Chapter 5

Artificial Pollination in Kiwifruit and Olive Trees

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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).

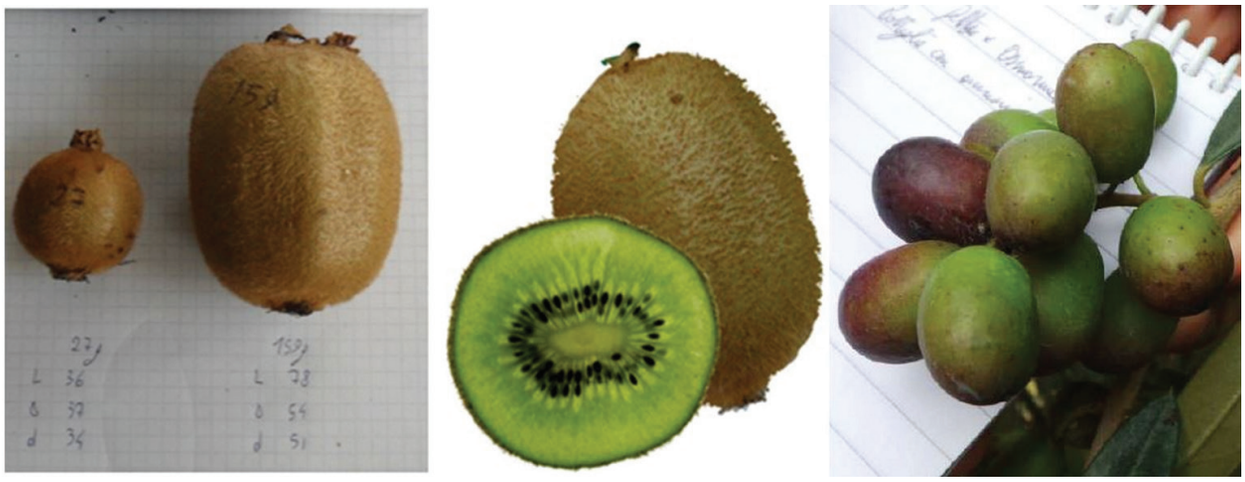

Figure 1. Kiwifruit with opposite size due to the pollination efficiency (left), perfect pollination with many pollinated ovules (center), and abundant pollination in olive (right).

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.

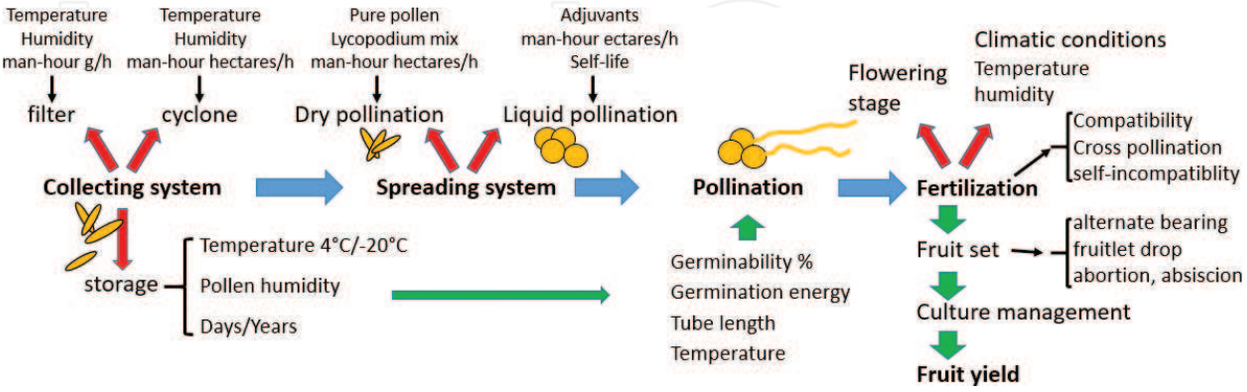

Figure 4. Flowchart of the main phases ranging from pollen collection to pollination and critical points analyzed in this chapter. Physiological aspects, climatic conditions, and human practices are integrated.

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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

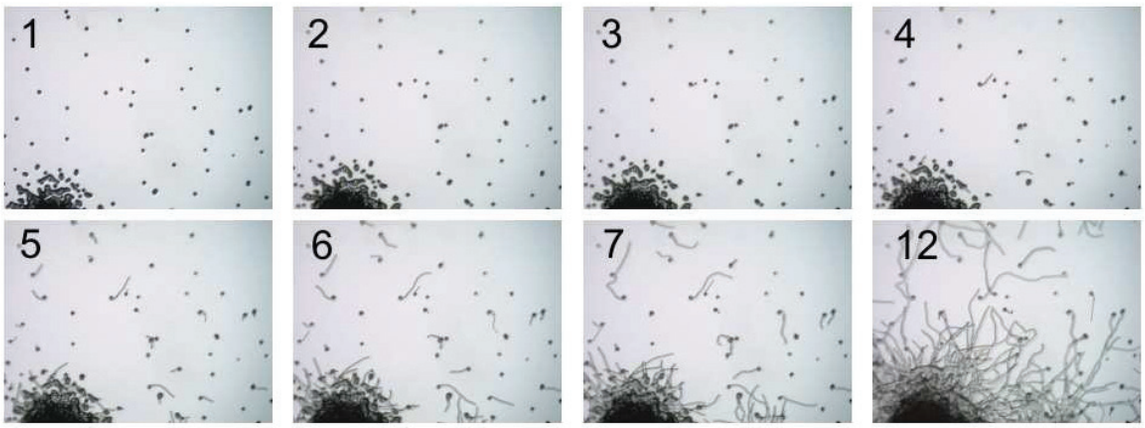

Figure 5. Time lapse during pollen germination at 20°C: the number indicates hours.

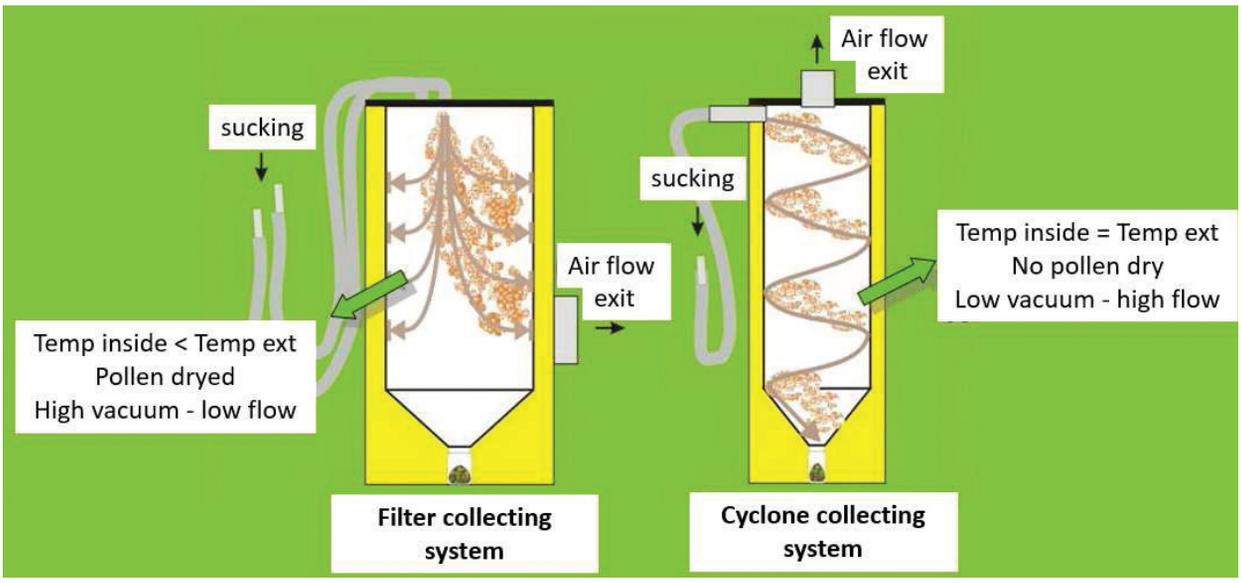

Figure 6. Schematic representation of the operating principle of the two pollen separation systems and related critical points.

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

			filter system					cyclone system				
			germ. start	2 h		12 h		germ. start	2		12 h	
N.	growth media	concentr.	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	0.9	90	8.5
3	Biotac sol.	20 ml/l	60	60	1.9	95	8.5	60	20	1.4	72	5.6
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	86	3.3	95	12	40	63	3	88	10
7	Biotac sol.	40 ml/l	60	74	2.9	94	12	60	58	2.7	88	10

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

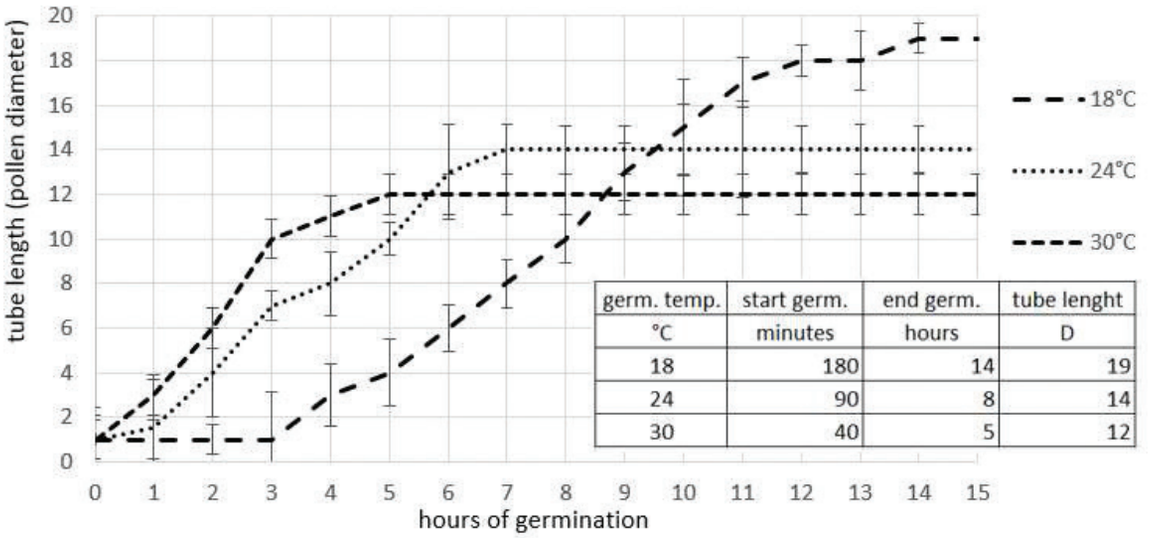

Figure 7. Dynamic of pollen germination at different temperatures and tube elongation.

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

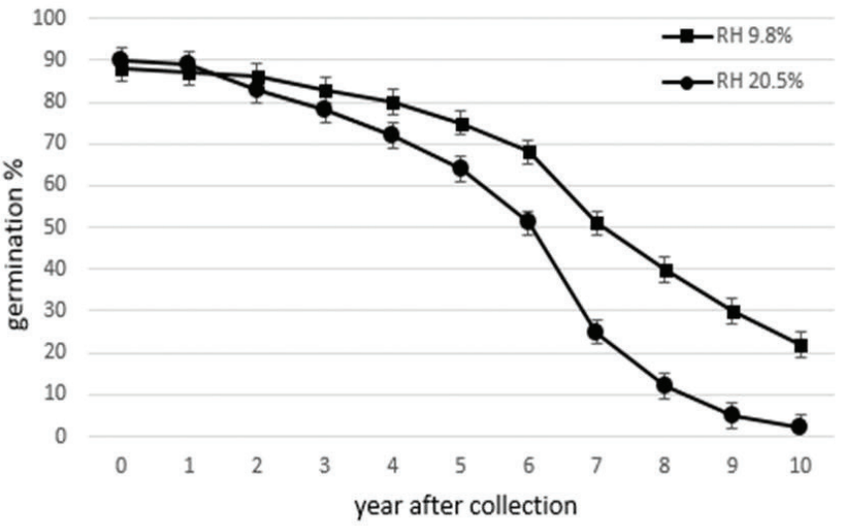

Figure 8. Pollen humidity related to collecting systems and environmental conditions (left) and effect of pollen humidity on germination during years of storage at −18°C (right).

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].

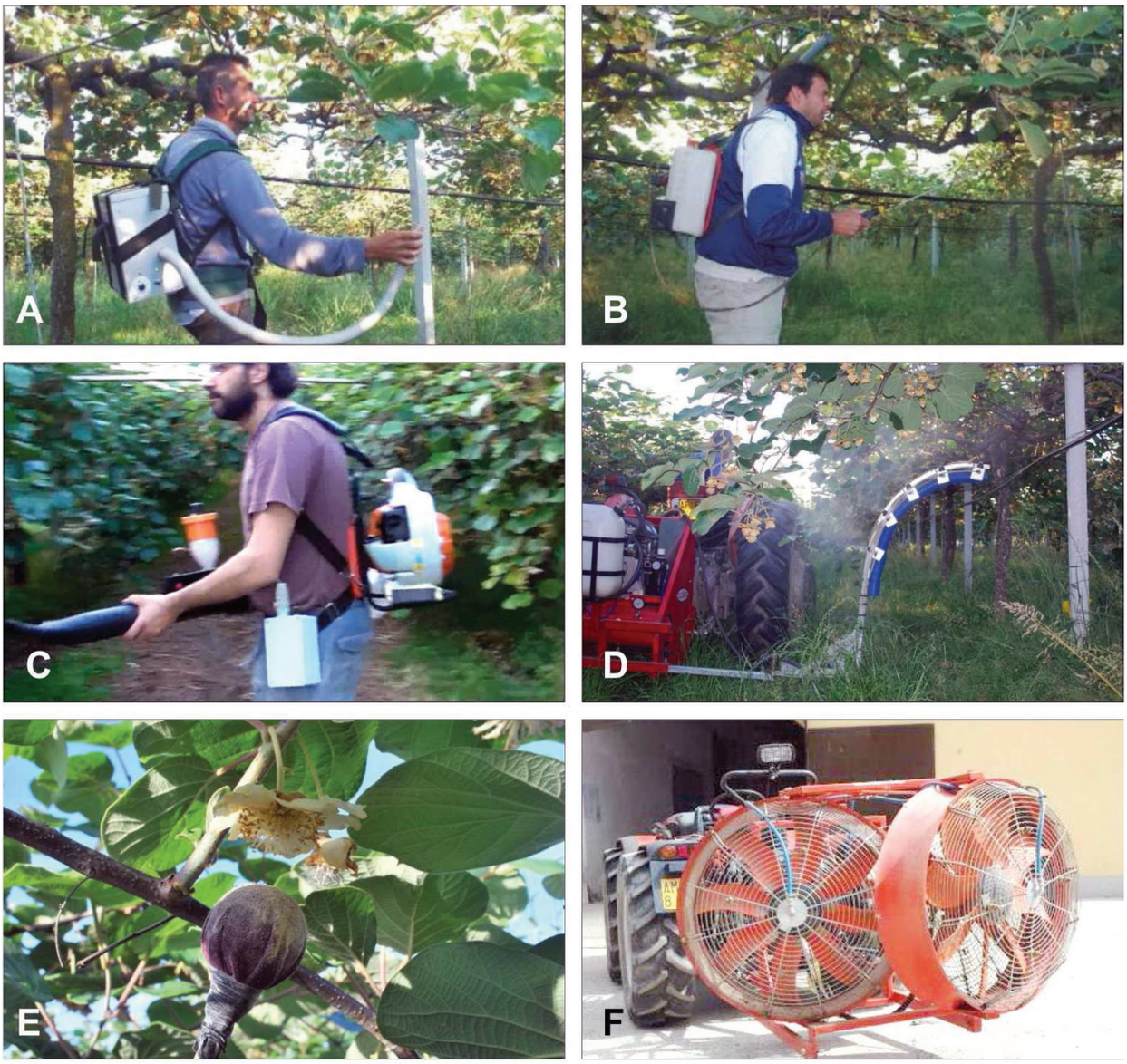

Figure 9. The commercial available equipments used in the pollination system's comparative test and their working capacity. (A) "speedy" (Dall'Agata, Forlì, Italy) is a battery dry distributor for pollen: *Lycopodium* (45–55%) mix (5–7 h/ha); (B) "ElettroEASY" (Volpi, Mantova, Italy) is a battery diaphragm 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,

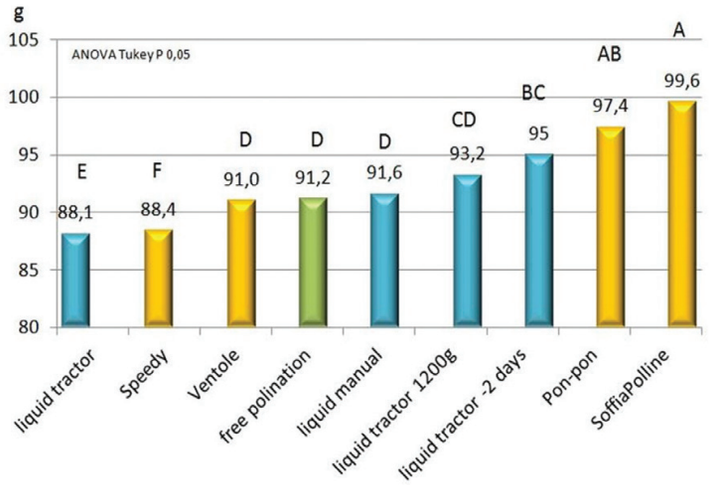

Figure 10. Average weight of the fruit pollinated with different equipments. Blue color is for liquid pollination systems: Spruzz@Polline TR 2x means double pollen dose, and Spruzz@Polline TR-2 means that the application was made 2 days before the other pollination. Yellow color is for dry pollination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

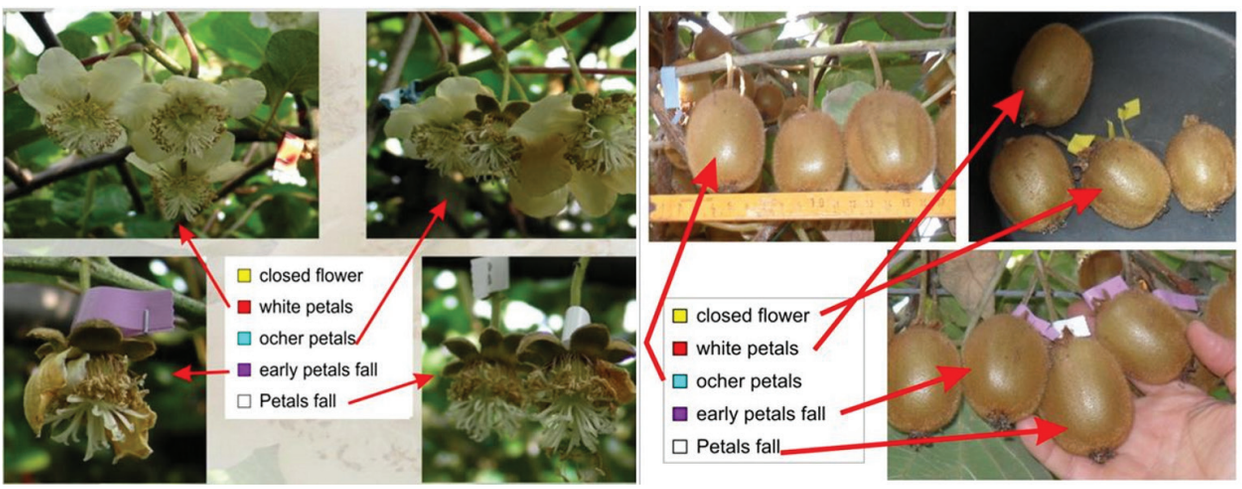

Figure 11. Fruits marked at the time of harvesting with the ribbon attached during pollination in order to go back the original flowering stage during pollination.

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

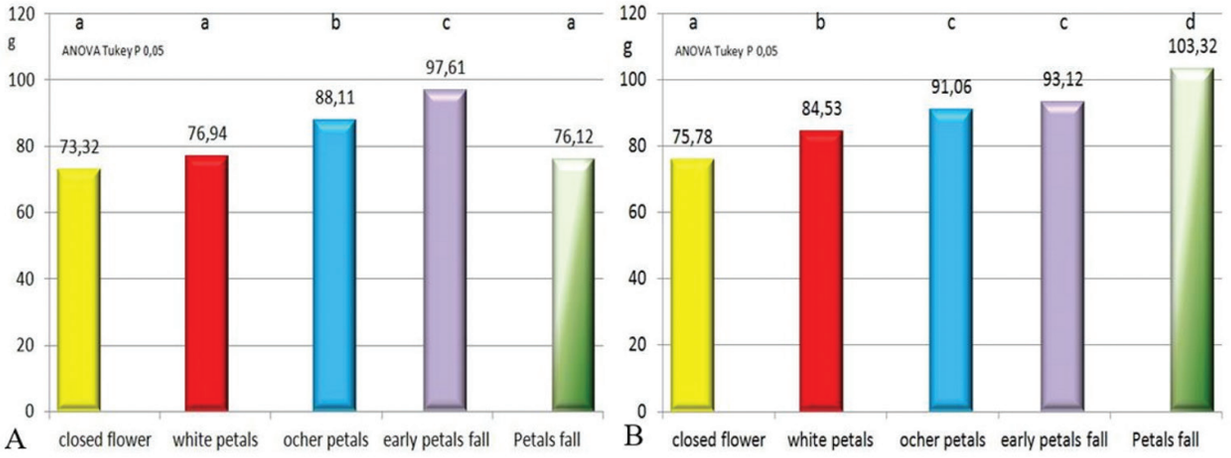

Figure 12. Average weights of the fruits pollinated by spray pollination system (A) and by dry pollination system (B). This experiment was repeated for 4 years in Verona, Cuneo, and Latina using Soffi@PollineZ for dry pollination and a sprayer diaphragm pump for liquid pollination. Different letters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

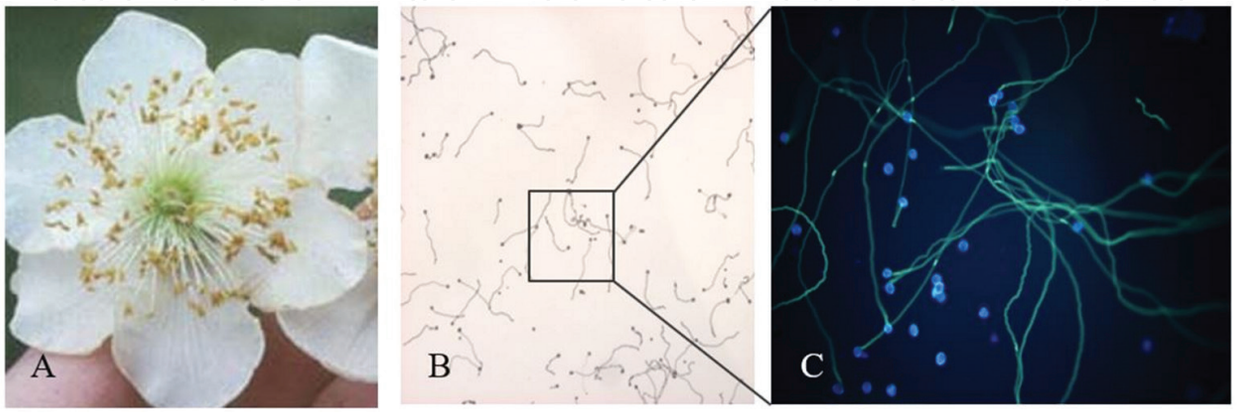

Figure 14. (A) Male flower, (B) pollen during germination under optical microscope (40×), and (C) magnification of germinated pollen stained with aniline blue under UV light (100×).

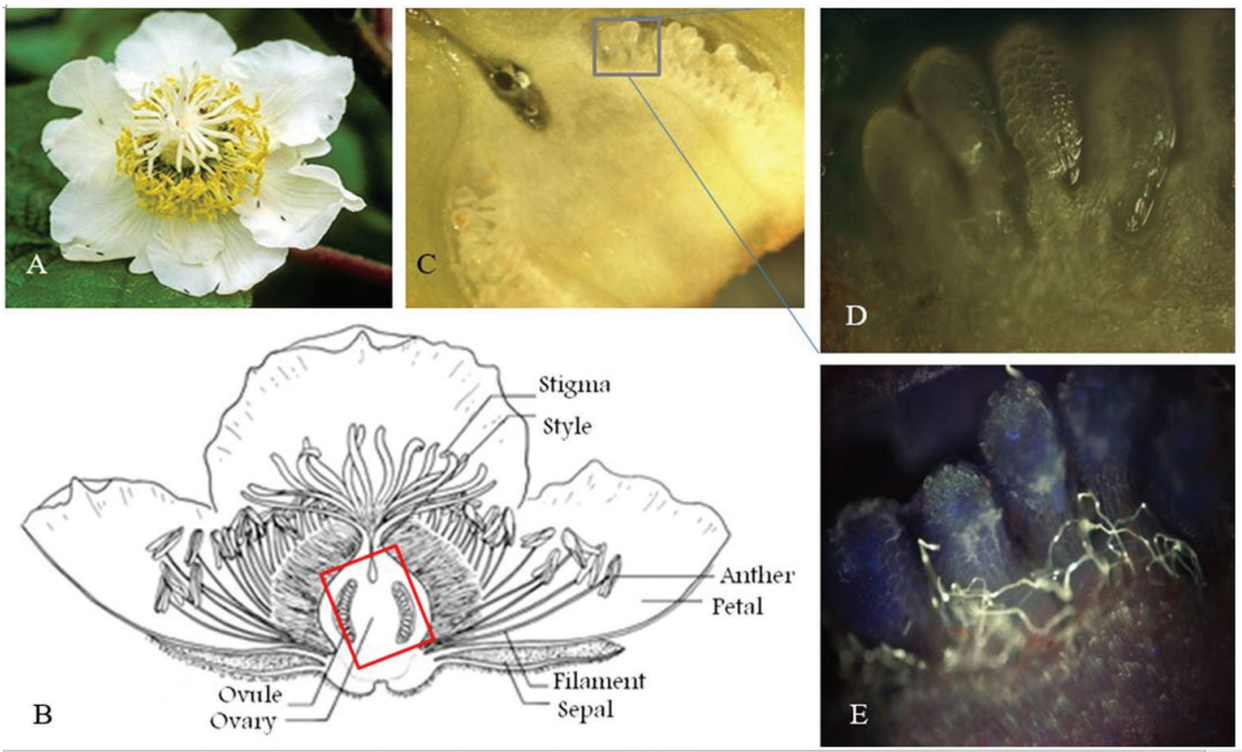

Figure 15. (A) Female flower, (B) schematic section and flower organs, (C) section of flower under optical microscope (40×), (D) magnification of ovules (100×), and (E) the same section stained with aniline blue under UV light 6 h after pollination.

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	3.68 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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