Natural pest management - genetic variation in volatile production in cabbage

Breeding for enhanced attraction of natural enemies

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Contents

Summary

Alternatives to pesticides, which have detrimental effects on humans, nature, and beneficial insects, are urgently needed. The use of biological pest control, i.e. natural enemies of pest insects that locate the pests on the plants and kill them, is a nature-friendly way to suppress pest survival and population development. Some plants release volatile organic compounds in response to attack by herbivores, that result in increased attraction of natural enemies. However, increasing indirect defense mechanisms in crop plants has received relatively little attention in breeding programs. In this report we explore the possibility of increasing the attraction of natural enemies by crop plants through breeding. For that, we investigated if natural variation for attraction is present in a crop and its wild relatives, in this case cabbage. In addition, we studied the variation in emission of VOCs in response to herbivore feeding. We used accessions of *Brassica oleracea*, including domesticated hybrids, landraces, and wild accessions, and accessions of the wild relatives *B. incana*, *B. villosa* and *B. montana*. Using a choice assay, we studied attraction of natural enemy *Cotesia vestalis* to plants that are fed on by *Plutella xylostella.* We compared each accession to the known attractive landrace Christmas Drumhead. We found that there is not one (wild) species, that is more attractive than the other species. In fact, we identified attractive accessions and non-attractive accessions in all studied species. In general, wild accessions and landraces seemed to be more attractive than domesticated hybrids. In addition, we measured herbivore-induced volatiles in selected accessions, and find considerable variation in VOCs between species. Relatively little variation was found between attractive and non-attractive accessions of the same species, suggesting that the attraction could be due to only a few volatiles. Attractive accessions were found to emit more alcohols and aldehydes. We conclude that there is variation for both attraction of natural enemies and volatile emission, that could potentially be used for breeding plants with enhanced attraction. Further studies should explore how enhanced attraction relates to the success of natural enemies, to investigate if it would indeed lead to a successful control of pest insects. Identified attractive and non-attractive accessions of the same species could be used to start breeding programs.

1 Introduction

1.1 Background

In agriculture, pesticides are used to deal with pest problems, but due to their detrimental effects on humans, nature, and beneficial insects, alternatives are needed. One of such alternatives is the use of biological pest control, which is based on ecological relationships between the plant, the pest and natural enemies of pests. In this system, naturally occurring enemies of pest insects, which parasitize or feed on the pests, are used to suppress survival and population development. Improved attraction of natural enemies to crop plants could improve biological control in open field environments. In response to attack by herbivores, many plants emit volatile compounds (VOCs), which can be recognized and used as localization signal by natural enemies. However, until now, the attraction of natural enemies of pests has been largely neglected as potential trait for breeding. Therefore, domesticated and cultivated plants may have lost these traits. Reintroduction of these traits in cultivars is possible via breeding for integration of tri-trophic interactions. However, there are several requirements for this to be feasible: a) There should be genetic variation for VOC emission and natural enemy attraction between accessions or species, which can be used for classical breeding and b) VOC production must be genetically rather simple (Stenberg et al., 2015). In addition, the circumstances in agricultural fields must be such that they support the presence and survival of natural enemies in the field or in the surrounding environment.

1.2 Project goal

In this project, we experimentally explore the possibilities for breeding for tri-trophic interactions by 1) investigating the natural variation in attraction of natural enemies in a crop, in this case cabbage, 2) studying natural variation in volatile production in selected accessions and 3) studying the connection of tri-trophic relationships with the quality of the ecosystem. The third point is discussed in another report. Here, we report on the experimental activities on variation in attraction of natural enemies and volatile production.

2 Material and methods

2.1 Study system

As study system we used cabbage (*Brassica oleracea*) and wild relatives. As herbivore, we used the Diamondback moth, *Plutella xylostella*, which is an important pest insect on cabbage worldwide. As natural enemy, the parasitoid *Cotesia vestalis* was used. *Cotesia vestalis* can respond to volatiles emitted by *B. oleracea* damaged by *P. xylostella* (Gols et al., 2012). Our goal was to determine how large the variation for attraction of this natural enemy is across *B. oleracea* and in three wild relatives: *Brassica villosa*, *Brassica incana* and *Brassica montana*. These three species are all crossable with *B. oleracea*, which is important for future breeding strategies. Our first aim was to identify attractive and non-attractive accessions. To this aim, we compare all tested accessions against *B. oleracea* var. *capitata* 'Christmas Drumhead', which is known to be attractive to parasitoids after feeding of *Pieris* caterpillars (Poelman et al., 2009; Aartsma et al., 2019). In addition, we asked if the variation is largest within or between species, and whether cultivated accessions are less attractive than wild accessions.

2.2 Approach

2.2.1 Selection of accessions

A large number of accessions of *B. oleracea* and wild relatives is present in the collection of the WUR Plant Breeding group. To be able to answer our questions, we aimed to test around 30 accessions. We included several accessions of each of the wild relatives. For *B. oleracea*, we included hybrids (cultivated accessions), landraces and wild accessions. In this selection, we aimed for variation in country of origin and type (broccoli, cabbage, white cabbage, red cabbage or kale) (Table 1). All accessions were obtained from the collection of Plant Breeding, except for the six cultivated hybrids, which were obtained from Bejo Zaden (Warmenhuizen). Our aim was to test three accessions of each wild species (*B. incana*, *B. montana* and *B. villosa*), but only one accession of *B. montana* germinated with enough plants to be used in the test.

Table 1 *Selection of accessions that were tested in this study. Shown are name of the accession, species, variety, whether the accession is wild, a landrace or a hybrid, type, and country of origin.*

Accession name	Species	Variety	CWR/LR/H[A]	Type	Origin
Bol2011-0219	*B. oleracea*	capitata	CWR		Turkey
Bol2011-0209	*B. oleracea*	italica	CWR		Germany
Bol2011-0446	*B. oleracea*		CWR		Germany
Bol2011-0214	*B. oleracea*	capitata	LR		China
Bol2011-0160	*B. oleracea*	acephala	LR	palm leaf kale	Italy
Bol2017-0002	*B. oleracea*	alboglabra	LR	chinese kale	Thailand
Christmas Drumhead	*B. oleracea*	capitata	LR	white cabbage	Great Britain
Bol2011-0131	*B. oleracea*	acephala	LR	kale	Croatia
Bol2011-0208	*B. oleracea*	italica	LR	broccoli	Italy
Bol2021-0005	*B. oleracea*	botrytis	H	cauliflower	The Netherlands
Bol2021-0002	*B. oleracea*	capitata	H	red cabbage	The Netherlands
Bol2021-0003	*B. oleracea*	capitata	H	white cabbage	The Netherlands
Bol2021-0001	*B. oleracea*	capitata	H	red cabbage	The Netherlands
Rivera	*B. oleracea*	capitata	H	white cabbage	The Netherlands
Bol2021-0006	*B. oleracea*	botrytis	H	cauliflower	The Netherlands
Bwi2011-0023	*B. incana*		CWR		Italy
Bwi2011-0024	*B. incana*		CWR		Italy
Bwi2011-0026	*B. incana*		CWR		Ukraine
Bwi2011-0037	*B. montana*		CWR		Italy
Bwi2012-0010	*B. villosa*		CWR		Italy
Bwi2011-0048	*B. villosa*		CWR		Italy
Bwi2011-0050	*B. villosa*		CWR		Italy

A:CWR: crop wild relative, LR: landrace, H: Hybrid.

2.2.2 Choice assay

Plants of all accessions were grown in greenhouse facilities of the university (Unifarm) in sets, from March 2021 to July 2021. All plants were tested when they had five real leaves. In a trial experiment, *P. xylostella*-damaged plants were shown to be chosen by *C. vestalis* over control plants. In our experiments, only plants undergoing feeding by *P. xylostella* were tested. To determine attractivity of each accession, plants of each accession were compared to plants of Christmas Drumhead in a Y-tube assay. For this, each plant was infested by 15-25 *P. xylostella* larvae (larval instar L2 and L3), which were allowed to feed on the plants for 24 hours. After 24 h of feeding, plants were used in the assay. In this assay, naïve *Cotesia vestalis* parasitoids were offered the choice between two plants and their choice was recorded (Figure 2.1). For each comparison, one replicate consisted of 10 responsive female wasps.

2.2.3 Volatile measurements

To measure volatiles and study differences in induced VOC between accessions, we chose from each used species several accessions that were either less or more attractive than Christmas Drumhead, based on the choice assay performed. In total, four accessions were selected that were classified as attractive: Christmas Drumhead, Bol2011-0131, Bol2011-0208, Bwi2011-0026, and four accessions were selected that were classified as non-attractive: Rivera, Bwi2011-0023, Bwi2011-0048 and Bwi2011-0050. The plants were grown until five weeks of age, when each plant was either infested by 15-25 *P. xylostella* larvae (larval instar L2 or L3) or not (uninfested plants). After 24 h of feeding, the amount of damage to the plants was scored, and leaves were harvested and immediately snap frozen in LN_2 and stored at -80 °C until further handling. Frozen leaves were ground under LN_2 to fine powder and 100 mg ground frozen material was weighed in 10 ml (precooled in LN_2) glass vials with screw cap. 1mL of a CaCl2/EDTA solution was added resulting in a 5M concentration. Volatile compounds were then trapped by SPME and analysed by GCMS as described by Diez-Simon *et al*. (2020). Raw data were processed using an untargeted metabolomics approach as described in detail by Diez-Simon *et al*. (2020).

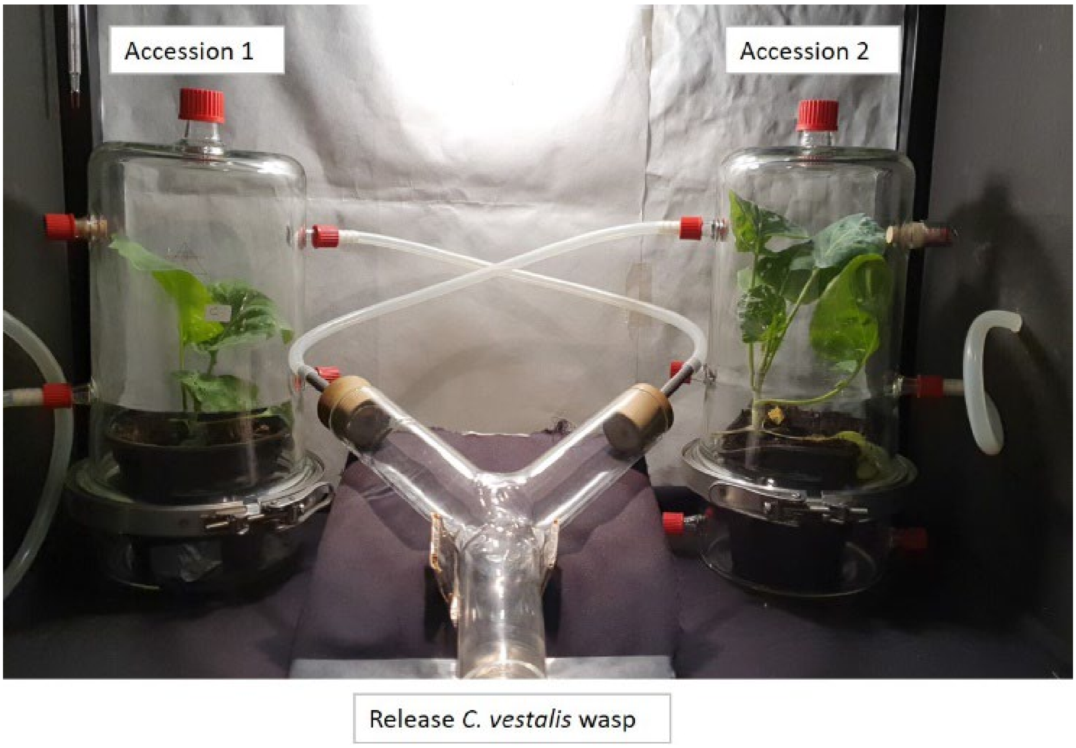

Figure 2.1 *Y-tube setup. In the behavioural experiment we used a glass Y-tube olfactometer based on the one described by Fatouros et al. (2012). The Y-tube is 3.5 cm in diameter and consists of two arms (12 cm long) connected to a stem with a length of 22 cm. Each arm is connected to a 5 L glass flask (diameter 15 cm and height of 30 cm) using silicone tubes. Four liter/min filtered air flow is led through the vessels. The plants are placed in the glass flasks. At the entrance of the Y-tube, a C. vestalis female is released. Each parasitoid was released individually, and in every replicate, the response of 10 responsive female wasps was recorded. A parasitoid was considered as respondent only when it entered the arm from which the blend of preference derived and passed the end line. Wasps that did not make a choice between the two arms within 5 minutes or did not cross the end line within 10 minutes were considered as non-responding.*

3 Results and Discussion

3.1 Attractiveness accessions

3.1.1 Choice assay comparison to Christmas Drumhead

In total, 18 of the selected accessions, were tested in a choice-assay comparison with Christmas Drumhead. The dataset of all comparisons can be found in Annex 1. The results are summarized in figure 3.1, in which the percentage of wasps that choose either odour source is shown. Our first aim was to determine if any of the accessions were more attractive than Christmas Drumhead. Indeed, in direct comparisons, several accessions were chosen by more wasps than this accession, most notably Bol2011-0208 and Bwi2012-0010. Several others seemed as attractive as Christmas Drumhead (50:50 choice). Next, we aimed to identify unattractive accessions. Previously, the hybrid Rivera was shown to be less attractive than Christmas Drumhead (Poelman et al., 2009). Here we confirmed this result, and show that also other commercially used hybrids seem non- attractive (all light green bars in figure 3.1). Also several landraces and wild accessions, i.e. Bwi2011-0050, Bol2011-0446 and Bwi2011-0048 were chosen much less than Christmas Drumhead.

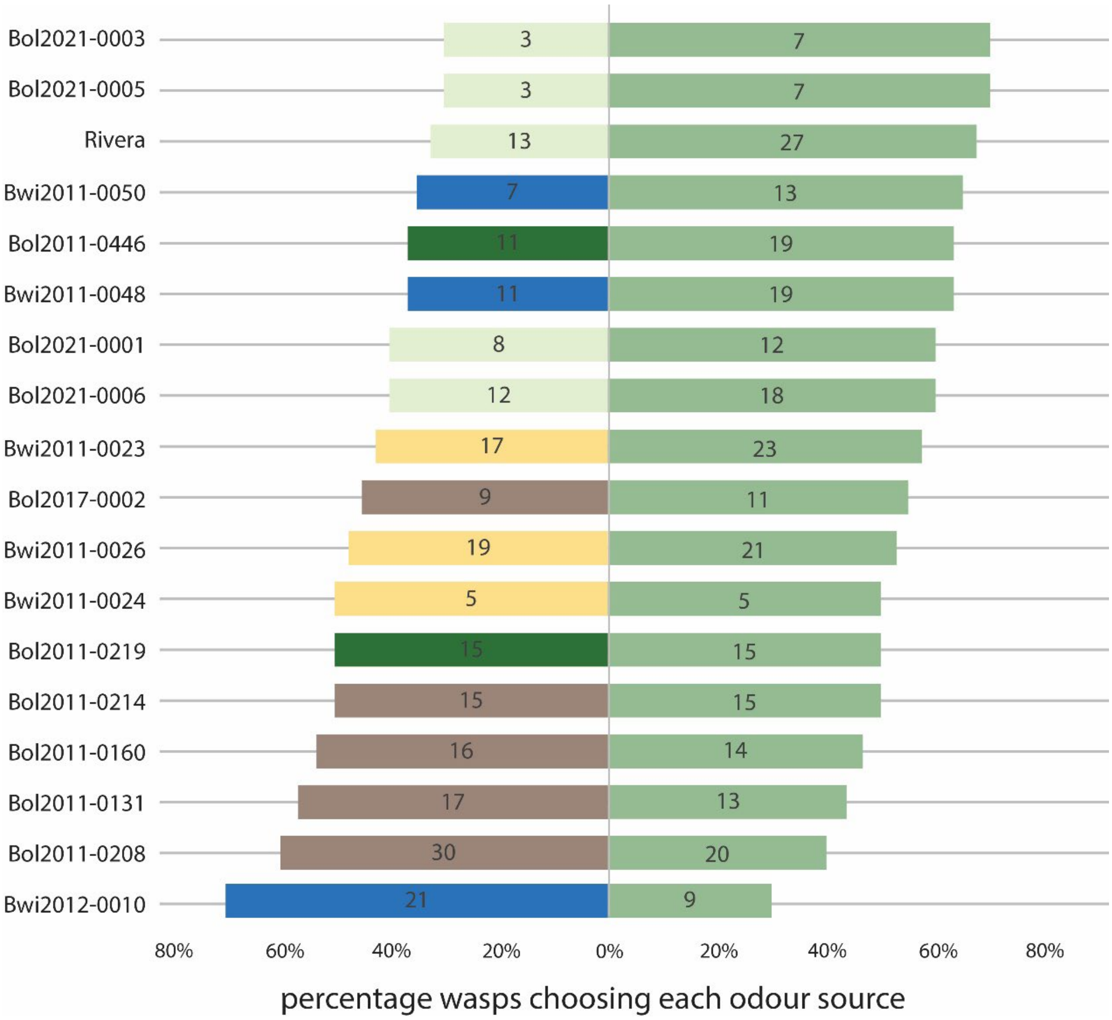

Figure 3.1 *Choice assay. For each accession, the percentage of wasps that choose it compared to Christmas Drumhead (right, green) is shown. The colours indicate the species or type that the accession belongs to. Blue: B. villosa, yellow: B. incana, grey: B. oleracea landraces, dark green: B. oleracea wild accessions, light green: B. oleracea hybrid cultivars. For this graph, all replicates were summed. The number in the bar is the number of wasps that chose the specific odour source. Some accessions were tested in more replicates than others and therefore have a higher number of wasps.*

3.1.2 Variation in attractiveness between species

If we compare variation of attraction between and within species, it is apparent that there is not one (wild) species, that is more attractive than the other species. In fact, in all species, we find attractive accessions and non-attractive accessions. This is most clear for *B. villosa*, where one accession was the most attractive (Bwi2012-0010), and two others were non-attractive (Bwi2011-0050 and Bwi2011-0048; blue bars in figure 3.1). We further tested these three accessions also in comparison to other accessions, and found that Bwi2012-0010 was preferred by wasps in each comparison it was in, while Bwi2011-0050 'lost' almost all comparisons (data not shown). For *B. montana*, we were not able to test enough accessions to draw conclusions. For *B. incana*, the difference within species seemed less clear in comparison with Christmas Drumhead: the accessions Bwi2011-0024 and Bwi2011-0026 were chosen by about the same number of wasps as Christmas Drumhead, and Bwi2011-0023 was chosen by less wasps. However, Bwi2011-0026 was also tested in comparison with other attractive accessions, and was chosen over the attractive Bol2011-0208. Bwi2011-0023 was also directly compared to Rivera, and chosen less (data not shown). In conclusion, in all tested *Brassica* species, there are some accessions that are attractive and some that are non-attractive to the parasitoid *C. vestalis* after feeding of *P. xylostella*.

3.1.3 Variation in attractiveness between domesticated and wild

For *B. oleracea*, landraces, hybrids and wild accessions were tested. In general hybrids seem to attract less parasitoids than other plants. The landraces (grey bars in figure 3.1) tested were all as attractive as Christmas Drumhead (also a landrace), or more attractive. For the wild species one was as attractive as Christmas Drumhead (Bol2011-0219), while one was less attractive. Based on our dataset, we would conclude that in the wild *B. oleracea*, there is variation for attraction of natural enemies, that most landraces do attract them, and that hybrids are not attractive to natural enemies, and may have lost this trait altogether. However, we would have to test more accessions to confirm this result, and increase the number of replicates for each accession tested.

3.2 Variation in volatiles

3.2.1 Variation in volatiles between species

In the choice assay, variation was found for attraction of the parasitoid *Cotesia vestalis* after feeding of *P. xylostella*, both in *B. oleracea*, and wild relatives *B. villosa* and *B. incana*. Next, the variation in (herbivore-induced) emitted volatiles between eight accessions (attractive and non-attractive) was assessed. For *B. oleracea* and *B. villosa*, attractive and non-attractive accessions were chosen. For *B. incana*, only accessions that were classified as non-attractive were available due to issues with seed germination. All plants were grown in a greenhouse, and half of the plants were infested with *P. xylostella* L2-L3 larvae. Leaves from both infested and non-infested plants were harvested after 24 hours and snap frozen. Samples were ground, volatiles were trapped, and then measured using GC-MS.

Raw data were processed using an untargeted metabolomics approach as described Diez-Simon *et al*. (2020), which led to the identification of 110 VOC in all samples. Analysis of the data showed that there is considerable variation in the VOC profiles of the different species (*B. oleracea*, *B. incana*, *B. villosa*), with B. *villosa* being the most different from the other two. There is also variation between accessions of each species, which was largest in *B. villosa* as well. Possibly, this is due to villosa still containing the most variable genetic material.

3.2.2 Variation in volatiles between attractive and non-attractive accessions

Interestingly, for each accession, there was only little variation between feeding-induced and non-induced samples. This would indicate that only a few VOCs that were measured here were induced after *P. xylostella* feeding. Next, the subset of Plutella-induced volatiles were analyzed, and attractive and non-attractive accessions were compared using an OPLS-DA analysis. This led to a selection of several volatiles that were characteristic of attractive accessions, and volatiles that were characteristic of non-attractive accessions. Attractive samples were shown to contain more alcohols and aldehydes. Possibly, these green leaf volatiles are attractive to the parasitoid, as has been found before (Reddy et al., 2002; Mumm & Dicke, 2010). However, this should be further explored by comparing VOCs in attractive and non-attractive accessions in more detail.

4 Conclusion and future research

In this project, we have investigated the natural variation in attraction of natural enemies in cabbage and wild relatives, and studied variation in volatile production in selected accessions. Importantly, we conclude that there is variation for attraction of the parasitoid *Cotesia vestalis* by plants after feeding of *P. xylostella*, both in *B. oleracea*, and wild relatives *B. villosa* and *B. incana*. Comparing wild accessions of *B. oleracea* and hybrid cultivars, indicated that landraces and wild accessions were more attractive to natural enemies than domesticated cultivars. If we compare variation of attraction between species, it is clear that there is not one (wild) species, that is distinctly more attractive than the other species. In fact, in all species tested, we have identified attractive accessions and non-attractive accessions.

However, what we have studied so far is limited to *one* natural enemy (parasitoid) and compared plants only after feeding by *P. xylostella*. We could further study the selected accessions to investigate whether the attraction is specific to *C. vestalis* or also to other parasitoids or predators, and could be used for general attraction of parasitoids and predators. Further, naïve parasitoids, as used here, should be compared to parasitoids that have learned to recognize plants infested by insects. It may be, that these parasitoids have learned to recognize specific volatiles. In addition, it is vital that the emission of volatiles takes place only after induction by herbivores, otherwise natural enemies may learn to not respond to the volatiles anymore, if no reward in the form of an prey or host is found. To be able to use this increased attraction of accessions in the field, it is also essential to study if increased attraction results in increased parasitisation of pest insects by means of a field or greenhouse assay. These experiments could determine how far the increased attraction of natural enemies found here, would result in successful control of pest insects.

We found considerable variation in emitted VOCs, especially between species. The dataset of all emitted volatiles could be explored further to compare attractive and unattractive accessions in more detail. Possibly, there are species-specific volatiles that are emitted by accessions that can be linked to attraction. In addition, the identified volatiles could be used in follow-up research investigating the diversity of volatile production and (genetic) causes of difference between attraction.

Our results suggest that within the *Brassica* complex, there is enough variation present that could potentially be used for breeding for enhanced attraction of natural enemies. Accessions with contrasting phenotypes can be crossed as a first step of generating populations that could be used to further study and map the trait in cabbage or wild relatives.

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Annex 1 Dataset choice assay comparison

The dataset is supplied as a seperate excel file.

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Report WPR-2021-11

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