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Is fennel crop dependent on insect pollination?

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Highlights

- Fennel (*Foeniculum vulgare*) is of food and economical particular interest
- Crop production were compared between insect pollinated flowers and bagged flowers
- The fennel fruit set dropped by 92% when reproduction occurred without insect
- The fennel fruit weight and their yield in *trans*-anethole increased with insect pollination
- Insects are essential to sustain aromatic fennel production

Abstract

The importance of insects for global food crop production is starting to be well-known but the level of dependence on insects is less referenced, especially for some aromatic crops. Fennel (*Foeniculum vulgare*) is an aromatic mass-flowering crop cultivated in the southern of France for its essential oil that contains *trans*-anethole of food and economical particular interest since it is used to elaborate anise drinks. Here, we have characterized the dependence of fennel on insect pollination. We used four controlled pollination treatments to compare the reproductive success (fruit initiation rate, fruit weight), and the fennel yield (weight of the fruits and amount of *trans*-anethole at the crop level) between insect pollinated flowers and bagged flowers. We also checked the ability of reproduction by autogamy in this species, and the existence of a reproductive limitation linked to insect activity. We have shown that pollination without insect

leads to reproductive failure in fennel which is not an autogamous plant species. At the crop level, insect pollination is fundamental to improve the fruit initiation rate, the weight of the fruits and the amount of *trans*-anethole leading to an 92% dependence of fennel on insect pollination. Finally, we showed no reproductive limitation with the current state of insect activity. We are thus providing interesting clues to managers and producers to support the production of fennel crops, showing once again that insects are essential to sustain global food production.

Keywords

Fennel crops; Insect diversity and abundance; Fruit set; Fennel yield; *Trans*-anethole

1. Introduction

Animal pollination enhances the sexual reproductive success of most wild and cultivated plants (Ollerton et al., 2011). While the importance of insects for crop yields, at least to some extent, is currently well-known (Bartomeus et al., 2014; Garibaldi et al., 2017; Klein et al., 2007; Williams, 2002), the question of the role of insects still arises for some major crops, such as aromatic crops. Due to this dependence, agricultural production overall has been shown to be reduced by three to eight percent without insect pollination (Gallai et al., 2009). Crop dependence on insects is defined by the proportion of the decrease in yield when pollination occurs without insects (Klein et al., 2007; Martin et al., 2019). Insect pollination is irrelevant for reproduction in more than half of the leading global crops (for example, quinoa, lentils, pepper, or vine grape; Klein et al. 2007). In contrast, some cultivated plants are highly dependent (40-90%) on insects for their reproduction such as cucumber, avocado, almond, and peach, and others ones are almost entirely dependent (more than 90%) such as melons,

zucchini, and apples (Klein et al., 2007). In contrast, the yield of many others depends less on insects with a decrease in production varying between 10-40% (eggplant, strawberry) and 0-10% (tomato, papaya ; Klein et al. 2007), since some cultivated plants are also capable of self-pollination or wind pollination (Culley et al., 2002). In the current context, where various pressures are known to lead to a decline of insects, such as soil degradation, loss of habitats and resources, the introduction of invasive species, and agricultural intensification (Sánchez-Bayo and Wyckhuys, 2019), it is important to understand the level of dependence of crop yields on insects.

Fennel (*Foeniculum vulgare*) is a generalist entomophilous plant (Schurr et al., 2021) of particular economic interest that is cultivated in the southern of France. Fennel essential oil (EO) contains an interesting organic compound with an anise flavor, *trans*-anethole, from the phenylpropene family. This compound is mostly produced by the fruits of fennel (Salami et al., 2016). To date, few authors have explored the importance of insects for the fruit set of fennel crops (Chaudhary, 2006; Meena et al., 2015; Schurr et al., 2021). We also suspected that the production of phytometabolite compounds was directly influenced by insect activity. Indeed, self-pollination compared to cross- or open-pollination positively influences the production of fennel essential oil and some of its secondary metabolites (Salami et al., 2016). The fennel literature mentions that the plant species may be able to self-pollinate (Meena et al., 2015), but protandry is also presumed (Meena et al., 2015; Németh et al., 1999). Furthermore, insect pollination has been shown to improve fennel seed set (Meena et al., 2015; Salami et al., 2016) and the production of essential oil (Schurr et al., 2021), but the question of the degree of dependence of fennel reproduction on insects has not been explored. To sustainably support fennel production, it is important to understand and quantify the role of insect pollination in the reproduction of fennel with regard to the supposed protandry of this species.

In this study, we analyzed the dependence of fennel on insect pollination by comparing fennel pollination with or without insects for (1) fruit initiation rate, (2) the weight of fruits and (3) the fruit weight per hectare, and (4) the amount of *trans*-anethol retrieved from fruits per hectare. Finally, we verified (a) whether fennel can be autogamous based on spontaneous autogamy and controlled manual autogamy, (b) whether fennel can reproduce without insect pollination by comparing free pollination and spontaneous autogamy, and (c) whether fennel reproduction is limited by insect activity by comparing free pollination and controlled manual saturation by allopollen added under open-pollination conditions.

2. Materials and methods

2.1. Study area and plant species

The fennel (*Foeniculum vulgare*, Apiaceae) populations used in this study were located in southern France (“Plateau de Valensole”, Alpes-de-Haute-Provence; Appendix A). In this area, fennel is cultivated for local anise spirit production. This species originally came from the Mediterranean basin but has been naturalized in many regions around the world, and fennel is now cultivated practically worldwide (Europe, Asia and North America; Badgujar et al. 2014). This species can achieve 2.5 m in height and be annual, biennial or perennial (Piccaglia and Marotti, 2001). Fennel has numerous small yellow flowers organized in umbellets forming large-flat inflorescences named umbels. Each fertilized fennel flower may produce an oblong to ovoid fruit, which is called a diachene. A diachene is composed of two achenes which each can hold one seed. The studied variety is “*Jupiter*”, developed by the Pernod-Ricard France® company. This variety is sown two times a year, resulting in two summer blooms. The first bloom occurs at the end of June, and it is harvested in early August (the plants being sown in the previous year). The second bloom occurs at the end of July until harvest in mid-September

(the plants being sown in March of the current year). Twelve crops were selected for our study (Appendix A), including six from the first bloom and six from the second bloom.

2.2. Flower number estimation

For each of the 12 studied crops (six from the July bloom, six from the August bloom), we counted the number of plants per m², three times, exhaustively (N = 36). We then estimated the number of plants per hectare. Per crop, respectively 10 fennel plants from the crops blooming in July, and 5 fennel plants from the crops blooming in August, were randomly selected. The number of bloomed umbels was exhaustively counted on each selected plant (N = 95 plants). We thus could estimate the number of umbels per hectare. On each plant, we selected three umbels for which we exhaustively counted the number of umbellets per umbel (N = 270 umbels). We then counted the number of flowers per umbellet on five umbellets for each selected umbel (N = 1 350 umbellets) to obtain the mean number of flowers per umbellet. Finally, the mean number of flowers per hectare was estimated.

2.3. Controlled pollination procedure and fruit set

To study the dependence of fennel on insects, we performed four controlled pollination treatments. Two pollination treatments with insects and two without insects (bagged flowers) : (1) “open pollination” [OP] (control): the flower left free to be visited by insects (no bagging); (2) “open pollination + supplementation” [OPS]: no bagging and manual saturation by allopollen from umbels of other fennel plants (at least three meters away, in the same crop); the pollen was added by rubbing the treated umbel with another umbel; (3) “spontaneous autogamy” [SA]: bagging of the umbel to avoid visits by insects and no manual pollen addition; and (4) “manual autogamy” [MA]: bagging of the umbel and manual self-pollination with

pollen from another umbel from the same plant (*i.e.*, geitonogamy); the pollen was added by rubbing the treated umbel with another umbel from the same plant.

From each of the 12 studied fennel crops, we randomly selected ten fennel plants to apply each of the four pollination treatments. The plants were selected from crops at least five meters from the field edge. On each plant, we selected four umbels at similar stages of development (Appendix B) that were identified by a colored wool thread, and we applied either OP, OPS, MA or SA pollination treatment. All flowers of each selected umbel were treated. There were four treatments hosted on each plant. In total, 480 umbels were treated (120 per treatment, of which 60 were from the July bloom, and 60 were from the August bloom). We treated the fennel flowers from the start of the flowering of umbel outer flowers. We then repeated the treatments at least three times and continued if needed every day until the end of the flowering of the inner flowers. Using our estimation of the number of flowers per umbellet, we estimated the number of pollinated flowers for each pollination treatment. The treated umbels were then collected just before harvest: August 8-19 for the July bloom and September 6-17 for the August bloom. The rate of fruit initiation (%) was calculated by dividing the number of initiated fruits by the estimated number of treated flowers per umbel. All fruits from each collected umbel were separated from the umbel remnants by hand and then they were weighed together for each umbel. The weight per fruit was then calculated by dividing this total weight of the fruits per treated umbel by the number of initiated fruits. We then estimated the fruit weight per hectare using our estimation of the number of umbels per hectare.

2.4. Phytometabolites content

Phytometabolites from the fruits of a subsample of 220 of the total treated umbels (N=51 for the SA treatment, N=52 for the MA treatment, N=57 for the OP treatment and N=58 for the OPS treatment) were obtained from the dried fruits placed in an ultrasound bath for a few

minutes before extraction with cyclohexane solvent. For each treated umbel, some fruits (the exact number was dependent on their mass) were selected to have a constant “*extraction volume/test sample*” ratio corresponding to 0.1 g of fruit. The fruit extracts were then analyzed using a gas chromatograph (GC, 6890N, Agilent Technologies®) coupled to a mass spectrometer detector (MSD 5973, Agilent Technologies ®). We injected 2.5 µL of the extraction solution into a capillary chromatographic column (HP5-MS, 30 m length, 0.25 mm diameter, 0.25 µm film thickness, Agilent Technologies®). The molecules were separated using a gradient temperature from 40°C to 280°C at a rate of 3°C min⁻¹ with a constant helium flow of 1.0 mL min⁻¹. The accuracy and precision of the method were determined through replicate analyses (dodecane). Molecule identification in chromatograms was achieved using spectral libraries (NIST library), spectra from injected standards of high purity (>99%, Sigma Aldrich®, France), and by comparison of our experimental and retention indices calculated using a homologous series of C8–C20 *n*-alkanes. As standards, the monoterpenes α -pinene, γ -terpinene, limonene, *trans*-anethole and 1,8-cineole were chosen to quantify the final concentrations of the measured terpenoids.

2.5. Statistical analysis

To explain the fruit initiation rate, the fruit weight, the fruit weight per hectare, and the amount of *trans*-anethol per hectare, based on the pollination treatments, we used linear mixed-effects models with “log+1” transformations, including a random effect for the identity of the experimental crops (lmer; R software version 4.0.2 2020-06-22 R Core Team 2020). The significance of these models was tested with an anova test.

We then conducted *a priori* tests (contrast analysis) between (1) the SA and MA treatments to determine if fennel can be autogamous, (2) the OP and SA treatment to test the

dependence of fennel reproduction on insect pollination, and (3) the OP and OPS treatments for testing for pollen limitations due to a lack of insect activity. We tested the difference between these treatments for fruit initiation rate, the weight of the fruit and the fruit weight per hectare, and the amount of *trans*-anethol from the fruit per hectare using the “emmeans” package (R software version 4.0.2 2020-06-22 (R Core Team, 2020)).

3. Results

3.1. Flower number estimation

We found a mean of 19.87 ± 7.55 flowers per umbellet (21.52 ± 7.35 for the July bloom and 16.57 ± 6.83 for the August bloom). We calculated a mean of 19.19 ± 6.54 umbellets per umbel (22.06 ± 5.62 in July and 13.43 ± 3.96 in August) and a mean of 7.74 ± 4.21 umbels per plant (6.07 ± 1.26 in July and 11.10 ± 5.81 in August). Finally, we estimated approximately $121,100 \pm 49,000$ plants per hectare ($118,900 \pm 37,900$ in July and $111,300 \pm 50,000$ in August). We then estimated $357,402,796 \pm 10,185,981$ fennel flowers per hectare ($342,624,426 \pm 1,972,575$ for the July bloom and $274,926,539 \pm 7,857,095$ for the August bloom).

3.2. Fruit initiation rate

We found an initiation rate of fennel fruits of 37.65 ± 20.71 for insect pollination treatment compared to 5.97 ± 10.41 for the treatment of pollination without insect (38.70 ± 19.63 for the OP treatment, 36.60 ± 21.78 for the OPS treatment, 6.41 ± 9.57 for the MA treatment, and 5.53 ± 11.22 for the SA treatment). The initiation rate of the fennel fruits dropped by 92% when pollination occurred without insect. The fennel fruit initiation rate for the MA and SA treatments was close to 0%.

The linear mixed-effects model explaining the variation of the fruit initiation rate according to pollination treatments has shown a difference between treatments (F value = 247.640, p -value < 0.001). The contrast analysis showed no difference between the SA and MA treatments ($E = 0.139$, $p = 0.243$). However, there was a significantly higher fruit initiation rate for the OP treatment than for the SA treatment ($E = 2.463$, $p < 0.001$; Fig. 1). Finally, there was a significant difference in the fruit initiation rate between the OP and OPS treatments ($E = 0.260$, $p = 0.027$).

Fig. 1. Fennel fruit initiation rate (%) with a log+1 transformation for open pollination (OP; red dots) and for spontaneous autogamy (SA; blue dots) - $E = 1.291$, $p < 0.001$. The vertical bars represent the 95% confidence interval.

3.4. Fruit weight

The linear mixed-effects model explaining the weight of the fruits according to pollination treatments has shown a difference between treatments (F value = 5.945, p -value < 0.001). The contrast analysis showed no difference in the weight of the fruits between the SA and MA treatments ($E = -0.001$, $p = 0.133$) or between the OP and SA treatments ($E = 0.001$, $p = 0.121$). Finally, there was a significant difference in the fruit initiation rate between the OP and OPS treatments ($E = 0.001$, $p < 0.001$).

3.5. The weight of the fruit per hectare

The linear mixed-effects model explaining the variation of the fruit initiation rate according to pollination treatments has shown a difference between treatments (F value =

119.380, p -value < 0.001). The contrast analysis showed no difference between the SA and MA treatments for the weight of the fruits per hectare ($E = 0.058$, $p = 0.781$). We found that the weight of the fruits per hectare was significantly greater for the OP treatment than the SA treatment ($E = 2.918$, $p < 0.001$; Fig. 2). Finally, there was a significant difference in the fruit initiation rate between the OP and OPS treatments ($E = 0.481$, $p = 0.012$).

Fig. 2. Difference in the weight of the fennel fruits per hectare (kg/ha) with a log+1 transformation between the open pollination (OP; red dots) and the spontaneous autogamy (SA; blue dots) - $E = 2.918$, $p < 0.001$

3.6. Amount of anethole per hectare

The linear mixed-effects model explaining the variation of the fruit initiation rate according to pollination treatments has shown a difference between treatments (F value = 20.837, p -value < 0.001). The contrast analysis showed no difference between the SA and MA treatments for the amount of anethole per hectare ($E = -0.043$, $p = 0.866$). We found that the amount of anethole per hectare was significantly greater for the OP treatment than the SA treatment ($E = 1.443$, $p < 0.001$; Fig. 3). There was no difference between the OP and OPS treatments in the amount of anethole per hectare ($E = 0.173$, $p = 0.474$).

Fig. 3. Difference between open pollination (OP; red dots) and spontaneous autogamy (SA; blue dots) for the weight of the fruits per hectare (kg/ha) - $E = 1.443$, $p < 0.001$. The vertical bars represent the 95% confidence interval.

4- Discussion

This study analyzed the degree of dependence of fennel on pollination by insects. This is the first study linking the presence of insects to fruit set and to a metabolite of high economic value: *trans*-anethole. Our results suggest that most of the time, spontaneous and manual autogamy lead to reproductive failure. From a yield point of view, insect pollination is fundamental to improve the number and the weight of fruits and the amount of *trans*-anethole per hectare. Finally, these results showed reproductive limitations when there is a lack of insect activity in fennel crops. Our results provide some clues to support and sustain the production of fennel crops, and we believe that these results will be of interest to fennel managers and producers.

4.1. Fennel dependence on insect pollination

Our results confirmed those of previous studies showing a positive influence of insects on crop yield (Garibaldi et al., 2016, 2013; Geslin et al., 2017; Woodcock et al., 2019), and especially for fennel seeds yield (Salami et al., 2016). There was no difference between spontaneous and manual autogamy. Both of which generally led to reproductive failure suggesting that *F. vulgare* self-pollinates poorly. This implies that fennel protandry could be a barrier to reproduction success in spontaneous as manual autogamy. By contrast, insects by visiting a lot of flowers at different stages thus may counter the problems linked to the protandry of fennel flowers and optimize the reproduction. Moreover, insect pollinated flowers showed better number of fruits and amount of anethole per hectare compared to bagged flowers. We can conclude that fennel is highly dependent on insects for reproduction. Many plant species rely heavily on insects for reproduction, both in wild species with implications for conservation (Schurr et al., 2019) and cultivated species with implications for global food sustainability (Gallai et al., 2009; Geslin et al., 2017, 2016; Klein et al., 2007). Klein et al. (2007) classified fennel, based on the literature, among highly insect dependent crops (40-90%) for seed

production We have shown that dependence degree could be superior (92% of reduction in the number of fruits without insect). Nevertheless, there were still a small number of initiated fruits in self-pollination treatments. We can propose the hypothesis that protandry in fennel could be more or less expressed at different levels in a plant (umbellets, umbels, plants). This could also result from minimal but possible wind pollination (Klein et al., 2007) or from insects infiltrating in the bags without being noticed (thrips). Especially since, interestingly, the weight of the fruits was similar between open pollination and self-pollination, meaning that when a fruit is initiated, the quality of the fruit is consistent regardless of the mode of pollination. However, at the crop level, we found a drop in the weight of the fruits per hectare in spontaneous and manual autogamy.

4.2. Reproduction limitation by insect activity

The initiation rate of the fruits was similar between open pollination and open pollination with allopollen supplementation. This is also the case of the amount of anethole produced per hectare. We expected an increase of these parameters with open pollination with allopollen supplementation. It seems that in our system, the fennel does not suffer any pollen limitation. Since the reproductive success of plants is linked to the amount of pollen received (Aizen and Harder, 2007), a greater abundance of insects could lead to pollen saturation of fennel flowers. This explanation would not be surprising given the great abundance and diversity of insects along fennel crops (Schurr et al. 2021) and within fennel flowers (Bharti et al., 2015; Chaudhary, 2006; Kumar and Rai, 2020; Meena et al., 2016, 2015; Ricciardelli D'Albore, 1986; Salami et al., 2016; Shilpa et al., 2014; Skaldina, 2020). However, not all insect species may be equal in their ability to carry a large amount of pollen or higher quality pollen, two other important factors in pollination (Aizen and Harder, 2007; Ollerton et al., 2011). Insect diversity could therefore be more important than insect abundance for the

reproduction of fennel with regard to the complementarity hypothesis of functional diversity (Frund et al., 2013; Gagic et al., 2015; Schurr et al., 2021; Woodcock et al., 2019). Nevertheless, the fruit weight, and the weight of the fruit per hectare appears to be higher for open pollination treatments without manual supplementation. As we used different donors, the possibility of inbreeding or incompatibility issues are excluded. We propose three explanations for this result. First, the supplementation technique used might not differentiate the quantity and quality or maturity of pollen transported, and either of the two factors could have a greater effect on fennel reproduction. But this explanation is closely dependent on the used number of different pollen donors' plants and flowers, pollen quality increasing with the increase in genetically different donors. Second, manual pollen saturation of flowers could lead to the obstruction of the not yet mature stigma, which would imply failure of fruit formation for one of the two available seeds. Seed set is not necessary maximised with the highest pollen deposition (Ornelas and Lara, 2009). Finally, hand manipulation could damage some parts of the flower, and if the style is damaged it can impact the fruit formation (Sáez et al., 2014), or leading the plant to allocate its energy to repair the flowers rather than to the fruit development.

4.3. Amount of *trans*-anethole

Reproduction of fennel is important because the fruits are the part of the plant containing the greatest amount of essential oil (Salami et al., 2016). Fennel is cultivated for its essential oil that contains *trans*-anethole, which is used to elaborate anise drinks. The positive effect of insects on crops regarding quantity and quality of seeds and fruits and by extension their economic value, has already been shown (Garibaldi et al., 2013; Woodcock et al., 2019). This has also been demonstrated for the amount of essential oil and some metabolic contents of certain plants, such as mint and fennel (Nazem et al., 2019; Schurr et al., 2021). The literature has previously reported discrepancies about the effect of insects on fennel essential oil yield.

Indeed, insect diversity had positive influence on the amount of essential oil produced by fennel (Schurr et al., 2021). Nevertheless, other authors found that cross-pollination by insects had a negative effect on fennel essential oil yield compared to self-pollination (Salami et al., 2016). The variability of results may be due to geographically contrasting insect communities, leading to variations in pollination efficiency. It could definitively also be due to varietal differences in fennel. This study showed that the amount of *trans*-anethole from fennel fruit per hectare highly increased with insect pollination vs self-pollination. Insects are thus essential to fennel crop production.

5. Conclusion

Here is the first study compared the fennel reproductive success and the subsequent yield in the amount of *trans*-anethole between bagged and unbagged fennel flowers. We showed that the fennel crops are highly dependent on insects for fruit formation, weight and anethole production. In agricultural landscapes, insects are more and more threatened due to pesticide use and landscape uniformization (Brittain and Potts, 2011; Hendrickx et al., 2007; Kleijn et al., 2009; Vanbergen, 2021). The preservation of insects can no longer be ignored in agro-ecosystems and should be considered in landscape managements. This study proves once again that insects are essential for us to sustainably maintain the quality and diversity of global food production. In this sense, protecting insect, and biodiversity globally also means enhancing livelihood and allowing food security which are the key challenges of our century (Garibaldi et al., 2016; Kremen and Merenlender, 2018).

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Appendices

Appendix A: The 12 studied fennel crops location and elevation in Alpes-de-Haute-Provence (France).

Appendix B: Stages of the fennel umbels' development used to choose the moment of bagging,
and of pollination treatments.

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Figures

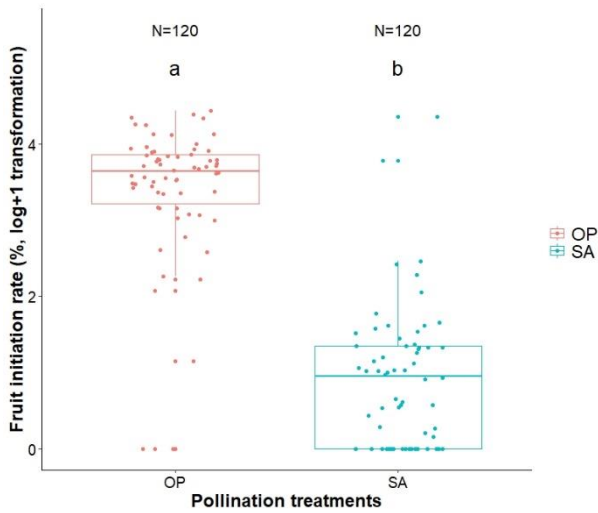


Fig. 1. Fennel fruit initiation rate (%) with a log+1 transformation for open pollination (OP; red dots) and for spontaneous autogamy (SA; blue dots) - $E= 1.291$, $p < 0.001$. The vertical bars represent the 95% confidence interval.

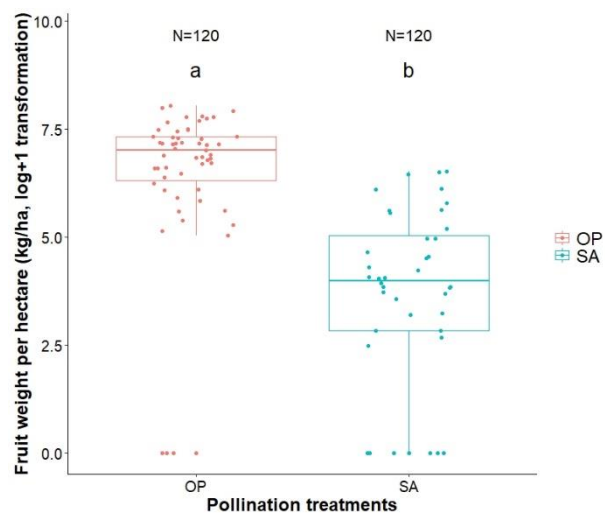


Fig. 2. Difference in the weight of the fennel fruits per hectare (kg/ha) with a log+1 transformation between the open pollination (OP; red dots) and the spontaneous autogamy (SA; blue dots) - $E = 2.918$, $p < 0.001$

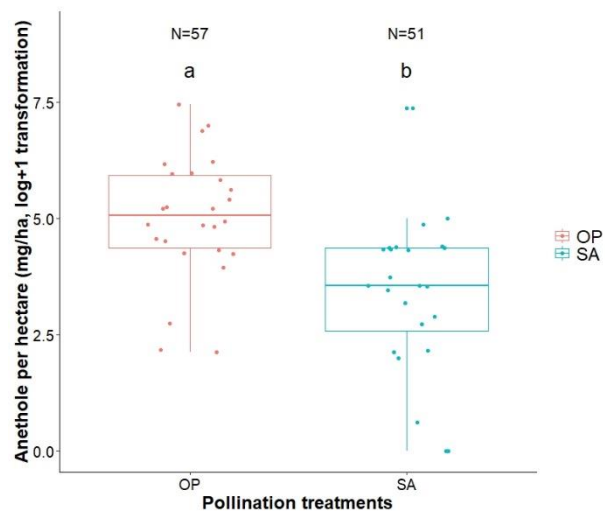
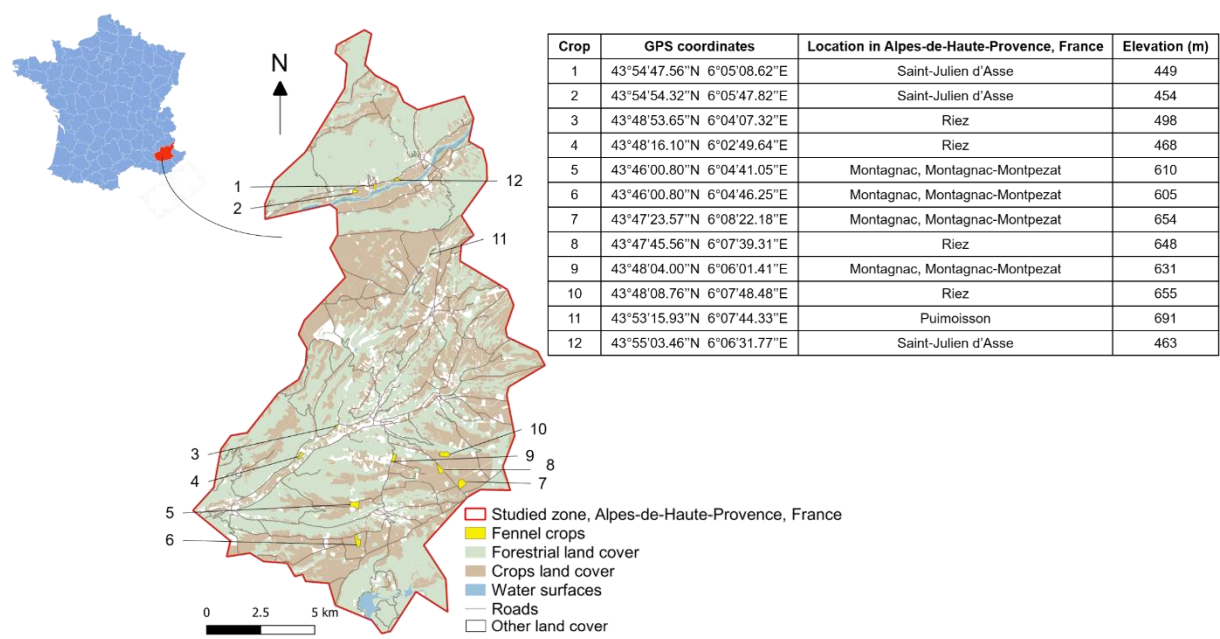
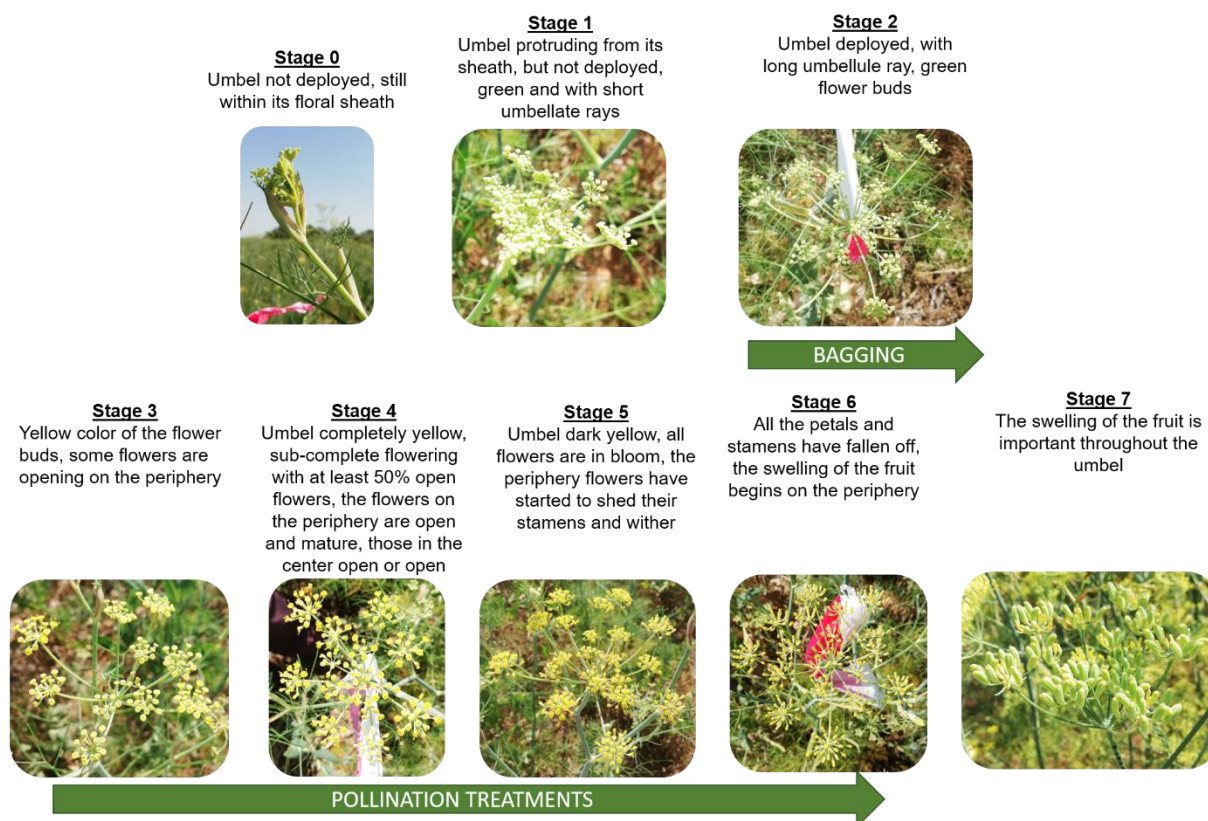


Fig. 3. Difference between open pollination (OP; red dots) and spontaneous autogamy (SA; blue dots) for the weight of the fruits per hectare (kg/ha) - $E = 1.443$, $p < 0.001$. The vertical bars represent the 95% confidence interval.



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Appendix A: The 12 studied fennel crops location and elevation in Alpes-de-Haute-Provence
526
(France).



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529 Appendix B: Stages of the fennel umbels' development used to choose the moment of bagging,
 530 and of pollination treatments.