Pollen load on thrips and its natural enemies

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Pollen load on thrips and its natural enemies

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Preface

Experiments in which genetically modified plants or animals are grown and studied under laboratory or greenhouse conditions have to comply with safety regulations that prescribe that genetically modified materials may not disperse outside the contained greenhouse or culture cabinet. Containment regulations also stipulate that no other organism than the genetically modified study species may be present in the growth cabinet or greenhouse. This specifically applies to pest species infesting the study plant, such as thrips, spider mite, aphids or other herbivorous insects. It is common practice to suppress such pests by chemical means. However, there are situations in which the use of chemical pesticides is undesirable, e.g. when the pesticide influences the physiological functioning of the plant in a way that is incompatible with the experiment. The use of biological control (predatory mites, parasitoid wasps, predatory bugs) may be a good alternative.

In a previous study commissioned by COGEM, an assessment was made on the question whether biological control used in greenhouses or growth cabinets requires additional containment measures. The authors concluded that the additional risk is likely negligible if biological control is effective, i.e. when there is hardly any pest population in the greenhouse and both pest and control agent are not associated with pollen. To verify this conclusion, a follow-up study was commissioned which consisted of an experimental test of the possible dispersal of GM plant material under conditions which were considered to involve the highest risk: flowering plants with a pest and its natural enemy, both highly associated with pollen. The data in this report support the earlier conclusion.

We are expecting that scientists will increasingly choose for biological control in greenhouse experiments. The use of natural enemies could also be considered in experiments with GM-plants. The present report will be a valuable aid with the risk assessment if COGEM is asked to give advice on applications for contained use under such conditions.

Nico M. van Straalen
Chair of the supervising committee

Summary

Regulation for experiments with GM crops in contained facilities such as greenhouses requires that spread of GM plant material into the environment is prevented. Therefore insect pests should be maximally controlled as they may carry pollen when they escape. In most cases chemical control is applied but for several reasons biocontrol of pests by natural enemies can be a good alternative. Introducing natural enemies however may change the risk of pollen transport as not only the pests but also natural enemies may contribute to pollen dispersal.

In order to quantify the potential pollen spread in a biocontrol setting, attachment of *Arabidopsis* pollen grains to Western Flower thrips (*Frankliniella occidentalis*) as pest and the frequently applied predatory mites (*Amblyseius swirskii*) and predatory bugs (*Orius laevigatus*) was investigated. The insects and mites were released separately or in combination on either flowering plants or detached flowers, creating opportunities to take up pollen during one or more days. After exposure they were recaptured and the number of pollen grains attached to the insect was counted. In different small scale settings pollen load on the insect body rapidly accumulated up to about 5 pollen grains per individual in *F. occidentalis*, 10-20 in *O. laevigatus* and about 10 in *A. swirskii*.

With these figures in mind, the contribution of pests and natural enemies to pollen transport and outcrossing is discussed relative to insect numbers and their risk of escape in different containment settings. Pollen load on insects is noticeable. However, based on expert guesses and taking other factors into account, the influence of using biological control to the final risk for pollen dispersal and outcrossing seems to be limited.

Samenvatting

Het doen van proeven met genetisch gemodificeerde planten in kassen en klimaatkamers is strikt gereguleerd. Daarbij is onder andere vereist om plagen te bestrijden zodat voorkomen wordt dat GM-materiaal zoals stuifmeel via ontsnappende insecten buiten de ingeperkte ruimtes terecht kan komen. Deze bestrijding vindt meestal met chemische middelen plaats. Omdat deze middelen echter niet altijd effectief zijn of om andere reden ongewenst zijn bij proeven, is er behoefte aan alternatieve methoden zoals het toepassen van biologische bestrijding met behulp van natuurlijke vijanden. Wanneer echter naast de plagen ook natuurlijke vijanden actief zijn, verandert daarmee het risico op transport van stuifmeel omdat zowel de plaaginsecten als de natuurlijke vijanden stuifmeel kunnen transporteren.

Om een meer kwantitatief beeld te krijgen van de potentiële verspreiding van stuifmeel bij biologische bestrijding, zijn in dit onderzoek proeven gedaan om de hechting van *Arabidopsis* stuifmeel te bepalen aan Californische trips (*Frankliniella occidentalis*) als plaag en aan twee veel toegepaste predatoren, namelijk roofmijten (*Amblyseius swirskii*) en roofwantsen (*Orius laevigatus*). Op kleine schaal werden daarvoor deze plagen en predatoren samen of apart losgelaten bij bloeiende *Arabidopsis* planten of losse bloemen. Na enkele dagen werden de tripsen en roofvijanden terug gevangen om de aangehechte stuifmeelkorrels te tellen. In deze setting bleken tripsen na blootstelling aan stuifmeel gemiddeld ongeveer vijf stuifmeelkorrels bij zich te dragen, de *Orius* roofwantsen 10-20 en de roofmijt *A. swirskii* ongeveer 10.

De betekenis van de verkregen resultaten wordt besproken tegen de achtergrond van de dynamiek van stuifmeelverspreiding wanneer biologische bestrijding wordt toegepast.
Het feit dat zowel plagen als predatoren aanzienlijke aantallen stuifmeelkorrels met zich mee kunnen dragen in het geteste worst-case scenario, betekent niet automatisch dat het risico op onbedoelde verspreiding buiten de ingeperkte ruimtes toeneemt bij biologische bestrijding. Verspreiding van stuifmeel hangt sterk af van de effectiviteit van de bestrijding, de ontsnappingskansen en het gedrag van de gebruikte natuurlijke vijanden. Mits effectief toegepast lijkt het risico van biologische bestrijding vooralsnog beperkt.

1 Introduction

Experiments with genetically modified plants have to comply with safety regulations. One important requirement from the regulations is that pests have to be absent or at least maximally controlled in order to prevent them from escaping while carrying GMO material from experimental units. Escape and transport of pollen is of main concern as this may lead to outcrossing with natural plant populations and hence undesired spread of modified genes.

There is substantial evidence that apart from pollinating insects also pest species may potentially contribute to pollen dispersal and many pollen can attach to a variety of insects (Pu *et al.* 2014). This potential contribution of insect to transport pollen is the main reason why they should be strictly controlled. Because of its assumed reliability, chemical control is common practice in GM plant experiments, which are done in high-quality greenhouses and climate rooms.

However, when chemical control is undesirable, for example when this is less effective or incompatible with experimental conditions, biological control by natural enemies such as predators or parasitoids could be a good alternative. By introducing more insect species in the systems however, additional risk for pollen transport may be introduced in case those species are also able to transport pollen. In a recent literature study (Booij and Messelink 2015), it was concluded that indeed several species used in biological control can carry pollen and may contribute to pollen spread.

The risk of additional pollen escape when using natural enemies instead of chemical control depends very much on the species used, the control efficiency and the crop itself.
The literature study and risk analysis of Booij & Messelink (2015) showed that this risk increases when:

1. crops produce abundant pollen,
2. numbers of prey and/or natural enemies are high, especially when they are highly mobile,
3. prey and/or natural enemies are flower visiting species,
4. prey and/or natural enemies tend to use pollen as main or alternative food source,
5. prey and natural enemies are relatively small and mobile and prone to escape or to transport.

Crucial for each specific crop-pest-biocontrol situation is whether and how much pollen is available, and whether and how much pollen is attached to the pests and natural enemies in the system. Information about all risk factors is needed to estimate the risk in various settings. Currently, knowledge on how much pollen can attach to the different insect pests and the natural enemies is virtually lacking or anecdotic, while this is crucial information to estimate potential effects of pollen spread.

A typical example of a high risk situation occurs when a small pollen-feeding insect pest is controlled by a pollen-feeding predator and both are prone to escape. Such a case is presented where the biocontrol of flower-visiting thrips by predators is concerned such as predatory mites or bugs that use pollen as alternative food. The latter combinations are commonly applied in several crops (Weintraub *et al.* 2011, Calvo *et al.* 2015). Such combinations are interesting worst-case scenarios to study potential pollen escape from GM crops with biocontrol of pests.

Whereas pollen feeding by thrips, predatory mites and predatory bugs is widely documented, very little information is available on how much pollen is attached to these organisms under natural conditions. Quantification of the potential pollen load of individual insects and mites is necessary as a starting point for estimating potential pollen spread.
In the current study pollen attachment was studied in small-scale experiments where pests and predators could move freely in a system of flowering *Arabidopsis*. Western flower thrips (*Frankliniella occidentalis*), predatory flower bugs (*Orius laevigatus*), and predatory mites (*Amblyseius swirskii*) were used.

2 Methods and Materials

2.1 General approach

The project focuses on a situation with an assumed high risk of pollen transport, using prey and natural enemies both with pollen feeding habits, exposure of insects during maximal flowering and small pollen size.

Selection of organisms
For the pollen load trials *Arabidopsis thaliana* var. Columbia (Col-0) was chosen as the pollen producing plant because:

- It is the most frequently used model plant species in genetic studies and genetic modification because of its fast growing and easy handling.
- In greenhouse situations undesired insect infestations are hard to prevent and chemical control is often not very effective. Options for integrated and biological control are used including the use of natural enemies.
- During its short but abundant flowering it produces abundant pollen which are small and potentially easy to be carried. Even though self-fertilization is the rule in this species, cross pollination occurs and theoretically pollen transport from experimental units to surroundings could occur.
- Thrips, but also other insects such as fungus gnats are a severe problem in *Arabidopsis* experiments, while these species are also known to be associated with flowers and pollen.
- In natural situations various Thrips species are (next to bees and flies) among the most frequently found flower visitors in *Arabidopsis* and are assumed to contribute to cross pollination (Hoffmann *et al.* 2003).
- There is ample evidence that biocontrol with natural enemies in *Arabidopsis* is effective in many cases. The species used for biological control (predatory bugs and predatory mites) are considered as flower visiting and potential carriers of pollen.

From the various pest species the Western flower thrips, *Frankliniella occidentalis* (Pergande) was chosen as a model pest for a worst case scenario, as this species is widespread in many crops, it has a fast reproduction that often leads to outbreaks, and it is known to be attracted to flowers and feeds on pollen. Moreover, it is already known to be able to carry pollen. Its abundance, small size, flight ability and dispersive character makes this species sensitive for escape. Moreover they easily adhere to cloths and hair of personnel working in the experimental units.

Two natural enemy species were chosen to study pollen load and transport, namely the predatory bug *Orius laevigatus* (Fieber) (Figure 1) and the predatory mite *Amblyseius swirskii* (Athias-Henriot). Both are successfully applied as a biocontrol agent against thrips and several other pests in a variety of crops (Weintraub 2011, Calvo *et al.* 2015). Both species are also feeding on pollen and are attracted to flowers. Adult *Orius* bugs are about 3 mm in size, good flyers, attracted to light and their ability to escape through small openings is well known. *Amblyseius* mites are inconspicuous (0.5 mm long) and prone to attach to clothing and transported by people working in the experimental units.

Figure 1 *Orius laevigatus, a predatory bug commonly used in biocontrol of spider mites (in picture), thrips, and other small insects such as aphids, psyllids and insect eggs.*
Photo © Edgardo González Carducci

2.2 Plants and Experimental units

For the experiments *Arabidopsis thaliana* var. Columbus was used. In order to have pollen-carrying plants available continuously over an experimental timeframe of 6 weeks plants were grown in 6 batches with weekly intervals. All plants were sown in plastic containers with three seeds per container. These were grown in thrips-free greenhouse environment at about 20°C with sufficient daylight (LD 16:8) for growth and initiation of flowering.
Pollen size varies between 20 and 40 µm; so they are relatively small compared to the natural enemies.

Plants were kept in the greenhouse until the emergence of flowers and transferred to climate cabinets to perform the pollen attachment experiments.

In the first and second experiment the plants were caged with transparent plastic cylinders to create single experimental units (Figure 2) in which insects and mites could be released. All units were placed on trays and watered regularly to maintain the quality of the plants with continuous flowering during the exposure experiment.
In a third –short duration- experiment devoted to predatory mites only, flowering tops of *Arabidopsis* were kept in water agar in petri dishes (Ø 5 cm) in upright position (Figure 3). Dishes were sealed to prevent escape of mites.

2.3 Insects used

Western flower thrips (*Frankliniella occidentalis*) (adults 1-1.5 mm, larvae 0.3-0.8 mm) was reared on French beans to sufficient numbers to be released into the experimental units. The initial thrips originated from a culture maintained on beans at Wageningen University, Plant Sciences Group, Wageningen University in the Netherlands. It was known to easily survive and reproduce also on *Arabidopsis* from previous experience. For the initiation of the rearing both larvae and adults were used. Next generation's young adults were used during the pollen exposure studies.

Predatory bugs (*Orius laevigatus*), flower bugs (size 3mm), were obtained in the adult stage from the commercial company Koppert Biological Systems, Berkel en Rodenrijs the Netherlands (purchased just before experimental use). They were kept in containers with ample food in order to have them in good and healthy condition at the start of the experiment.

Predatory mites (*Amblyseius swirskii*) (size 0.5 mm) were initially also obtained in batches from Koppert Biological Systems, Berkel en Rodenrijs, the Netherlands together with flour mites as food. However, during the first trials separating the mites from the food appeared to be difficult and time consuming. Due to low numbers virtually/almost no mites could be recaptured after exposure. For the third trial freshly separated mites were obtained from Entocare Wageningen, The Netherlands on the start day of the experiment.

Treatments were initially designed to measure pollen load of single organisms (thrips, mites and bugs) and combinations of thrips with either mites or bugs, in order to determine possible interaction effects between prey and predators on pollen load, due to assumed potential density effects of food switching (see discussion). Because of experimental constraints the assessment of interaction effects appeared too ambitious.

2.4 Exposure trials

Three successive trial were performed to measure *Arabidopsis* pollen attachment during a limited time mimicking an experimental situation where a predatory bugs and mites were released in order to control pests (thrips). At the start of the trials the procedure of release-recapture and pollen extraction from the insects was tested in a few additional units. Pollen extraction and staining techniques were adopted from pollinators research methods (Ham *et al.* 1999)

Trial 1

The original setup aimed at testing potential pollen load for the pest and predators apart and combined is described below and shown in Figure 2. The experiment consists of 5 treatments:

1. Thrips (20 per cage)
2. Mites (10 per cage)
3. Predatory bugs (20 per cage)
4. Thrips with mites (20 + 10 per cage)
5. Thrips with predatory bugs (20 + 10 per cage)

All treatments were replicated five times (giving five cages per treatment with three plants per cage, see Figure 2). At day 1 thrips were released; mites and flower bugs were released at day 14. At day 17 the experiment ended and thrips and natural enemies were recaptured as far as possible. Thus, thrips were exposed to pollen for 16 days and the natural enemies were exposed to pollen for 3 days.

Figure 2 *Experimental units used in trial 1 and trial 2 consisting of potted Arabidopsis plant with plastic cylinders, placed in a climate cabinet.*

Trial 2

Only a single treatment (twenty *Orius laevigatus* per cage) was applied to obtain more accurate estimates of pollen load in flower bugs. The treatment was replicated five times (i.e. five units with three plants per cage). The predatory bugs were carefully released in the experimental unit and recaptured after three days of exposure to flowering plants. Part of the recaptured bugs was transferred to non-flowering plants and stayed there for another two days to see if pollen-loss occurred. From the other part of the recaptured bugs the attached pollen were extracted, stained with fuchsine and counted immediately. The pollen load of the transferred bugs was determined two days later.

In order to recapture insects and mites with pollen attached, but to avoid undesired contamination with pollen due to recapture techniques, the animals had to be recaptured with great care. This was done by applying CO_2 to the plants after cages had been removed carefully, which caused the insects to drop from the plants without further disturbance. Recovery of mites in Trial 1 without disturbance did not appear to be feasible because numbers were low and they are very inconspicuous.

The collected thrips and bugs were carefully stored in Eppendorf tubes for further determination of pollen load. Pollen load was determined by making preparations of bugs or thrips with Kaiser's glycerol gelatine and fuchsine. The slides were covered with Permacel tape. In this way attached pollen numbers could be counted under the microscope (Zeiss Axioscop 200x).

Trial 3

To measure the pollen attachment in the commonly used predatory mite *Amblyseius swirskii* a switch was made from the potted cages to a small scale trial using flowering stems in petri dishes with water agar to keep the flowers vital. In such a small scale trial it appeared to be much easier to recapture mites after exposure to pollen.

In this setting the mites had no prey and fed on the available pollen as they usually do in natural conditions. In this case exposure was assumed to be maximal. In the experiment ten mites were released in each of the five petri dishes by transferring them from stock to a piece of filter paper under cooled conditions. The piece of filter paper was then transferred to the petri dish with flowers. After that the mites were allowed to move freely and feed at 20°C and LD 16:8 light conditions. After 2 days most of the mites could be recollected by searching the petri dishes under a binocular microscope(Zeiss Stereomicroscope SR 40x). Subsequently, mites were stained individually with Kaiser's glycerol gelatin and fuchsin and mounted on slides for pollen counting under the microscope (Zeiss Axioscop 200x).

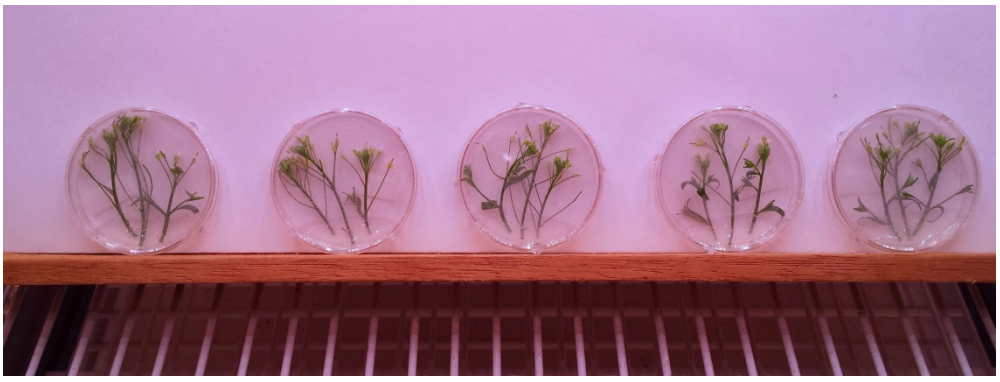


Figure 3 *Experimental units, petri dishes with flower tops, used in trial 3.*

3 Results

Trial 1

The first trial was set up to assess the pollen attachment on thrips, predatory bugs and predatory mites while foraging on *Arabidopsis* and getting in contact with flowers and pollen. The CO_2 method appeared to be effective in recollecting part of the thrips and predatory bugs at the end of the exposure time. However, due to the hidden life style, the small size and because of the transparent and inconspicuous appearance of the mites, we were unable to recapture the low numbers of mites from the units.

The recovery of thrips and bugs was successful but time did not allow assessing the number of pollen per individual insect. So insects were pooled per unit and after centrifuge treatment total numbers of pollen attached to the insects were determined. As Table 1 shows thrips (adults and larvae) were recaptured in sufficient numbers to estimate the average pollen load. In the treatments where only thrips was present 67 specimens were recaptured, together carrying 311 pollen grains, which means an average of about 4.6 grains per thrips.

In the units where both thrips and *Orius* bugs were present totally 503 pollen grains were found on 30 thrips specimens giving an average of 16.7 grains per thrips. The difference in average number of pollen grains/thrips between the single and combined units is just significant (Students t-test $P<0.05$) despite the big variance. An explanation could be that thrips shelter more in the flowers when predators are present or that their increased activity caused more contact with pollen.

The number of predatory bugs recaptured was low (only 4 % of those released) and varied per unit. Probably many bugs were lost due to high humidity and condensation in the type of cages used. The number of pollen grains attached to their bodies after 3 days ranged from 2 to 47 per individual (average about 21 pollen grains per bug). The data variability does not allow for a further statistical comparison between units with and without prey.

Table 1

The average number of Arabidopsis pollen on thrips and predatory bugs after staying on flowering plants for 10 and 3 days respectively. Averages are based on total pollen divided by the number of insects recollected.

Only thrips			Only *Orius* bugs			Thrips and *Orius* bugs combined					
cage	# thrips	avg # pollen	cage	# bugs	avg # pollen	cage	# thrips	avg # pollen	cage	# bugs	Avg # pollen
1	10	2.30	1	3	13.0	1	8	7.6	1	0	..
2	14	5.35	2	1	47.0	2	6	35.6	2	1	17.0
3	5	7.80	3	0	..	3	3	27.6	3	1	34.0
4	9	9.00	4	1	2.0	4	4	55	4	1	31.0
5	29	3.20	5	0	..	5	9	90	5	0	..
	67	4.64		5	17.6		30	16.76		3	27.3

Trial 2

Trial 2 aimed at estimating pollen load on predatory bugs to get more information on the individual variation. It also aimed to get a first estimate of the loss of pollen grains when no new pollen is available in the environment.

Therefore part of bugs were analysed immediately after three days of exposure to flowering *Arabidopsis* while part of the bugs were transferred to non-flowering plants. The bugs were recaptured after two days and the remaining pollen grains were counted.

Table 2 shows that the pollen attachment to predatory bugs in trial 2 was a bit lower than in trial 1 but still in the same order of magnitude, confirming the significant acquisition of pollen by the bugs. Bugs carry about ten pollen grains after two days of exposure.

The lower average number of pollen grains per bug two days after stopping pollen exposure indicates that pollen grains are gradually lost. This is what one expects, but the numbers are too variable to give a firm conclusion on this aspect. However, it shows that pollen may keep attached to the predators body for a couple of days.

Table 2

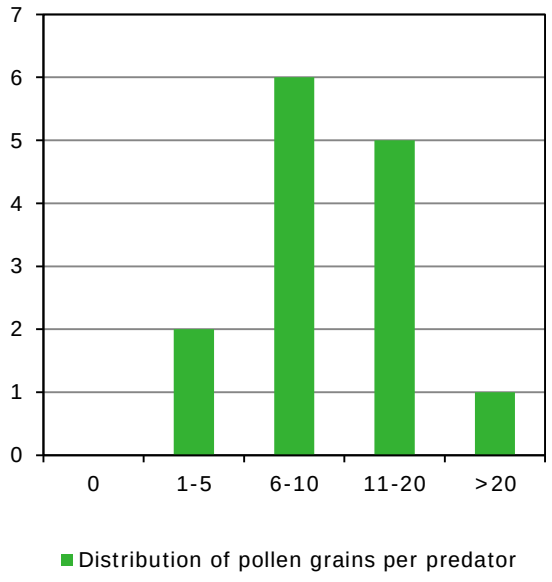
Average number of Arabidopsis pollen grains attached to predatory bugs after 2 days exposures to flowering plants (cages 1a, 2a and 3a) and after 2 subsequent days without pollen exposure (cage 1b, 2b and 3b).

Cage	# Bugs recaptured / analysed / transferred	# pollen grains per bug average ± s.d. after exposure	range	Cage	# Bugs recaptured / analysed	# pollen grains per bug average ± s.d. after exposure	range
1a	15/ 7 / 8	9.9 ± 5.3	3-19	1b	0 / 0	-	-
2a	9 /4 / 5	10.2 ± 7.1	4-20	2b	5 / 5	5.0 ± 4.8	1-13
3a	3 /3 / 0	12.3 ± 10.1	6-24	3b	0 / 0	-	-

Table 3

Raw data for the numbers of pollen grains attached to individual bugs after exposure to flowering Arabidopsis and according distribution histogram.

Unit	Bug	# pollen	average	s.d.
1a	1	9	9.9	5.3
	2	13		
	3	3		
	4	19		
	5	7		
	6	12		
	7	6		
2a	1	4	10.2	7.1
	2	20		
	3	6		
	4	11		
3a	1	24	12.3	10.1
	2	6		
	3	7		
2b	1	3	5.0	4.8
	2	1		
	3	2		
	4	13		
	5	6		



Trial 3

While the recapture of predatory mites failed in the original set-up in trial 1, the experimental approach of trial 3 appeared to be more successful. About half of the released mites could be recollected after two days. By using individual mites and pollen staining, pollen load was relatively simple to assess.

The microscopic preparation and examination indicated most pollen were on the outside of the body though it cannot be excluded that some freshly consumed pollen grains were inside the body. As pollen was both present in flowers and fallen on the petri dish mites may have picked up the grains in different ways.

Pollen density in the petri dishes may have affected the average pollen loads measured in each dish. The results in Table 4 show that the mites can carry considerable numbers of pollen (20 grains per individual). These numbers are comparable to those found on the predatory bugs in trial 1 and trial 2, even though mites are considerably smaller than the bugs.

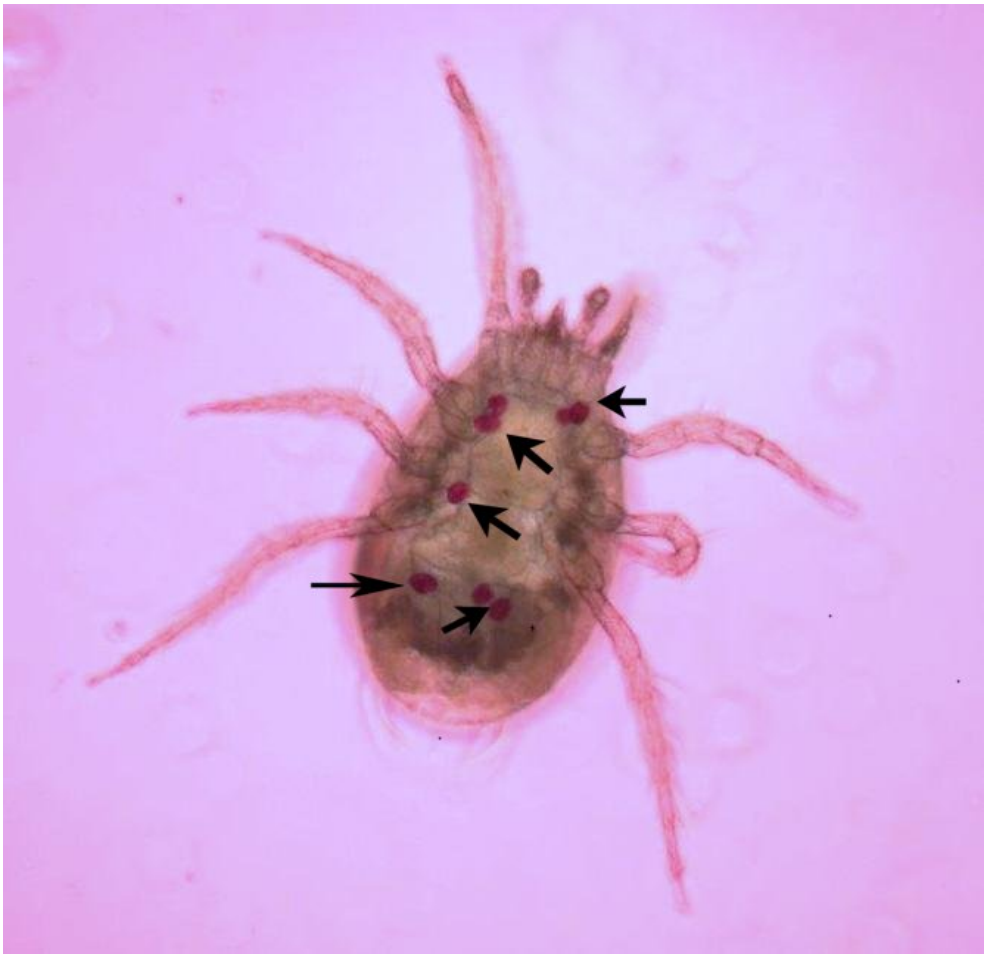
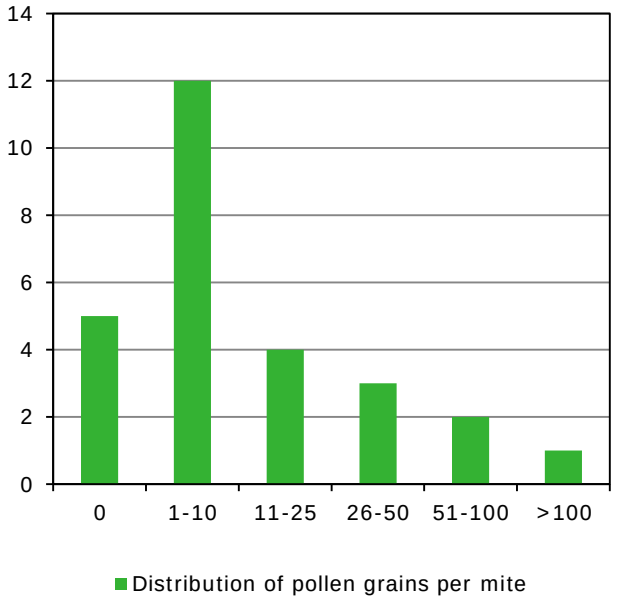


Figure 4 *Amblyseius swirskii predatory mite with pollen grains (arrows) attached to the back.*

Table 4

Number of Arabidopsis pollen grains attached to predatory mites after 2 days of exposure to Arabidopsis flowers and according distribution histogram.

Dish	Mite	# pollen	avg.	s.d.
1	1	10	19.1	34.9
	2	2		
	3	0		
	4	7		
	5	6		
	6	90		
2	1	0	17.2	15.6
	2	39		
	3	4		
	4	32		
	5	10		
	6	18		
3	1	33	12.8	14.1
	2	4		
	3	0		
	4	5		
	5	22		
4	1	0	20.0	25.0
	2	7		
	3	8		
	4	69		
	5	20		
	6	16		
5	1	4	29.0	54.0
	2	0		
	3	110		
	4	2		



4 Discussion

Pollen attachment to pest insects and their natural enemies in a biocontrol system is a dynamic process that varies with experimental conditions and time.
Our experiments focused on gathering the first basic information about such a process in a model system and getting hold on the factors that play a role in the attachment of pollen grains to insects in such a system. The case chosen was assumed to be representative by studying interaction between *Arabidopsis*, thrips as a pest, and flower bugs and predatory mites, mimicking real world combinations in research.
But realizing the dynamic and complex nature of the pollen attachment and transport process, the experimental results should be considered as preliminary.

Answering a simple question such as: 'how much pollen do natural enemies transport when feeding on prey that lives on flowering plants', appeared to be quite complex when broken down to a specific case and translated into an amenable and relevant experimental setting.

Beforehand it should be realized that even clear-cut data from a good case study cannot be translated directly to other insect-plant combinations because:

- Every insect species will be associated with flowers and pollen in a different way; some spending much time in and feeding on pollen, some less. Attachment of pollen to and loss from the insect body will depend on morphological characteristics of the species.
- Plant species vary in the abundance and time frame of pollen production. Size and morphological characteristics of pollen such as surface structure and stickiness will impact the adherence of pollen to insects.
- Switching between prey and pollen feeding, as is done by many predators in relation to prey density, may impact the attachment rate of pollen substantially.
- Pollen load (attached pollen per insect) will vary in time, being high during maximal flowering and lower before and after the flowering phase (due to pollen loss).
- Pollen load may diminish when pollen is no longer available. Maximal pollen load is not likely to be retained when the insect leaves the pollen rich experimental setting.

So this experimental setting required the following conditions:

- Defined exposure to pollen in a time frame when pollen is abundant.
- Pollen load after a defined period of feeding in a pollen rich micro-environment is likely to reflect worst case situation in term of pollen dispersal potential.
- The presence and abundance of prey is likely to influence the contact with pollen as natural enemies may switch to the most rewarding food source.
- During recapture and pollen assessments care should be taken to avoid loss of pollen from the insects or detection of extra pollen due to contamination.

Despite the experimental constraints the results however indicate that noticeable numbers of pollen are attached to both pests and predators when there is affinity with pollen as alternative food as was the case in the species chosen for the experiment.

The dynamic nature of the pollen attachment process is illustrated by the fact that pollen will only be acquired during the flowering stage of the experimental plants. It is likely that pollen density strongly affects the attachment of pollen grains. After flowering pollen attachment stops and the results indicate that a substantial number of pollen is lost from the insect body after some days. Hence the total numbers of attached pollen in the experimental system depends on the growing stage of the plants, the species that are present, their affinity to pollen and the (fluctuating) numbers of active insects.

In our case study, during maximal flowering of *Arabidopsis*, thrips on average carried about five pollen grains per individual, *Orius* bugs about ten to twenty grains and *Amblyseius* mites about ten grains. Hence natural enemies may carry noticeable amounts of pollen, at least in a worst-case situation such as the one studied here.

Facing the experimental constraints one may wonder how realistic the experiments are compared to a normal experimental situation in a greenhouse or climate room. The main weakness may be that the data were from small units where only the predators were active, forcing them to feed on pollen. In the combined *Orius*/thrips units we were unable to establish (and check) a realistic predator/prey situation in terms of numbers. In all cases it seems likely that forcing pollen feeding promoted the attachment of pollen to the insects. But from the species used in this study it is known that they are frequent pollen feeders, so it seems likely that the results at least confirm that pests and natural enemies can carry noticeable numbers of pollen grains in a pollen rich environment. The numbers of pollen are also similar to the number of rice pollen near rice fields observed for *Orius* and Thrips under natural conditions (Pu *et al.* 2014). Therefor care should be taken when pollen transport in contained GMO settings has to be minimized.

The relative contribution and impact of pollen attachment to pests and natural enemies for potential gene flow from contained experiments to natural environments remains hard to estimate. Many more factors are important such as the amount of escaping insects, the survival after escape, the rate of pollen loss after exposure, the chance of these non –specific flower visitors to reach the same plant species elsewhere, and that pollen survive and compete with other pollen transported by wind and specific pollinators.

Based on what is known now the breakdown of the process into single steps may help to quantify the potential pollen grain flow and potential gene-flow on this and similar cases. At several points in the process we can only make expert guesses to compare the potential pollen grain flow from chemically or biologically controlled experiments.

Assuming that during sub-optimal chemical control short outbreaks of thrips can occur that are 10-100 times higher than the continuous thrips levels under a proper biocontrol regime. In such a case pollen attachment to predators (that can be twice that of the pest) is still is low compared to pollen attached to thrips pressure under partly effective chemical control.

We assume that in the case of *Arabidopsis* biocontrol is considered to be effective and applicable when thrips densities remain below one individual per plant. Such levels can be maintained by one predatory bug or five predatory mites per ten plants. When thrips, predatory bug and predatory mites on average carry five, ten and fifteen pollens grains respectively, this means that in a containment unit with 1000 plants 7000-15000 grains are transportable in worst case in a flowering crop. When 1 in 100 of the insects escape by active flight or by attachment to cloths 100 pollen grains may escape from the containment unit of which half is estimated to be lost in 2 days. This results in the distribution of approximately 50 grains from an experiment into the environment.

In the case where flowering *Arabidopsis* is growing nearby the experimental unit, the chance that a thrips, bug or mite will reach such a plant is likely to be low even though thrips is an active disperser and frequently visits *Arabidopsis* (Hoffmann 2003). When such chance would be 1 in 50 it would mean that from each experiment only one pollen grain may come in contact with the stamen in one of the flower. The chance of fertilizing such a flower may be one in a thousand because of competition with the many natural pollen grains present. Additionally, in natural populations of *Arabidopsis* about 99% of the seeds arise from self-pollination and only 1% from cross pollination.

This results in a chance of 1 in 200.000 that one experiment will lead to one single cross fertilization and a viable seed may be formed. In the case one in 100 seeds germinates and leads to a new flowering plant, two million experiments have to be done to result in one such a plant. Though based on a number of assumptions, such an occasion can be considered as extremely unlikely

Hence the risk of outcrossing seems to be extremely low at first sight. In settings similar to the one studied here and when biocontrol is applied with similar effectiveness as with chemical control, risks are unlikely to be very different, as natural enemies contribute less to pollen dispersal than pests will do. However, these conclusions may not be valid in settings that differ strongly from the combination of pests and natural enemies studied here.

We tend to conclude that in a biocontrol system where pests and natural enemies are in dynamic interaction, and assuming that biocontrol is effective, pest numbers tend to be low. The absolute contribution of the natural enemies to the amount of pollen ‘on the move’ is likely to be lower than that of the contribution of the pest itself. Therefor it may be safer to apply effective biocontrol than using a partly effective chemical control system.

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