**Research**

**Different pollination approaches to compare the seed set of diploid and tetraploid red clover *Trifolium pratense* L.**

Shuxuan Jing, Per Kryger and Birte Boelt

In red clover seed production, low seed yield is limiting the commercial exploitation of tetraploid red clover. To explore if pollination is the limiting factor for the seed yield in tetraploid red clover, we investigated pollinator behaviour and plant reproductive success in diploid (2x) cultivar 'Rajah' and tetraploid (4x) cultivar 'Amos' using honey bee and hand pollination approaches. We measured the seed set at the flower head level with the increasing visitation rate of honey bees (one bee, two bees and open pollination) and pollen diversity (one and two pollen donors) of hand pollination. We found no difference in honey bee pollination behaviour (visitation rate) between the diploid and tetraploid red clover. Surprisingly, the seed set in two bees was significantly lower than that of one bee in 'Rajah'. We suggest that honey bees prefer to visit already tripped florets or to visit fewer florets due to scent marks left by the previous honey bees. In open pollination, seed number per flower head in 'Amos' (on average 11) was half of 'Rajah' (on average 20). Seed set in 'Amos' was lower than 50% of 'Rajah' in honey bee (one bee treatment) and hand pollination (one and two pollen donors treatments). There were no differences in stigma receptivity whereas pollen viability in 'Amos' was lower than 'Rajah' after the peak pollen viability was reached (six days after the onset of flowering). We suggest that the fertility problems such as low pollen viability may be the reason for the low seed set in tetraploid red clover. This conclusion should be verified by investigating other fertility traits during the ovary, embryo and seed development with a broader range of diploid and tetraploid cultivars.

Keywords: hand pollination, honey bees, pollination, red clover, seed set

**Introduction**

Red clover *Trifolium pratense* L. is an important perennial forage legume grown in most of the temperate regions of the world (Taylor and Quesenberry 1996, Boller et al. 2010). The main breeding goal for grain legumes is improving seed yield, while the main breeding goals for forage legumes, by contrast, are improving persistence and forage yield (Boller et al. 2010, Boelt et al. 2015). The persistence and forage yield in red clover have been significantly improved by inducing the polyploidy from diploid (2n = 2x = 14) to tetraploid (2n = 4x = 28) red clover (Boller et al. 2010). Despite the high forage yield, the low seed yield has been limiting the commercial
exploitation of tetraploid red clover (Boller et al. 2010, Vleugels et al. 2019a).

Seed production of red clover requires cross pollination by insect pollinators because of the high self-incompatibility in red clover (Westgate and Coe 1915, Free 1965, 1993). The pollinator performance of bumble bees Bombus spp. and honey bees Apis mellifera L. in red clover seed production was comprehensively reviewed during the last century (Bohart 1957, Holm 1966, Dennis and Holm 1977, Free 1993). Managed honey bee hives are often used as supplementary pollination approach to improve the pollination conditions in areas with inadequate bumble bees (Free 1993). In southern Sweden, red clover fields were pollinated by 60% of bumble bees and 40% of honey bees (Rundlöf et al. 2018). Four to five honey bee colonies per hectare in red clover seed production in dry and warm summers and placing honey bee hives within up to 1 km from the field were suggested by Brodersgaard and Hansen (2002). Nevertheless, Wermuth and Dupont (2010) found the abundance of long-tongued bumble bees was negatively affected by the presence of honey bee hives in red clover fields. To improve pollination management (e.g. adding honey bee hives) in seed production, more direct measures of pollinator performance (e.g. flower visitation rate) are needed (Garibaldi et al. 2020).

Previous studies described that there were more honey bees visiting diploid compared to tetraploid red clover (Dennis and Haas 1967, Free 1993), which can be due to the difficulties for honey bees reaching nectar in the long corolla tube of tetraploid red clover (Julén 1950, Dennis and Holm 1977, Boller et al. 2010). Recent studies suggested that neither the corolla tube length nor pollinator (e.g. Bombus pascuorum Scopoli) preference can explain the seed yield differences between diploid and tetraploid red clover due to the complex pollination conditions (Vleugels et al. 2015, 2019a, Vanommeslaeghe et al. 2018). A recent review suggested that plant ploidy may lead to the shifts in pollinator behaviour (Rezende et al. 2020). Evaluation of pollinator performance based on single visits is critical in connecting pollination success and plant reproductive success (Ne’eman et al. 2010). Given that a variety of bee species are available in red clover pollination, monitoring honey bee pollination behaviour under confined conditions can provide a base line in studying the relative role of different pollinator species in diploid and tetraploid red clover seed production.

Hand pollination is a supplementary pollination approach in studying the breeding systems of red clover, which requires controlling of pollen donors (Jing et al. 2019, Vleugels et al. 2019b). When cross-pollinated, a seed set of 75% in diploid and 50% in tetraploid red clover can be expected through hand pollination (Boller et al. 2010). However, hand pollination studies often neglect pollen quality and stigma receptivity. Stone et al. (1995) reviewed 283 articles with hand pollination experiments, and half of the authors mentioned the stigma receptivity and only 30% mentioned the pollen freshness. Further, using low-diversity pollen (e.g. pollen grains from one pollen donor) may reduce the reproductive success of hand pollination studies compared to natural pollination (Young and Young 1992). Previous studies found the trend of lower pollen viability and pollen germination in tetraploid compared to diploid red clover, which may result in low seed set in tetraploid red clover (Büyükkartal 2003, Grebenisan and Savatti 2011, Vleugels et al. 2019a, b). Each flower head of red clover have on average 100 florets which open sequentially over the duration of six to ten days (Williams 1930, Free 1993). Stigma receptivity is a limiting factor for the pollination period of the flowering plants (Egea and Burgos 1992). The floret fertility decreased rapidly after the opening of florets on the flower head in red clover (Free 1965). At present limited information is available on temporal difference of pollen viability and stigma receptivity in diploid and tetraploid red clover during the flowering time.

The current study followed the honey bee experiment setup in a previous study, which showed that honey bees had similar visitation rate (i.e. floret number visited per flower head) between diploid and tetraploid red clover under caged conditions (Jing 2017). In addition to monitoring the visitation rate of honey bees, we exploited the potential of using honey bee pollination and hand pollination as supplementary pollination approaches to compare the plant reproductive success of seed set (i.e. proportion of ovules that develop into seeds per pollinated floret) between the diploid and tetraploid red clover cultivars (Wesselingh 2007, Ne’eman et al. 2010). The current study is based on the following hypotheses: 1) the visitation rate of honey bees differ in diploid and tetraploid red clover; 2) tetraploid red clover has lower seed set compared to the diploid red clover regardless of pollination approaches (honey bee and hand pollination); 3) increasing visitation rate and pollen diversity increase the seed set of red clover; 4) the pollen viability and stigma receptivity of red clover vary among days after onset of flowering (DAF) and daytime.

Material and methods

Experimental site and plant material

Experiments were performed at Aarhus University, Flakkebjerg, Denmark (55°19′52″N, 11°24′29″E) during the 2017 summer. The experiment used the two most commonly grown diploid and tetraploid red clover cultivars for seed production in Denmark: ‘Rajah’ (diploid or 2x) and ‘Amos’ (tetraploid or 4x). Information of the chosen cultivars can be found in the European Plant variety database (<https://ec.europa.eu/>, accessed December 2020). Thirty plants of each red clover cultivar were collected from a seed production field trial. Plants were transplanted to 5-l pots which were placed in a semi-field area protected from rain and wind during April 2017. Plants in the semi-field were watered daily with an automatic drip irrigation system. We conducted the experiment with honey bee pollination in a plastic-covered tunnel supplied with one honey bee colony in a Kirchhain mating nuc (approximately 1500 honey bees). We conducted the experiments of hand pollination, pollen
viability test and stigma receptivity in a climate chamber. The climate chamber was set with 14-h daylight (between 08:00 and 22:00) and temperature of 20°C (day) and 15°C (night) to supply the favourable environment for the flowering (Bowley et al. 1987).

Pollination experiment

For honey bee pollination, we used 11 plants from each cultivar. On each plant, we applied three treatments on nine flower heads (three flower heads per treatment). For hand pollination, we used ten plants per cultivar. On each plant, we applied two treatments on six flower heads (three flower heads per treatment). Description of the treatments in honey bee pollination and hand pollination is shown in Table 1.

Honey bee pollination experiments started from 09:00 to 16:00 of each experimental day for a total of four days for ‘Rajah’ and eight days for ‘Amos’. Flower heads with already opened florets were removed from the plants in the semi-field, and red clover plants with flower head buds were moved into the climate chamber for flower development. When the florets on the flower heads were fully opened, we transferred plants into the tunnel. In order to compare the seed set of honey bee pollination with hand pollination, we controlled the pollen diversity in the tunnel that honey bees had access to three pollen donor plants from the same red clover cultivar.

Hand pollination experiments were conducted in the climate chamber. We removed keel and wings of the recipient florets prior to the hand pollination without removing the stamens (Boller et al. 2010). Pollen grains were collected from the flower heads of the pollen donor plant by tripping the floret with a plastic toothpick. Pollen grains were then applied directly to the stigma to the recipient florets with the toothpick. The toothpicks were replaced for each flower head. After the pollination experiments, we moved plants to the semi-field and the pollinated flower heads were carefully bagged by light/air permeable nonwoven fabrics to avoid further pollination and labelled until harvest and registration of plant reproductive success. The number of seeds per tested flower head was counted after the flower heads of both cultivars had been harvested, air-dried, hand-threshed and cleaned in October 2017. As each red clover floret can produce two ovules, each floret has the potential of producing two seeds (Lorenzetti 1993). Plant reproductive success of seed set was calculated for the treatments of one bee and two bees in honey bee pollination and hand pollination: seed set (\%) = the number of harvested seeds/(the number of pollinated florets per flower head \times 2) \times 100. Plant reproductive success in open pollination was presented as the number of seeds per flower head.

Pollen viability

Pollen viability was measured with the tetrazolium test (TTC) and presented as the percentage of the viable (stained with red colour) pollen grains among the total amount of pollen grains (Dafni 1992). A pilot study was conducted and the feasibility of the TTC test for red clover was verified, since not all the plant species are sensitive to the method, which may give intermediate results (Dafni 1992). Pollen grains were collected from the florets using a toothpick, carefully suspended in a drop of TTC solution on the slide, and covered with a coverslip. The slides were put in the petri dish with wet filter paper, incubated at 35°C for 15 min. Afterwards, the slides were put under the microscope with \( \times 30 \) magnification to be photographed. The percentage of the viable pollen grain was evaluated using the image processing software FIJI Ver. 1.0 (Schindelin et al. 2012). In each image, three \( 90 \times 90 \) pixel squares were selected as replicates among the area with visible pollen grains. Both stained and unstained pollen grains were recorded and calculated as: viable pollen grain (%) = the number of stained pollen grains/the number of stained pollen grains + the number of unstained pollen grains) \times 100.

Stigma receptivity

High enzyme activity is correlated to the stigma receptivity, and hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) accelerates the oxygen bubbles due to the peroxidase enzyme of the stigma (Zeisler 1933, Dafni et al. 2005). The evaluation of the stigma receptivity of red clover followed the method developed by Dafni (1992), using \( \text{H}_2\text{O}_2 \) (3%) and observing bubbles released from the receptive stigma. The stigmas were picked using a fine forceps and were immediately immersed on the slide with the reagent. The observation was operated under the microscope with \( \times 30 \) magnification. A pilot study established a subjective assessment score (0–3) of stigma receptivity (Fig. 1). During the observation, reaction levels were scored immediately after the stigma was submerged in the reagent.

Table 1. Treatment description in honey bee pollination and hand pollination.

| Pollination approach | Treatment | Description |
|----------------------|-----------|-------------|
| Honey bee pollination| One bee   | Tested flower head was available for the visit of one honey bee. The floret number visited per flower head by the honey bee was recorded. |
|                      | Two bees  | As in the one-bee treatment, and floret number visited per flower head by the second honey bee was recorded separately. |
|                      | Open pollination | Tested flower head was available for pollination without limitation of honey bee numbers within two hours. |
| Hand pollination     | One pollen donor | Ten florets of each tested flower head were hand pollinated using pollen from one external red clover plant. |
|                      | Two pollen donors | Ten florets per tested flower head were hand pollinated using mixed pollen from two external red clover plants. |
We compared the seed number per flower head in the open pollination of the diploid and tetraploid red clover cultivars by fitting GLMM (Poisson distribution with a log link). We used the fixed effect of cultivar (‘Rajah’, ‘Amos’) and the random effect of flower head ID (n = 65) nested in plant ID (n = 22). Further, we compared the visitation rate between the first and second visiting bees in the treatment of two bees (honey bee pollination) by fitting GLMM (Poisson distribution with a log link). We included the fixed effects of cultivar (‘Rajah’, ‘Amos’), bee (first, second) and their interactions and we included the random effect of flower head ID (n = 130) nested in plant ID (n = 22).

To study the temporal differences of pollen viability and stigma receptivity, we fit linear mixed models (LMMs). For the DAF variability of pollen viability (square-root transformed) and stigma receptivity, we included the fixed effects of day (1–9), cultivar (‘Rajah’, ‘Amos’) and their interactions. For the daytime variability of pollen viability and stigma receptivity, we included the fixed effect of time (8:30, 10:30, 12:30, 14:30, 16:30), cultivar (‘Rajah’, ‘Amos’) and their interactions. For both DAF and daytime variability tests, we averaged the data points of flower heads per plant, and included plant ID (n = 14) as random effect.

All statistical analyses were performed using R ver. 4.0.3 (www.r-project.org). The statistical significance of effects were determined with type III Wald $\chi^2$ ANOVA tests in the package car (Fox and Weisberg 2011). The assumptions of normality was examined using Shapiro–Wilk test and the homogeneity of variance was examined by using Bartlett’s test. Post hoc analyses were conducted by using packages of emmeans (Mangiafico 2020), multicompView (Graves et al. 2019) and emmeans (Lenth 2020).

Results

Floret number visited per flower head and seed set

We found a significant effect of treatment on the visitation rate (GLMM, Treatment $\chi^2 = 84.829, p = 0.001$, Table 2) and

| Effect                  | Floret number visited per flower head | Seed set (%) |
|-------------------------|--------------------------------------|--------------|
|                         | df   | $\chi^2$ | p-value | df   | $\chi^2$ | p-value |
| Treatment               | 3    | 84.829  | < 0.001 | 3    | 7.184   | 0.066   |
| Cultivar                | 1    | 1.787   | 0.181   | 1    | 40.070  | < 0.001 |
| Treatment $\times$ Cultivar | 3    | 16.070  | 0.0011  |
we found a significant interaction effect between treatment and cultivar on the seed set (GLMM, Treatment × Cultivar $\chi^2 = 16.070$, p < 0.0011, Table 2). The floret number per flower head visited by the two bees was significantly (p < 0.0001) higher compared to the one bee treatment regardless of cultivars (Fig. 2a). However, the seed set in two bees was significantly (p < 0.032) lower than that of one bee in 'Rajah'. The seed set in the two bees was similar to the seed set in one bee in 'Amos'. Honey bees visited similar numbers of florets per flower head between the two cultivars, and the seed set from one bee treatment was significantly (p = 0.0029) higher in 'Rajah' compared to 'Amos' (Fig. 2b).

When ten florets per flower head were hand-pollinated, 'Rajah' obtained significantly higher seed set compared to 'Amos' (p < 0.032) lower than that of one bee in 'Rajah'. The seed set in the two bees was similar to the seed set in one bee in 'Amos'. Honey bees visited similar numbers of florets per flower head between the two cultivars, and the seed set from one bee treatment was significantly (p = 0.0029) higher in 'Rajah' compared to 'Amos' (Fig. 2b).

DAF and daytime differences in pollen viability and stigma receptivity

The pollen viability and stigma receptivity varied during different DAF and daytime in 'Rajah' and 'Amos' (Table 3, 4, Fig. 4). For DAF variability, we found a significant interaction effect between cultivar and day in pollen viability (LMM, Day × Cultivar $\chi^2 = 9.627$, p = 0.002, Table 3). The peaks of pollen viability was day 5 for 'Amos' and day 6 for 'Rajah' (data not shown), followed by a rapid decline (Fig. 4). The pollen viability in 'Rajah' was significantly (p < 0.05) higher compared to 'Amos' on day 6 to day 8 (Fig. 4). Overall, the duration of the pollen viability in 'Rajah' was longer than that of 'Amos'. There were decreasing trends of the stigma receptivity during the eight days for both cultivars (Fig. 4). However, no significant effect of DAF variability in stigma receptivity was detected between 'Rajah' and 'Amos' (LMM, Cultivar $\chi^2 = 1.641$, p = 0.2, Table 3). It is noticeable that the average DAF for the florets per flower head were fully opened was day 5 for 'Rajah' and day 4 for 'Amos' (data not shown), both of which were one day before the peak pollen viability reached (Fig. 4). For daytime variability, we found significant effect of the time on pollen viability (LMM, Time $\chi^2 = 6.192$, p = 0.013, Table 4) and stigma receptivity (LMM, Time $\chi^2 = 5.407$, p = 0.02, Table 4). 'Rajah' showed a higher pollen viability after 10:30 compared to 'Amos' (Fig. 4), but was not statistically significant (LMM, Cultivar $\chi^2 = 0.063$, p = 0.803, Table 4). Similar to the trend in the DAF variability, the daytime variability of stigma receptivity decreased significantly (p = 0.02) over time. Noticably, slightly increasing
trends of pollen viability and stigma receptivity during 14:30 to 16:30 were observed in both cultivars (Fig. 4).

Discussion

When receiving higher number of visits from the two honey bees compared to the one honey bee, the seed set in ‘Amos’ was not increased with the visitation rate and the seed set in ‘Rajah’ was even decreased (Fig. 2b). One hypothesis can be that the following honey bee re-visited the same florets that have been visited by the first honey bee, making a limited contribution to seed set. Honey bees sometimes visit those already tripped flowers for collecting the pollen from the previous pollinators in plants such as Scotch broom Cytisus scoparius L. Link, the pollination of which requires pollinators with large body sizes, to release the styles and anthers inside the keel petals by tripping the flowers (Parker 1997). The already tripped red clover florets may reduce the difficulties for honey bees to enter the florets. However, the increase of visits to the florets may not increase the seed set accordingly. Frequent visits may damage the stigma or the style of the pistil, leading to the poor seed development in the ovary. For example, high visitation rate of honey bees increased the pollen deposition on stigmas of raspberry Rubus idaeus L., but reduced the drupelet set by damaging 80% of the flower styles (Sáez et al. 2014). A hand pollination study of red clover showed that an increasing number of pollen grains deposited on the stigma did not increase the seed set, suggesting the importance of the pollen quality (pollen viability and pollen source) to the red clover seed set (Jing et al. 2019).

The second honey bee visited a significantly lower number of florets per flower head compared to the first honey bee in the two bees treatment, which can be related to the above mentioned re-visit hypothesis. When the second honey bee found inadequate resources (pollen and nectar) in those already tripped florets, they may give up visiting more florets on the same flower head to avoid the emptied florets. Another hypothesis may explain the behaviour of the second honey bee. The discrimination behaviour of honey bees for increasing the forage efficiency was reported in a study of birds'-foot trefoil Lotus corniculatus L., with the mechanisms of either smelling nectar odour or bee scent marks left by the previous pollinators (Wetherwax 1986). The repellent scent mark was further demonstrated by using the air extractor device to remove the scent marks from the artificial flower (Giurfa and Núñez 1992). Future studies may use techniques such as video recording to track the specific behaviour of the honey bees on each floret.

A similar number of florets per flower head were visited by honey bees between the diploid and tetraploid red clover (Table 2), showing that tetraploid was as attractive as diploid red clover in the controlled condition, which is consistent with the previous honey bee pollination experiments conducted under the similar experimental set-up (Jing 2017). The result of the current study shows that seed set in the tetraploid red clover ‘Amos’ was lower compared to the diploid red clover ‘Rajah’ under both honey bee pollination and hand pollination approaches (Fig. 2b). The seed number per flower head in ‘Amos’ (on average 11) was significantly (p = 0.016) lower compared to ‘Rajah’ (on average 20) in the open pollination treatment (Fig. 3), which is in agreement with the previous studies (Vleugels et al. 2016, Amdahl et al. 2017). Our results of seed number per flower head in ‘Amos’ was consistent with the study conducted by Vleugels et al. (2016), where on average 12.7 seeds per flower head were obtained in ‘Amos’ under field conditions. Nevertheless, the number of seeds per flower head in the current study can be limited by the pollination time (restricted to two hours for each flower head) and the pollen diversity (restricted to three pollen donor plants). Our study was conducted under the condition that only one cultivar was available in the tunnel each time. Wild pollinators have been shown to enhance the fruit or seed set of crops more efficiently compared to honey bees (Garibaldi et al. 2013). The competence and balance between commercial honey bees and wild pollinators should be investigated in large-scale red clover seed production. Further studies may include the comparison of pollinator performance of different pollinator species under field conditions where both diploid and tetraploid red clover are

---

Table 3. Type III ANOVA table of LMMs for pollen viability (%) and stigma receptivity during different days after onset of flowering. Probability less than 0.05 are shown in bold.

| Effect          | Pollen viability (%) | Stigma receptivity |
|-----------------|----------------------|--------------------|
|                 | df  x^2  p-value     | df  x^2  p-value   |
| Day             | 1  20.154  0.001     | 1  59.342  0.001   |
| Cultivar        | 1  0.0008  0.978     | 1  1.641  0.200    |
| Day × Cultivar  | 1  9.627  0.002      | 1  0.113  0.736    |

Table 4. Type III ANOVA table of LMMs for pollen viability (%) and stigma receptivity during different time of the day. Probability less than 0.05 are shown in bold.

| Effect          | Pollen viability (%) | Stigma receptivity |
|-----------------|----------------------|--------------------|
|                 | df  x^2  p-value     | df  x^2  p-value   |
| Time            | 1  6.192  0.013      | 1  5.407  0.020    |
| Cultivar        | 1  0.063  0.803      | 1  0.021  0.885    |
| Time × Cultivar | 1  2.764  0.096      | 1  1.376  0.241    |
grown and available at the same time. Both pollination success and plant reproductive success should be investigated in a broader range of diploid and tetraploid red clover cultivars.

Free (1965) demonstrated that the florets of each flower head in red clover open over six to eight days and the florets must be pollinated within two to four days after opening because of the rapid decrease in fertility. In white clover *Trifolium repens* L., it was found that the gynoecia aging (zero, three and five days after onset of flowering) led to the decrease of seed set (Jakobsen and Martens 1994). Our study also found that the favourable pollination period for a flower head can be at the beginning of the flowering due to the rapid decrease of stigma receptivity afterwards (Fig. 4). However, we also found that the peak of pollen viability was reached between one to two days after the florets were fully opened on the flower head, indicating a mismatch of timing for a flower head to reach the high stigma receptivity and high pollen viability. Therefore, the timing for optimal pollination for the seed set is yet to be determined. Higher pollen viability of ‘Rajah’ compared to ‘Amos’ was found from day 6 to day 8 in the DAF variability and also found in the daytime variability (Fig. 4). This is in agreement with Grebenisan and Savatti (2011), reporting that the percentage of viable pollen in tetraploid red clover (77.8%) was less than in diploid red clover (99.1%). However, the highest percentage of pollen viability in the current study was 43% in ‘Rajah’ and 34% in ‘Amos’, which was not as high as the study from Grebenisan and Savatti (2011). The reason can be due to the different staining methods used to assess the pollen quality. The trends of reaching the full flowering, peak of pollen viability and the decreasing pollen viability of ‘Amos’ were found earlier than that of ‘Rajah’ in the current study (Fig. 4). The current study was conducted at the flower head level, the influence of flowering duration on the seed yield may depend on the number of flower heads per plant. Amdahl et al. (2017) registered the floral phenology of diploid and tetraploid red clover cultivars under field conditions, the number of flower heads per plant differed among cultivars at the early flowering stages but not at the time harvest, indicating the start date of flowering may not strongly influence the seed yield.

Contrary, the pattern of stigma receptivity was similar between ‘Rajah’ and ‘Amos’, indicating that the low pollen viability might be the main reason for the low seed set of tetraploid red clover. This is in agree with the previous studies (Büyükkartal 2003, Grebenisan and Savatti 2011, Vleugels et al. 2019a, b), showing that fertility problems are the main limiting factor for the seed yield in tetraploid red clover.

**Conclusions**

We hypothesized that visitation rate of honey bees differ between the diploid and tetraploid red clover. However, similar visitation rate was observed between ‘Rajah’ and ‘Amos’. Higher visitation rate did not increase the seed set in two cultivars whereas higher pollen diversity only increased the seed set in ‘Amos’. Seed set in ‘Amos’ was less than 50% of the ‘Rajah’ regardless of the pollination approaches. We found a rapid decrease of stigma receptivity after the beginning of flowering, but the stigma receptivity was not different between two cultivars. Six days after the onset of flowering, we found lower pollen viability in ‘Amos’ compared to ‘Rajah’. Further studies, exploring other reproductive traits during the development stages of ovary, embryo and seed may further reveal the reasons for the low seed set of tetraploid red clover.

**Acknowledgements** – We would like to thank DLF Seed for providing basic seed of the red clover cultivars used in the experiment.

**Funding** – This work was supported by GUDP (Grønt Udviklings- og Demonstrations Program) project J. no. 34009-13-0726, the Danish Agricultural Agency under the Ministry of Environment and Food of Denmark.

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

Shuxuan Jing: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Visualization (equal); Writing – original draft (equal). Per Kryger: Methodology (equal); Resources (equal); Supervision (equal); Visualization (equal); Writing – review and editing (equal). Birte Boelt: Conceptualization (equal); Funding acquisition (equal); Methodology (equal); Project administration (equal); Resources (equal); Supervision (equal); Visualization (equal); Writing – review and editing (equal).

Data availability statement

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.dbfrv15f3> (Jing et al. 2021).

References

Amdahl, H. et al. 2017. Seed yield components in single plants of diverse Scandinavian tetraploid red clover populations (Trifolium pratense L.). – Crop. Sci. 57: 108–117.
Boelt, B. et al. 2015. Legume seed production meeting market requirements and economic impacts. – CRC Crit. Rev. Plant Sci. 34: 412–427.
Bohart, G. E. 1957. Pollination of alfalfa and red clover. – Annu. Rev. Entomol. 2: 355–380.
Boller, B. et al. 2010. Red clover. – In: Boller, B. et al. (eds), Fodder crops and amenity grasses. Springer, pp. 439–455.
Bowley, S. R. et al. 1987. Photoperiodic response and heritability of the pre-flowering interval of two red clover Trifolium pratense populations. – Ann. Appl. Biol. 111: 455–461.
Brodsgaard, C. J. and Hansen, H. 2002. Pollination of red clover in Denmark. – Danish Inst. of Agricultural Sciences.
Büyükkartal, H. N. 2003. In vitro pollen germination and pollen tube characteristics in tetraploid red clover (Trifolium pratense L.) – Turk. J. Bot. 27: 57–61.
Dafni, A. 1992. Pollination ecology: a practical approach. – Oxford Univ. Press.
Dafni, A. et al. 2005. Practical pollination biology. – Enviroquest Ltd.
Dennis, B. A. and Holm, S. N. 1977. Recent trends in red clover pollination. – Pczcle. Zeszty Nauk. 21: 149–157.
Dennis, B. A. and Haas, H. 1967. Pollination and seed-setting in diploid and tetraploid red clover (Trifolium pratense L.) under Danish conditions II. Studies of floret morphology in relation to the working speed of honey- and bumble-bees. – Arsskr. Kgl. Vet. Landbohojsk. 1967: 118–133.
Egea, J. and Burgos, L. 1992. Effective pollination period as related to stigma receptivity in apricot. – Sci. Hortic. 52: 77–83.
Fox, J. and Weisberg, S. 2011. An R companion to applied regression. – Sage Publications.
Free, J. B. 1965. The ability of bumblebees and honeybees to pollinate red clover. – J. Appl. Ecol. 2: 289–294.
Free, J. B. 1993. Insect pollination of crops. – Academic Press.
Garibaldi, L. A. et al. 2013. Wild pollinators enhance fruit set of crops regardless of honey bee abundance. – Science 339: 1608–1611.

Garibaldi, L. A. et al. 2020. Crop pollination management needs flower-visitor monitoring and target values. – J. Appl. Ecol. 57: 664–670.
Giurfa, M. and Núñez, J. A. 1992. Honeybees mark with scent and reject recently visited flowers. – Oecologia 89: 113–117.
Graves, S. et al. 2019. multcompView: visualizations of paired comparisons. – R package ver. 0.1-8.
Grebenshan, M. and Savatti, M. 2011. The comparative effects of the micro- and macroporogenesis of the red clover with different levels of ploidy, in relation with its fertility. – Bull. Univ. Agric. Sci. Vet. Med. 68: 138–143.
Holm, S. N. 1966. The utilization and management of bumble bees for red clover and alfalfa seed production. – Annu. Rev. Entomol. 11: 155–182.
Jakobsen, H. B. and Martens, H. 1994. Influence of temperature and ageing of ovules and pollen on reproductive success in Trifolium repens L. – Ann. Bot. 74: 493–501.
Jing, S. 2017. Pollination and seed setting of diploid and tetraploid red clover (Trifolium pratense L.). – Aarhus Univ.
Jing, S. et al. 2019. Is red clover seed set potential influenced by pollen quality? – In: Anderson, N. P. (ed.), Proceedings of the 10th international herbage seed conference. IHS0, pp. 51–54.
Jing, S. et al. 2021. Data from: Different pollination approaches to compare the seed set of diploid and tetraploid red clover (Trifolium pratense L.). – Dryad Digital Repository, <http://dx.doi.org/10.5061/dryad.dbfrv15f3>.
Julén, U. 1950. Fertility conditions of tetraploid red clover. – Hereditas 36: 151–160.
Lenth, R. 2020. Emmeans: estimated marginal means, aka least-squares means. – R package ver. 1.5.1.
Lorenzetti, F. 1993. Achieving potential herbage seed yields in species of temperate regions. – In: Baker, M. J. et al. (eds), Proceedings of the 17th international grassland congress. Kelly and Mundy Ltd., pp. 1621–1628.
Mangiafico, S. 2020. rcompanion: functions to support extension education program evaluation. – R package ver. 2.3.25.
Ne’eman, G. et al. 2010. A framework for comparing pollinator performance: effectiveness and efficiency. – Biol. Rev. 85: 435–451.
Parker, I. M. 1997. Pollinator limitation of Cytisus scoparius (Scotch broom), an invasive exotic shrub. – Ecology 78: 1457–1470.
Rezende, L. et al. 2020. Can plant hybridization and polyploidy lead to pollinator shift? – Acta Bot. Bras. 34: 229–242.
Rundlöf, M. et al. 2018. Annual flower strips support pollinators and potentially enhance red clover seed yield. – Ecol. Evol. 8: 7974–7985.
Sáez, A. et al. 2014. Extremely frequent bee visits increase pollen deposition but reduce drupelet set in raspberry. – J. Appl. Ecol. 51: 1603–1612.
Schindelin, J. et al. 2012. Fiji: an open-source platform for biological-image analysis. – Nat. Methods 9: 676–682.
Stone, J. L. et al. 1995. Assessment of pollen viability in hand-pollination experiments: a review. – Am. J. Bot. 82: 1186–1197.
Taylor, N. L. and Quesenberry, K. H. 1996. Red clover science. – Kluwer Academic Publishers.
Vanommeslaeghe, A. et al. 2018. Influence of pollinator abundance and flower visitation on seed yield in red clover. – Arthropod Plant Interact. 12: 339–349.
Vleugels, T. et al. 2015. Influence of flower and flowering characteristics on seed yield in diploid and tetraploid red clover. – Plant Breed. 134: 56–61.
Vleugels, T. et al. 2016. Models with only two predictor variables can accurately predict seed yield in diploid and tetraploid red clover. – Euphytica 209: 507–523.
Vleugels, T. et al. 2019a. Factors underlying seed yield in red clover: review of current knowledge and perspectives. – Agronomy 9: 829.
Vleugels, T. et al. 2019b. Seed yield in red clover is associated with meiotic abnormalities and in tetraploid genotypes also with self-compatibility. – Euphytica 215: 79.
Wermuth, K. H. and Dupont, Y. L. 2010. Effects of field characteristics on abundance of bumblebees (Bombus spp.) and seed yield in red clover fields. – Apidologie 41: 657–666.
Wesselingh, R. A. 2007. Pollen limitation meets resource allocation: towards a comprehensive methodology. – New Phytol. 174: 26–37.
Westgate, J. M. and Coe, H. S. 1915. Red clover seed production studies. – Bull. US Dept Agric.
Wetherwax, P. B. 1986. Why do honeybees reject certain flowers? – Oecologia 69: 567–570.
Williams, R. D. 1930. Some of the factors influencing yield and quality of red clover seeds. – Bull. Welsh Plant Breed. Stn. Ser. H 11: 60–91.
Young, H. J. and Young, T. P. 1992. Alternative outcomes of natural and experimental high pollen loads. – Ecology 73: 639–647.
Zeisler, M. 1933. Über die Abgrenzung der eigentlichen Narbenfläche mit Hilfe von Reaktionen. – Beitr. Bot. A 58: 308–318.