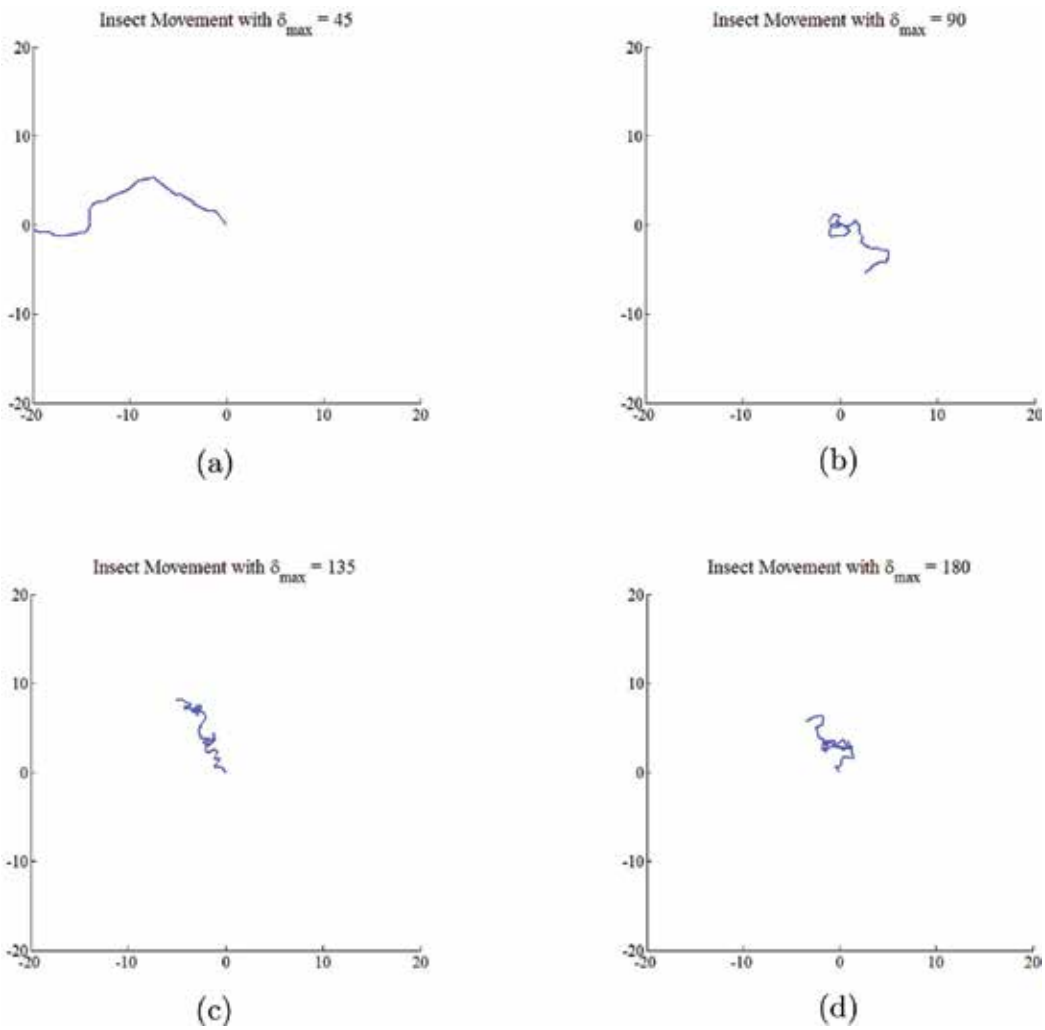

Figure 1. Sample paths based on δ_{max}.

As a pollinator visits multiple flowers, the chances that it deposits pollen from a previous flower diminishes with each successive flower visited [19]. It was shown by [20] that from a given flower, a pollinator will deposit roughly $\gamma(1-\gamma)^{k-1}$ pollen grains onto the k^{th} flower visited, where γ depends upon the type of pollen as well as the type of pollinator. In this study, the probability that an pollinator distributes pollen from one tree to another tree is given by

$$P_\kappa = \begin{cases} \rho(1-\rho)^{\kappa-1} & \text{if } \kappa \leq \kappa_{\max} \\ 0 & \text{otherwise} \end{cases} \tag{4}$$

where κ is the number of previously visited flowers, and ρ is the chance of pollination when $\kappa = 1$. For the simulation work we used $\rho = 0.30$ based on work by [21].

2.5. Statistics

To characterize pollen movement and how it responds to the parameters of the models, pollination graphs were constructed. The connectivity network is based upon the physical location of individual trees and the pattern of spatial pollen movement created by the pollinators. In this network, each tree is represented as a node and the edges designate the movement of pollen from donor (paternal individual) to recipient (maternal individual), creating a directed pollination graph.

The parameters we vary in constructing these networks include tree density, ω, pollination carryover, κ, and pollinator maximum turning angle, $\delta_{\max}$. To determine the effect of these parameters on the landscape pattern of connectivity, we estimated the *number of fathers per mother*, Φ_m, *connectance*, L, *average weighted diversity of fathers*, E, *average pollination distance*, $\overline{D}$, *average maximum pollination distance*, $\tilde{D}$, and the *weak and strong clustering coefficients of fathers*, C_{weak} and C_{strong}.

Each tree has the ability to contribute pollen to other trees and to accept pollen from other trees. When applicable, we will refer to a tree as a father tree, f, if that tree contributes pollen to another tree. We will refer to a tree as a mother tree, m, if that tree is accepts pollen from another tree. Let ϕ_m be the set of trees which father seeds on tree m, then the number of fathers for each tree m in the graph is $|\phi_m|$, $m = 1, \ 2, \ \dots, \ \tau$, where $|\cdot|$ denotes cardinality. The set containing the *number of fathers per mother* for all trees in the graph is

$$\Phi_m = \{|\phi_1|, \ |\phi_2|, \ \dots, \ |\phi_\tau|\}. \tag{5}$$

This value is similar to the degree distribution in the studies by Ramos-Jilberto et al. [22] and Valdovions et al. [23].

From this construct, we then create the $\tau \times \tau$ adjacency matrix, $\mathbf{A} = [a_{i,j}]$, where $a_{f,m} = 1$ if tree f fathers at least one seed on tree m, and 0 if not. Thus $\mathbf{A}$ is a binary representation of the connectance of the graph. Since the trees do not self-pollinate, the number of possible interactions on this matrix is $\tau(\tau - 1)$.

The *connectance*, L, of a graph is defined as the proportion of realized pollination events to the number of possible pollination events [24]. The connectance of the graph is given by

$$L = \frac{\sum_{f=1}^{\tau} \sum_{m=1}^{\tau} a_{f,m}}{\tau(\tau - 1)}. \tag{6}$$

As with the previous parameters, connectance has been used in a number of settings, see [23, 25–28], where the graphs is between species. In this study the focus is on individuals, and so the connectance is the proportion of realized pollination events between individual plants.

Furthermore, if there is an edge between tree m and tree f where tree $f \in \phi_m$, then denote $b_{f,m}$ to be the weight of that edge, which is equal to the number of times tree f fathers seeds on tree m. With this we create the matrix $\mathbf{B} = \left[b_{i,j}\right]$, which is a $\tau \times \tau$ matrix such that $b_{f,m}$ is the number of seeds that tree f fathers on tree m. The weighted diversity of fathers for a mother tree m is a weighted measurement of the number of fathers that contribute pollen to seeds on m, accounting for the various number of seeds fathered by each father tree. The weighted diversity of fathers, $\widehat{F}_m$, is computed for each m in the graph by the formula

$$\widehat{F}_m = \frac{\left(\sum_{f=1}^{|\phi_m|} b_{f,m}\right)^2}{\sum_{f=1}^{|\phi_m|} \left(b_{f,m}\right)^2}. \tag{7}$$

The *average weighted diversity of fathers* is the mean average of the weighted diversity of fathers over all mother trees, and is given by the formula

$$E = \frac{1}{\mu} \sum_{m=1}^{\mu} \widehat{F}_m, \tag{8}$$

where $0 \le \mu \le \tau$ is the total number individuals in the graph.

The average pollination distance for an pollinator i is the average of the distances between any two trees mated by i. Let $\mathbf{Y}^{(f^{(i)})} = \left(y_1^{(f^{(i)})},\ y_2^{(f^{(i)})}\right)$ be the location of father tree $f^{(i)}$ and $\mathbf{Y}^{(m^{(i)})} = \left(y_1^{(m^{(i)})},\ y_2^{(m^{(i)})}\right)$ be the location of mother tree $m^{(i)}$ where pollinator i delivers pollen from tree $f^{(i)}$ to tree $m^{(i)}$. Then the average pollination distance, $\overline{D}^{(i)}$, achieved by pollinator i for all such pairings is

$$\overline{D}^{(i)} = \frac{1}{\mu^{(i)}} \sum_{m=1}^{\mu^{(i)}} \sum_{f=1}^{\phi_m^{(i)}} \frac{1}{\phi_m^{(i)}} \sqrt{\left(y_1^{(m^{(i)})} - y_1^{(f^{(i)})}\right)^2 + \left(y_2^{(m^{(i)})} - y_2^{(f^{(i)})}\right)^2}, \tag{9}$$

where $\mu^{(i)}$ is the number of mother trees pollinated by pollinator i, and ϕ_m^i are the number of father trees pollinating tree m by pollinator i. The average pollination distance, $\overline{D}^{(i)}$, for each

pollinator is averaged over the total number of pollinators, β, to obtain the *average pollination distance*, $\overline{D}$, for the graph

$$\overline{D} = \frac{1}{\beta} \sum_{i=1}^{\beta} \overline{D}^{(i)}. \tag{10}$$

The *maximum pollination distance* for an pollinator i is

$$\tilde{D}^{(i)} = \max_{m^{(i)} \le \mu^{(i)},\ f^{(i)} \le \phi_m^{(i)}} \left(\sqrt{\left(y_1^{(m^{(i)})} - y_1^{(f^{(i)})} \right)^2 + \left(y_2^{(m^{(i)})} - y_2^{(f^{(i)})} \right)^2} \right). \tag{11}$$

The maximum pollination distance for each pollinator is averaged over all of the pollinators to obtain the *average maximum pollination distance* for the graph

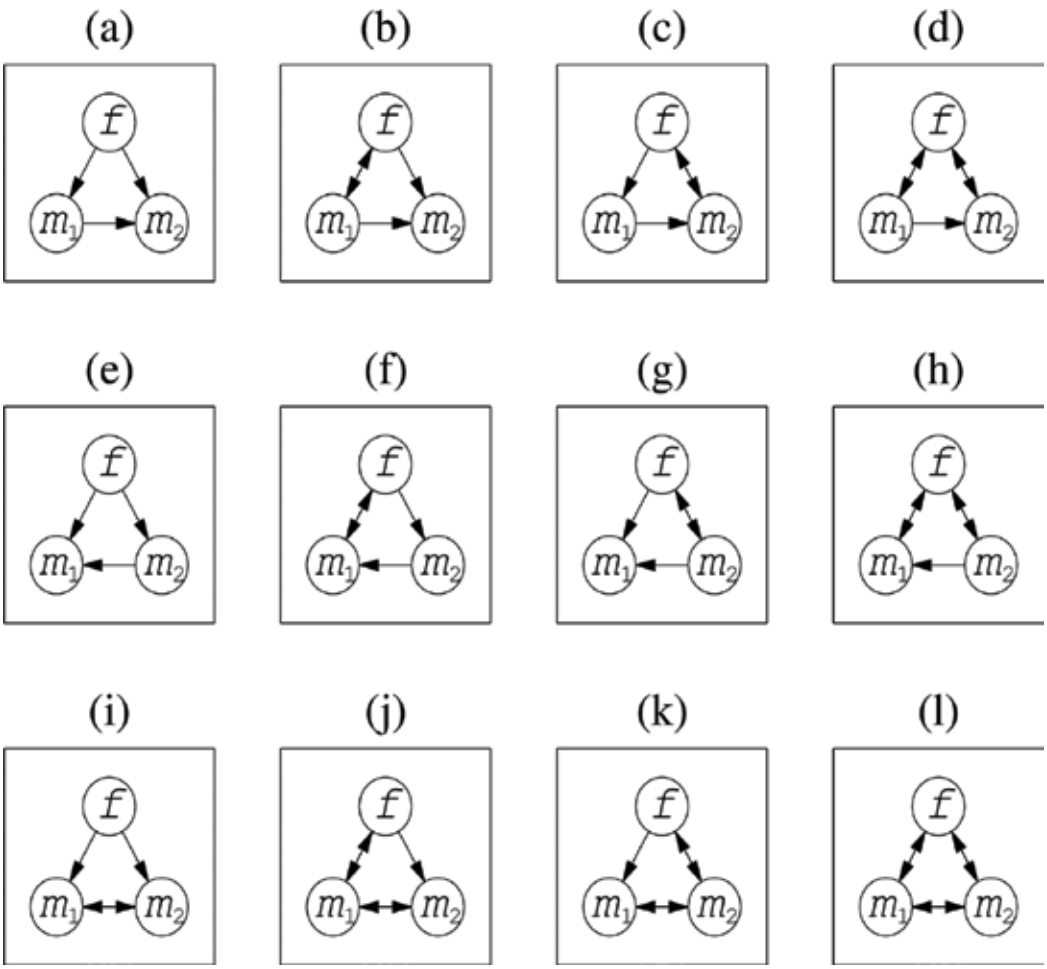

Figure 2. Fathering triangles. Arrows indicate direction of gene flow.

$$\tilde{D} = \frac{1}{\beta} \sum_{i=1}^{\beta} \tilde{D}^{(i)}. \tag{12}$$

A fathering triplet is the relationship between three trees such that tree f is a father to seeds on both tree m_1 and tree m_2. These seeds are half-siblings on the paternal side. In particular a weak fathering triangle, as a subset of fathering triplets, is one such that m_1 also fathers seeds on m_2 (**Figure 2(a)–(d)**), m_2 fathers seeds on m_1 (**Figure 2(e)–(h)**), or both (**Figure 2(i)–(l)**). A strong fathering triangle is defined as fathering triangle where f, m_1, and m_2 all father seeds on each other (**Figure 2(l)**).

The *weak clustering coefficient of fathers*, C_{weak}, is the number of weak fathering triangles in the pollination graph over the total number of fathering triplets

$$C_{\text{weak}} = \frac{\text{number of weak fathering triangles}}{\text{number of fathering triplets}}. \tag{13}$$

The *strong clustering coefficient of fathers*, C_{strong} is the number of strong fathering triangles in the pollination graph over the total number of fathering triplets

$$C_{\text{strong}} = \frac{\text{number of strong fathering triangles}}{\text{number of fathering triplets}}. \tag{14}$$

C_{weak} and C_{strong} are measurements of the tendency of parent trees to be clustered together in densely connected groups.

3. Results and discussion

We examine the effect of varying parameters on the graph statistics: the number of fathers per mother, Φ_m, connectance, L, the average weighted diversity of fathers, E, the clustering coefficient of fathers, C, the average pollination distance, $\overline{D}$, and the average maximum pollination distance, $\tilde{D}$. The model was simulated a total of 10 replicate runs for each unique combination of parameters. The results follow.

3.1. Number of fathers

One way to analyze the genetic structure and connectivity within a local plant population is to examine the number of different fathers per mother tree, Φ_m. In **Figure 3**, the number of different fathers per mother in a randomized placement of trees is distributed in a Gaussian-like distribution. This is an expected outcome since gene flow should be directly proportional to the distance traveled by a pollinator. It has been shown, see [29], that the distribution range resulting from a CRW would necessarily result in Gaussian-like behavior for a large number of pollination events.

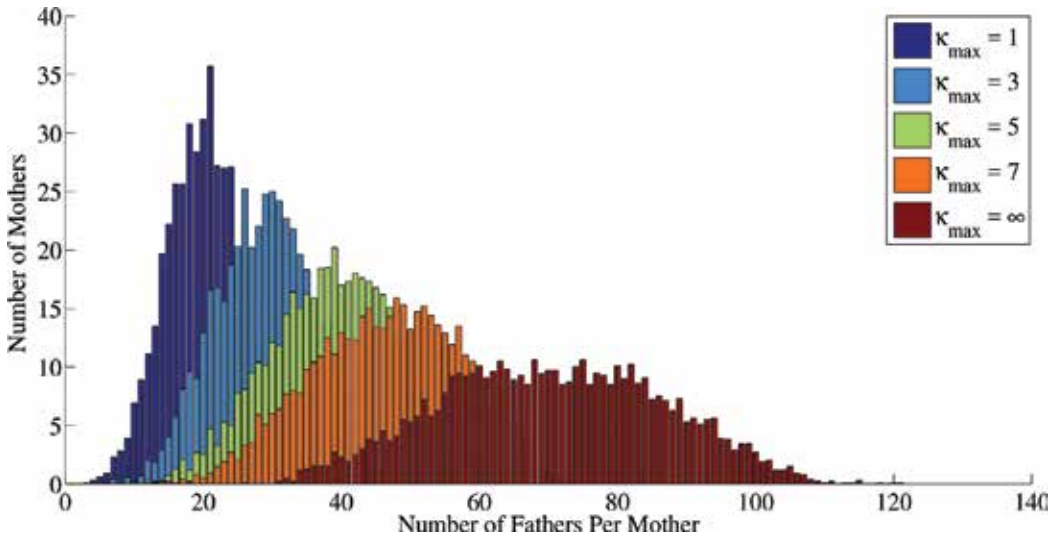

Figure 3. Number of fathers per mother. Tree density $\omega = 0.071552$ trees per square unit. Maximum pollinator turning radius $\delta_{\max} = 45^{\circ}$. Pollination chance diminishing with larger carryover. Maximum pollen carryover $\kappa_{\max} = \{1, 3, 5, 7, \infty\}$.

Of interest here is that as the maximum pollen carryover increases, the mean number of fathers increases. This is due to the increase in the variability in pollen that each pollinator can distribute, which would increase diversity. The variance in the distribution also increases as pollen carryover increases, which is also due to this increase in diversity.

However, when this distribution is compared with the tree placement at the Rice Center, see **Figure 4**, we observe a bi-modal distribution of the number of fathers per mother. This distribution

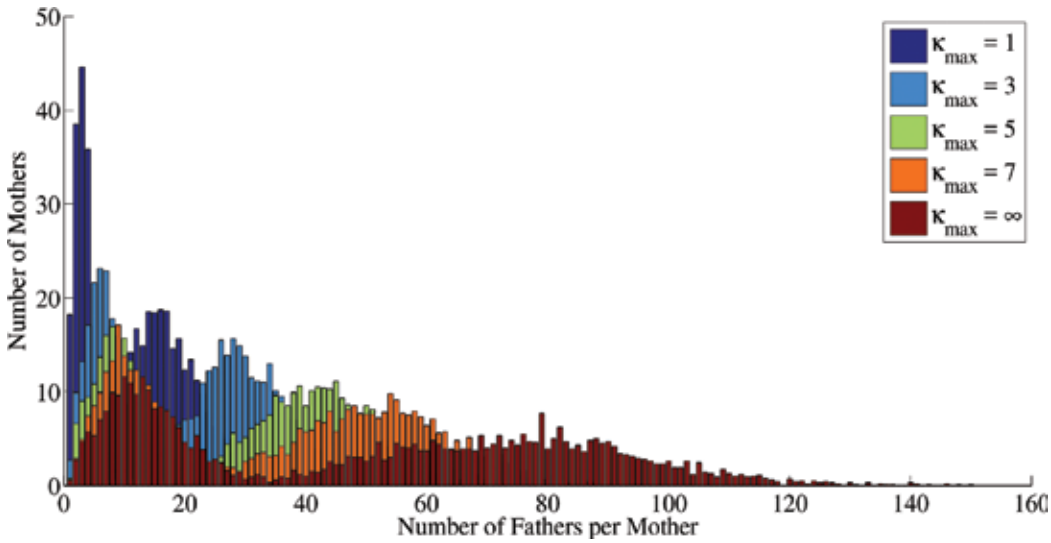

Figure 4. Number of fathers per mother. Field size 133.2794×47.2927 units. Tree density $\omega = 0.071552$ trees per square unit. Maximum pollinator turning radius $\delta_{\max} = 45^{\circ}$. Pollination chance diminishing with larger carryover. Maximum pollen carryover $\kappa_{\max} = \{1, 3, 5, 7, \infty\}$.

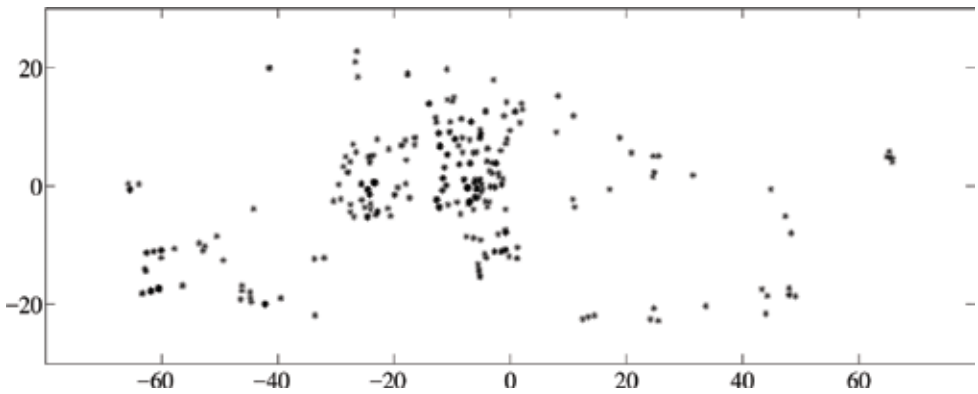

Figure 5. *Tree coordinates (scaled) at the VCU Rice Center*. Field size 133.2794×47.2927 units. Tree density $\omega = 0.071552$ trees per square units.

is attributed to the spatial heterogeneity of the research site. This influences the genetic structure and connectivity in *C. florida* populations [11] at the Rice Center.

The spatial heterogeneity is evident in **Figure 5**. The bimodal distribution of the number of fathers per mother is caused by the variation of tree density across the landscape. There is a high density group of trees in the center of the region, and the density decreases toward the boundary of the region. This region is bounded by a river and a lake on two sides, and a major road and a farm on the other two sides, which are not pictured here.

These two different density regions create the two peaks shown in **Figure 4**. In particular when $\kappa_{\max} = \infty$, the first peak is approximately 3 fathers per mother on 29 mother trees, and the second peak is 32 fathers per mother on 12 mother trees. This bimodality is present whether an pollinator is restricted to moving in a straight line, i.e., $\delta_{\max} = 0^\circ$, or with pure dispersal, i.e., $\delta_{\max} = 180^\circ$. Clearly, knowing the locations of tree is critical in understanding the gene flow in a particular area (**Figure 5**).

3.2. Connectance

The connectance is a measure of how complete a pollination graph is in terms of individuals mating with others. In **Figures 6** and **7**, we see the effect of density and maximum turning angle. With higher density, and thus more individuals, the connectance is reduced due to a much larger number of potential pairs of individuals. As the maximum turning angle increases the connectance also decreases. If an pollinator travels in a straight line, it will cover a greater spatial distance as it visits more different trees than it would if it just spun around in circles locally. Smaller $\delta_{\max}$ increases the potential for mating to occur between trees with greater distance between them. The graph connectance is three to five times greater with small $\delta_{\max}$ than it is with large $\delta_{\max}$.

If an pollinator does not venture very far from its starting location, as would be the case when $\delta_{\max}$ is close to 180°, the effect of maximum pollen carryover on the connectance of the graph decreases. When $\delta_{\max} = 0$, the connectance of the graph is nearly four times greater if the

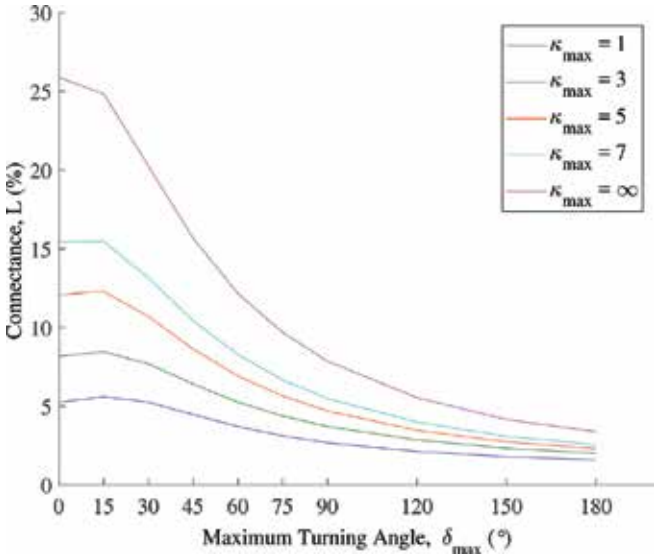

Figure 6. Connectance. Field size 133.2794×47.2927 units. Tree density $\omega = 0.071552$ trees per square unit. Pollination chance diminishing with larger carryover. Maximum pollen carryover $\kappa_{\max} = \{1, 3, 5, 7, \infty\}$.

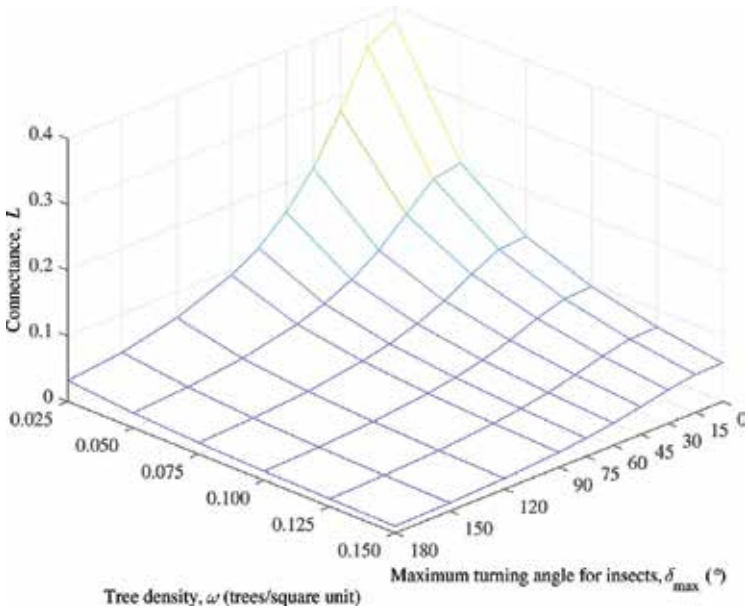

Figure 7. Connectance. Field size 100×100 units. Tree density in trees per square unit, $\omega = \{0.025, 0.050, 0.075, 0.100, , 0.125, 0.150\}$ ($\tau = \{250, 500, 750, 1000, 1250, 1500\}$ randomly-placed trees). Pollination chance diminishing with larger carryover. Maximum pollen carryover $\kappa_{\max} = \infty$.

maximum pollen carryover is unlimited versus the case when pollen carryover is limited to only one flower. However, when $\delta_{\max} = 180^\circ$, the connectance of the graph is only about twice if the maximum pollen carryover is unlimited.

Pollen carryover is important in connectance as well. In **Figure 7**, it is clear that if the maximum pollen carryover is limited, the connectance of the pollination graph is also limited due to the diversity of pollen an individual would have access to via the pollinator. With smaller κ_{max} the diversity of pollen distributed is greatly decreased and thus the diversity of pollination events is reduced as well.

When considering the actual tree locations at the Rice Center, the connectance of the graph is close to half of the connectance value with randomly-placed trees. The differences between the graphs is greatest when $\delta_{\max}$ is close to 0°. Connectance values for simulations run with the Rice Center data are shown in **Figure 8**.

3.3. Average weighted diversity of fathers

The average weighted diversity of fathers is clearly affected by density and the maximum turning angle, though the effects of maximum turning angle are more pronounced. In **Figure 9**, as the maximum turning angle increases, the average weighted diversity of fathers decreases. The greatest change occurs between 15 and 90° and tends to even out at the extremes. As described earlier, with greater potential for a variety of fathers, the average number of fathers contributing pollen to mother trees increases as the maximum turning angle of pollinators decreases. This adds a greater genetic diversity to the tree population. Pollinators that travel in straighter paths not only distribute pollen greater distances, but also with greater diversity. The effects of density are more pronounced at smaller maximum turning angles, where we see a reduction of the average weighted diversity of fathers as the density increases.

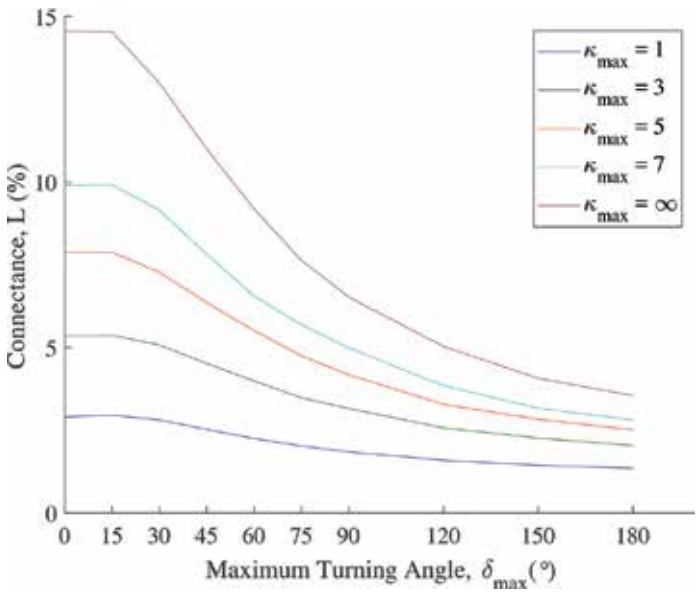

Figure 8. Connectance. Field size 133.2794 × 47.2927 units. Tree density $\omega = 0.071552$ trees per square unit. Pollination chance diminishing with larger carryover. Maximum pollen carryover $\kappa_{\max} = \{1, 3, 5, 7, \infty\}$.

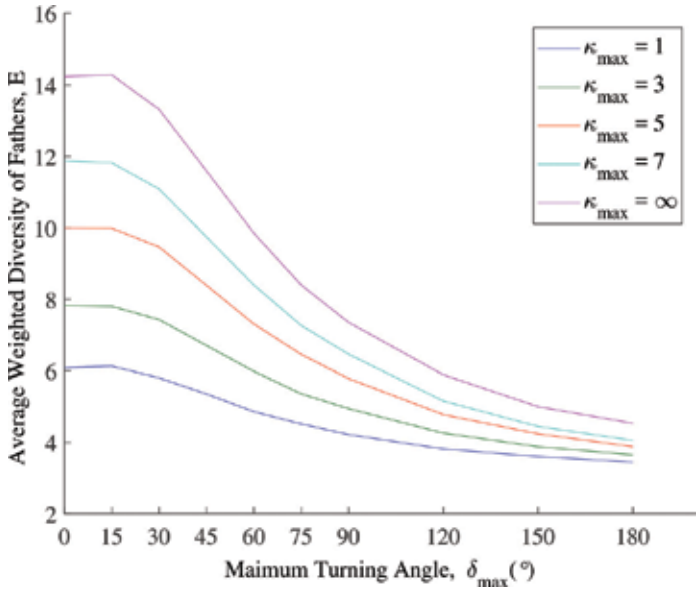

Figure 9. Average weighted diversity of fathers. Field size 100×100 units. Tree density in trees per square unit, $\omega = \{0.025, 0.050, 0.075, 0.100, 0.125, 0.150\}$ ($\tau = \{250, 500, 750, 1000, 1250, 1500\}$ randomly placed trees). Pollination chance diminishing with larger carryover. Maximum pollen carryover $\kappa_{max} = \infty$.

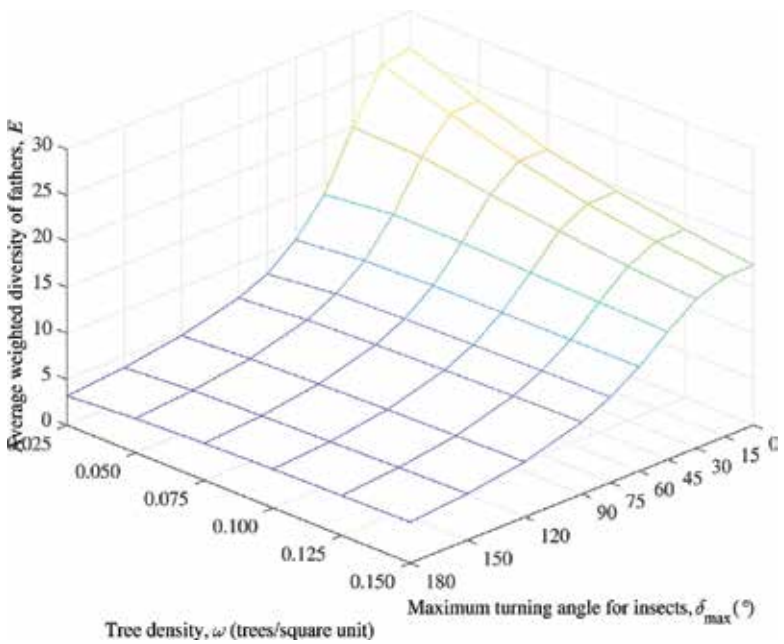

Figure 10. Average weighted diversity of fathers. Field size 133.2794×47.2927 units. Tree density in trees per square unit, $\omega = 0.071552$ ($\tau = 451$ Rice Center trees). Pollination chance diminishing with larger carryover. Maximum pollen carryover $\kappa_{max} = \{1, 3, 5, 7, \infty\}$.

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The differences in average weighted diversity of fathers as pollen carryover increases are exactly as one would expect, see **Figure 10**. With higher κ_{max}, the larger the diversity in pollen increases the average weighted diversity in fathers. This increase is small for large δ_{max} due to the increase in the number of multiple visits by pollinators to the same trees.

When comparing the random tree distribution to that of the Rice Center, the random distribution has a larger average weighted diversity of fathers (not shown here) the average weighted diversity of fathers at the Rice Center is about 60% of the randomly placed trees. Trees that are in densely packed groups are going to be greatly influenced by surrounding trees, but trees at greater distances will have less of a comparative impact on the fatherhood of seeds.

3.4. Average and maximum average pollination distances

The average and maximum pollination distances behave similar to the connectance. As with the connectance, as the density increases both distances decrease, which is due to a greater number of shorter pollination events lowering the averages, see **Figures 11** and **12**. The change in density has a smaller effect on the maximum pollination distance. Also as the maximum turning angle increase both distances decreases as well since the pollinators do not travel as far. This decrease is more dramatic at lower densities.

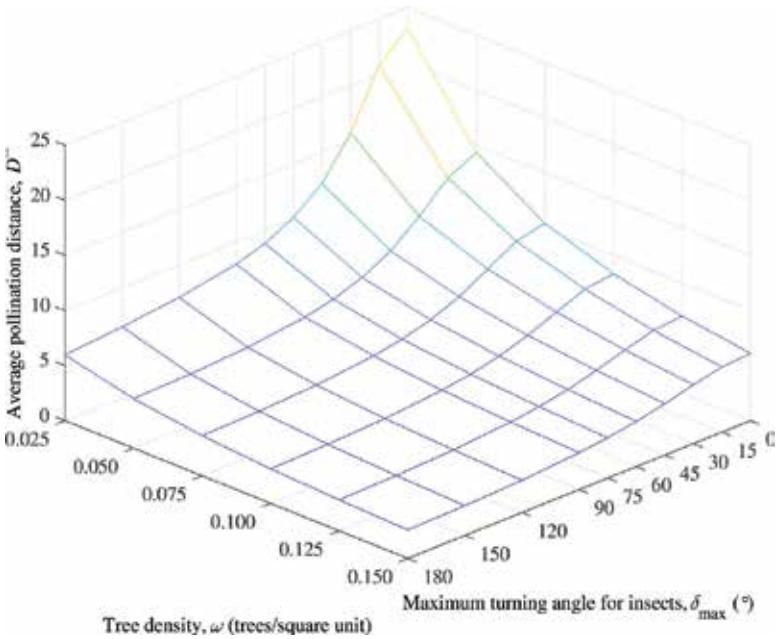

Figure 11. Average pollination distance. Field size 100×100 units. Tree density in trees per square area, $\omega = \{0.025, 0.050, 0.075, 0.100, 0.125, 0.150\}$ ($\tau = \{250, 500, 750, 1000, 1250, 1500\}$ randomly placed trees). Pollination chance diminishing with larger carryover. Maximum pollen carryover $\kappa_{\max} = \infty$.

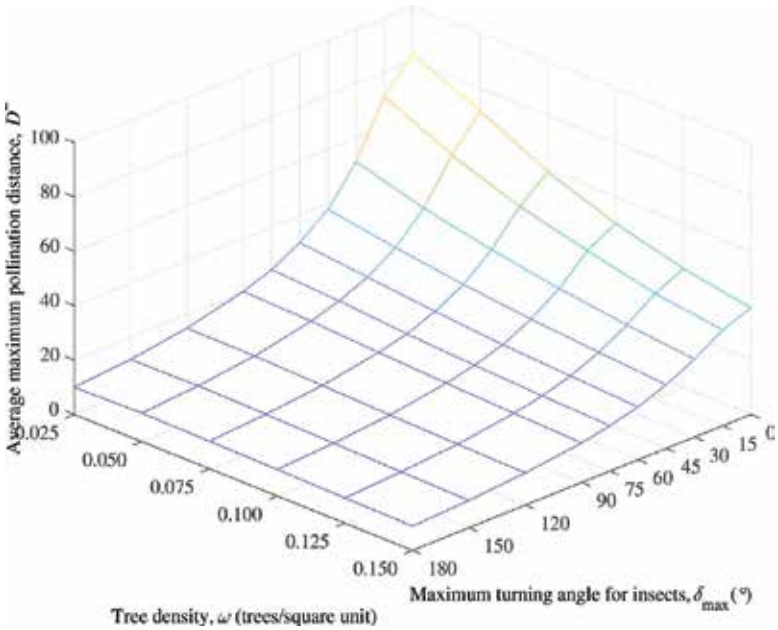

Figure 12. Average maximum pollination distance. Field size 100×100 units. Tree density in trees per square unit, $\omega = \{0.025, 0.050, 0.075, 0.100, 0.125, 0.150\}$ ($\tau = \{250, 500, 750, 1000, 1250, 1500\}$ randomly placed trees). Pollination chance diminishing with larger carryover. Maximum pollen carryover $\kappa_{max} = \infty$.

Using Rice Center locations (not shown here) if δ_{max} is close to 0°, the average pollination distance is greater than that of randomly placed trees. However, if δ_{max} is close to 180°, the average pollination distance using Rice Center data is less than that of randomly placed trees. This is again due to the combination of the low and high density distribution of trees at the Rice Center. At low δ_{max} pollinators interact with both densities of trees, whereas at high δ_{max} the interaction between these groups is greatly diminished.

We see the effects of pollen carryover in the average pollination distance is shown in **Figure 11** and in the maximum pollination distance in **Figure 12**. As expected, increasing κ_{max} increases the average pollination distance. If the pollen carryover increases that allows pollen grains to travel further since pollen can be deposited after visiting several intermediate trees.

The results are similar with the Rice Center model. The results at the Rice Center are slightly higher in distances due to the potential longer distances at the edges of the region.

3.5. Clustering coefficients of fathers

The clustering coefficient is a measure of the interconnectedness of the graph, as well as how the genes are shared within the graph. For a field of randomly placed trees, there is a maximum value for the weak clustering coefficient of fathers, C_{weak}, see **Figure 13**, that occurs between 60 and 90° for all maximum pollen carryover values, κ_{max}. This is explained by examining the extreme values of δ_{max}. For small δ_{max}, pollinators travel across the landscape

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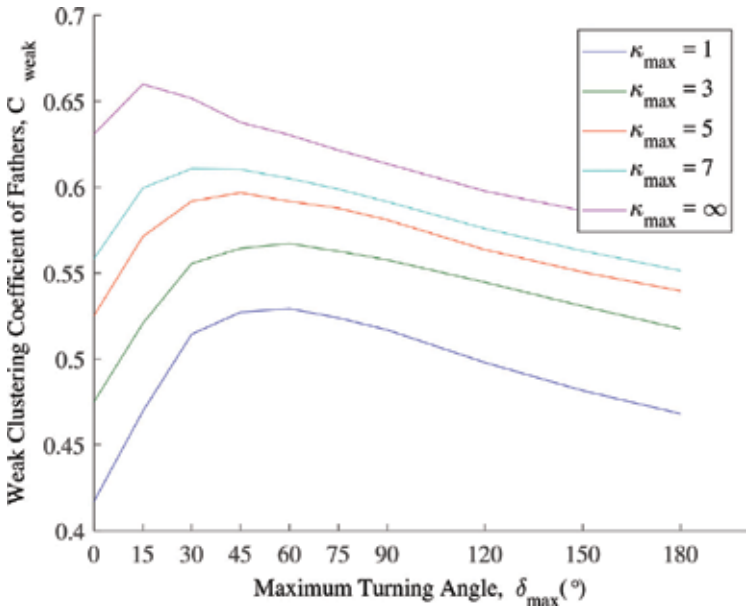

Figure 13. Weak clustering coefficient of fathers. Field size 133.2794×47.2927 units. Tree density $\omega = 0.071552$ trees per square unit ($\tau = 451$ randomly placed trees). Pollination chance diminishing with larger carryover. Maximum pollen carryover $\kappa_{\max} = \{1, 3, 5, 7, \infty\}$.

and do not stay in a small neighborhood. Since the weak clustering coefficient of fathers is an average measure of clustering at a local level, it is natural for C_{weak} to be low if pollinators do not remain in a small neighborhood.

At the other extreme, for large $\delta_{\max}$, pollinators do not move around enough to increase the value of C_{weak}. When $\delta_{\max}$ is not at an extreme value, the displacement of the pollinators is high enough to visit many trees, but low enough so more of the trees that it visits are within a closer proximity to one another.

Modeled data on the weak clustering coefficient of fathers, C_{weak}, from the Rice Center is shown in **Figure 14**. The C_{weak} values slowly increase as $\kappa_{\max}$ increases over the entire interval from 0 to 180˚, mostly flattening out for $\kappa_{\max} > 75$˚. As $\delta_{\max}$ approaches 180˚, pollinators remain in the same general area. Thus, in locally dense patches of trees, clustering will naturally be higher.

Unexpectedly, varying the tree density, ω, did not have a major effect on C_{weak}. It would seem that varying ω would have the same quantitative effect on C_{weak} as varying the maximum pollinator turning angle, $\delta_{\max}$. We suspect that the reason for the relative consistency of values for C_{weak} is due to the having both low and high density regions. The values for C_{weak}, varying ω and $\delta_{\max}$, are shown in **Figure 15**.

Figures 16–18 are corresponding plots for strong clustering coefficient of fathers, C_{strong}. In these plots we see similar results as with the weak clustering coefficient. Numerically the C_{strong} results are a magnitude smaller than the C_{weak} results since they are a measure of a subset of possible combinations of C_{weak}. This is however a greater increase in these values with increasing density, which allows for these fathering triangles to occur.

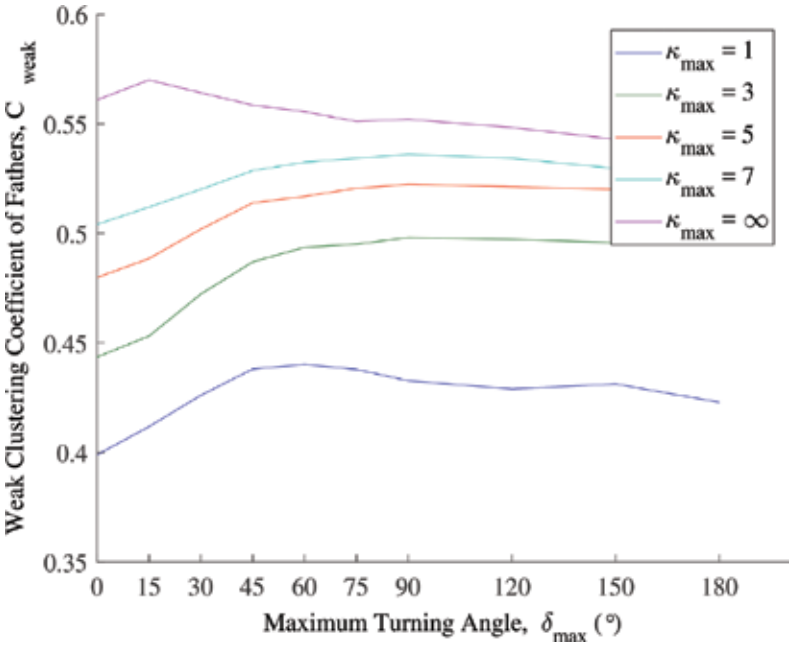

Figure 14. Weak clustering coefficient of fathers. Field size 133.2794×47.2927 units. Tree density $\omega = 0.071552$ trees per square unit ($\tau = 451$ Rice Center trees). Pollination chance diminishing with larger carryover. Maximum pollen carryover $\kappa_{max} = \{1, 3, 5, 7, \infty\}$.

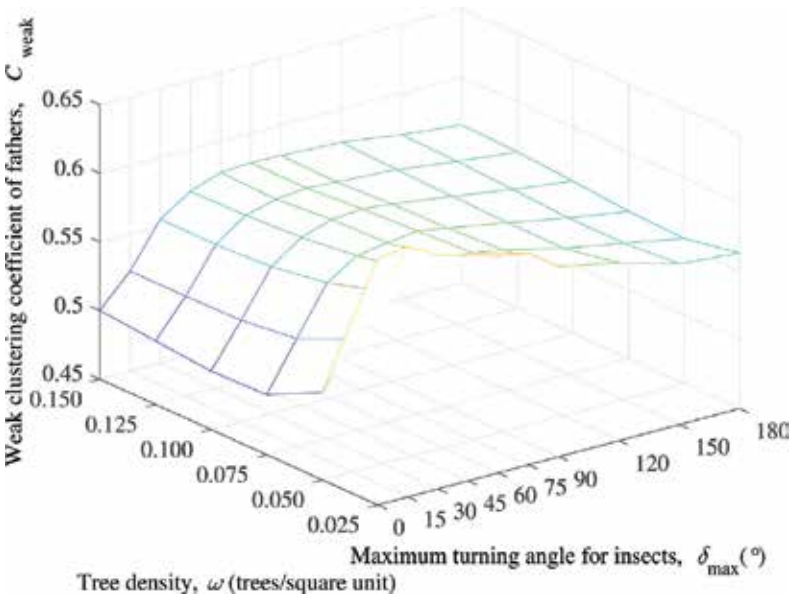

Figure 15. Weak clustering coefficient of fathers. Field size 100×100 units. Tree density in trees per square unit, $\omega = \{0.025, 0.050, 0.075, 0.100, 0.125, 0.150\}$ ($\tau = \{250, 500, 750, 1000, 1250, 1500\}$ randomly placed trees). Pollination chance diminishing with larger carryover. Maximum pollen carryover $\kappa_{max} = \infty$.

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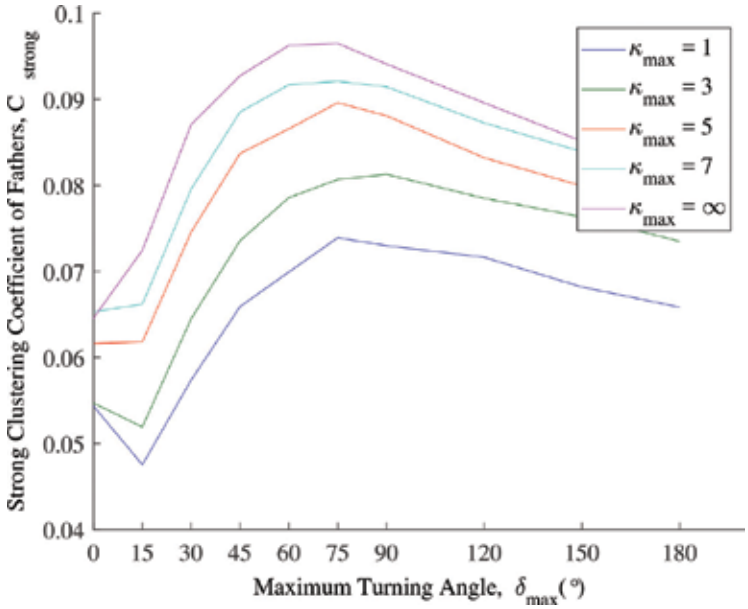

Figure 16. Strong clustering coefficient of fathers. Field size 133.2794 × 47.2927 units. Tree density $\omega = 0.071552$ trees per square unit ($\tau = 451$ randomly placed trees). Pollination chance diminishing with larger carryover. Maximum pollen carryover $\kappa_{max} = \{1, 3, 5, 7, \infty\}$.

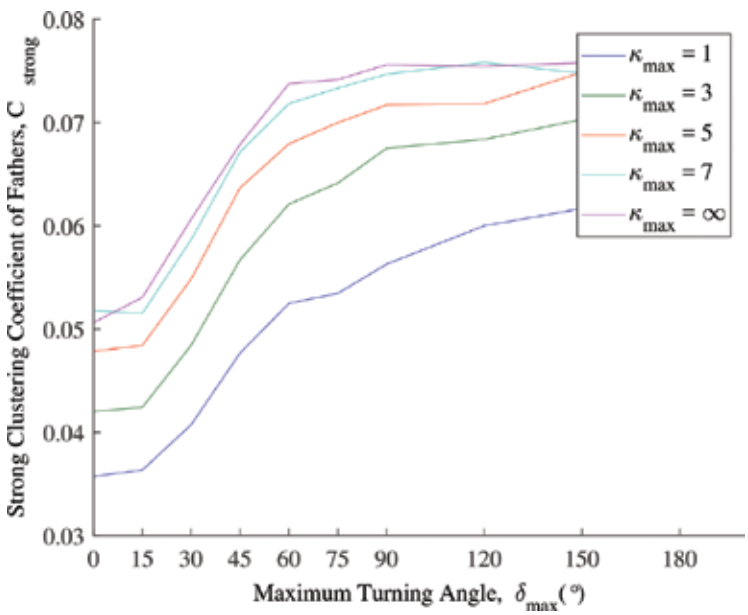

Figure 17. Strong clustering coefficient of fathers. Field size 133.2794 × 47.2927 units. Tree density $\omega = 0.071552$ trees per square unit ($\tau = 451$ Rice Center trees). Pollination chance diminishing with larger carryover. Maximum pollen carryover $\kappa_{max} = \{1, 3, 5, 7, \infty\}$.

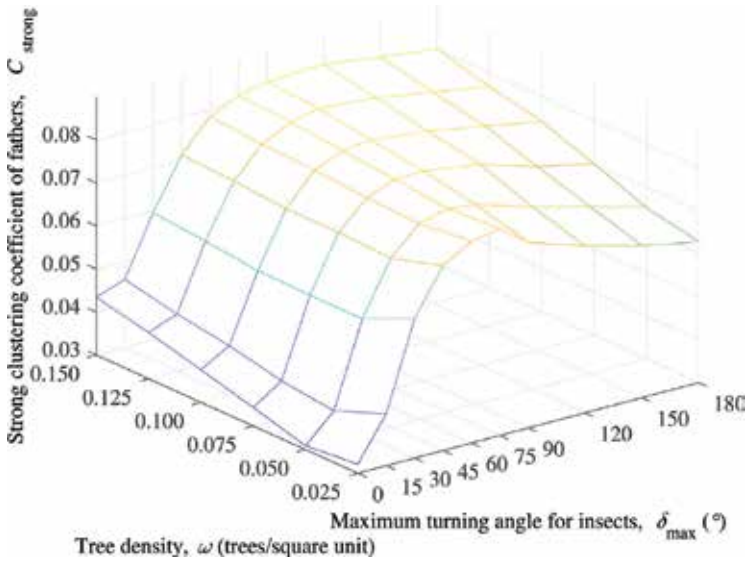

Figure 18. Strong clustering coefficient of fathers. Field size 100×100 units. Tree density in trees per square unit, $\omega = \{0.025, 0.050, 0.075, 0.100, 0.125, 0.150\}$ ($\tau = \{250, 500, 750, 1000, 1250, 1500\}$ randomly placed trees). Pollination chance diminishing with larger carryover. Maximum pollen carryover $\kappa_{\max} = \infty$.

4. Conclusions

From the agent-based, correlated random walk model presented, we observe the effects of varying the maximum pollen carryover, κ_{max}, the maximum pollinator turning area, $\delta_{\max}$, and the density of trees, ω, on the distribution of pollen within a population of *Cornus florida*.

When $\kappa_{\max}$ increases we see that the mean number of fathers per mother, the connectance, the average weighted diversity of fathers, the average and maximum average pollination distances, and the clustering coefficients all increase. The percentage increase varied between the measured, though the largest effect was seen in the connectance of the pollination graph. These increases are due to the increased capability of the pollinators to carry pollen farther from their source.

Changing the pollinator movement by increasing the maximum turning angle, $\delta_{\max}$, affected each of the measures as well. With most of the measures decreasing with increasing $\delta_{\max}$. These include the connectance, the average weighted diversity of fathers, and the average and maximum average pollination distances. On the other hand, the weak and strong clustering coefficients had maximal values between angles of 45 and 90°.

When $\delta_{\max} = 180°$, pollen is distributed in a purely random walk, and is more representative of pollen dispersal by wind. When $\delta_{\max} = 0°$, pollinators travel in a straight line, only changing direction when bouncing off of the boundary. This leads to a greater spatial displacement for each pollinator and thus a greater distance that pollen travels, resulting in greater genetic

diversity in the *C. florida* population. While neither of these extremes may be biologically relevant in *C. florida* populations, we note that the clustering coefficient of fathers, *C*, is maximized when δ_{max} is between 60 and 90˚, which could help illuminate some of the biological processes at work in the system.

Major changes are observed when comparing simulations using randomly-placed trees with simulations using the tree-placement at the Rice Center. When using the Rice Center data, we see a bimodal distribution in the number of fathers per mother, the connectance values are halved, the average weighted diversity of fathers is lower, the average pollination distance is lower when δ_{max} is close to 0˚ and higher when δ_{max} is close to 180˚, and the clustering coefficient of fathers exhibits both quantitative and qualitative differences. All of these differences highlight the need for specificity in describing the tree locations within a specific ecosystem in order to truly understand how pollen is distributed within that ecosystem. The differences in these graph indicators is due to the non-uniform distribution of trees at the Rice Center.

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Plants are the basic source of food for both humans and animals. Most of the food is made of fruits and seeds. For these to be formed, pollination must first take place. This process is the transfer of pollen grains from the anther, which is the male structure of the flower, to the sigma on the female structure of the flower. The transfer process requires agents to be carried out. The agents can be either biotic or abiotic. Nature perfected this arrangement between the pollination agents and the plants. As ecosystems and agricultural systems are changing, this balanced arrangement becomes disturbed. This makes it necessary that pollination systems be studied so that necessary measures can be undertaken to ensure productivity.

The chapters of this book present results in research undertaken to improve productivity in crops such as *Actinidia chinensis* (the kiwifruit), *Theobroma cacao* (cocoa), and *Manicaria saccifera* (a tropical forest palm). Some results are presented on tests to check the viability of pollen grains and the delivery of sperm cells through pollen tubes to the embryo sac. These results can serve as guidelines to any person seeking to improve pollination and productivity or to check the efficiency on pollination in ecosystems or agricultural production systems.

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