Effects of Pollination on Floral Scent

Investigating how floral volatile compounds change in red clover variety Peggy after pollination treatments

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Effects of Pollination on Floral Scent. Investigating how floral volatile compounds change in red clover variety Peggy after pollination treatments

Effekter av pollinering på blomdoft. Undersöka hur blomdoftämnen ändras I rödklöversorten Peggy efter pollineringsbehandlingar

Sheelan Barazanji

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Abstract

Floral scent plays a crucial role in plant-pollinator interactions, influencing pollinator attraction and reproductive success. As red clover is dependent on cross pollination for seed production, it is important to understand how floral volatile organic compounds (VOCs) change in response to pollination. This study investigated the effects of various pollination treatments on the emission of floral VOCs in the red clover variety Peggy. Specifically, whether the quantity of VOCs changed in response to different pollination methods, including cross-pollination, self-pollination, mechanical shaking, and a control group, inside a greenhouse chamber at SLU. The emission of floral VOCs was also examined outside at SLU garden laboratory with two treatments. One treatment acted as a control where flower heads were covered to avoid pollination, and the other treatment was to allow pollination by locally occurring pollinators for 24 hours. Additionally, variations in flower head development from bud to full bloom to wilting among three red clover varieties: Peggy, Holly, and Yngve was also examined inside the greenhouse chamber. Finally, a seed count was conducted after the different pollination treatments from the greenhouse chamber to investigate whether pollination treatments were successful.

The results show that there were significant differences between some of the varieties in flower head development from bud to full bloom, but not from full bloom to wilting stages. This indicates that the flower head development differences between the varieties might be genetically regulated. VOCs were collected before and after the treatments, revealing a significant decline across all treatments, with the most substantial reductions observed in the cross-pollination and mechanical disturbance treatments. This was also true for the control flower heads that showed a decline in floral VOC emission. These findings indicate that the decline in floral VOCs was possibly affected by environmental factors inside the greenhouse chamber. Furthermore, the analysis of seed count showed that cross-pollination of 15 florets resulted in the greatest seed production between all treatments. In contrast, the mechanical disturbance by shaking flower head had almost no seeds. This reinforced the necessity of cross-pollination in red clover due to self-incompatibility. Future research should investigate the biochemical mechanisms behind VOC emissions and their potential implications for agricultural pollination success.

Keywords: Floral scent, red clover, *Trifolium pratense* L.*,* floral volatile organic compounds, pollination treatments, reproductive success, flowering time, cross-pollination, self-incompatibility

Sammanfattning

Blomdoft spelar en avgörande roll i samspelet mellan växter och pollinatörer genom att påverka pollinatörers attraktion och växternas reproduktiva framgång. Eftersom rödklöver är beroende av korspollinering för fröproduktion är det viktigt att förstå hur avgivningen av de volatila organiska föreningar (VOCs) från blommorna förändras som en respons på pollinering. Denna studie undersökte effekterna av olika pollineringsmetoder på avgivningen av blommornas VOCs i rödklöverblommor av sorten Peggy. Specifikt undersöktes huruvida mängden flyktiga ämnen förändrades som svar på olika pollineringsmetoder, inklusive korspollinering, självpollinering, mekanisk skakning samt en kontrollgrupp, i en växthuskammare vid SLU. Avgivning av blomdoft (VOCs) studerades även utomhus vid SLU:s trädgårdslaboratorium med två behandlingar. I en av behandlingarna täcktes blomhuvudena för att undvika pollinering och fungerade därmed som kontroll, medan den andra behandlingen innebar att blommorna var öppna för lokalt förekommande pollinatörer under 24 timmar.

Utöver detta studerades även utvecklingen av blomhuvuden från knoppstadiet till full blomning och vidare till vissning hos tre rödklövervarianter: Peggy, Holly och Yngve, i växthuskammaren. Slutligen genomfördes en fröräkning efter de olika pollineringsbehandlingarna i växthuskammaren.

Resultaten visade signifikanta skillnader mellan vissa av varianterna i utvecklingen från knopp till full blomning, men inte mellan full blomning och vissning. Detta tyder på att skillnaderna i blomhuvudets utveckling mellan varianterna kan vara genetiskt reglerade. Blomdoftprover samlades in både före och efter behandlingarna, och analysen visade en signifikant minskning av VOC-avgivning i samtliga behandlingar, med den största nedgången vid korspollinering och mekanisk störning. Detta gällde även för kontrollblommorna, som visade en minskning i blomdoftens avgivning. Dessa resultat tyder på att nedgången i VOC-emissioner kan ha påverkats av miljöfaktorer i växthuskammaren. Vidare visade fröräkningen att korspollinering av 15 småblommor resulterade i den högsta fröproduktionen av alla behandlingar. Däremot gav mekanisk skakning av blomhuvudet nästan inga frön, vilket ytterligare bekräftar behovet av korspollinering i rödklöver på grund av dess självinkompatibilitet. Framtida forskning bör undersöka de biokemiska mekanismerna bakom VOC-avgivning och deras potentiella konsekvenser för pollinationsframgång inom jordbruket.

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Abbreviations

SLU	Swedish University of Agricultural Sciences
VOC	Volatile organic compound
HP5	Hand cross-pollination of 5 florets
HP15	Hand cross-pollination of 15 florets
HPS	Hand self-pollination of 5 florets
KB	Control flower head
SK	Shaking flower head

1. Introduction

Flower scent plays a crucial role in plant pollinator interactions, serving as a key mechanism for attracting pollinators and ensuring reproductive success (Robacker et al. 1988; Lo et al. 2024; Primante 2015). Scent offers an invisible signal that can extend over distances, guiding pollinators to flowers even in dense vegetation or low visibility conditions (Raguso & Pellmyr 1998). Understanding the dynamics of floral scent composition is therefore essential for studying plant ecology and evolution, as it can reveal insights into how plants adapt their signals to changing environments and pollinator behaviours.

1.1 Red Clover

Red clover (*Trifolium pratense*) is originally from south Europe, Western Asia and North Africa, and it has since spread to temperate regions worldwide owing to its value in agriculture (Taylor & Quesenberry 1996). Its ability to fix atmospheric nitrogen, as many other leguminous plants belonging to the *Fabaceae* family, enhances soil fertility, which in turn makes it an important component in sustainable farming practices (Taylor & Quesenberry 1996). In 2013 red clover fields dedicated to seed production covered approximately 2,000 hectares of Sweden's agricultural land, making the country among the leading producers of red clover seeds in Europe (Lundin 2013). Red clover is mostly cultivated as a forage crop, offering high quality fodder for livestock (Taylor & Quesenberry 1996). This plant also offers advantages in agriculture due to its positive use in crop rotation and a high market price per kilogram of seeds (Lundin 2013).

The plant can reach 60-70 cm in height, with thin roots close to the soil surface and strong taproot from the primary seedling root, exhibiting a semi-erect growth habit (Jing et al. 2021). The plant is characterized by its trifoliate leaves. The flowers consist of densely packed florets often in a rounded arrangement and has single or double seeds in its fruit (Boller et al. 2010). It is a diploid species with a gametophytic self-incompatibility system but also bred as a tetraploid through sexual or asexual chromosome doubling (Annicchiarico et al. 2015).

Red clover has a limited lifespan due to its greater vulnerability to pests and diseases if compared to other leguminous crops (Taylor 2008). However, research suggests that tetraploid cultivars exhibit better persistence, resistance to disease and vegetative vigour (Boller et al. 2010). Additionally, they produce higher forage yields and contain greater amounts of sugars and proteins than diploid cultivars (Hedström 2019; Boller et al. 2010). Nevertheless, the widespread adoption of tetraploid red clover cultivars is limited because of its lower seed

production rates, which is 20–50% lower than of diploid cultivars (Boller et al. 2010). This limitation presents a challenge for their large-scale distribution despite their agronomic benefits (Lundin 2013).

The flower head of red clover is compact and generally found at the tip of the stems and contains between 55 and 275 florets (McGregor 1976a). It takes between 6-8 days for the flower to reach full bloom, meaning all florets fully developed (Free 1970). The development of florets starts from the base of the flower head and moving upwards (Free 1970). Understanding flower head development is important because it can affect pollinator attraction which in turn benefit seed production (Free 1970). Individual florets, can range from 7.5 to 12.4 mm in length but are only 1.6 to 2.5 mm in diameter, and each floret contains an ovary with two ovules, though typically only one develops into a seed (McGregor 1976a). Research by Dijkstra (1969) indicates that the occasional development of two seeds per ovary does not significantly impact overall seed yield (Dijkstra 1969).

Each floret consist of a staminal column, with 10 stamens and a longer stigma extending to the corolla tube's opening but remains enclosed within the keel petals (McGregor 1976a). When a bee visits a flower for nectar or pollen, it presses on the keel petals of the floret with its head. The stigma and anthers are then pushed out, and contact with the bees head is made. When the bee releases the pressure, the staminal column retracts but stays flexible and can bent multiple times (McGregor 1976a).

At the base of each of the corolla tubes of the florets nectar is produced and stored (Free 1970). Tetraploid red clover produces more nectar per floret than diploid red clover (McGregor 1976); however, due to a longer corolla tube, the nectar remains difficult for honeybees to access. This means that red clover nectar is better accessed by bees and bumblebees with longer tongues (McGregor 1976a;Free 1970).

Red clover is generally unable to self-fertilize due to a gametophytic self-incompatibility system (Taylor & Quesenberry 1996; Ulloa et al. 2003), which prevents successful self-pollination. This mechanism promotes genetic diversity by favouring cross-pollination over selfing, aligning red clover with the breeding patterns of many other outcrossing species (Ulloa et al. 2003). Being an allogamous species, diploid red clover has a strong self-incompatibility system controlled by a single S-locus, which emphasize the need for cross-pollination to produce seeds (Taylor & Quesenberry 1996). However, certain tetraploid genotypes have exhibited high self-pollination rates, likely due to intensive breeding efforts aimed at increasing seed yield (Vleugels et al. 2019). Abundant flowering was associated with higher seed yield per plant, making it a helpful indirect indicator for selecting plants with greater seed production (Boller et al. 2010). Large-scale seed production of red clover depends on insect pollinators,

particularly bumblebees and honeybees (Free 1970) and lack of pollination affects how many seeds a plant can produce (McGregor 1976b; Bommarco et al. 2011).

1.2 Floral VOCs

One important factor affecting pollinators, and consequently seed production, is floral scent (Robacker et al. 1988). This is especially true for the relationship between angiosperms and pollinating insects and their mutual dependence, which is of significant ecological importance. The production of floral volatiles, designed to selectively attract pollinators, is essential for ensuring successful pollination that leads to seed production, which in turn is of great significance in agriculture (Robacker et al. 1988).

Studies show that floral scent emission varies throughout a flower's lifespan. Typically, the highest levels of scent are produced when flowers are ready for pollination, and as the flowers begin to senesce, the emission of volatiles decreases (Lo et al. 2024). Pollination success often results in a decline (Schiestl & Ayasse 2001; Rodriguez-Saona et al. 2011) or change of floral VOC emissions to prevent further visits from pollinators (Lo et al. 2024), and this in turn direct them toward other unpollinated flowers (Schiestl and Ayasse 2001; Rodriguez-Saona, Parra, et al. 2011). Negre et al. (2003) investigated the molecular mechanisms underlying the reduction of the floral VOC methylbenzoate in petunia and snapdragons. The author explained that the reduction was regulated by different mechanisms where one was regulated by a transcriptional gene and the other by enzymatic activity. These findings emphasize the intricate regulatory processes plants employ to enhance their reproductive success and resource allocation post-pollination (Negre et al. 2003).

The release of floral scent is limited to specific flower tissues and is carefully controlled by developmental stages and timing (Lo et al. 2024). While petals are the main source of volatile compounds in flowers, other floral structures can also contribute to their emission, such as nectar, pistil and stamen (Muhlemann et al. 2014). As Raguso and Pellmyr (1998) highlights, modern techniques such as dynamic headspace analysis allow for precise measurement and identification of VOCs emitted by flowers. This helps in providing a clearer picture of these complex olfactory signals and their ecological impacts (Raguso & Pellmyr 1998).

Several studies have found that increased floral scent emission is linked to higher fitness (Raguso 2008; Schiestl 2010; Parachnowitsch et al. 2012). However, the success of pollination depends on multiple factors, including the availability and activity of pollinators, as well as flower longevity (Free 1970; Xu & Servedio 2021). Understanding what influences pollinator attraction and flower viability is therefore crucial for improving seed production.

Furthermore, variations in floral scent emissions can significantly impact pollinator preferences and visitation rates, which in turn affect plant fitness (Vega-Polanco et al. 2023). Floral longevity, the length of time that a flower remains open and functional, is a key trait influencing reproduction because it governs a plant's mating opportunities (Spigler & Woodard 2019; Xu & Servedio 2021).

1.3 Purpose and aim

Despite the agricultural importance of red clover, seed production remains a challenge (Hedström 2019), largely due to limitations in pollination success. Factors such as pollinator availability, flower longevity, and reproductive mechanisms all might contribute to this variability. Understanding how these factors interact is crucial for improving pollination efficiency and maximizing seed yield. This study aims to address some of these challenges by investigating how different pollination treatments influence floral VOC emissions. A second aim is to investigate flower head development from bud to bloom and bloom to wilt to investigate if there are differences between the three varieties.

1.3.1 Rationale for the study's research questions

Red clover seed production is highly dependent on successful pollination (Free 1970), yet multiple challenges affect pollination efficiency. These challenges include limited pollinator availability, which can lead to incomplete pollination, variations in floral longevity, which may influence how long flowers remain receptive to pollinators, and differences in reproductive mechanisms across varieties, which could impact seed set and yield.

One key aspect influencing pollination success is the emission of floral VOCs, which serve as chemical signals to attract pollinators. To understand how pollination influence floral VOC emissions, it is necessary to investigate if different pollination treatments influence floral scent production differently. Additionally, the developmental stages of the flower, from bud formation to bloom and eventual senescence, may differ between red clover varieties, potentially affecting their attractiveness to pollinators and overall seed production. Investigating these developmental patterns can help clarify whether certain varieties have advantages in terms of longevity or reproductive timing.

To address these knowledge gaps, this study explores how pollination treatments, mechanical disturbances, and environmental conditions influence floral VOC emissions and seed production. Furthermore, it examines whether flower development varies between red clover varieties, which could have implications for breeding and agricultural management strategies.

By answering the formulated research questions, this study aims to provide a better understanding of red clover's reproductive biology and identify factors that can help improve seed production efficiency.

1.4 Research questions

To explore these aspects in greater detail, this study formulates central research questions aimed at finding the relationship between pollination treatments, floral VOC emissions, and seed production. By addressing these questions, the study seeks to clarify how red clover responds to different pollination conditions and whether floral scent plays a role in signalling reproductive status.

The following specific research questions are addressed in the thesis:

1. To determine whether certain varieties exhibit advantages in longevity or reproductive timing the following question needed to be answered: Are there any differences between varieties of red clover in flowering duration, from bud development to full bloom and from full bloom to the onset of senescence?

2. Can different pollination treatments, such as cross-pollination, self-pollination and mechanical disturbance influence the emission of floral VOCs differently before and after conducting the treatments?

3. At what point can floral VOC emission get affected? Does it happen after lower or higher pollen transfer in cross-pollination processes?

4. Does the self-pollination of flowers also affect the amount of floral VOCs produced before and after?

5. Does mechanical disturbance, such as shaking the flower head, influence floral scent emission, indicating that factors other than pollination can alter VOCs?

6. How does pollination in an outdoor garden setting influence floral VOC emission compared to greenhouse conditions, and does natural insect pollination result in different emission patterns?

7. How does pollination success vary across treatments, and which treatment results in the highest seed production?

2. Method

2.1 Clover varieties and individuals

Three red clover varieties were used in this study, Yngve a diploid (2n) variety, Holly and Peggy tetraploid (4n) varieties. However, only the variety Peggy was used in the pollination treatments. The plants were two years old and dug up in May 2024 at different fields used for clover seed production in Skåne province of southern Sweden. All plants were transplanted into 10 Liter pots filled with a fertilized peat soil mixture. Ten pots of each variety were numbered and placed randomly on watering tables in a greenhouse chamber at SLU. The pots were watered twice a week. The remaining pots were placed in the garden laboratory at SLU with an automated watering program and fenced to protect from herbivores.

2.2 Comparing floral development of the three varieties

To examine if there were differences in the development of flower heads between the three varieties Yngve, Peggy and Holly, ten replicates of each variety were marked and observed daily inside the greenhouse chamber. The marking with date and individual plant and flower head was done when flower buds began to develop their first pink petals. The flower heads were observed daily and floral development documented *Appendix 2*. They were observed until they reached full bloom, where all florets fully developed. They were then observed until they reached the senescence stage by noting the first five wilting florets. The flower heads used in this experiment were not handled during observation to avoid disturbing the natural floral development. Marking flower heads was done when the florets were still undeveloped.

2.3 Pollination effects on floral VOCs

To examine the effects of pollination on volatile compound emission in red clover, four pollination treatments were conducted on the variety Peggy inside the greenhouse chamber, along with a control treatment. Each treatment was applied to eight replicate flower heads and several replicates were made on different days but at the same time of day. The same treatment did not occur on different flower heads on the same plant. All treatments were conducted manually by hand and no

pollinators were used inside the greenhouse chamber. To examine how natural pollination affect floral VOCs, two pollination treatments were conducted on the plants outside at the garden laboratory. Each of the two treatments from the garden laboratory had eight replicates. The aim of having the treatments outside was to compare how effects of pollination by natural pollinators on floral VOCs differed from manual hand pollination in the greenhouse.

2.3.1 Pollination treatments in the greenhouse chamber

Pollination experiments were conducted manually in three of the four treatments inside the greenhouse. This meant transferring pollen manually by hand from one flower head to another. For cross-pollinated florets, pollen was gathered from the flowerheads of a different plant individual. For self-pollinated florets, pollen was gathered from flowers of the same individual plant. The fourth manual pollination treatment was conducted by shaking a flower head.

By gently scraping pollen of the stamens, with a wooden toothpick, the pollen was placed on a microscope glass slide prior to each pollination procedure (figure 1). The keel on the recipient florets was carefully bent down prior to the hand pollination without removing the stamens (Boller et al. 2010). Pollen grains were then applied directly to the stigma of the recipient florets with a new toothpick. The toothpicks were replaced for each flower head. After each hand-pollination treatment, the flowerheads were left untouched for 24 hours before collecting flower scent, to allow time for fertilization to occur, which takes place between 17 to 26 hours after pollination (Bowley et al. 1984).

Figure 1. Pollen extraction and transfer onto a microscope glass slide prior to hand pollination. The two smaller pictures on the left show tetraploid red clover floret showing the wing and keel of the floret and a picture of the stamen and stigma. On the right is closeup of a sample of fresh pollen

The following treatments were conducted, (figure 2):

1. Cross-pollination of 5 florets (**HP5**)
   Five florets per flower head were cross-pollinated by hand using pollen from a different plant. This treatment was conducted to simulate a lower visitation rate level of cross-pollination, mimicking natural insect visits with limited pollen transfer. This treatment was conducted to investigate whether the pollination of five florets was enough to minimize the amount of floral volatile compounds emitted.

2. Cross-pollination of 15 florets (**HP15**)
   Fifteen florets per flower head were manually cross-pollinated, representing a higher level of cross-pollination to assess whether increased pollen transfer influences volatile emissions.

3. Self-pollination of 5 florets (**HPS**)
   Five florets per flower head were manually pollinated with pollen from the same plant. This treatment aimed to evaluate the effect of self-pollination on floral volatile production.

4. Flower head shaking (**SK**)
   Flower heads were gently shaken for 60 seconds to mimic mechanical disturbance caused by pollinators or weather conditions, assessing whether non-pollination-related movement influences volatile emissions.

5. Control flower head (**KB**)
   The control flower heads were only handled during the sampling of volatile compounds and were not exposed to any manual pollination.

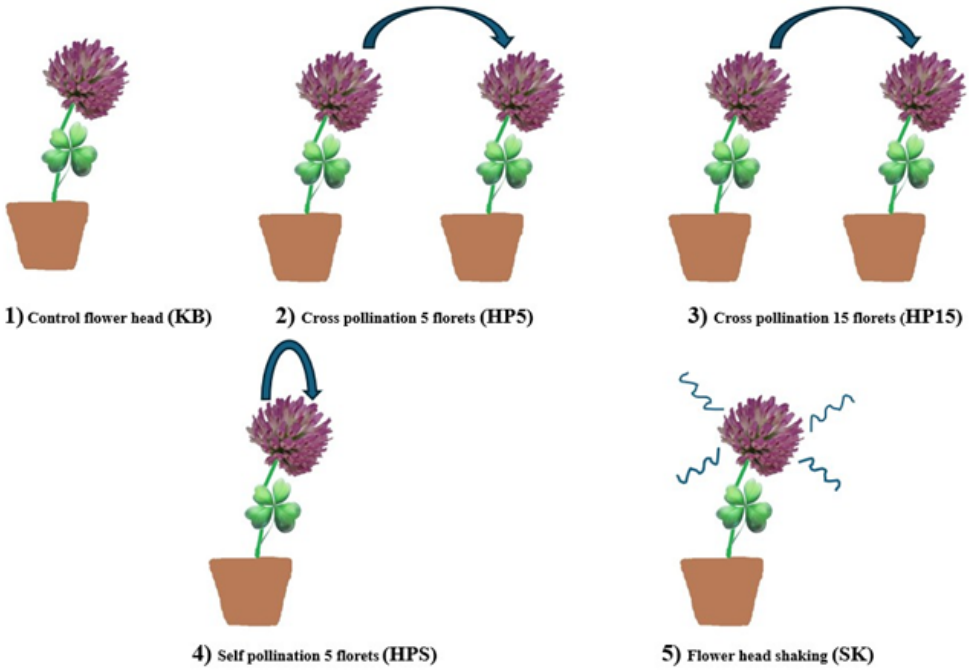


Figure 2. A graphic view of the different treatments performed inside the greenhouse chamber at SLU

2.3.2 Pollination treatments at the garden laboratory

To compare if flowers pollinated naturally by insects differ from the flowers hand-pollinated in the greenhouse, two treatments were conducted on the variety Peggy outside at the garden laboratory (figure 3). To focus on unpollinated flower heads, green buds without visible pink petals were enclosed in polyvinyl chloride mesh bags (0.2 mm mesh size), to prevent pollinator access until they reached the desired developmental stage. After the experiment, the mesh bags were reapplied to the flower heads and kept in place until the heads ripened and were harvested for seed counting.

The following treatments were conducted:

1. Protected from pollinators (**Covered**)
   Eight flowers from different plant individuals were covered throughout the whole experiment with mesh bags, except for when collecting floral VOCs. However, no pollinators were able to have direct contact with these flowers at any time.

2. Exposed to pollinators (**Open**)
   Eight flowers from different plant individuals were first covered until they reach full bloom. Pollinators had access for 24 hours only.

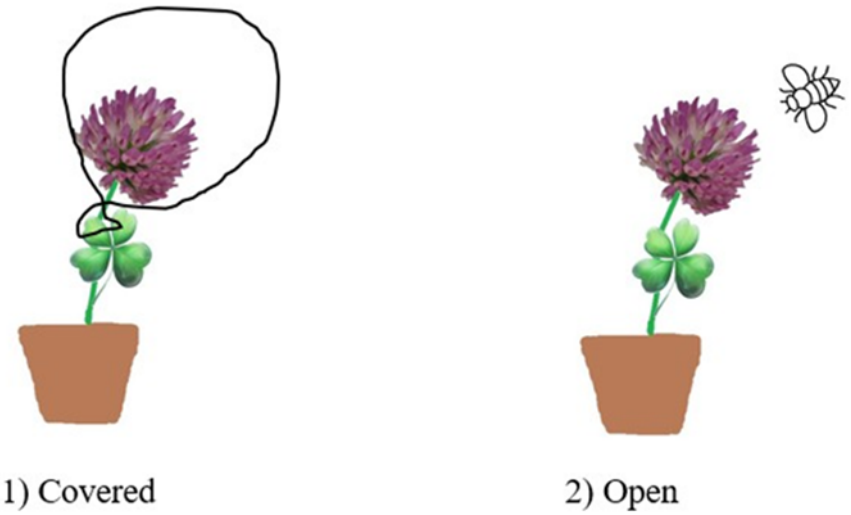


Figure 3. A graphic view of the two treatments performed outside at the garden laboratory at SLU

2.4 The design of the experiments from the greenhouse chamber and the garden laboratory

2.4.1 Collecting floral VOCs inside the greenhouse chamber

Several flower heads of the variety Peggy in different potted plants were marked with different treatments. Every plant had 5 treatments and a total number of 8 plants were used. VOCs were collected during the stage of full bloom in the summer of 2024, which occurred in May-June. Initially, potted plants were relocated to a separate greenhouse chamber to prevent contamination from other neighbouring red clover flowers that could affect the scent collection.

VOCs were collected twice from each marked flower head to be able to compare between before and after each treatment. The first collection occurred as the flower head was in full bloom (figure 4), 24 hours prior to performing the different treatments. The VOCs were collected again 24 hours after treatment application. This means that VOC collection from each flower head happened twice, first 24 hours before the treatment and 24 hours after the treatment.

Figure 4. The setup of floral VOC collection inside a greenhouse chamber at SLU

2.4.2 Collecting floral VOCs at the garden laboratory

VOCs were collected from the marked flowers at the garden laboratory during the stage of full bloom in the summer of 2024, which occurred in July.

For the flowers that would have access to pollinators, eight green buds were enclosed in mesh bags until reaching full bloom (figure 5). Floral scent was collected, then the flowers were left open for pollination for 24 hours. The floral scent was then collected after 24 hours of being uncovered and the flower heads were covered again.

For the flowers that would remain covered, eight green buds were enclosed in the same type of mesh bag until reaching full bloom. Floral scent was collected, and the flowers were covered again to prevent pollinators from accessing. After 24 hours, floral scent was collected again, and the flower heads were covered with the same mesh bags.

Figure 5. The setup outside in the garden at SLU garden lab.

2.5 Collection method of floral VOCs

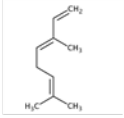
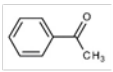
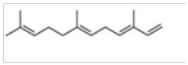
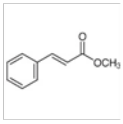
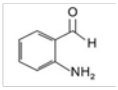
The method for collecting VOCs from flower heads involved several key steps to ensure purity and accuracy in measurements. The extraction technique is crucial in shaping the composition of the isolated volatile mixture (Stashenko & Martínez 2008). For the collection of scent from flower heads, a dynamic headspace method was employed as described by Larsson et al. (2021). The flower heads used to collect the VOCs were enclosed in large polyamide oven bags (25 x 38 cm), secured at the base of each head with a rubber coated wire. One bag was used for each flower head and the bag was discarded after VOC collection. Air was extracted from the bags using a 12V diaphragm air pump connected through PVC tubing, which drew air at a rate of 150 ml/min from one side of the bag. A small opening was cut on the opposite side of the bag allowing ambient air to enter through, ensuring continuous air exchange (Larsson et al. 2021).
The air pump was connected to a filter system within the bag, utilizing teflon tubing and filled with Porapak Q adsorbent material. The teflon (TFE) tubing, with an inner diameter of 3 mm and a length of circa 50 mm, was used to contain 30 mg of the adsorbent polymer Porapak Q (50–80 mesh). To secure the Porapak in place, rolled balls of polypropylene wool were used, along with short segments of smaller Teflon tubing (inner diameter 1.5 mm, length 2 mm) inserted into the main tube on either side of the adsorbent material. Blanks were sampled where an empty air bag was attached to a filter at the same time of each sampling occasion. This was done to determine the background contribution from the sorbent material, the surrounding air or the bag. All floral VOC collection, both from

inside and outside, was conducted between 1000-1500 h, which meant a total of five hours collection time.

2.6 Pre-selected floral VOCs

Based on previous research, this study focused on a subset of pre-selected floral VOCs from red clover, specifically those shown in (table 1). These compounds were chosen due to their consistent detection in red clover flowers across multiple studies (Buttery et al. 1984; Valentin 2022;Svensson unpublished) and as some had potential significance in affecting pollinators (Bisrat & Jung 2022).

Table 1. The floral VOCs identified in several studies of red clover flowers and are used in this study.

VOC	Cas-nr	Structure
E-Ocimene *(3E)-3,7-dimethylocta-1,3,6-triene* Monoterpene (Buttery et al. 1984)	3779-61-1	
Acetophenone *1-Phenylethanone* Aromatic ketone (Soucy 2014)	98-86-2	
E-E-a-farnesene (3E,6E)-7,11-dimetyl-3,6,10-dodekatrien sesquiterpene (Arimura and Maffei 2016)	502-61-4	
Methyl cinnamate Methyl 3-phenylpropenoate ester (Effmert et al. 2005)	103-26-4	
2-amino-benzaldehyde 2-Aminobenzaldehyde aldehyde (Lv et al. 2024)	529-23-7	
Indole 1H-Benzo[b]pyrrole aromatic heterocycle (Mathada et al. 2021)	120-72-9	

2.7 Preparing volatile extracts

After the scent collection, each filter was eluted with 2 x 150 µl of hexane with the help of a carefully introduced slow flow of nitrogen gas. Although hexane is a nonpolar solvent, which may limit its effectiveness in eluting polar compounds,

several studies had successfully used it for preparing volatile extracts (Martínez-Díaz et al. 2023; Pimienta et al. 2023). However, the prepared solutions were transferred to 1.5 ml screw-cap glass vials (figure 6) and stored at -20°C in the freezer for later analysis. Each filter was reused for multiple scent collections and carefully washed with 2 x 150 µl of hexane after each use. 10 µl Anethole solution, at a concentration of 100 ng/µl, for a total of 1 µg per sample, was added to the sample to serve as an internal standard for quantification of volatile components in the extracts.

Figure 6. Collecting the floral VOCs from the filters with hexane into 2 ml glass vials

2.8 GC-MS analysis

Before analysis, 50 µl of each of the eluted samples, including internal standard, were moved into glass vials with conical insert and secured with caps with septa (figure 7). The samples were injected via an autoinjector. N-Alkane standard solution (C7–C30) was injected before each batch of samples for calculating retention indexes.

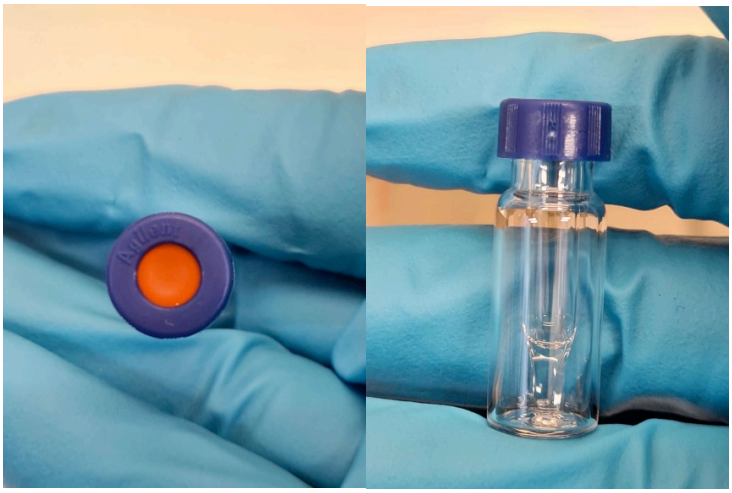


Figure 7. The glass vial with conical insert and silicon cap allowing injection.

The Gas Chromatograph was coupled with a 5977A MSD Mass Spectrometer with a polar DB-Wax capillary column with an autoinjector. The method settings were applied with an injector temperature of 225°C, an initial program temperature of 40°C held for 3 minutes, followed by an 8°C per minute increase until reaching 225°C and maintained for 10 minutes.

The gas chromatograph separates the complex extracts into individual components according to their specific retention times in the column. As the compounds elute from the column, they are immediately directed to the mass spectrometer, where they are analysed based on the unique mass fragmentation patterns of their molecules. The data analysis program used for the identification of volatile compounds was Agilent MSD ChemStation software (ver. F.01.00.1903).

The quantitative data for each compound was obtained from calculating compound peak area divided by internal standard (Anethole) peak area. This was done for each compound before and after for each of the treatments.

The chromatogram of each flower head before treatment was compared to the one after treatment (figure 8). The samples were paired so each head had two chromatograms. The peaks of VOCs in one chromatogram were compared to the same peaks in the pairing one. Blanks were also run to determine the background contribution. All flower head chromatograms were compared to the blanks chromatogram to be able to identify if any of the compounds were caused by the background.

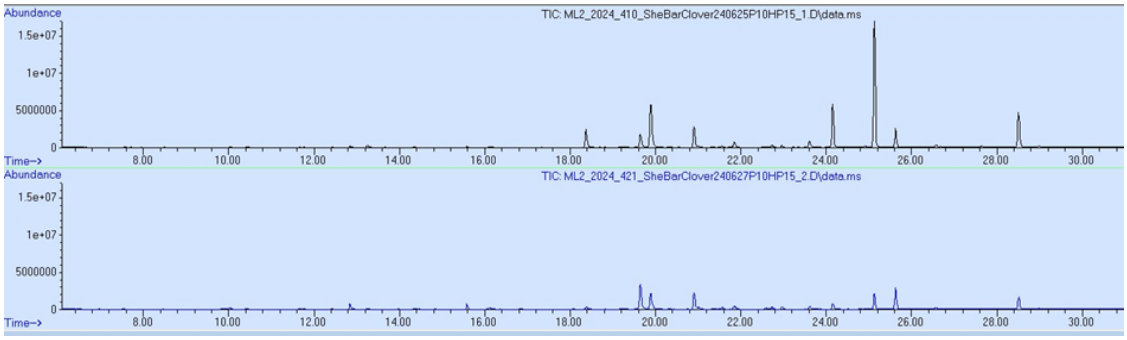


Figure 8. Chromatogram with overlay function showing peaks before and after treatment

The identification was conducted by comparing mass spectra of chromatogram peaks with reference spectra from commercial and custom mass spectral databases Alnarp 11, NIST 20 and Wiley 12 as shown in (figure 9).

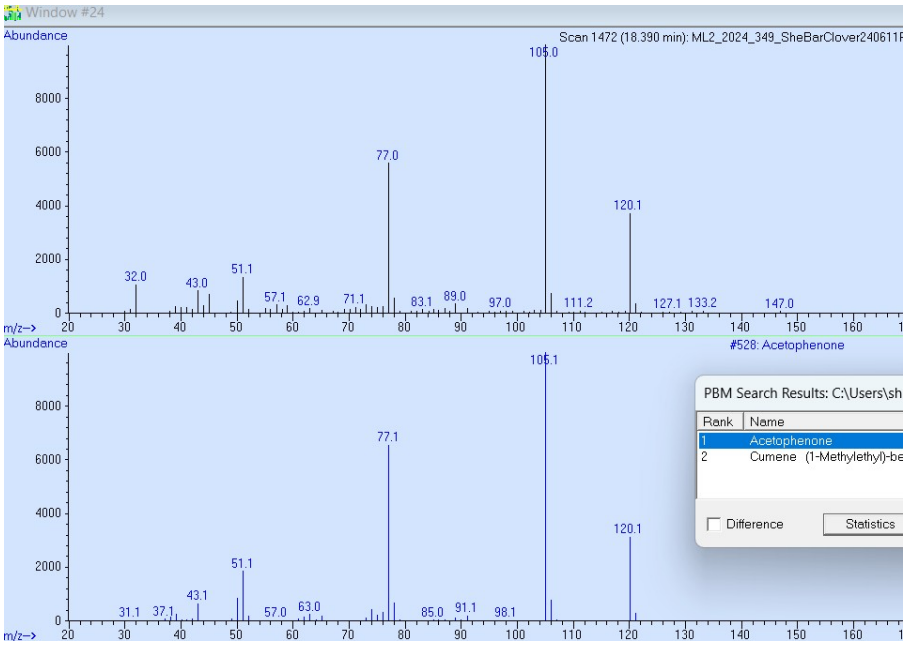


Figure 9. Comparing a peak with a reference spectrum from a database

2.9 Seed counting

To investigate the degree of seed set as a presumed effect of pollination, the seeds were counted in each treated flower head. As some flowers may have the potential to self-pollinate, treated flower heads were compared with those of controls. Flower heads were harvested once they reached full maturity, which is when they were dry, down to few centimetres of the stem. This typically occurred around 3 weeks after the florets began to wilt. After collecting, the mature flower heads

were stored in a cool and dry place for approximately three months until seed counting took place. During the counting process, florets were separated from the stem and pressed with a finger to determine the presence of a developed seed. Furthermore, the florets that contained seeds were examined under the microscope to determine if the seeds were damaged or not. The seeds could have been damaged by insects feeding on them, which may be indicated by visible bite marks, holes, or partially consumed seed tissue. Based on this assessment, the number of florets containing seeds and the number of empty florets were recorded. Unfortunately, no seed count could be carried out from the flowerheads outside from the garden laboratory since most flower heads were not found upon collection.

2.10 Statistical analysis

All statistical tests were conducted in RStudio, Version 2024.12.0 Build 467. All the boxplots were also conducted in RStudio, and the tables were created in Microsoft Excel.

To investigate if there was any significant difference between the three varieties in terms of floral development from bud to flower and flower to wilt, a one-way analysis of variance (ANOVA) was conducted. To determine between which varieties there were significant differences, a Tukey's HSD post hoc test for pairwise comparisons was used after the ANOVA. This test allowed the comparison of the means between different varieties to determine which specific groups are significantly different from each other.

For the floral VOCs data, a Shapiro-Wilk test was conducted to determine the normality of the data. Because the data was not normally distributed, non-parametrical tests were used. Wilcoxon signed-rank non-parametric test was run for the data of the identified floral VOCs before and after treatments. This test determined whether the amount of each floral VOC significantly differed before and after each of the treatment. The results from the tests were considered significant if the p-value was < 0.05. The results were presented in boxplots and tables of p-values.

The percentage reduction in floral VOCs between before and after the treatment was calculated using the formula: (VOCs before – VOCs after) / VOCs before. Another non-parametric test, the Kruskal-Wallis test, was then used to determine whether at least one treatment differed significantly from another. However, this test does not indicate which specific treatments are different. To further investigate this, Dunn’s post hoc test with Bonferroni correction was conducted to identify the differences while controlling for multiple comparisons.

To assess how the seed count differed between treatments, a Dunn's test was performed. A non-parametric post-hoc test used to compare multiple treatment

groups following a Kruskal-Wallis test, to identify which specific pairs of treatments show significant differences.

3. Results

3.1 Floral development of the three varieties

The ANOVA test showed a statistically significant effect of variety on bud to bloom time ($F = 7.329$, $p = 0.00287$), while no significance of variety on bloom to wilt ($F = 1.301$, $p = 0.289$). Furthermore, Tukey's post-hoc test showed that the significant effect of variety was between Peggy (b) – Holly (a), and Yngve (b) – Holly (a) from bud to bloom, however, no significance was noted between Peggy (b) – Yngve (b). As seen in (table 2), no significance was noted between the three varieties Peggy (a), Holly (a), and Yngve (a) from bloom to wilt.

Table 2. Summary of flower head development for Peggy, Holly and Yngve varieties, including the mean times (in days) from bud to bloom and from bloom to wilt, along with the associated standard deviations (±SD). The statistical groupings for both stages indicate that different letters (a and b) show significant differences between the varieties.

Variety	N	Mean bud to bloom ±SD	Mean bloom to wilt ±SD	Statistical grouping bud to wilt	Statistical grouping bloom to wilt
Peggy	10	7.0 ± 0	5.10 ± 0.32	b	a
Holly	10	7.70 ± 0.49	5.20 ± 1.23	a	a
Yngve	10	6.90 ± 0.70	6.0 ± 2.0	b	a

3.2 Analysis of floral VOCs

The samples processed in the GC-MS system showed that several different VOCs were collected from the flower heads of the cultivar Peggy. The VOCs included both known and unknown compounds. However, this study focused on examining only pre-selected compounds. There were however other peaks that could not be assigned because their mass spectra were those of mixed state or related to the green parts of the plant. Nonetheless, the VOCs that could be identified were analysed for quantitative variation among the different treatments. E-ocimene, acetophenone, E-E-α-farnesene, methyl cinnamate, 2-amino-Benzaldehyde and indole were used to investigate the differences in floral scent before and after the performed treatments in this study.

The analysis of floral VOCs from the flowers of red clover treatments in the greenhouse revealed an overall decline in compound concentrations following treatment (figure 10). Across all treatments, a reduction in emission levels was observed for many of the identified volatiles.

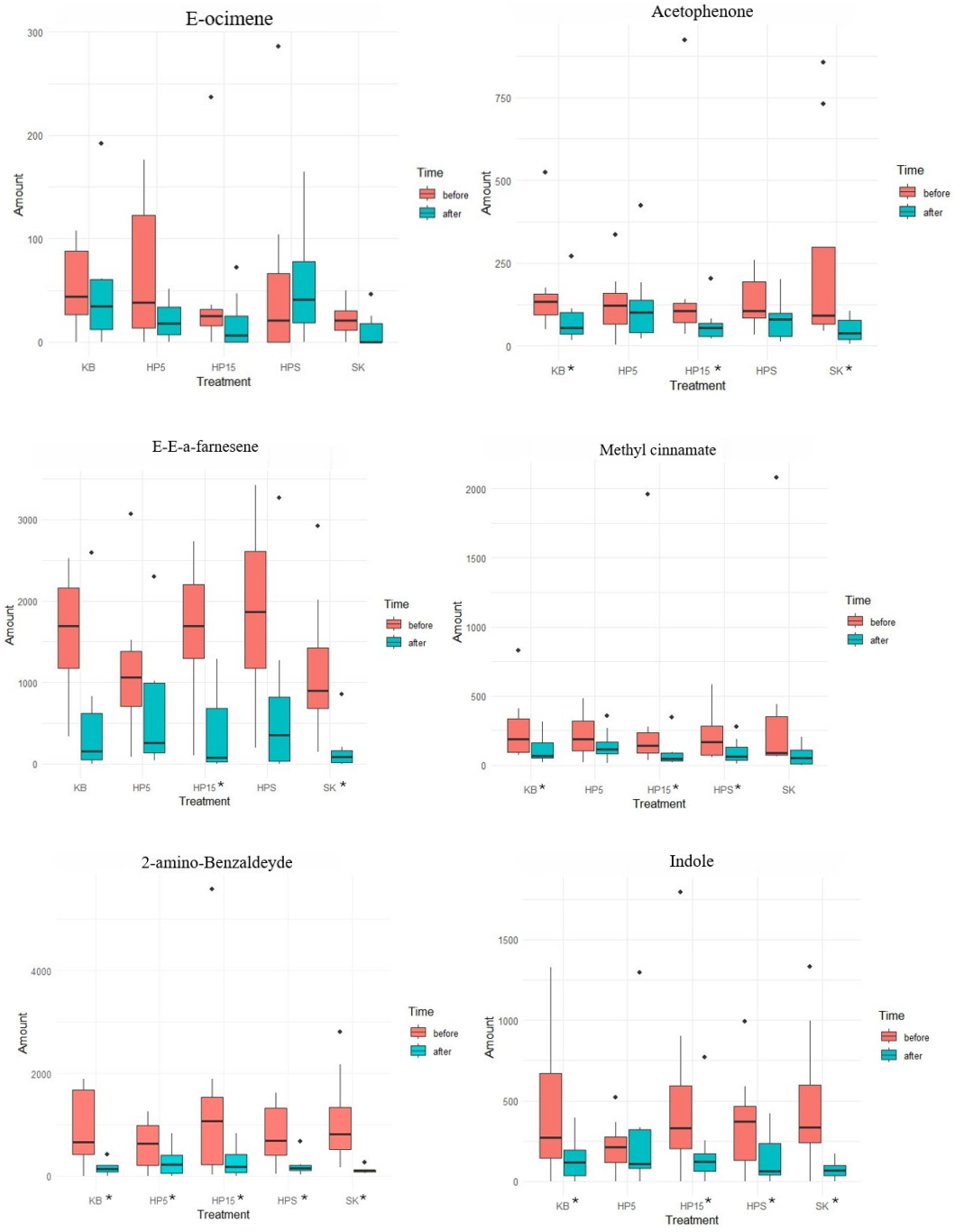


*Figure 10. Boxplot showing the amount of VOCs before and after the different treatment inside the greenhouse chamber at SLU. * indicates treatments with significance ($p < 0.05$) between before and after. Treatment IDs: KB = Control, HP5 = Hand pollination with 5 florets, HP15 = Hand pollination with 15 florets, HPS = Hand self-pollination with 5 florets, , SK = Shaking flower head.*

When investigating how each floral VOC varied between before and after treatment, certain VOCs exhibited a more evident decline than others after treatment. The Wilcoxon signed-rank non-parametric test confirmed significant differences before and after cross pollination of 15 florets (HP15) treatment for acetophenone, E-E-α-farnesene , methyl cinnamate, 2-amino-Benzaldehyde and indole, with p-values ranging from 0.0391 to 0.00781 (table 3).

The most substantial decline was observed for acetophenone and 2-amino-Benzaldehyde in shaking flower head treatment (SK) and for E-E-α-farnesene and methyl cinnamate in cross pollination of 15 florets treatment (HP15) ($p = 0.00781$).

Furthermore, cross pollination of 5 florets (HP5) and self-pollination of 5 florets (HPS) displayed a more moderate decline. However, since the control flowers (KB) also showed significant differences for acetophenone, methyl cinnamate, 2-amino-Benzaldehyde and indole with p-values ranging from 0.0156 to 0.036, it seems that it is not possible to conclude that any differences were due to the treatments.

*Table 3. Results from Wilcoxon-signed rank test showing p-values of the treatments from inside the greenhouse chamber for each of the identified floral VOCs. * indicates statistically significant differences between before and after each treatment. Treatment IDs: KB = Control, HP5 = Hand pollination with 5 florets, HP15 = Hand pollination with 15 florets, HPS = Hand self-pollination with 5 florets, , SK = Shaking flower head.*

VOC	KB	HP5	HP15	HPS	SK
Eocimene	0.675	0.0759	0.529	0.529	0.205
Acetophenone	0.0156*	0.383	0.0391*	0.0547	0.00781*
E-E-a-farnesene	0.0781	0.25	0.00781*	0.0547	0.0391*
Methyl cinnamate	0.0156*	0.0547	0.00781*	0.0391*	0.109
2-amino-Benzaldehyde	0.036*	0.0346*	0.0156*	0.00781*	0.00781*
Indole	0.036*	0.673	0.0225*	0.0225*	0.0225*

Nevertheless, the Kruskal-Wallis test performed for each compound to assess whether the decrease in emission levels differed significantly between treatments, indicated that for most compounds, there were no statistically significant differences among treatments ($p > 0.05$). But, for indole, a significant difference was observed ($\chi^2 = 12.396$, $df = 4$, $p = 0.01463$), suggesting that its reduction in emission varied depending on the treatment applied. Dunn's post-hoc test with Bonferroni correction showed that the decrease in indole emission was significantly different between HP5 and SK (adjusted $p = 0.00617$).

Results for the remaining compounds, including E-ocimene, acetophenone, E-E-α-farnesene, methyl cinnamate and 2-amino-benzaldehyde, did not reveal statistically significant differences among treatments, although some compounds, such as 2-amino-benzaldehyde, approached significance. These results suggest that while a general decline in floral VOC emissions was observed, the extent of

reduction was largely consistent across treatments, apart from indole, which showed a treatment-dependent response.

Observations of the treated flower heads showed that the florets used in pollination treatments (HP15, HP5 and HPS) wilted within 24-48 hours after the treatment. Furthermore, the SK treatment flowers also showed wilting of most florets 24-48 hours prior to the treatment application. The control flowers (KB) did not show any signs of wilting within 24-48 hours (figure 11).

Figure 11. Wilting patterns occurring 24-48 hours after HP5=hand pollination with 5 florets, HP15= hand pollination with 15 florets, HPS= hand self-pollination, and SK=shaking flower head treatments. KB=Control flower not showing signs of wilting within 24-48 h compared to the rest of the treatments.

Unlike the greenhouse flowers, the control (Covered) flowers in the garden laboratory at SLU, which remained unpollinated, did not exhibit a general reduction in floral VOC emissions over time. However, results from the Wilcoxon test revealed that significant differences in all floral VOCs were observed exclusively in flower heads that were exposed to insect pollinators (Open) for 24 hours, between before and after pollination as seen in (table 4) and (figure 12).

*Table 4. Results from Wilcoxon-signed rank test showing p-values of the treatments from the garden laboratory for each of the identified floral VOCs. * indicates statistically significant differences between before and after each treatment. Treatment IDs: Covered= no pollination was allowed, Open= open for pollination by locally occurring pollinators for 24 h.*

VOC	Covered	Open
Eocimene	0.0391*	0.0156*
Acetophenone	0.195	0.0156*
E-E-a-farnesene	0.0592	0.036*
Methyl cinnamate	0.641	0.00781*
2-amino-Benzaldehyde	0.547	0.00781*
Indole	0.208	0.00781*

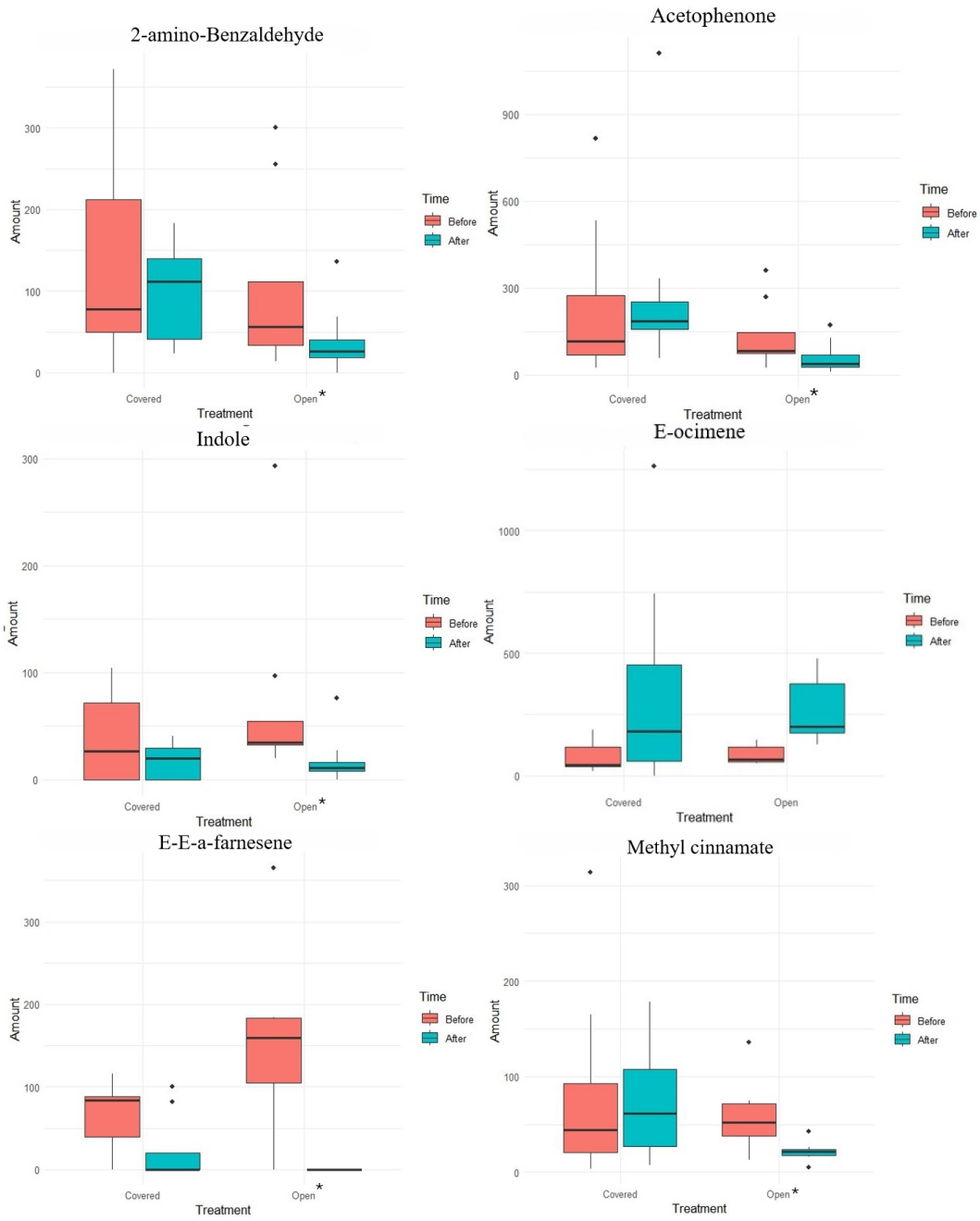


*Figure 12. Boxplot showing the amount of VOCs before and after the different treatment at the garden laboratory at SLU. * indicates treatments with significance ($p < 0.05$) between before and after. Treatment IDs: Covered= no pollination was allowed, Open= open for pollination by locally occurring pollinators for 24 h.*

However, The Kruskal-Wallis test to assess whether the reduction in floral VOC emissions differed between treatments in the outdoor environment, showed statistically significant differences between treatments (Covered and Open) for acetophenone, methyl cinnamate and 2-amino-Benzaldehyde.

3.3 Seed count

The results of Dunn's test to assess seed count (figure 13) revealed that amount of seeds after the treatment HP15 was significantly higher than HPS, KB, and SK, while all other treatment comparisons showed no significant differences. The HP5 treatment gave lower seed count than HP15, and higher seed count than the treatments HPS, KB and SK. However, HP5 was not significantly different from any of the other treatments. The significance ($p < 0.05$) was as follows, HP15 and HPS ($p = 0.000435$), HP15 and KB ($p = 0.000080$), and HP15 and SK ($p = 0.00392$).

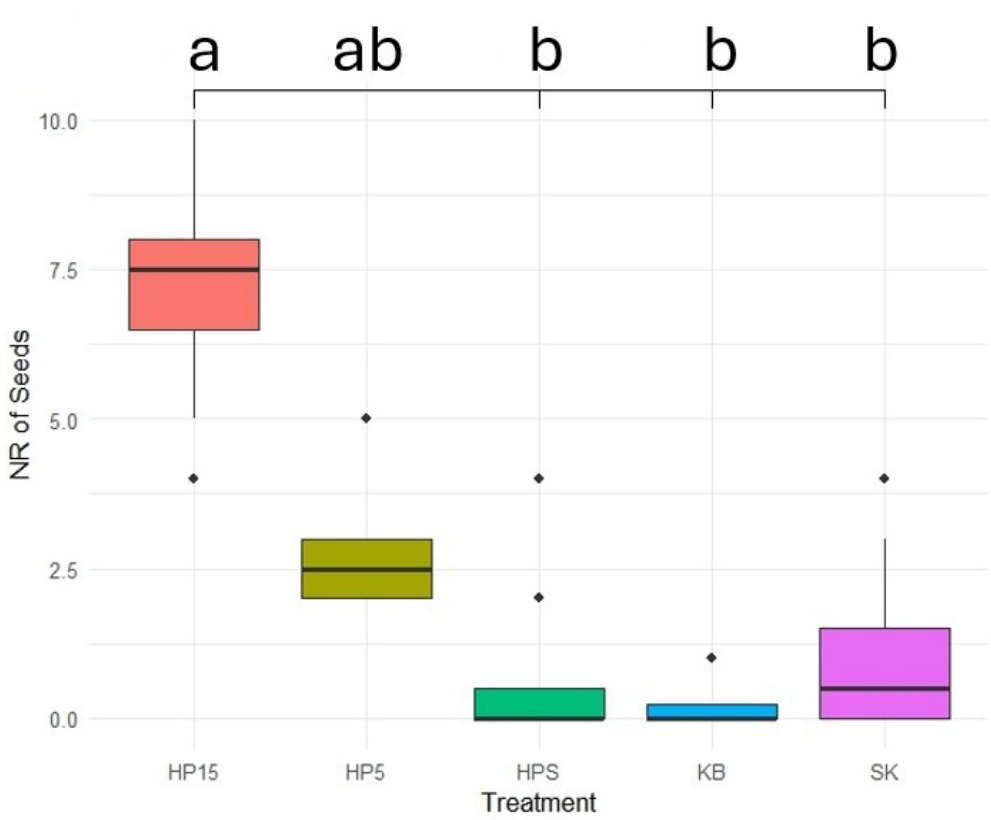


Figure 13. Boxplot showing seed count differences between the different treatments. HP15 (a) significantly different from HPS, KB, and SK (b), while HP5 (ab) not significantly different from any of the other groups. Treatment IDs: KB = Control, HP5 = Hand pollination with 5 florets, HP15 = Hand pollination with 15 florets, HPS = Hand self-pollination with 5 florets, , SK = Shaking flower head.

4. Discussion

The purpose of this study was to investigate the reproductive biology of red clover, focusing on variations in flowering duration among different varieties, the effects of pollination treatments on floral VOC emissions, and the relationship between pollination success and seed production. By examining how flowering time and how different pollination methods impact floral scent modulation, the study aimed to provide insights into plant-pollinator interactions and understand how complex these processes are. The findings contribute to a better understanding of pollination efficiency, floral signalling, and breeding strategies to optimize red clover cultivation. Each research question is addressed below, with a discussion of the key results and their implications.

The differences noted in flowering time between varieties, specifically, the delayed transition from bud to bloom in Holly compared to Peggy and Yngve, suggest that there is genetic variability between the varieties. Genetic factors influence not only the timing of flowering but also other developmental processes, such as flower longevity and wilting (Spigler & Woodard 2019). This could have implications for agricultural practices, as variety selection may influence not only yield potential but also the efficacy of pollination processes (Xu & Servedio 2021). Understanding how these timing differences affect pollinator behaviour and subsequent fertilization success could help refine cultivation strategies for red clover and similar crops. The absence of significant differences during the bloom-to-wilt phase suggests that, once fully developed, all three varieties have similar flower longevity. This is important because it indicates that none of the varieties are particularly sensitive to an early end of receptivity or flowering.

Interestingly, the results from the garden laboratory show that the control (Covered) did not show a significant reduction in scent, while those exposed to pollinators displayed a significant difference in the levels of floral VOCs emission before and after pollination. Notably, this is the first documentation of floral scent reduction occurring within 24 hours after pollination in a natural setting of a red clover cultivar. Previous studies have primarily focused on the 48-hour interval (Svensson unpublished; Emelianova 2024) meaning these results contribute new insights into how quickly this process can take place in natural environments. This observation also supports the hypothesis that flowers actively regulate their scent in response to pollination and that this mechanism may be adapted to optimize resource allocation (Lo et al. 2024). Studies on Nicotiana attenuata and Petunia axillaris have shown similar patterns, where scent diminishes following successful pollination (Kessler & Baldwin 2007; Muhlemann et al. 2014). These studies provide a theoretical basis for the results of this study and emphasize the importance of further investigating this process in natural settings. Future research

could investigate whether this rapid response is a general phenomenon or specific to certain plant-pollinator interactions.

However, in the greenhouse chamber there was a substantial decline in VOC emissions across all treatments inside the greenhouse, particularly following cross-pollination and mechanical disturbance. This aligns with existing theories that suggest a decrease in floral scent after pollination acts as a signal to redirect pollinators to unpollinated flowers (Schiestl & Ayasse 2001). Nevertheless, the decline of floral VOCs in the control (KB) suggests that the decline was affected by the time factor or other factors such as the climate inside the chamber, rather than the different treatments.

This study also confirmed the self-incompatibility nature of red clover, which necessitates cross-pollination for successful fertilization (Taylor & Quesenberry 1996; Ulloa et al. 2003). The lack of seed formation in the self-pollination and shaking treatment reinforced this, as insufficient pollination prevented the reproductive process. This also indicated that mechanical disturbances alone are insufficient for successful fertilization in this species. The lack of seeds after the treatments though, emphasizes the importance of ecological interactions and pollinator visits in the reproductive success of red clover (Free 1993).

Previous research has shown that self-incompatibility mechanisms in plants, such as those found in red clover, are crucial for maintaining genetic diversity and avoiding inbreeding depression (Barrett 2002). Studies on *Trifolium pratense* have demonstrated that pollinator-mediated cross-pollination increased seed set and overall reproductive success (Taylor & Quesenberry 1996).

Considering these findings, future research should focus on the biochemical mechanisms that drive the observed changes in floral VOCs and how environmental factors interact with these processes. By expanding our understanding of how floral traits influence pollination outcomes, we can develop more effective agricultural practices that promote biodiversity and sustainability.

Overall, this study contributes valuable knowledge to the field of horticultural science, offering a foundation for enhanced practices in crop management and a deeper appreciation of the ecological processes that underpin plant reproduction.

4.1 Limitations

Although the study provides valuable insights into the relationship between pollination treatments and floral VOC emissions in red clover, several limitations should be noted.

One primary limitation is the exclusive focus on a single red clover variety, Peggy, for the pollination treatments. This approach facilitated controlled comparisons of VOC responses; however, it remains uncertain whether the observed trends would be replicable in other red clover varieties. Different

cultivars, such as tetraploid and diploid types, may possess distinct floral traits or scent profiles that could influence post-pollination VOC emissions. Future research should aim to explore these variations across different cultivars to develop a more comprehensive understanding of VOC dynamics in red clover.

Environmental factors in the experimental setting also present a limitation. The pollination treatments were conducted in a greenhouse chamber where light, and temperature were variables that might have influenced the outcomes. The VOC emissions observed were specific to the conditions present during the experiments, and these factors could affect both flower development and subsequent VOC emissions. The challenge of conducting successful hand pollinations in the field is also a substantial limitation. Ensuring that manual pollination effectively mimics natural pollination conditions is difficult, which may introduce variability in results.

Additionally, these findings highlight the potential difficulty of performing pollination studies in controlled environments, such as greenhouses, where external pollinators are excluded. Although greenhouse studies can be valuable for isolating specific variables, it is important to confirm that flowers exhibit natural physiological responses under these conditions before drawing conclusions. If flowers do not behave as they do in the field, the results of controlled pollination experiments may not accurately reflect natural reproductive processes. Future studies should verify that floral scent production, receptivity, and fertilization success remain consistent across different experimental settings before applying greenhouse-derived conclusions to field conditions.

Furthermore, the methodology for VOC collection posed challenges in excluding leaves and stems from the sampling process. For example, E-ocimene, a compound that may be emitted not only by flowers but also significantly from leaves and seed pods (Buttery et al., 1984), was present in the collection bags. Svensson unpublished (2025) found E-ocimene in the green buds of the red clover variety Peggy. Since the flower heads were attached to the plant and small leaves as well as stems were included in the collection setup, it is likely that E-ocimene detected was derived from both floral and foliar sources, which could have skewed the results.

Finally, time constraints limited the ability to test synthetic compounds that could have confirmed the identified VOCs. This absence of testing hinders the validation of the emitted compounds and their specific contributions to the observed patterns.

In conclusion, while the study provides foundational insights into floral VOC emissions in response to pollination in red clover, these limitations highlight the need for caution when interpreting the results. Future research should address these concerns by including a broader range of varieties, refining VOC collection methods, and undertaking comprehensive environmental controls.

References

Annicchiarico, P., Barrett, B., Brummer, E.C., Julier, B. & Marshall, A.H. (2015). Achievements and Challenges in Improving Temperate Perennial Forage Legumes. *Critical Reviews in Plant Sciences*, 34 (1–3), 327–380. https://doi.org/10.1080/07352689.2014.898462

Barrett, S.C.H. (2002). The evolution of plant sexual diversity. *Nature Reviews Genetics*, 3 (4), 274–284. https://doi.org/10.1038/nrg776

Bisrat, D. & Jung, C. (2022). Roles of flower scent in bee-flower mediations: a review. *Journal of Ecology and Environment*, 46. https://doi.org/10.5141/jee.21.00075

Boller, B., Schubiger, F.X. & Kölliker, R. (2010). Red Clover. I: Boller, B., Posselt, U.K., & Veronesi, F. (red.) *Fodder Crops and Amenity Grasses*. Springer. 439–455. https://doi.org/10.1007/978-1-4419-0760-8_18

Bommarco, R., Lundin, O., Smith, H.G. & Rundlöf, M. (2011). Drastic historic shifts in bumble-bee community composition in Sweden. *Proceedings of the Royal Society B: Biological Sciences*, 279 (1727), 309–315. https://doi.org/10.1098/rspb.2011.0647

Bowley, S.R., Taylor, N.L. & Dougherty, C.T. (1984). Physiology and Morphology of Red Clover. I: Brady, N.C. (red.) *Advances in Agronomy*. Academic Press. 317–347. https://doi.org/10.1016/S0065-2113(08)60457-5

Buttery, R.G., Kamm, J.A. & Ling, L.C. (1984). Volatile components of red clover leaves, flowers, and seed pods: possible insect attractants. *Journal of Agricultural and Food Chemistry*, 32 (2), 254–256. https://doi.org/10.1021/jf00122a019

Dijkstra, J. (1969). The importance of two-seeded pods in red clover (Trifolium pratense L.). *Euphytica*, 18 (3), 340–351. https://doi.org/10.1007/BF00397781

Emelianova, M (2024). Can ageing flowers produce scent? Identification and comparison of floral scent compositions in “fresh” and “old” unpollinated flower heads in five varieties of red clover (*Trifolium pratense* L.). Department of Plant Protection Biology. Swedish University of Agricultural Science. Alnarp, Sweden

Free J.B (1970). Insect Pollination of Crops. Academic Press, London and New York.

Hedström, V (2019). Ecology of Pollinators, Pests and Natural Enemies in Agricultural Landscapes "Doctoral thesis". Faculty of Landscape Architecture, Horticulture and Crop Protection Science. Department of Plant Protection Biology. Swedish University of Agricultural Science. Alnarp, Sweden

Jing, S., Kryger, P. & Boelt, B. (2021). Different pollination approaches to compare the seed set of diploid and tetraploid red clover *Trifolium pratense* L. *Nordic Journal of Botany*, 39 (6). https://doi.org/10.1111/njb.03006

Kessler, D. & Baldwin, I.T. (2007). Making sense of nectar scents: the effects of nectar secondary metabolites on floral visitors of *Nicotiana attenuata*. *The Plant Journal*, 49 (5), 840–854. https://doi.org/10.1111/j.1365-313X.2006.02995.x

Lo, M.-M., Benfodda, Z., Molinié, R. & Meffre, P. (2024). Volatile Organic Compounds Emitted by Flowers: Ecological Roles, Production by Plants, Extraction, and Identification. *Plants*, 13, 417. https://doi.org/10.3390/plants13030417
Lundin, O. (2013). Ecology and Management of Crop Pollination and Pest Control. Department of Ecology, Swedish University of agricultural sciences. Uppsala, Sweden.
Martínez-Díaz, Y., Cuevas, E., Espinosa-García, F., García-Rodríguez, Y.M. & Martén-Rodríguez, S. (2023). Floral attractants in an alpine environment: linking floral volatiles, flower size and pollinators. *Alpine Botany*, 134, 1–14. https://doi.org/10.1007/s00035-023-00303-7
McGregor, S.E. (1976a). *Insect Pollination of Cultivated Crop Plants*. Agricultural Research Service, U.S. Department of Agriculture.
McGregor, S.E. (1976b). *Insect Pollination of Cultivated Crop Plants*. Agricultural Research Service, U.S. Department of Agriculture.
Muhlemann, J.K., Klempien, A. & Dudareva, N. (2014). Floral volatiles: from biosynthesis to function. *Plant, Cell & Environment*, 37 (8), 1936–1949. https://doi.org/10.1111/pce.12314
Negre, F., Kish, C.M., Boatright, J., Underwood, B., Shibuya, K., Wagner, C., Clark, D.G. & Dudareva, N. (2003). Regulation of Methylbenzoate Emission after Pollination in Snapdragon and Petunia Flowers. *The Plant Cell*, 15 (12), 2992–3006. https://doi.org/10.1105/tpc.016766
Parachnowitsch, A.L., Raguso, R.A. & Kessler, A. (2012). Phenotypic selection to increase floral scent emission, but not flower size or colour in bee-pollinated Penstemon digitalis. *New Phytologist*, 195 (3), 667–675. https://doi.org/10.1111/j.1469-8137.2012.04188.x
Pimienta, M., Salazar, D. & Koptur, S. (2023). The Nighttime Fragrance of *Guettarda scabra* (Rubiaceae): Flower Scent and Its Implications for Moth Pollination. *Molecules*, 28. https://doi.org/10.3390/molecules28176312
Primante, C. (2015). The role of floral traits in structuring plant-pollinator interactions "Doctoral thesis". Autonoma de Barcelona University, Spain
Raguso, R.A. (2008). Wake Up and Smell the Roses: The Ecology and Evolution of Floral Scent. *Annual Review of Ecology, Evolution, and Systematics*, 39 (Volume 39, 2008), 549–569. https://doi.org/10.1146/annurev.ecolsys.38.091206.095601
Raguso, R.A. & Pellmyr, O. (1998). Dynamic Headspace Analysis of Floral Volatiles: A Comparison of Methods. *Oikos*, 81 (2), 238–254. https://doi.org/10.2307/3547045
Robacker, D.C., Meeuse, B.J.D. & Erickson, E.H. (1988). Floral Aroma: How Far Will Plants Go to Attract Pollinators? *BioScience*, 38 (6), 390–398. https://doi.org/10.2307/1310925
Rodriguez-Saona, C., Bardehle, L., Quiroz, A. & Isaacs, R. (2011). Variation in highbush blueberry floral volatile profiles as a function of pollination status, cultivar, time of day and flower part: Implications for flower visitation by bees. *Annals of botany*, 107, 1377–90. https://doi.org/10.1093/aob/mcr077
Schiestl, F.P. (2010). The evolution of floral scent and insect chemical communication. *Ecology Letters*, 13 (5), 643–656. https://doi.org/10.1111/j.1461-0248.2010.01451.x
Schiestl, F.P. & Ayasse, M. (2001). Post-pollination emission of a repellent compound in a sexually deceptive orchid: a new mechanism for maximising reproductive success? *Oecologia*, 126 (4), 531–534. https://doi.org/10.1007/s004420000552
Spigler, R.B. & Woodard, A.J. (2019). Context-dependency of resource allocation trade-offs highlights constraints to the evolution of floral longevity in a

monocarpic herb. *New Phytologist*, 221 (4), 2298–2307. https://doi.org/10.1111/nph.15498
Taylor, N.L. (2008). A Century of Clover Breeding Developments in the United States. *Crop Science*, 48 (1), 1–13. https://doi.org/10.2135/cropsci2007.08.0446
Taylor, N.L. & Quesenberry, K.H. (1996). Tetraploid Red Clover. I: Taylor, N.L. & Quesenberry, K.H. (red.) *Red Clover Science*. Springer Netherlands. 161–169. https://doi.org/10.1007/978-94-015-8692-4_13
Ulloa, O., Ortega, F. & Campos, H. (2003). Analysis of genetic diversity in red clover (*Trifolium pratense* L.) breeding populations as revealed by RAPD genetic markers. *Genome*, 46 (4), 529–535. https://doi.org/10.1139/g03-030
Valentin, I. (2022). - A study on seed production, pollinators across regions and landscapes, and floral scent. Department of Plant Protection Biology. Swedish University of Agricultural Science. Alnarp, Sweden
Vega-Polanco, M., Solís-Montero, L., Rojas, J.C., Cruz-López, L., Alavez-Rosas, D. & Vallejo-Marín, M. (2023). Intraspecific variation of scent and its impact on pollinators' preferences. *AoB PLANTS*, 15 (4), plad049. https://doi.org/10.1093/aobpla/plad049
Vleugels, T., Amdahl, H., Roldán-Ruiz, I. & Cnops, G. (2019). Factors Underlying Seed Yield in Red Clover: Review of Current Knowledge and Perspectives. *Agronomy*, 9 (12), 829. https://doi.org/10.3390/agronomy9120829
Xu, K. & Servedio, M.R. (2021). The evolution of flower longevity in unpredictable pollination environments. *Journal of Evolutionary Biology*, 34 (11), 1781–1792. https://doi.org/10.1111/jeb.13936

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Appendix 1

Table 5. Nr of days from bud to bloom and bloom to wilt of the three red clover varieties

Variety	nr days bud to bloom	nr days bloom to wilt
Peggy	7	5
Peggy	7	6
Peggy	7	5
Peggy	7	5
Peggy	7	5
Peggy	7	5
Peggy	7	5
Peggy	7	5
Peggy	7	5
Peggy	7	5
Holly	8	4
Holly	7	4
Holly	7	7
Holly	7	7
Holly	8	4
Holly	8	5
Holly	8	6
Holly	8	6
Holly	8	4
Holly	8	5
Yngve	6	9
Yngve	6	10
Yngve	8	4
Yngve	6	4
Yngve	7	6
Yngve	7	6
Yngve	7	5
Yngve	8	6
Yngve	7	5
Yngve	7	5

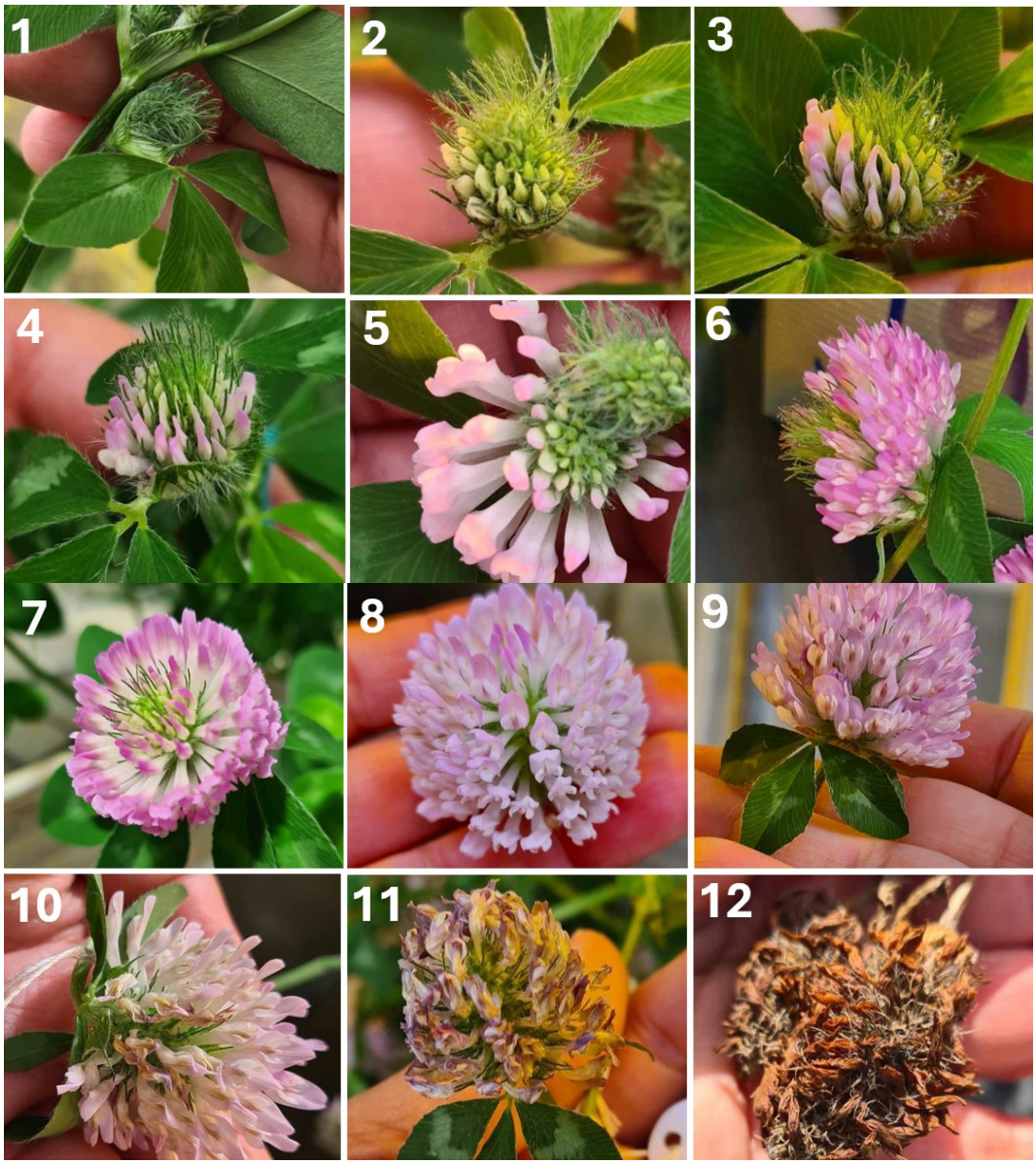
Appendix 2

Table 6. Seed count all treatments inside the greenhouse

ID	Treatment	Seeds	no seeds, undamaged floret
KB P3	KB	1	115
KB P9	KB	0	94
KB P5	KB	0	137
KB P4	KB	1	91
KB P6	KB	0	109
KB P1	KB	0	81
KB P2	KB	0	83
KB P10	KB	0	87
HP5 P3	HP5	2	108
HP5 P9	HP5	3	93
HP5 P5	HP5	2	144
HP5 P4	HP5	2	74
HP5 P6	HP5	3	106
HP5 P1	HP5	2	56
HP5 P2	HP5	3	89
HP5 P10	HP5	5	64
HP15 P3	HP15	8	92
HP15 P9	HP15	7	86
HP15 P5	HP15	7	129
HP15 P4	HP15	8	78
HP15 P6	HP15	4	107
HP15 P1	HP15	5	83
HP15 P2	HP15	8	77
HP15 P10	HP15	10	73
HPS P3	HPS	4	92
HPS P9	HPS	0	92
HPS P5	HPS	0	118
HPS P4	HPS	0	93
HPS P6	HPS	0	138
HPS P1	HPS	2	84
HPS P2	HPS	0	87
HPS P10	HPS	0	85
SK P3	SK	3	60
SK P9	SK	0	114
SK P5	SK	0	126
SK P4	SK	1	96
SK P6	SK	1	111
SK P1	SK	0	90
SK P2	SK	0	94
SK P10	SK	4	64

Appendix 3

Figure 14. Observation of floral development stages inside the greenhouse chamber at SLU. Pictures 1-12 (from bud to totally wilted flower) show the different development stages a flower head goes through without being objected to any treatments.



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