WAGENINGEN UNIVERSITY
LABORATORY OF ENTOMOLOGY

Plant mediated effects of *Brassica oleracea* infested with *Brevicoryne brassicae* on the performance and behaviour of *Diaeretiella rapae*

No: 821116-324-080

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Summary

Many aphid species are major agricultural pests. Infestation by these piercing and sucking insects can cause serious loss of yield or a reduction of the economical value of crop plants. Parasitoids are the most commonly used natural enemy for the control of aphids as an attractive alternative to the application of pesticides. Defensive strategies of plants against herbivores may play an important role in the attraction of parasitoids and may also affect parasitoid performance. In this study plant mediated effects of four white cabbage (*Brassica oleracea)* cultivars infested with cabbage aphids (*Brevicoryne brassicae*) on the performance and behaviour of the parasitoid wasp *Diaeretiella rapae* were investigated. Manipulation of direct and indirect plant defenses may provide a means of improving performance of *D. rapae* on crops in the Brassicaceae and making the crops more attractive to the wasp. Determining which crop cultivar and chemical blend induced by *B. brassicae* is most attractive to *D. rapae* and examining if certain cultivars support this parasitoid's population better, could therefore result in more effective use of *D. rapae* for the control of this aphid pest on white cabbage and other Brassicaceous crops.

Although no significant differences were found for the total amount of glucosinolates, a group of secondary chemicals characteristic for Brassicaceae, some differences in the amount of individual glucosinolates were found for *B. brassicae* reared on the four different cultivars and the phloem of those cultivars. However, the performance of *D. rapae* was equal on aphids reared on different cultivars. Our findings suggest that *D. rapae,* considered a specialist for aphids that have Brassicaceae as host, is not affected by glucosinolates sequestered by *B. brassicae.* However, differences in glucosinolate content of the aphids were not very pronounced and it cannot be concluded with any certainty that *D. rapae* is not affected by high levels of glucosinolates because a lack of variation in the wasp's performance could simply be the result of the lack of variation in glucosinolate levels found in the four cultivars.

Diaeretiella rapae was attracted more to *B oleracea* plants infested with *B. brassicae* than uninfested plants in a two choice olfactometer. However, although the trend was similar for al the cultivars, the results were only significant for two of the four cultivars tested. No differential attraction of *D. rapae* to the volatiles emitted by the four cultivars of *B. oleracea* infested with *B. brassicae* was found. Preliminary analysis of the headspace of the cultivars revealed that the volatile blends emitted by infested and uninfested plants are very similar and that the blends emitted by the four different cultivars are also very similar to each other. These results show that *B. brassicae* probably does not induce a strong response in *B. oleracea* and that little variation exists between the four cultivars.

In a pilot experiment *D. rapae* was found to significantly parasitize *B. brassicae* less on one cultivar of *B. oleracea* with a possible role for honeydew or plant architecture. However, more research needs to be done before any conclusions can be drawn.

Physical and chemical direct defenses of plants against insect herbivores may also affect predators and parasitoids negatively. When predators or parasitoids are attracted more to plants that are suboptimal in terms of its own fitness, a conflict may arise. In the system studied here, there were no indications of a conflict between direct and indirect defenses of *B. oleracea* for *D. rapae.*

The results showed that little variation existed between the cultivars used in this study. The plant mediated effects of wild plant species on the performance and behaviour of parasitoids are likely to be much more pronounced. A system made up of wild and cultivated brassicaceous plant species will provide the variation needed to study plant mediated effects on the performance and behaviour of *D. rapae.* Therefore, further research should focus on the comparison of wild and cultivated brassicaceous plant species.

Acknowledgements
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1. Introduction

Aphids are probably best known as insect pests. The insects can cover entire cars parked under infested trees in the sugary honeydew they excrete and, more importantly, many species are major agricultural pests. In fact almost every major crop is a host for at least one aphid species (Blackman and Eastop 2000). Infestation by these piercing and sucking insects can cause serious loss of yield or a reduction of the economical value of crop plants (Liu *et al*. 1994).

The application of chemical pesticides is the most commonly used method of aphid control for the protection of various crops (Vu *et al*. 2007). However, most pesticides may also affect non-target organisms and human health and have a severe negative impact on the environment, and the use of insecticides on vegetable crops has caused increasing concern amongst growers, markets and consumers (Ellis *et al*. 1996). Additionally aphids may develop resistance against these pesticides (Vu *et al*. 2007). Therefore, the use of pesticides for the control of aphids is not sustainable and the development of alternative methods is urgently needed. Biological control of aphids is an attractive alternative method because it is environmentally friendly, and in most cases very specific to aphids. Biological control agents for aphids may include pathogens such as fungi, predacious insects such as ladybirds, syrphid flies and lacewings, and parasitoid wasps. The use of natural enemies to control aphids is now widespread and many of these are now becoming commercially available (Nordlund *et al*. 2001; Emden and Harrington 2007).

Parasitoids from the subfamily Aphidiinae are the most commonly used natural enemy for the control of aphids and although they often parasitize a wide range of aphid species, they have the advantage of preying exclusively on aphids (Emden and Harrington 2007). Several are produced commercially in large numbers, particularly for use in glasshouses. Economic constraints associated with mass rearing and release has often interfered with the use of these bio-control agents in the more challenging protection of outdoor crops (Emden and Harrington 2007). Defensive strategies of plants against herbivores may play an important role in the attraction of parasitoids and may also affect parasitoid performance. Understanding of these matters is needed for more effective control of aphids using parasitoids in glasshouses and especially for the protection of outdoor crops.

1.1 Resistance to insect herbivory in plants

1.1.1 Plant defenses

Plants are under constant threat from a wide array of herbivores including herbivorous insects. Together phytophagous insects feed on every part of a plant imaginable. Biting and chewing insects known as mandibulates may feed on plant structures such as roots, stems, leafs, flowers, pollen, fruits, and seeds while piercing and sucking insects, or haustellates, feed on various plant liquids such as the phloem, parenchyma or xylem sap. Although plants are amazingly resilient and are often able to compensate for and tolerate the detrimental effects of herbivory, insect feeding can have dramatic effects on plants. The most obvious result of insect feeding is the loss of plant biomass and even the more subtle feeding mechanism of sucking insects may cause symptomatic damage to the plant such as stunting of the roots and the shoot, wilting and chloriosis. Additionally, insect feeding may transmit several pathogens that may cause severe plant diseases (Schoonhoven *et al.* 2005; Emden and Harrington 2007).

Not surprisingly plants have evolved a wide array of defenses against herbivore attackers in addition to tolerance of herbivory. Plant defenses may directly negatively affect the performance of the herbivorous insects (direct defense) or positively affect the performance of the herbivore's natural enemies such as predators or parasitoids (indirect defense) (Schoonhoven *et al.* 2005). Both direct and indirect defense mechanisms can be present all the time (constitutive defense) or only in response to herbivory (inducible defense) (Karban and Baldwin 1997; Schoonhoven *et al.* 2005).

1.1.2 Induced responses

Plants are constantly interacting with various other organisms. Some of these may pose a threat, such as herbivorous insects or pathogens, while others may be beneficial to the plant, such as insect pollinators or growth-promoting rhizobacteria. Constitutive defense against attack from many different organisms that each may require specific defensive strategies can be metabolically costly. To solve this problem many plant species have evolved the ability to respond to an attack (Pieterse and dicke 2007). This induced response can be observed as changes in a set of traits that will reduce the effect the attacking organism has on the fitness of the plant (Poelman *et al.* 2008b). In many cases the response is both local and systemic in nature (Schoonhoven *et al.* 2005). In order to effectively protect themselves against attackers and at the same time accommodate beneficial organisms, plants are capable of fine tuning their induced response to the organism involved. To do this, plants need to 'perceive' an attack (Pieterse and dicke 2007). In the case of insect herbivory, mechanical damage caused by insect feeding or oviposition in combination with herbivore derived elicitors, such as saliva or oviduct secretions (Kessler and Baldwin 2002), play an important role in the induction of defensive responses (Dicke *et al*., 2003; Schoonhoven *et al.* 2005). The plant hormones salicylic acid, jasmonic acid and ethylene are key players in the regulation of induced plant defense. These hormones are involved in different signal transduction pathways: the shikimic acid pathway, the octadecanoid pathway and the ethylene pathway respectively (Schoonhoven *et al.* 2005; Pieterse and dicke 2007). Although various other regulatory mechanisms contribute to attacker specific responses, the production of salicylic acid, jasmonic acid and ethylene has been shown to vary in *Arabidopsis* depending on the organism that is attacking the plant and plays an important primary role in the fine tuning of the defensive responses (Pieterse and dicke 2007).

As a result of fine tuning of plant responses and variation between and within plant species the induced response may vary with the herbivore and plant species or cultivar involved (Geervliet *et al.* 1997; Turlings, Bernasconi *et al*. 1998; Pieterse and dicke 2007) and even which part of the plant is damaged (Coleman, Barker *et al*. 1997). Additionally the response of a plant may also be affected by abiotic factors such as light, moisture and the availability of nutrients (Schoonhoven *et al.* 2005).

1.1.3 Direct defense

Direct plant defenses against insects are any plant traits that increase the plants fitness in an environment with insect herbivores by affecting performance of and/or susceptibility to these insects (Kessler and Baldwin 2002). Physical barriers such as trichomes, spines, wax layers and strengthened cell walls may reduce insect feeding while at the same time they may prevent insect herbivores from settling on a plant (Schoonhoven *et al.* 2005). Even the colour of the leaves may influence insect behaviour. For example Radcliffe and Chapman (1965, 1966) showed that alatae (winged form) of the cabbage aphid *Brevicoryne brassicae* do not settle well on red cabbage varieties compared to green ones, even though apterae (wingless form) caged on such varieties have a better growth rate than those on normal green varieties. Chemical plant defenses against insect herbivores may include repellents, antifeedants, toxins and chemicals that reduce digestibility of plant parts such as protein inhibitors and tannins. These chemicals negatively affect the insect's performance by reducing survival, growth and fecundity, and extending development time (Kessler and Baldwin 2002; Schoonhoven *et al.* 2005). Repellents, antifeedants and toxins include many different classes of primary and secondary plant metabolites from various plant species such as hydroxamic acids (Hansen 2006), alkaloids (Cai *et al*. 2004), glucosinolates and their breakdown products (Agrawal and Kurashige 2003; Fahey *et al* 2001; Halkier and Gershenzon 2006; Mewis, 2006; Poelman *et al.* 2008a) terpenes (Aharoni *et al*. 2003) and C-6 aldehydes (Vancanneyt *et al*. 2001).

1.1.4 Indirect defense

Indirect defenses against insect herbivores are plant traits that attract and increase the foraging success of the natural enemies of the herbivores (Kessler and Baldwin 2002). Plant structures, such as domatia and extrafloral nectar, provide food and shelter for predators and parasitoids and can indirectly affect the occurrence of insect herbivores on a plant (Schoonhoven *et al.* 2005). Additionally, herbivory induced plant volatiles such as sesquiterpene, methyl salicylate and cis-jasmone can promote the presence of predators and parasitoids. Changes in the amount and the composition of the chemical blend emitted by a plant upon herbivory provide predators and parasitoids with reliable information on herbivore availability and when this results in increased predation pressure on the herbivore this results in indirect defense (Du 1996; Du 1998; Geervliet 1998; De Moraes 1998; Turlings 1998; Dicke 1999; Yan 2006; Heil 2008; Poelman *et al.* 2008b).

As a result of genetic variation the production of volatiles can differ widely between conspecific plants induced by the same herbivore species. Individual plants with increased volatile emissions have often been found to be more attractive to parasitoids under controlled conditions (Poelman *et al.* 2008b) and although only few studies have shown that parasitoids are differentially attracted to plants that produce different mixtures and amounts of volatiles under field conditions Poelman *et al.* (2009) did show that laboratory assays on differential attraction of parasitoids to plants differing in their volatile emissions reliably predicted the relative rate of parasitism in the field. It appears that as a result of variation in indirect defenses some genotypes within a plant species will be more enemy dense and therefore better protected against herbivores than others.

1.1.5 Interactions between direct and indirect defenses

The numerous kinds of plant defenses do not act alone and direct defenses may interact with the indirect defenses. Plant metabolites can be transmitted through the food chain and, besides protecting a plant against herbivore attackers, also negatively affect the natural enemies of attacking herbivores (Gols *et al.* 2008). Similarly, morphological plant features such as slippery wax layers and trichromes may reduce insect herbivore feeding, but at the same time slow the searching rate of predators and parasitoids or even make the herbivore inaccessible (Schoonhoven *et al*. 2005). A conflict for the predator or parasitoid may arise when it is attracted more to a plant that is suboptimal in terms of its own fitness (Gols *et al.* 2008)

1.2 Biology of aphids

1.2.1 Aphids

The insect family of *Aphididae* comprises about 4700 species all of which are specialized to feed on the phloem sap of plants. Aphids occur throughout the world but they are most abundant in the temperate zones of the Northern hemisphere. Unlike most other insects, female aphids of at least a few generations reproduce clonally and are viviparous (Emden and Harrington 2007). The embryonic development of an aphid begins even before their mother is born and generation times are consequentially very short (Goggin 2007). Aphids display a diverse range of relatively complicated life cycles. Typical aphid life cycles are divided into distinct stages and each stage is characterized by one or more morphs, specialized in sexual or asexual reproduction, dispersal, and surviving severe or less favorable climatic or nutritional conditions. For example when aphid densities are high some wingless individuals (apterae) may give rise to winged progeny (alatae) which are able to migrate to new hosts. These alatae are adapted for dispersal and migration but have a reduced capacity to reproduce: wingless parthenogenetic morphs reproduce at a rate up to 70% greater than their winged counterparts. Most aphid lifecycles can be divided into two major groups: host alternating (heteroecious) or non-host-alternating (monoecious). Heteroecious aphids live on a primary host plant species in the winter and migrate to a secondary and unrelated host plant species in summer and migrate back to the primary host

in autumn (Emden and Harrington 2007). This enables these aphids to reproduce rapidly through parthenogenesis in the summer on their secondary host and produce an overwintering egg stage from mid-summer to autumn on their primary host through sexual reproduction (Goggin 2007). Monoecious aphids remain either on the same host species or migrate between closely related species throughout the year but they display similar characteristics as the host alternating aphids producing sexual morphs in autumn that mate and produce overwintering eggs. The embryos remain in diapause inside the eggs until they have experienced cold winter conditions to insure they hatch the following season. The change from asexual to sexual reproduction can be induced by factors such as short day length, low temperatures or quality of nutrition depending on the species involved. However, under certain conditions sexually reproducing morphs never occur and some aphid species never produce an egg and overwinter as parthenogenic females (Emden and Harrington 2007).

1.2.2 Aphid feeding

Aphids feed using their stylets. These specialized mouthparts enable them to pierce plant parts and suck up the plant sap. Phloem sap requires very little digestive processing because it is principally made up of sugars and amino acids and the assimilation efficiency of aphids is usually very high. However, the sap is nutritionally unbalanced: it is low in essential amino acid content and of high C:N content. Aphids are able to meet their nutritional needs because of their symbiotic relationship with several microorganisms. Nearly all aphids of the family Aphididae contain the bacterium *Buchnera aphidicola* and often other bacteria at lower densities (Emden and Harrington 2007). *Buchnera aphidicola* is an obligate endosymbiont which is transmitted vertically from female aphids to their offspring (Beauman 2005) and provides the aphids with the deficient amino acids and riboflavin (vitamine B_2) (Goggin 2007). The importance of these endosymbionts to the aphids is revealed when aphids are treated with antibiotics at a dose that kill the bacteria but apparently do not affect the aphids directly. Treated aphids stop producing offspring within 3 days but they show no known specific abnormalities, for example in feeding or embryogenesis (Wilkinson 1998). This suggests that the role of *Buchnera* is probably exclusively nutritional (Emden and Harrington 2007).
When probing a host plant with their stylets the aphids may puncture epidermal, mesophyll and parenchyma cells, but for the most part they penetrate the plant's tissues through an intercellular route. Aphids secrete a proteinaceous salivary sheath that lines the stylet path and a watery saliva that contains enzymes such as oxidases, pectinases, cellulases and calcium-binding proteins which help prevent calcium triggered phloem occlusion by the plant in response to wounding (Goggin 2007; Will *et al*. 2007). Negative impacts on the aphid's hosts, which because of their rapid reproduction can be dramatic, are thought to occur mainly due to assimilate withdrawal and the injection of saliva (Miles 1999). Aphid infestation can cause symptomatic injury to plants which may include chlorosis, necrosis, wilting, plant stunting, and malformation of new growth such as misshapen fruits or leaves. Aphids may also cause injury that is not obvious: phloem feeding does not cause any apparent damage to the plant but it does reduce the growth of shoot and roots of their host (Emden and Harrington 2007). Additionally aphids are common vectors of viral disease in plants and about 200 species are known to transmit phytopathic viruses (Ng and Perry 2004) and in some species the indirect damage caused by the transmission of a virus outweighs the direct damage done by the aphid (Goggin 2007).

1.3 Biology of parasitoids

1.3.1 Parasitoids

Parasitoids are insects whose larvae develop in four stages, egg, larvae, pupa and adult, inside or attached to the egg, pupa or adult body of an arthropod host, usually an insect. The carnivorous larvae partly or almost completely consume the host, killing it in the process (Godfray 2004). However, as adults they will usually feed on substances with high sugar

content, such as nectar or honeydew, only occasionally feeding on their host or pollen for their protein content (Lucchetta *et al.* 2008). Parasitoids are abundant in most terrestrial ecosystems and most species have broadly similar life histories. Most parasitoids are wasps (Hymenoptera) or flies (Diptera), but a small number of beetle (Coleoptera) families also contain some parasitoid species. Unlike some solitary wasps, parasitoids never move their host to a nest or cache. Solitary parasitoids develop as a single embryo inside their host, and, although they sometimes develop from just one or two eggs, gregarious species may produce up to as much as 3000 embryos inside their host. However, hosts are usually not negatively affected until the larvae develop into the final instar to insure maximum food intake for the larvae (Godfray 2004).

1.3.2 Host localization

Parasitoid larvae are completely reliant on their host for their sustenance and shelter, this makes it very important for adult females to find and choose the right host for their offspring. Parasitoids are able to perceive vibrations and parasitoids may also use visual information to find their hosts, some pupal parasitoids are even able to detect hidden pupa using emitted vibrations (vibrational sounding) (Broad and Quicke 2000**)**. However, especially chemical information plays an important role in host searching (Vet and Dicke 1992). Chemical cues may be derived from the host itself such as feces, kairomones from the host's cuticle, exuviae, secretions of mandibular and accessory glands, pheromones, honeydew, body scales, or hemolymph or from organisms associated with the host such as microbes (Ayal 1987; Vet and Dicke 1992; Powell 1998). Stimuli derived from the herbivore itself are generally the most reliable sources of information. However, insects are constantly under selective pressure from parasitoids and predators for inconspicuousness in an already complex environment. This makes it hard to detect these chemicals, apart from pheromones emitted by the hosts, especially at long distances (Vet and Dicke 1992). Plant derived cues are less reliable but are more readily available and easier to detect at long distances. Consequently, these plant volatiles mediate the host searching behaviour of parasitoids mostly at longer distances, while herbivore derived chemical cues become more important at shorter distances from the host (Ayal 1987; Vet and Dicke 1992; Powell 1998; Bradburne and Mithen 2000). Long range localization may involve the constitutively emitted plant volatiles and volatiles induced by herbivore infestation of the plant. The production of most volatiles such as terpenoids is widespread among plants, so it is likely that parasitoids also use compounds more specific to the taxon of the plant species involved to locate their host (Bradburne and Mithen 2000). Analyses of volatile plant compounds have shown that different herbivores such as caterpillars or aphids induce different chemical profiles in the same plant species and even between different aphid species a difference in profiles occurs (Du 1996; Du 1998; De Moraes 1998; Turlings 1998; Dicke 1999; Yan 2006). While generalist parasitoid species appear to be less selective, specialist parasitoids respond to very specific plant volatile blends induced by their host (Du 1996; Geervliet 1998; De Moraes 1998; Powell 1998; Shiojiri 2000; De Boer 2005; Takabayashi 2006; Yan 2006).

Responses of parasitoids to the various cues they encounter during foraging can change with experience and especially learning with odours during host searching has received a lot of attention (Vet and Dicke 1992). However, learning with visual cues may play an important role in foraging for non-host food (Lucchetta *et al*. 2008). The response of parasitoids to odours can be influenced by pre-adult learning and is dependent on the host the parasitoid developed in, and even the plant species or genotype the host was feeding on during development. However, adult learning is more pronounced. Adult parasitoids are thought to learn associatively while foraging for a host by linking cues, such as herbivore induced plant volatiles, with a host reward, such as an oviposition experience or cues from the host itself (Vet and Dicke 1992; Wäckers and Lewis 1999; Lucchetta *et al*. 2008).

1.4 Study system

1.4.1 Brassicaceae

Vegetables from the plant family Brassicaceae are very important economically and as a food source, are grown throughout the world and make up a large proportion of all vegetables grown (Gabrys *et al*. 1998). The genus *Brassica* includes vegetables such as cabbage, cauliflower, broccoli, brussels sprouts and many allied species. Glucosinolates, or mustard oils, are the main secondary metabolites accumulated by cruciferous plants for defense against herbivores. Cruciferous plants employ a very distinctive defensive mechanism: myrosinases that catalyze the hydrolysis of glucosinolates (Kazana *et al*. 2007) are spatially divided from glucosinolates within the plant tissue. When the tissue is damaged by an insect the two come together and various secondary compounds are formed such as isothiocyanates, thiocyanates and nitriles (Bones and Rossiter 1996). Most of the biological activity of glucosinolates such as toxicity, growth inhibition, and feeding deterrence to a wide range of herbivores and plant pathogens is attributed to these breakdown products (Halkier and Gershenzon, 2006). Paradoxically, specialized insect herbivores may use these compounds as token stimuli in host acceptance (Schoonhoven *et al.* 2007).

In this study four cultivars (Lennox, Rivera, Badger Shipper and Christmas Drumhead) of white cabbage (*Brassica oleracea convar. capitata var. alba*) will be used to represent the Brassicaceae. Cultivars from highly cultivated crops often have reduced levels of glucosinolates compared to wild conspecifics, although levels of glucosinolates may vary with the cultivar involved (Moyes *et al*. 2000; Kushad *et al*. 2004; Gols 2008). This may have an impact on direct and indirect defenses and these plants may be more susceptible to attack by insect herbivores (Gols 2008).

1.4.2 *Brevicoryne brassicae*

The cabbage aphid *Brevicoryne brassicae* is specialized on plant species of the genus *Brassica*. The aphid infests the leaves of their hosts and these monoecious aphids remain on herbaceous cruciferous plants (Brassicaceae) year round. *B. brassicae* is able to produce overwintering eggs which hatch in February to March but often it is the parthenogenic nymphs and adults that overwinter (Emden and Harrington 2007).

Although the glucosinolates found in cruciferous plants are a potent defense against most insect herbivores, both *B. brassicae* and the Turnip aphid *Lipaphis pseudobrassicae,* which is also specialized on Brassica, are adapted to mimic the plants defensive mechanism (Bridges *et al*. 2002). Both aphid species are able to accumulate glucosinolates ingested from their cruciferous hosts and have evolved a specific myrosinase which is distinctly different from plant myrosinase (Jones *et al*. 2001; Pontoppidan *et al*. 2001; Bridges *et al*. 2002; Jones *et al*. 2002 ; Husebye *et al*. 2005). Aphid myrosinase is localized to the sarcoplasm of non-flight muscle and is compartmentalized into crystalline microbodies. Consequently, hydrolysis of glucosinolates and the production of toxic secondary compounds only occurs when tissue of the aphids is damaged as a result of predator attack (Francis *et al*. 2001; Kazana *et al*. 2007). In contrast to *B. brassicae* the green peach aphid *Myzus persicae* lacks myrosinase activity and appears to excrete ingested glucosinolates in the honeydew (Merritt 1996). It is unable to produce toxic hydrolysis products when attacked (Francis *et al*. 2001). Larva of the aphidophagous two spot ladybird *Adalia bipunctata* have been shown to have a lower survival rate when they were fed with *B. brassicae* reared on a range of crucifer host-plants, while they were able to develop normally when fed *M. persicae* aphid reared on the same host (Francis *et al*. 2001). These results suggest that the myrosinase–glucosinolate system may be central to the aphids' defense against this natural enemy (Francis *et al*. 2001) and is probably effective against most, but not all, generalist predators. For example the seven-spot ladybird *Coccinella septempunctata* is commonly found predating on *B. brassicae* (Acheampong and Stark 2004).

B. brassicae is adapted to cope with high levels of glucosinolates, however, other direct plant defenses in cruciferous plants may affect the aphids. Broekgaarden *et al.* (2008) showed that the *B. oleracea* cultivars Rivera and Lennox supported a slower population

growth of *B Brassicae* than the cultivars Badger Shipper and Christmas Drumhead and revealed a possible defensive role for a trypsin-and-protease inhibitor.

1.4.3 *Diaeretiella rapae*

The solitary Braconid wasp *Diaeretiella rapae* is an important primary endoparasitoid of a wide range of aphids including *B. brassicae* and *Myzus persicae* and also including other major aphid pests such as the Russian wheat aphid *Diuraphis noxia*, the cotton aphid *Aphis gossypii*, the bird cherry-oat aphid *Rhopalosiphum padi* and the corn leaf aphid *Rhopalosiphum maidis* (Elliott *et al*. 1994; Pike *et al*. 1999). Although the wasp's potential host range is greater than 60 species of aphid, *D. rapae* is often regarded as a specialist parasitoid of Brassicaceae feeding aphids because of its ability to parasitize a range of these specialist aphids. Additionally, the number of *D. rapae* accounted for 82.5% of all aphid parasitoids in cruciferous vegetable fields collected in the USA (Pike *et al*. 1999).

The hydrolysis of glucosinolates in cruciferous plants results in various volatile and non-volatile compounds and especially isothiocyanates are thought to provide parasitoids specialized on Brassicaceae feeding hosts with cues that can be utilized in the host foraging process. Bradburne and Mithen (2000) showed that *D. rapae* is differentially attracted to *Brassica oleracea* cultivars differing in the amount of But-3-enyl isothiocyanate. The parasitoids were more attracted to white cabbage cultivars that produced more But-3-enyl isothiocyanate in a two choice olfactometer test and parasitism rates of aphids were higher on *Brassica napus* plants that produced more of the chemical. Infestation with *B. brassicae* is likely to induce a very specific volatile blend in cruciferous host plants which may provide *D. rapae* with the long range cues to find these aphids. The response to infestation with *B. brassicae* and the volatile blend produced as a result may vary with the plant species or cultivar involved. As a result *D. rapae* may show a preference for certain plants and cultivars while foraging. Additionally, the levels of glucosinolates may differ between Brassicaceae species or cultivars and consequentially levels of glucosinolates found in *B. brassicae* may vary with the host plant on which they live. High glucosinolate levels in the aphids may negatively affect the performance of *D. rapae* on these aphids (Hunter 2003; Ode 2006). However, parasitoids that parasitize generalist herbivores have been shown to be more affected by toxins in the diet of their host than specialist parasitoids that attack hosts feeding only on Brassicaceae plants (Sznajder and Harvey 2003; Gols, Witjes *et al*. 2008a) and it is likely that *D. rapae* is not very strongly affected by glucosinolate levels found in their aphid hosts. However, the performance of a host and its parasitoid are often positively correlated (Gols 2008). The differential performance of *B. brassicae* on the four cultivars also used in this study (Broekgaarden *et al.* 2008) may therefore also affect the performance of *D. rapae.*

1.5 Research

Vegetables from the plant family Brassicaceae are very important economically and make up a large proportion of all vegetables grown (Gabrys *et al*. 1998). Infestation with aphids can cause serious yield and market value reduction of the crops (Liu *et al*. 1994) and in addition to direct damage, *B. brassicae* is a vector for several the plant diseases such as *Turnip mosaic virus* and *Cauliflower mosaic virus* that can cause further damage to crops (Emden and Harrington 2007). *Diaeretiella rapae* can potentially be used as a biological control agent of aphid pests of *Brassica* crops as an attractive alternative to the application of pesticides.

Variations in direct and indirect defenses may influence the attractiveness of crops to *D. rapae* and the insect's performance on these crops. Manipulation of direct and indirect plant defenses may provide a means of improving performance of *D. rapae* on crops in the Brassicaceae and making the crops more attractive to the wasp. Determining which crop cultivar and chemical blend induced by *B. brassicae* is most attractive to *D. rapae* and examining if certain cultivars support this parasitoid's population better, could therefore result in more effective use of *D. rapae* for the control of this aphid pest on white cabbage and other *Brassica* crops

2. Research questions and hypotheses

2.1 Research questions

Direct defence of white cabbage cultivars:

1. Are there differences in glucosinolate levels in *B. brassicae* reared on different cultivars?

2. Are there differences in performance of *D. rapae* on *B. brassicae* reared on different cultivars?

Indirect defence of white cabbage cultivars and attraction of *D. rapae*:

3. Are there differences in the attractiveness of different white cabbage cultivars infested with *B. brassicae* to the parasitoid *D. rapae*?

4. Can the differences in attractiveness of the different cultivars be explained by differences in volatile chemical blend emitted by the different white cabbage cultivars infested with *B. brassicae*?

Interaction of direct and indirect defence of white cabbage cultivars:

5. Is there a conflict between direct and indirect plant defense?

2.2 Hypotheses

1. *Brevicoryne brassicae* obtains and sequesters glucosinolates from its diet (Pontoppidan 2001) and because different cultivars may vary in glucosinolate levels (Poelman 2008a), the glucosinolate levels in *B. brassicae* will vary with the cultivar it feeds on.

2. *Diaeretiella rapae* is specialized on aphids that feed on *Brassica* and it is likely that it has adapted to the glucosinolates found in these aphids. Therefore variations in the glucosinolate levels in *B. brassicae* feeding on different cultivars probably do not affect the performance of *D. rapae*. However, *B. brassicae* has better performance on the cultivars Badger Shipper and Christmas Drumhead than on the cultivars Rivera and Lennox (Broekgaarden *et a*l. 2008). In many cases the performance of the parasitoid and its host are positively correlated (Gols 2008). Therefore the performance of *D. rapae* may also be suboptimal on aphids reared on Lennox and Rivera compared to Badger Shipper and Christmas Drumhead.

3. Bradburne and Mithen (2000) showed that *D. rapae* is differentially attracted to white cabbage cultivars differing in the amount of But-3-enyl isothiocyanate. Additionally, differential attraction of several other parasitoid species by different cultivars of white cabbage, including the ones used in this study, has been shown (Poelman, 2009). Therefore, it is likely that *D. rapae* will be differentially attracted to the cultivars of white cabbage infested with *B. brassicae* used in this study.

4. The response of white cabbage to infestation with *B. brassicae* will vary with the cultivar involved and some produce a volatile chemical blend or higher quantities of chemicals which are more attractive to *D. rapae* than the blends or quantities produced by others.

5. If *D. rapae* is attracted more to Lennox and Rivera compared to Badger Shipper and Christmas Drumhead and its performance is affected by the aphid's performance on these cultivars this will lead to a conflict between direct and indirect plant defense.

3. Materials and methods

3.1 Insects and plants

Four different white cabbage (*Brassica oleracea var. alba*) cultivars were used: Christmas Drumhead and Badger Shipper (Centre for Genetic Resources, CGN, Wageningen, The Netherlands), representing open pollinated cultivars, and Lennox and Rivera (Bejo Zaden BV, The Netherlands), representing more recently cultivated F1 hybrids.
Seeds were germinated on peat soil (Lentse potgrond, no. 4, Lent, The Netherlands). After two weeks, individual seedlings were transferred to peat soil in 1.45 l pots. Plants were watered daily, and were provided with SON-T light (500 µmol/m^2/s; L16:D8). The plants were grown under 18-26 ℃ and 40-70% r.h. When the plant s reached an age of four weeks, they were fertilized weekly by applying Christalon Blauw (Hydro Agri Rotterdam, The Netherlands) (N-P-K) with an EC of 2.2. The plants were used in the experiments when they were 7 weeks old, and were watered daily.

The aphids used in the experiments were collected from a field in the vicinity of Wageningen during the summer of 2008. They are maintained on Brussels Sprouts (*B. oleracea* var. *gemmifera* cv *Cyrus*) in a greenhouse compartment (L16:D8; 22±2 C°; 60% r.h.). *D. rapae* was collected from *B. brassicae* mummies from a field in the vicinity of Wageningen during the winter of 2008. The parasitoids are maintained on *B. brassicae* feeding on Brussels Sprouts in a climate room (L16:D8; 22±2 C°; 60% r.h.).

3.2 Glucosinolate levels

In order to examine glucosinolate levels in *B. brassicae* feeding on different cultivars, 400 adult aphids were allowed to larviposit for two days on a single plant, after which they were removed using a fine paint brush. For each cultivar 10 plants were infested in this way. Three days after the removal of the larvipositing adults a phloem sample and approximately 300 L3 stage nymphs were collected from each plant. After 13 days another phloem sample and approximately 300 adults were collected from each plant. Phloem samples were collected by cutting the third fully expanded young leaf in the whorl of cabbage leaves from each plant and extracting the phloem into 2 ml of EDTA solution for two hours in the dark. All samples were frozen after collection at -20 degrees Celsius.

Glucosinolate extraction and analysis were performed at the NIOO (Netherlands Institute of Ecology). Glucosinolates were extracted from the aphid samples as described previously by Poelman *et al.* (2008a). Glucosinolates were extracted from the phloem samples slightly differently than from the aphid samples (appendix 6.1).The aphid samples were freeze dried, weighed and ground into a fine powder. The ground material and 1 ml from the EDTA solution was dissolved in 1ml 70%MeOH. The extract was desulfated on a DEAE-Sephadex A25 column (SigmaAldrich Chemie BV, Zwijndrecht, The Netherlands) and the glucosinolate content was assessed by high performance liquid chromatography (HPLC), using the method described by van Dam *et al.* (2004). Five concentrations of sinigrin (sinigrin monohydrate; Sigma, St. Louis, MO, USA) were desulfated following the same protocol as the aphid samples and were used as an external standard. Glucosinolate detection was performed with a Photodiode Array detector (200–350 nm) with 229 nm as the integration wavelength. Desulfoglucosinolate peaks were identified by comparison of HPLC retention times and ultraviolet spectra with standards provided by Patrick Kabouw from the NIOO and concentrations of the glucosinolates were calculated using the programme Chromeleon.

3.2 Performance of *Diaeretiella rapae*

Experiments to determine the performance of *D. rapae* on *B. brassicae* raised on different cultivars have already been performed by Martine kos and the results and conclusions will be used in combination with the results from the glucosinolate analysis of the aphids. Adult *B. brassicae* were allowed to larviposit overnight on each of the four white cabbage cultivars. The next day, the adults were removed, while the young nymphs were kept on the plants. When the nymphs reached the right age (second instar), they were individually parasitized by *D. rapae*. A vial with a single mated female was placed over a leaf with a single *B. brassicae* nymph until the nymph was seen to be parasitized, after which the nymph was transferred to its host plant with a fine paintbrush. Each female wasp was used to parasitize up to ten individual host nymphs. In total for each cultivar, 300 parasitized aphids were evenly distributed over 6 cages, with two undamaged plants in each cage. The time of oviposition was recorded per experimental cage. The nymphs were allowed to develop into either adults which produced progeny (unparasitized aphids) or mummies (parasitized aphids) inside the cages, while being able to move around freely between the plants in one cage. The mummies were removed from the cages and transferred individually to labeled glass vials. The unparasitized aphids were counted and removed from the plants. Upon emergence of the wasps, vials were checked every two hours, and when adult wasps emerged, the egg-to-adult development time and the sex of the wasps was recorded. Wasps were frozen immediately, dried for 72 hours at 80 ℃, and weigh ed on a Cahn C-33 microbalance (Cahn instruments, USA). All experiments were conduced in a greenhouse compartment, L16:D8, 22 ± 2 C°, 60% r.h.

3.4 Choice experiments with *Diaeretiella rapae*

3.4.1 Two choice olfactometer experiments and volatile profiling

For the preference tests plants of the four cultivars infested with 400 mixed instar aphids 4 days prior to the experiments were used. We use *D. rapae* wasps that are 2 to 4 days of age. These wasps emerged from mummies individually taken from Brussels sprouts leaves and placed in a cage separate from the main rearing cages to ensure the wasps have no experience with cabbage plants which might influence the preference for the cultivars.

The four cultivars are tested in pairs of two different cultivars (6 combinations) in a y-tube olfactometer (figure 1.). Additionally, before the cultivars were tested against other cultivars they were tested against control plants from the same cultivar not infested with aphids (4 combinations) in a control experiment to test if the wasp is indeed attracted to infested plants. For all experiments two plants are placed in individual 30 litre pots and sealed air tightly, each pot is supplied with a constant flow of 2,1 L/min. air at the bottom, cleaned through a charcoal filter. Each of the separate arms of the y-tube olfactory meter

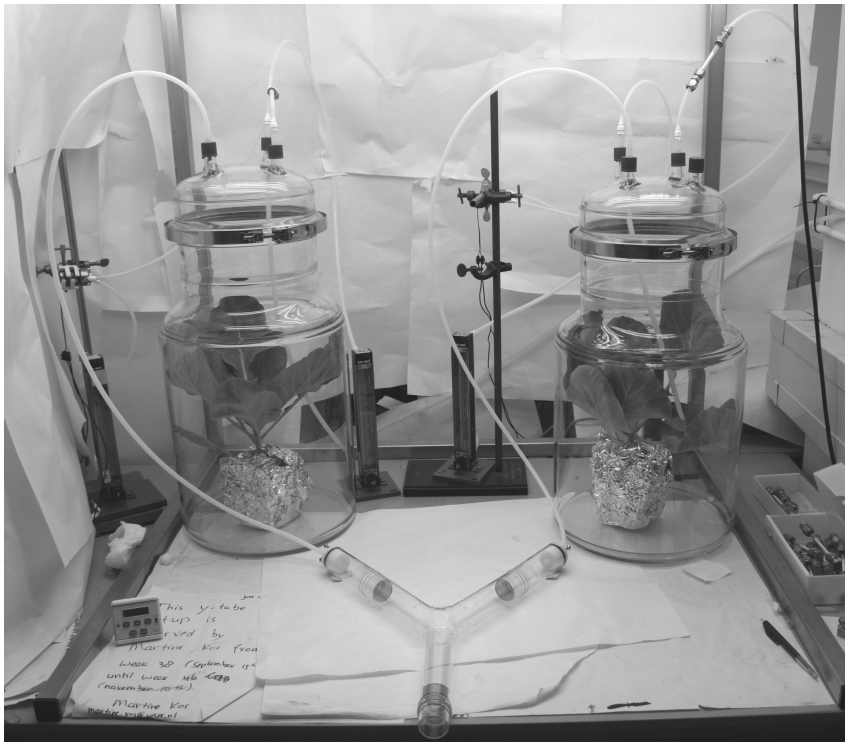


Figure 1. The experimental set up for preference testing and volatile trapping

receives an airflow of 2 L/min (the remaining 0,1 L/min was required for volatile trapping) from the top from one of the pots (Fig. 1). For the control experiments 10 to 11 replications were done for each combination and for the cultivar versus cultivar experiments 6 replications were done for each combination. For each replication 10 naïve female wasps are tested one by one. The wasps were transferred from a cage supplied with water and honey to the y-tube in a small test tube and released. During each replication the air supplying tubes from the two pots containing the cultivars were switched to the opposite arm of the y-tube after 5 wasps had been tested to compensate for unforeseen asymmetry in the setup. For all experiments each combination was tested on different days and on one day two different combinations were tested.

The test started when the wasp left the test tube and entered the y-tube (3.5 cm in diameter Fig. 2). it was given 10 minutes to choose one of the opposite arms of the y-tube. Wasps that did not choose within 10 minutes were considered as non responsive. When the wasp passed the first line at the beginning of the arms of the y-tube within 10 minutes this was considered as a first choice and the time of the first choice was recorded. When the wasp passed the second line at the end of one of the arms of the y-tube and it did not pas the first line again within 15 seconds, this was considered as a final choice and the time of the final choice was recorded.

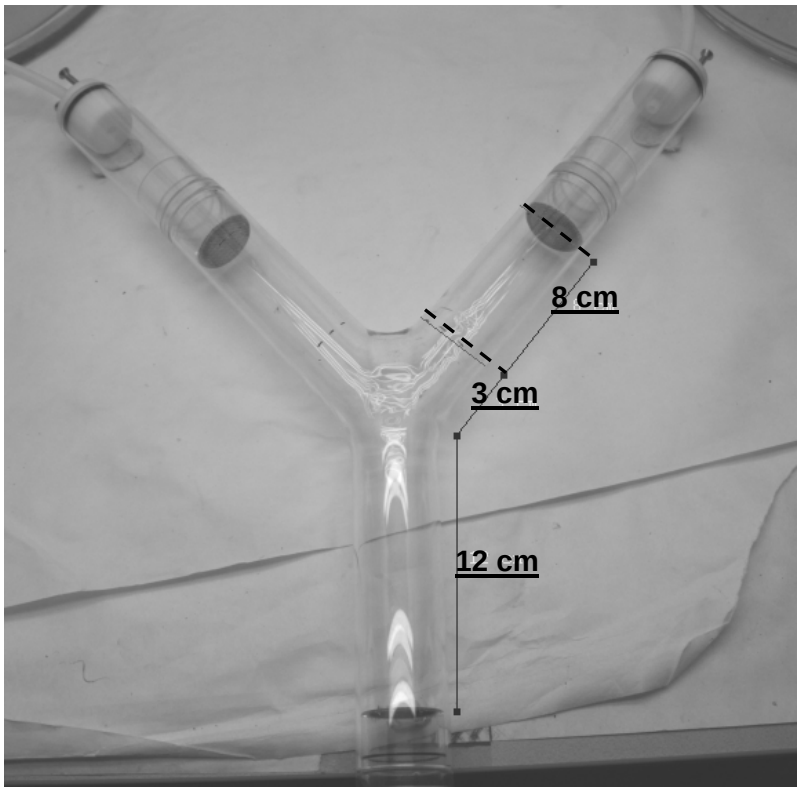


Figure 2. The y-tube set up, interrupted lines indicate first and final choice marks.

If no final choice was made within 15 minutes this was considered as non responsive. Additionally if the wasp switches from one arm to the other without making a final choice this was considered as a switch and the number of switches was recorded.

During the experiments volatiles emitted by the plants were entrained using an adsorbing polymer, Tenax-TA 20/35 (Grace-Alltech, USA). The soil surrounding the roots was wrapped in aluminium foil, and plants were handled with latex gloves to prevent contamination of the plants volatiles as much as possible. Headspace volatiles were collected with a flow rate of 100 ml/min for 3 h on a cartridge containing approximately 100 mg Tenax TA connected to the 30 litre pots (Fig.1). Samples were analyzed by thermodesorption followed by gas chromatography - mass spectrometry (GC-MS), on a Markes International Ltd/Thermo Scientific system consisting of a thermal desorption autosampler (Model Ultra 50:50, Markes, UK), a electrically cooled cold trap for focusing (general purpose cold trap, Unity, Markes), a Trace GC Ultra (Thermo Scientific, Waltham, USA) coupled to a Trace DSQ quadrupole mass spectrometer (Thermo Scientific). Before analysis 1µl of 0.05mg/ml methyl octanoate (Sigma-Aldrich) dissolved in methanol (MeOH) was injected on each cartridge as internal standard. Samples were purged for minimal 6 min with (He, 5.0 grade) at room temperature to remove the MeOH, moisture, and oxygen. Tube

desorption was performed at 240℃ for 5 min and the volatiles were focused on a cold trap at 0℃. Injection onto the analytical column (30 m x 0 .25 mm ID x 1.0 µm film thickness, ZB-5MSi, Zebron, Phenomenex, USA) was achieved by heating the cold trap to 270℃ for 7 min in splitless mode (flow rate of 1.0 mL/min). The temperature of transfer lines between the thermodesorption unit and GC-MS was set to 160℃. T he GC temperature was programmed as follows: 7 minutes at 40℃ followed by a gradien t of 5℃/min to 200℃, then 20℃/min to 280℃ (2 min hold),. the column effluent was ionize d by electron impact (EI) ionization at 70 eV, scanning in positive mode from 33-280 m/z with a scan speed of 4.2 scans/s. A solvent delay of 3 min was set. The ion source temperature was 250℃ and the transfer line between the GC and MS was set to at 275℃. Peak identificat ion was performed using Xcalibur software (Thermo Electron Corporation, San Jose, USA). The eluted compounds were identified using Xcalibur software (Thermo, Waltham, USA) by comparing the mass spectra with those of authentic reference standards or with NIST 05 and Wiley library spectra. Linear retention indices were calculated for each compound according to van den Dool and Kratz (1963) and were compared with those published in the literature. Peak surface estimates performed using the software Xcalibur (Thermo Electron Corporation, San Jose, USA). Peak areas were divided by the total volume of air (ml) that was passing over the traps.

The entire experimental setup (y-tube, pots and volatile traps) was placed in a climatized room. The temperature ranged from 20 to 25 degrees Celsius and in addition to daylight it was illuminated with four fluorescent tube lights (FTD 32 W/84 HF, Pope, The Netherlands) that were positioned 90 cm above the setup.

3.4.2 Parasitism pilot experiments

In addition to the choice experiments using the y-tube olfactometer we monitored the parasitism rates of *B. brassicae* by *D. rapae* on the four cabbage cultivars in a pilot experiment. Adult aphids were allowed to larviposit on Brussels sprouts plants for two days. On each of the white cabbage cultivar plants approximately 100 nymphs were placed four days prior to the experiments. Before the experiment the number of aphids still present on the plants was counted and recorded to account for mortality of the nymphs. Two different cultivars were placed in opposite corners in a gauze Bugdorm® cage of 35x35x60 cm (6 combinations in total). Because the number of aphids on the plants varied from about 50 to 100 as a result of mortality, plants with approximately the same number of aphids were put together as much as possible. One naïve female wasp 2 to 4 days of age was placed in the middle of the floor of the cage, and allowed to parasitize the aphids for 24 hours before it was removed again. Before the experiments the wasps were provided with water and honey and additionally a drop of honey was placed in the middle and on the top of the gauze cage. After the wasp was removed the plants were placed in another room, and the mummies (parasitoid cocoons) were counted after they appeared. For each combination 8 replications were done, however one replicate was lost for the combinations Lennox-Christmas Drumhead, Rivera-Badger Shipper and Christmas Drumhead-Badger Shipper due to infestation with *Plutella* caterpillars. The actual experiments were conduced in a compartment, L16:D8, 22 ± 2 C°, 60% r.h. However, d ue to lack of space the cabbage plants were placed in rooms without precise climate control after the wasps were removed. Additionally, for half of the experiments the cultivars were infested with aphids inside a greenhouse compartment and kept there for the four days, for the second half this was done in the rooms without climate control.

3.5 Statistical analysis

For the statistical analysis the following tests have been performed. For the olfactometer choice experiments chi squared tests were performed. Wilcoxon signed rank tests were performed for the differences in parasitism rates between cultivars. Redundancy analysis (RDA), ANOVA, ANOVA post hoc tukey, Kruskal Wallis and Mann whitney tests were performed for the differences of glucosinolate profiles between cultivars. The RDA's were performed using canoco for Windows, all other tests were performed using SPSS 15.0 for Windows.

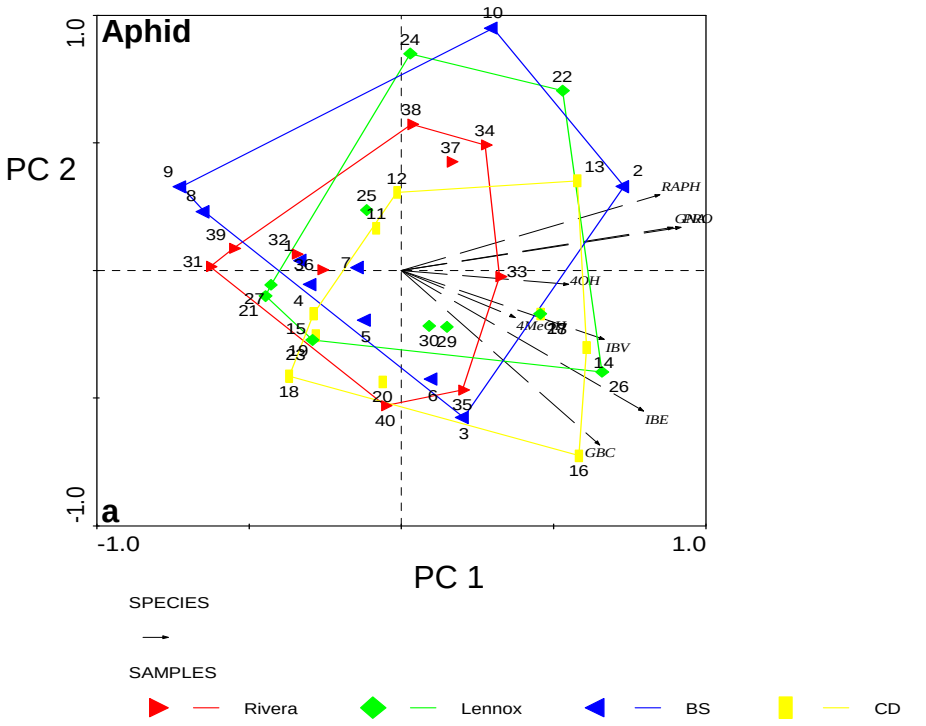
4. Results

4.1 Glucosinolate levels

The glucosinolate content of adult stage *B. brassicae* is still being analyzed and are not yet available and only the results for L3 stage aphids are presented here. The glucosinolate content of L3 stage *B. brassicae* feeding on different cultivars of *B. oleracea* and the glucosinolate content in the phloem of those cultivars (induced with the aphids for four days) did not differ between cultivars when all chemicals where analysed as a group. The same kinds of glucosinolates where found in the four cultivars and the aphids that fed on them (fig. 1a-1b). The first Principal Component (PC) axis explained most of the variation in both analyses. There was no significant separation between the cultivars on the first axis (*B. brassicae*: RDA, p= 0.59; phloem: RDA, p=0.95) or the other axis (*B. brassicae*: RDA, p= 0.64; phloem: RDA, p=0.92).

However, when we looked at individual glucosinolates, the levels of several glucosinolates where found to differ between the cultivars, although the differences were sometimes very small (Fig.2a-2b). The levels of progoitrin (Pro) were significantly higher in aphids feeding on Christmas Drumhead than on Rivera (ANOVA post hoc Tukey test: p=0.008), levels of glucoraphanin (Raph) were significantly higher in aphids feeding on Badger Schipper than on Rivera (ANOVA post hoc Tukey test: p=0.030), levels of gluconapin (GNA) were significantly higher in aphids feeding Christmas Drumhead than on Lennox or Rivera (ANOVA post hoc Tukey test: p=0.044 and p=0.029 respectively) and the levels of 4-hydroxyglucobrassicin (4 OH) were significantly higher in aphids feeding on Christmas Drumhead than on Rivera (ANOVA post hoc Tukey test: p=0.011). There were no significant differences between aphids feeding on the different cultivars for the glucosinolates glucoiberin (IBE), 3-methylthiopropyl (IBV), glucobrassicin (GBC) and 4-methoxyglucobrassin (4-MeOH) (ANOVA, p>0,05) (Fig 2a).

The level of 4-MeOH was significantly lower in the phloem of Lennox than in the phloem of Badger Shipper and Rivera (Mann Whitney test, p=0,001) although the difference was very small (9,56E-5 micromol/gram or less) (Fig. 2b). The levels of sinigrin (Sin), gluconasturtiin (NAS), neo-glucobrassicin (NEO), GNA, 4-OH, GBC, 4-MeOH and two unidentified glucosinolates did not differ between the cultivars (Kruskal Wallis test, p>0.05) (Fig 2b).



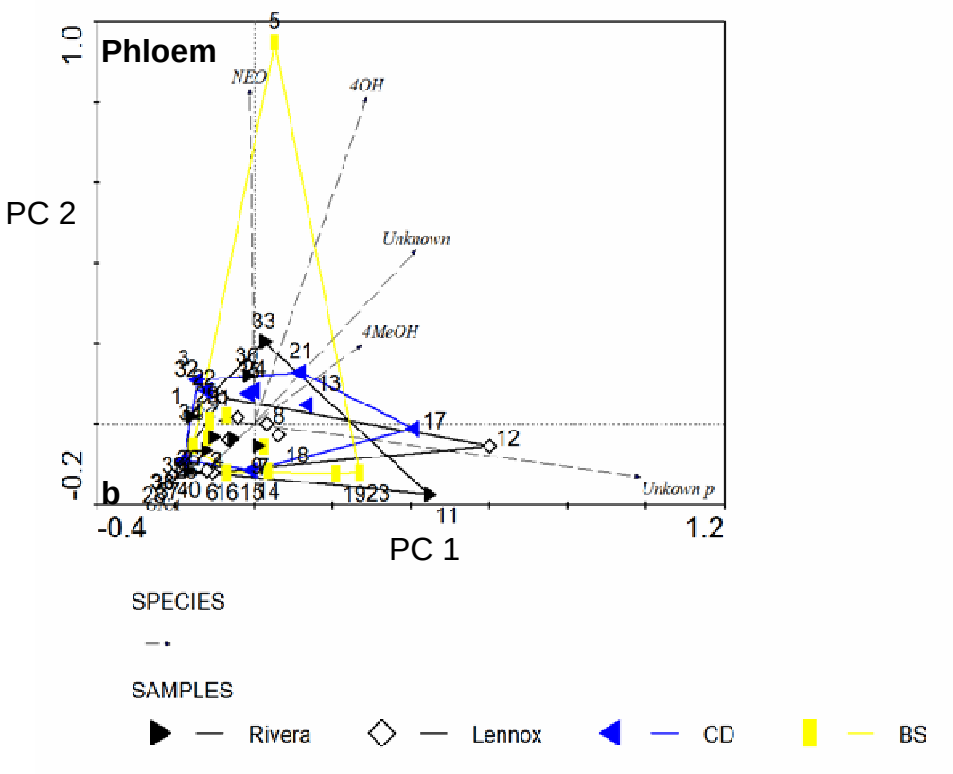


Figure 1a-1b. PCA plots of the glucosinolates found in L3 stage *B. brassicae* feeding on different *B. oleracea* cultivars (a) and the glucosinolate levels in the phloem of those cultivars (b). Cultivars are: Badger Shipper (BS), Rivera, Christmas Drumhead (CD) and Lennox. In both analyses the separation was strongest on the first Principal Component axis. In both analyses no significant separation was found on the first axis (a: RDA, p= 0.59; RDA, b: p=0.95) or the other axis (a: RDA, p= 0.64; b: RDA p=0.92).

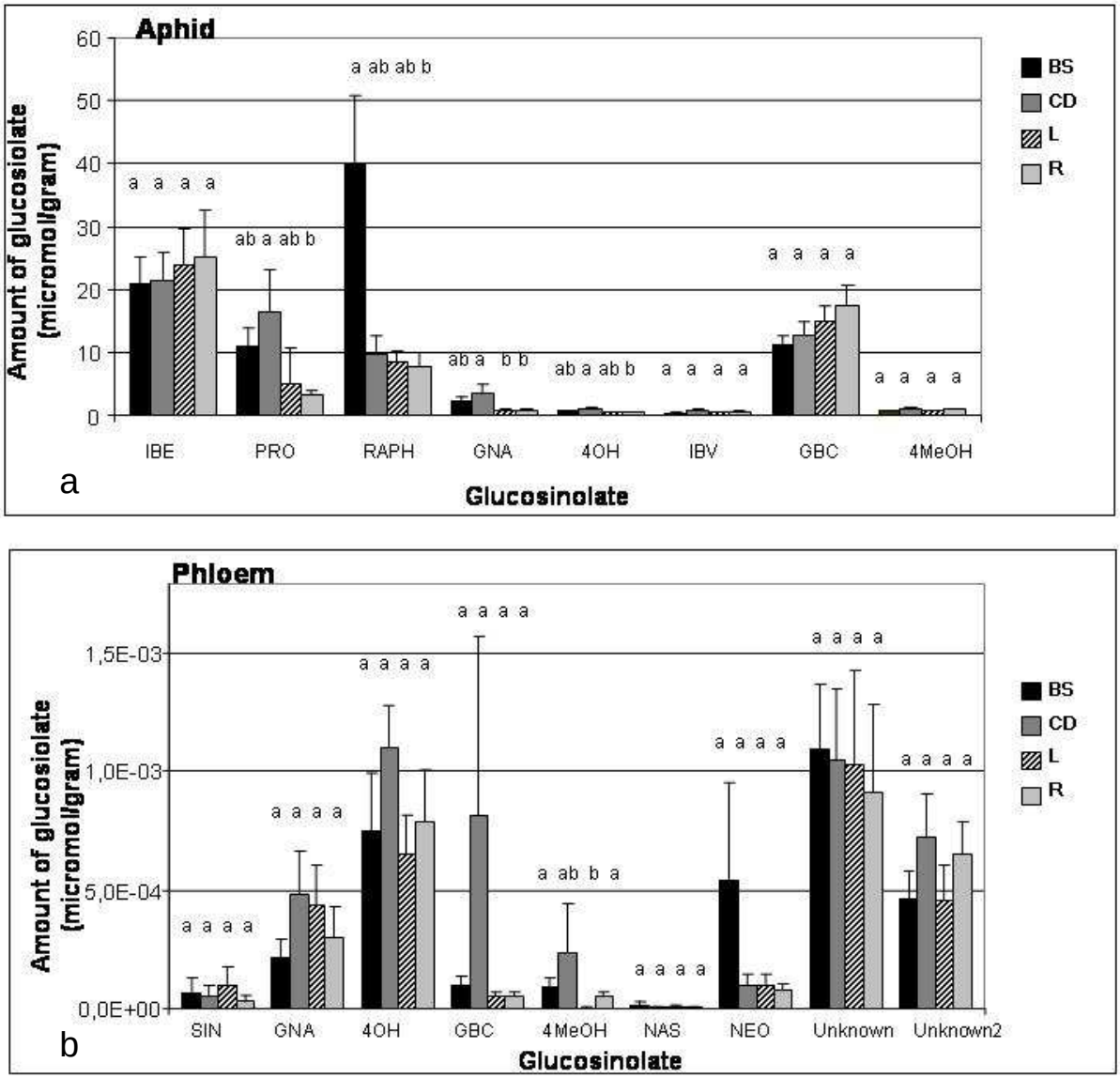


Figure 2a-2b. The levels of various glucosinolates ± S.E. in L3 stage *B. brassicae* feeding on different *B. oleracea* cultivars (upper graph) and the glucosinolate levels in the phloem of those cultivars (lower graph). Cultivars are: Badger Shipper (BS), Rivera (R), Christmas Drumhead (CD) and Lennox (L). Different letters above the bars indicate significant differences at the level of $p<0.05$ (ANOVA post-Hoc Tukey test) in Graph 2a and at the level of $p<0.001$ (Mann Whitney U test) in Graph 2b between cultivars only for that particular glucosinolate, the letters do not indicate significant differences between different glucosinolates.

The total amount of glucosinolates found in the aphids and in the phloem was higher for Badger Shipper and Christmas Drumhead than in Rivera and Lennox but these differences between the cultivars were not significant for the aphids (Anova, p=0.215) or for the phloem (ANOVA, p=0.39) (Fig.3a-3b).

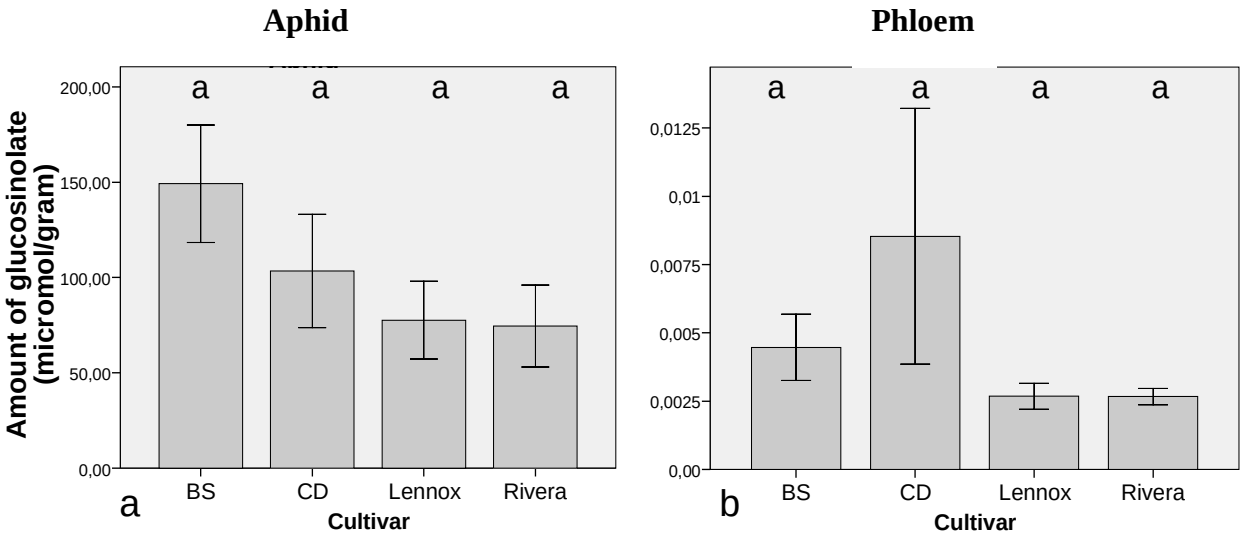


Figure 3a-3b. The total amount of glucosinolates ± S.E. in L3 stage *B. brassicae* feeding on different *B. oleracea* cultivars (Graph a) and the glucosinolate levels in the phloem of those cultivars (graph b). Cultivars are: Badger Shipper (BS), Rivera (R), Christmas Drumhead (CD) and Lennox (L). Similar letters above bars indicate no significant differences between cultivars (ANOVA p>0.05)

4.2 Performance of Diaeretiella rapae

All results concerning the performance of *D. rapae* were provided by Martine Kos. There was no difference in the performance of wasps that developed in aphids that lived on the four different cultivars. There was no significant difference in survival (Fig. 4) development time (Fig. 5) or adult dry mass (Fig. 6) of the wasps between the different cultivars (ANOVA $p>0.05$).

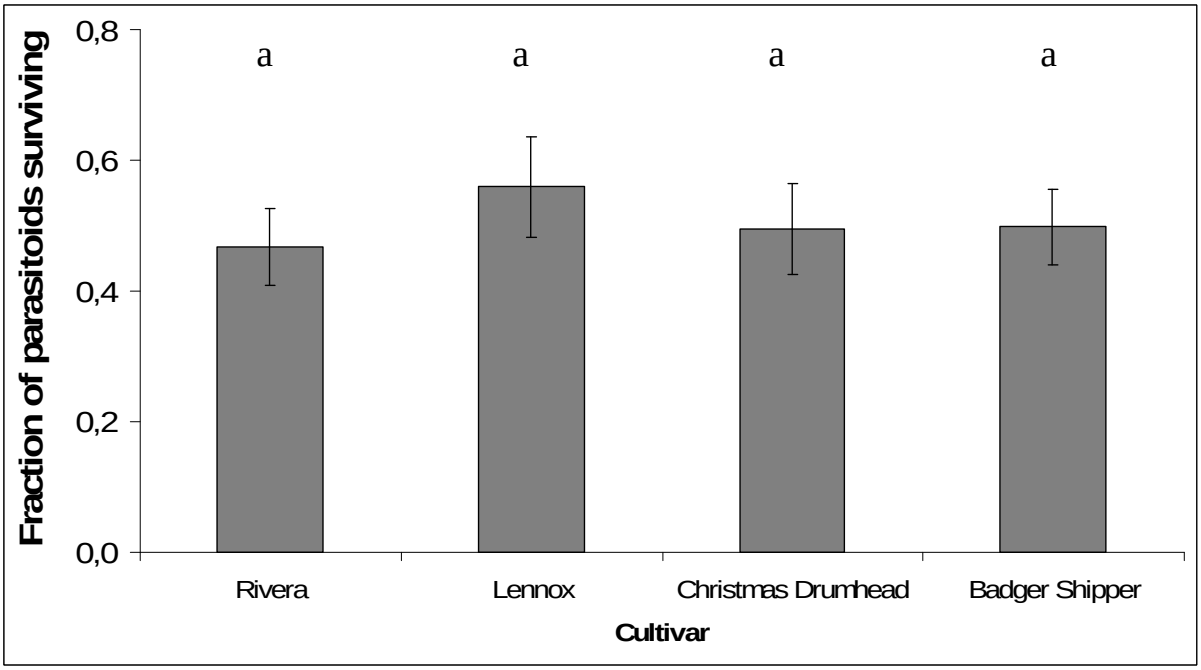


Figure 4. Average fraction of *D. rapae* surviving ± S.E. when developing in *B. brassicae* feeding on four different *B. oleracea* cultivars. Similar letters above bars indicate no significant differences between cultivars (ANOVA $p>0.05$)

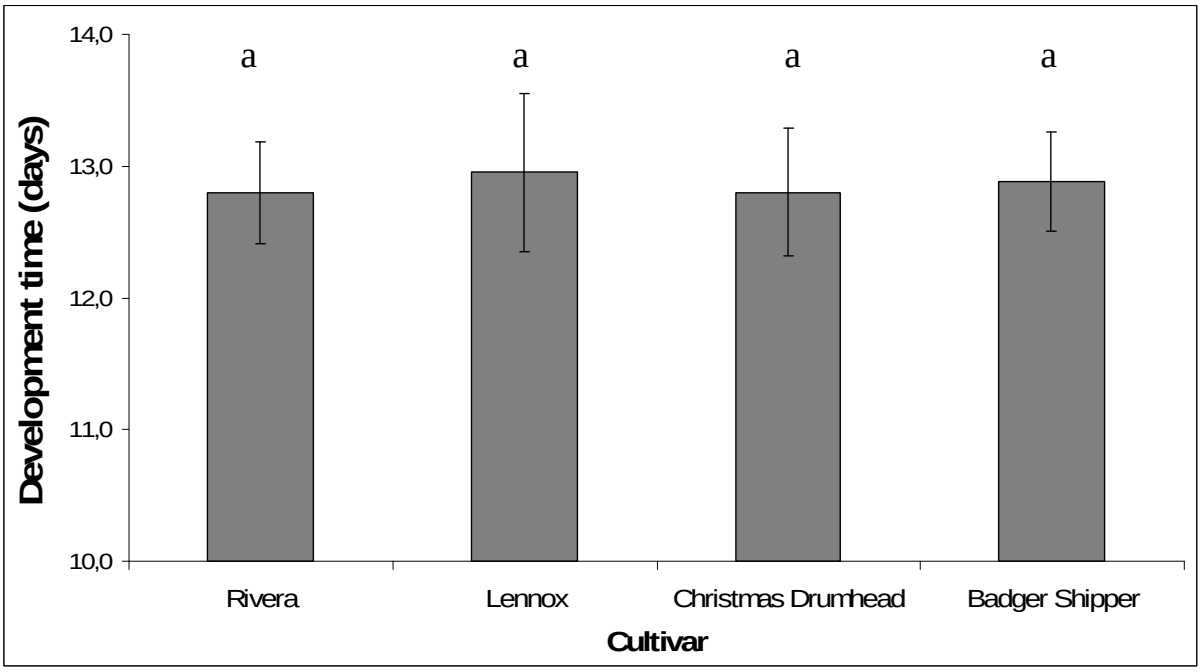


Figure 5. Average development time in days ± S.E. of *D. rapae* developing in *B. brassicae* feeding on four different *B. oleracea* cultivars. Similar letters above bars indicate no significant differences between cultivars (ANOVA $p>0.05$)

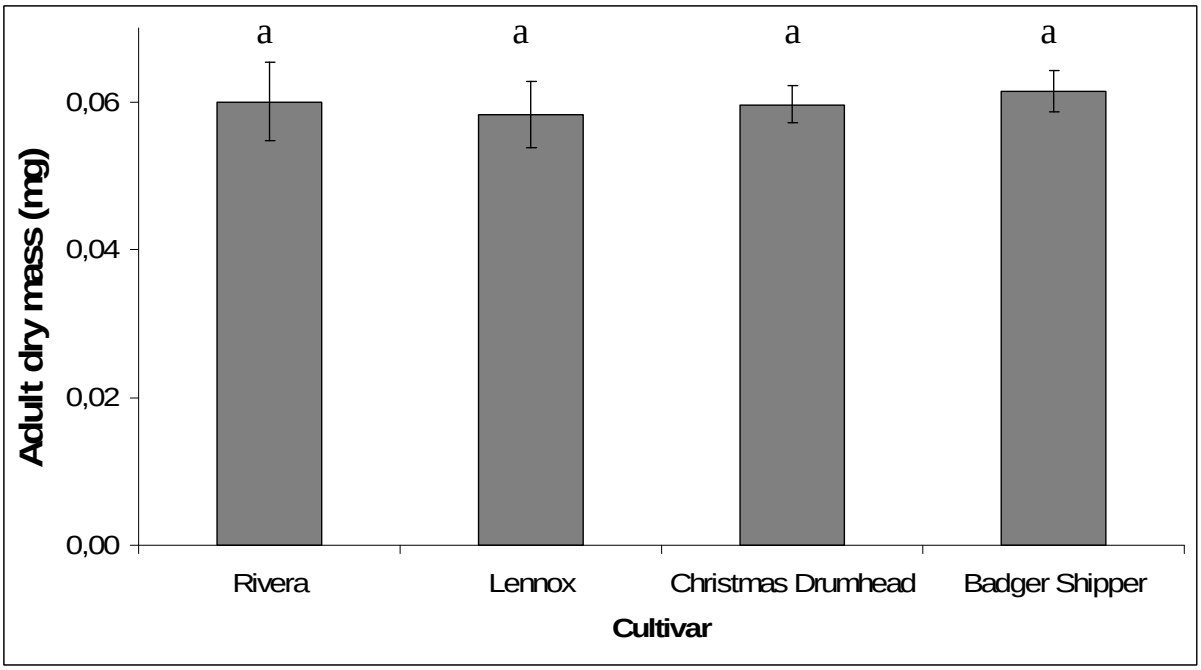


Figure 6. Average dry weight (mg) ± S.E. of adult *D. rapae* that developed in *B. brassicae* feeding on four different *B. oleracea* cultivars. Similar letters above bars indicate no significant differences between cultivars (ANOVA p>0.05)

4.3 Volatile profiling: preliminary results

Results from the volatile profiling taken from the headspace of *B. brassicae* infested (induction of four days) or uninfested cultivars of *Brassica oleracea* are still being analyzed and only the preliminary results can be shown here. Volatiles were trapped during the y-tube olfactometer experiments. The preliminary results suggest that there was little difference in chemical blend from infested and uninfested plants from the same cultivar. The GC-MS profiles from infested and uninfested plants were very similar in both the position and the surface area of the peaks, although the peaks for the infested plant in the lower graph had a slightly larger surface area (Fig.7). Additionally, infested and uninfested plants clustered close together in a principal component analysis plot based on 20 compounds (Fig. 8). The first Principal component axis explained most of the variation in the dataset and the chemical blend emitted by Christmas Drumhead seemed to differ slightly from the blends emitted by the other cultivars, while the blends of the other cultivars remained relatively similar to each other (Fig. 8). Additionally, the results showed clear day effects: the chemical blend emitted by infested and uninfested plants taken on one day clustered close together (not indicated in the graph).

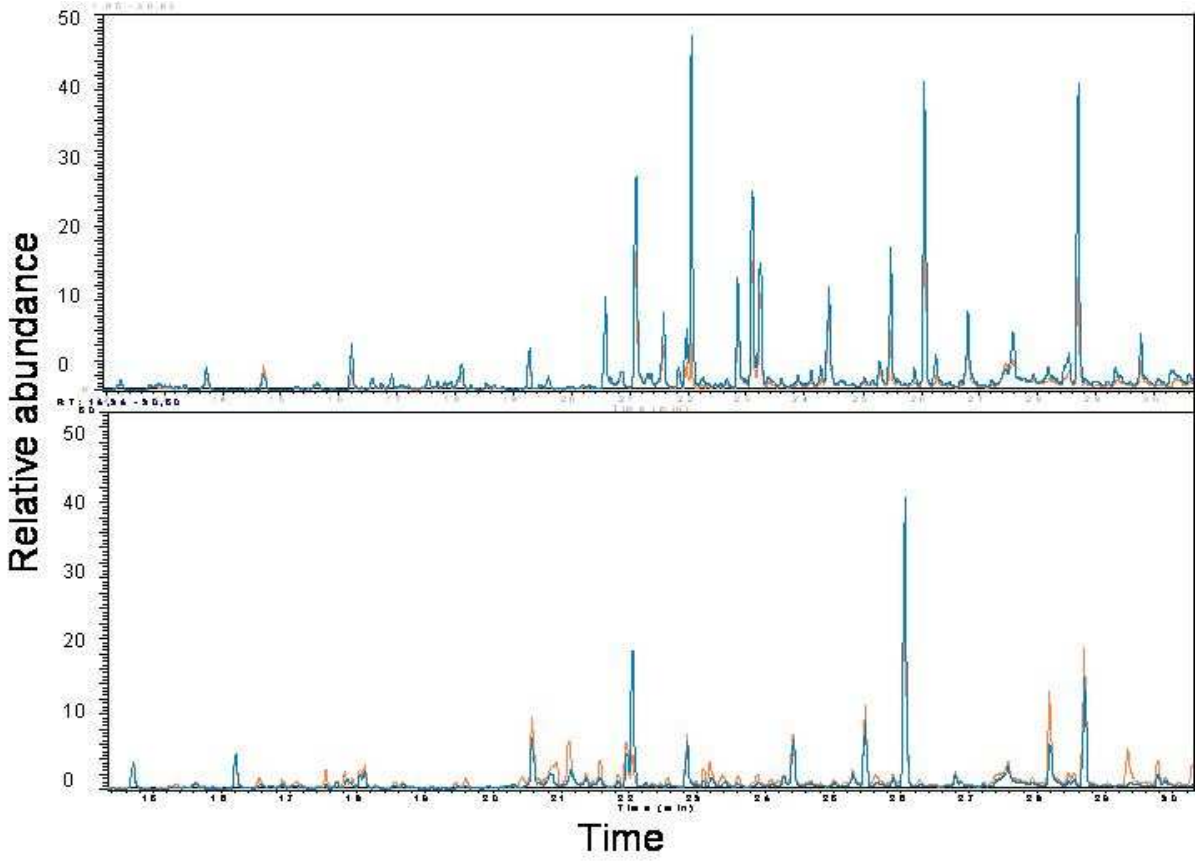


Figure 7. Graphs show examples of GC-MS profiles of volatiles emitted by cultivars of *B. oleracea.* Upper graph shows uninfested Lennox in blue and Lennox infested with *B. brassicae* in orange.
Lower graph shows uninfested Christmas Drumhead in blue and Christmas Drumhead infested with *B. brassicae* in orange. Note that peaks are very similar in position and surface area for infested and uninfested plants of the same cultivar.

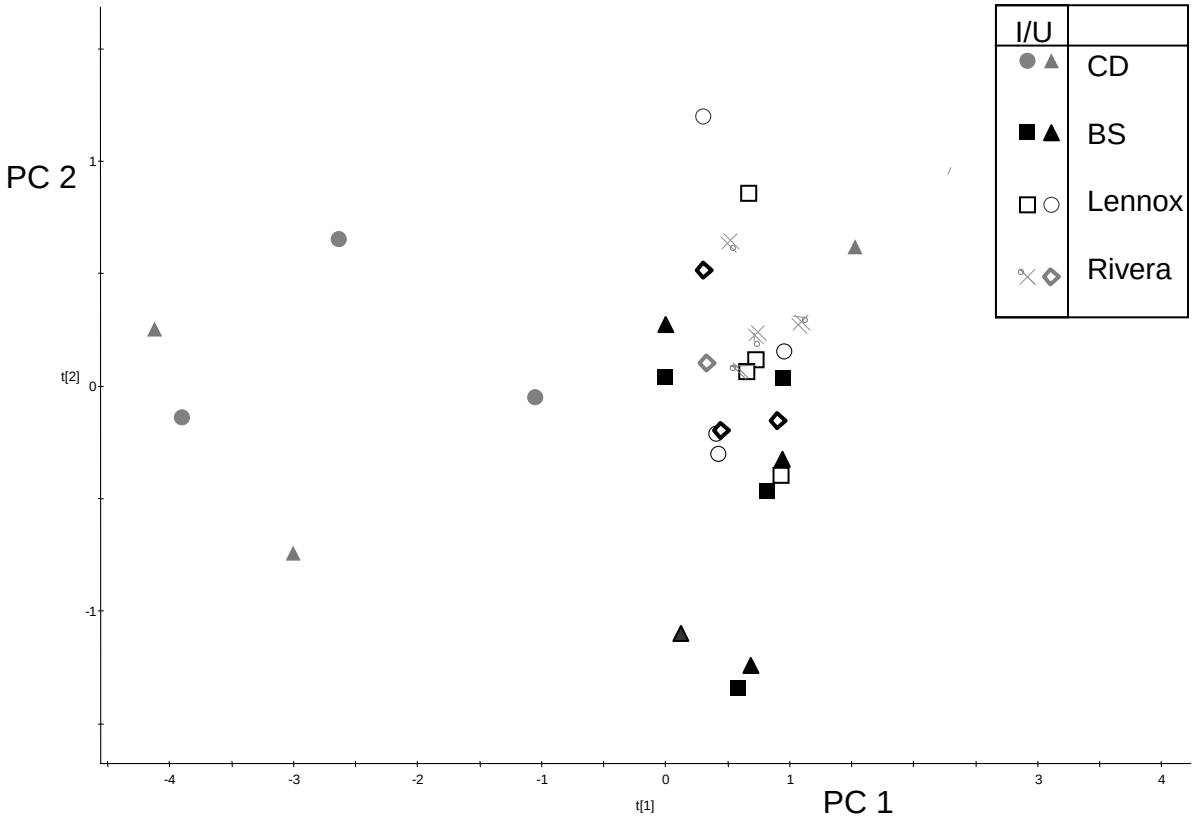


Figure 8. Preliminary PCA plot of headspace of *B. brassicae* infested (I) or uninfested (U) cultivars of *Brassica oleracea.* The plot is based on 20 compounds, mainly green leaf volatiles and terpenoids. Separation between the cultivars was strongest on the first axis. Note the slight separation of Christmas Drumhead (CD) from Badger Shipper (BS), Lennox and Rivera while uninfested and infested plants show almost no separation.

4.4 Dual choice olfactometer experiments

As expected *D. rapae* chose *B. brassicae* infested cultivars of *Brassica oleracea* over uninfested plants when given a choice between the two (Fig. 9). However, a significant difference could only be found for the cultivars Lennox (P= 0,005 Chi squared test) and Badger Shipper (P= 0,042 Chi squared test). No significant differences where found for Rivera (P= 0.096, Chi squared test) and Christmas Drumhead (P= 0.100, Chi squared test). Overall *D. rapae* was very responsive, the wasps made a choice approximately 60% to 79% of the time.

Unexpectedly, *D. rapae* did not show a preference for any of the cultivars infested with B. brassicae when given a choice between two different cultivars (Fig. 10). No significant differences were found for any of the combinations (P>0.75, Chi squared test). Again, the wasps were very responsive and made a choice approximately 66% to 81% of the time.

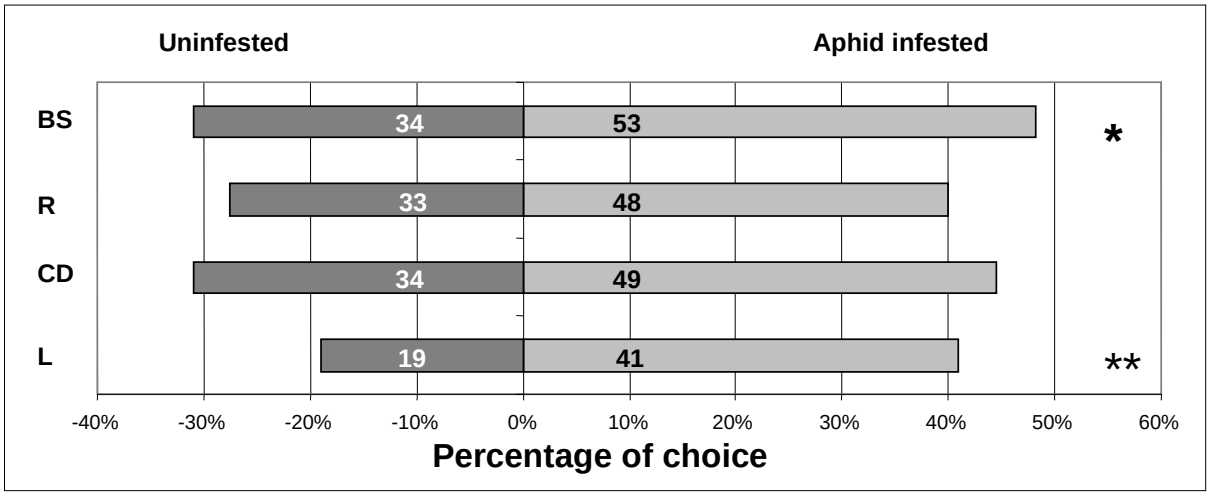


Figure 9. Preference of *D. rapae* for cultivars of *Brassica oleracea* in dual choice y-tube olfactometer tests. The bars represent a choice test between an uninfested (left) and a *B. brassicae* infested plant (right) for Badger Shipper (BS), Rivera (R), Christmas Drumhead (CD) and Lennox (L). Numbers in the bars indicate the number of wasps that made a choice for that treatment. * or ** indicates significant difference; * P<0.05; ** P< 0.01 (Chi squared test)

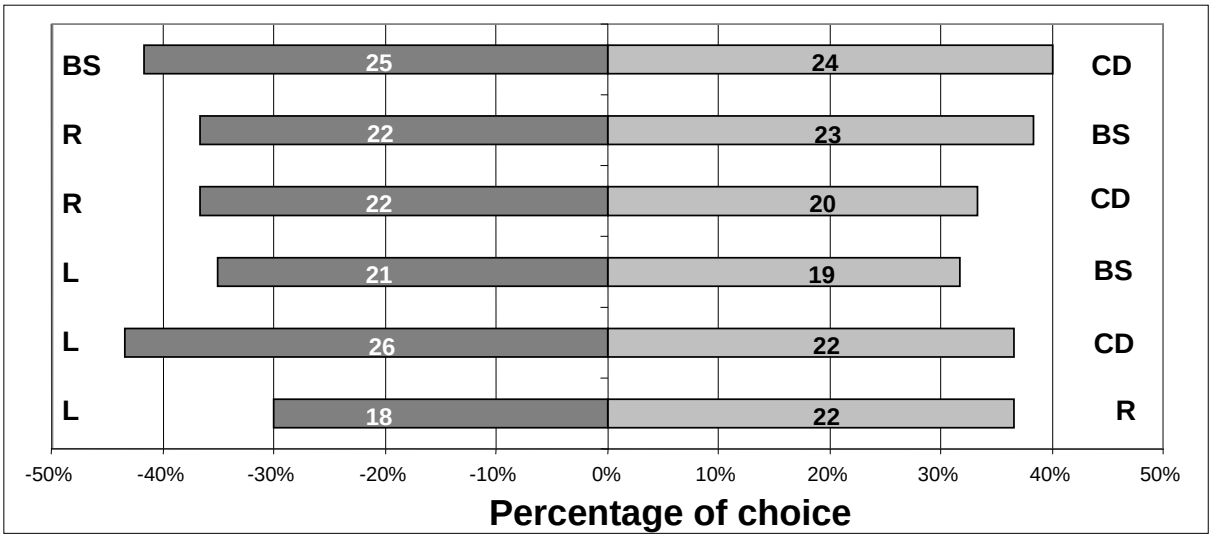


Figure 10. Preference of *D. rapae* for cultivars of *B. oleracea* in dual choice y-tube olfactometer tests. The bars represent a choice test between two different cultivars infested with *B. brassicae* for Badger Shipper (BS), Rivera (R), Christmas Drumhead (CD) and Lennox (L). Numbers in the bars indicate the number of wasps that made a choice for that treatment. There were no significant differences between the cultivars for al combinations (Chi squared test P>0,05)

4.5 Parasitism pilot experiments

In a dual choice situation between two different cultivars of *B. oleracea* infested with *B. brassicae*, *D. rapae* parasitized a significantly higher percentage of aphids on Christmas Drumhead than on Badger Shipper (P= 0.028, Wilcoxon signed rank test) (Fig. 11). For all other combinations no significant differences was found (P>0.65, Wilcoxon signed rank test). Because this was a pilot experiment and some plants were completely eaten by *Plutella* caterpillars only 7 or 8 replications were performed for each combination, which makes the results less reliable.

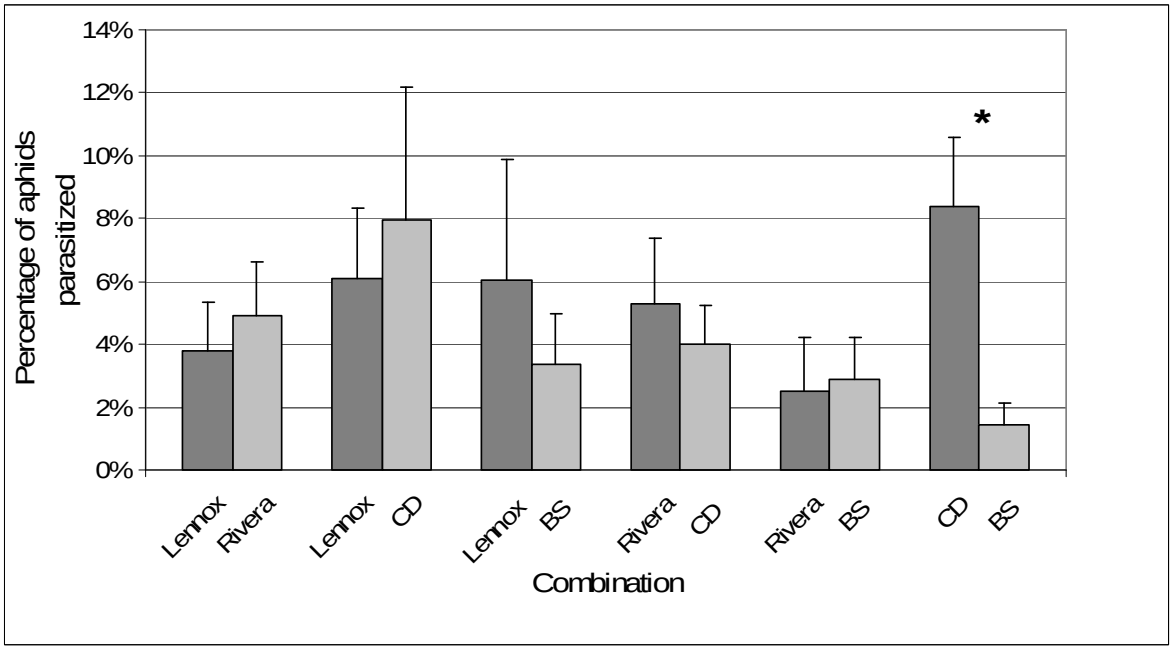


Figure 11. Oviposition preference of *D. rapae* for cultivars of *Brassica oleracea* in dual choice tests. The bars represent the mean ± S.E. percentage of aphids parasitized on cultivars infested with *B. brassicae* when presented to the wasp in pairs of two different cultivars. Combinations were made with Badger Shipper, Rivera, Christmas Drumhead and Lennox. * indicates significant difference
(Chi squared test P<0,05)

5 Discussion

Direct plant defense and performance of *Diaeretiella rapae*

Total glucosinolate levels in the phloem of the *Brassica oleracea* cultivars were higher for Christmas Drumhead and Badger Shipper than for Lennox and Rivera. Interestingly, the total levels of glucosinolates were also higher for L3 stage *Brevicoryne brassicae* feeding on Christmas Drumhead and Badger Shipper than for those feeding on Lennox and Rivera. These results seem to suggest that glucosinolate levels in the plants might determine the amount of total glucosinolate found in the aphids. However, no significant differences in total amount of glucosinolates in the phloem or the aphids could be found between cultivars, and no final conclusions can be drawn from these results. The levels of the glucosinolates in *B. brassicae* increase with age (Kazana *et al.* 2007) and the differences between adult aphids reared on the four cultivars might be more pronounced.

In contrast to the total amount of glucosinolates, the levels of several individual glucosinolates did differ significantly between aphids feeding on the four different cultivars. Most notably, levels of progoitrin, gluconapin and 4-hydroxyglucobrassicin were higher in aphids reared on Christmas Drumhead than in aphids reared on Rivera, and aphids reared on Rivera also contained lower levels of glucoraphanin than those reared on Badger shipper. However, these differences could not be found for the same individual glucosinolates in the phloem of the cultivars and in some cases glucosinolates found in the aphids were not found in the phloem at all. The levels of glucosinolates in the phloem were very low and it is possible that concentrations of several glucosinolates were to low to detect in the phloem and were only detectable after sequestration by the aphids. Kazana *et al* (2007) and Pratt *et al*. (2007) showed that the concentration of the glucosinolate sinigrin in the diet of *B. brassicae* determined the concentration of this chemical in the tissue of *B. brassicae*, and it is very likely that the differences in individual glucosinolates found in the aphids in this study is also determined by the cultivar they fed on.

The performance of parasitoids may be negatively affected by high glucosinolates in their host (Hunter 2003; Ode 2006). Additionally, the performance of a host and its parasitoid are often positively correlated (Gols 2008). Broekgaarden *et al.* (2008) showed that the *B. oleracea* cultivars Rivera and Lennox supported a slower population growth of *B. Brassicae* than the cultivars Badger Shipper and Christmas Drumhead with a potential role for a trypsin-and-protease inhibitor as a defensive secondary compound. However, even though its larvae are obligate tissue feeders and cannot avoid ingesting secondary plant compounds that might be present in the aphid's tissue, performance of *D. rapae* on *B. brassicae* was not affected by the cultivar the aphids fed on. Parasitoids that parasitize generalist herbivores have been shown to be more affected by toxins in the diet of their host than specialist parasitoids that attack hosts feeding only on Brassicaceae plants (Sznajder and Harvey 2003; Gols, Witjes *et al*. 2008a) and it is likely that *D. rapae* is simply not very strongly affected by glucosinolate levels found in *B. brassicae.* Additionally, plant mediated effects on performance are usually less pronounced in parasitoids than in their herbivore hosts, because host detoxification or excretion may dilute the effect of secondary plant compounds on the performance of parasitoids even if the larvae are obligate tissue feeders (Gols 2008) which could explain why the performance of *D. rapae* is not affected by the differential performance of *B. brassicae* on the four cultivars used in this study. However, because differences in glucosinolate levels between the aphids feeding on the different cultivars were not very pronounced it cannot be concluded with any certainty that *D. rapae* is not affected by high levels of glucosinolates and a lack of variation in the wasp's performance could simply be the result of the lack of variation in glucosinolate levels found in the four cultivars. A second explanation for the lack of differences in performance could be that the effects of more glucosinolates in Badger Shipper and Christmas Drumhead and reduced performance of *B. brassicae* on Lennox and Rivera balance out any differential effects on the performance of *D. rapae*. However, because differences in glucosinolate level of aphids feeding on the four cultivars were not very pronounced this seems unlikely.

Indirect plant defense and the attraction of *Diaeretiella rapae*

Several studies have shown that *D. rapae* is able to differentiate between the volatiles emitted by plants infested with aphids and uninfested plants, and, while being attracted to uninfested plants in the absence of infested plants, it prefers the infested plants over uninfested plants (Girling *et al.* 2006; Agbogba and Powell 2007).

Although *D. rapae* also preferred *B. brassicae* infested *B. oleracea* plants over uninfested ones in a two choice olfactometer in this study, and this trend was found for all cultivars, the difference was only significant for two of the four cultivars. Blande *et al*. (2007) have shown that the response of *D. rapae* can be improved by giving them an ovipositioning experience. However, a pilot in which experienced wasps were compared with inexperienced wasps revealed no improvement of the response in this study (unpublished data). The preliminary results from the volatile analyses indicate that there are little qualitative or quantitative differences between the volatile blends emitted by infested or uninfested cabbage plants. Glucosinolate levels of highly cultivated crops, such as cabbage, are often reduced compared to wild conspecifics because of selection for taste and yield rather than plant defenses, although glucosinolate levels may vary with the cultivar involved (Gols 2008). Similarly, the response of cultivated crops to herbivory may also be reduced. Additionally, only few volatiles are emitted by plants in response to phloem feeders compared to tissue feeding insects and the volatiles are sometimes even undetectable (Du *et al*. 1998;Turlings *et al*. 1998). Aphids are known to suppress plant defenses (Broekgaarden *et al.* 2008) and this is likely due to the minimal damage inflicted by aphid feeding and introduction of salivary effectors that deter volatile synthesis (Walling 2008). Both crop cultivation and suppression of plant defenses by aphids could explain the lack of differences between volatiles emitted by the infested and uninfested cabbage cultivars used in this study and consequentially the only slight attraction of *D. rapae* to infested plants compared to uninfested plants.

This study also revealed that *D. rapae* was not differentially attracted to the *B. oleracea* cultivars Lennox, Rivera, Badger Shipper and Christmas Drumhead in a two choice olfactometer. The volatile blends emitted by conspecific plants may vary widely as a result of genetic variation and individual plants with increased volatile emissions have often been found to be more attractive to parasitoids (Poelman *et al.* 2008b) However, preliminary analyses showed that in this study, although some variation existed, the blends emitted by the cultivars were very similar. This in combination with the reduced induction of volatile production in these cultivars as a response to *B. brassicae* infestation explains why the wasps show no preference for the volatiles emitted by any of the cultivars.

A pilot experiment revealed that a higher percentage of *B. brassicae* were parasitized on Christmas Drumhead than on Badger Shipper when *D. rapae* was given a choice between the two. In this experiment the parasitoids were in close contact with the plants and the aphids while in the olfactometer experiment the wasps never got close to the plants or aphids. It is known that for parasitoids plant volatiles mediate the host searching behaviour mostly at longer distances, while herbivore derived chemical cues become more important at shorter distances from the host (Ayal 1987; Vet and Dicke 1992; Powell 1998; Bradburne and Mithen 2000). Honeydew that was excreted by the aphids might play a role in the possible differential attraction revealed in this alternative experiment. D. rapae may alight anywhere on a host plant and when it encounters honeydew, it is arrested and begins to move higher on the plant to find aphids (Ayal 1987). Badger Shipper plants used in this experiment were much larger than the Christmas Drumhead plants, and it is possible that the aphids on Christmas Drumhead were closer to each other and therefore the concentration of honeydew could have been higher on Christmas Drumhead leaves. Additionally, the lower number and the smaller size of the leaves of Christmas Drumhead mean that honeydew drips to lower parts of the of Christmas Drumhead plants more easily where it is caught by higher leaves in Badger Shipper. These factors could make the aphids easier to find for the parasitoid on Christmas Drumhead than on Badger Shipper. An additional factor could be that *D. rapae* also has a harder time finding the aphids because of the plants architecture. Badger shipper has more folds and curls in its leaves (personal observation), which makes it easier for an aphid to hide. Although the parasitism rate of the aphids was generally low on

Badger shipper, no significant differences were found in combination with cultivars other than Christmas Drumhead, but because this was a pilot experiment and some plants were eaten by *Plutella* caterpillars, only 7 or 8 wasps were tested for each combination of cultivars. More replications are needed to make sense of these results.

Interactions between direct and indirect defenses

Physical and chemical direct defenses of plants against insect herbivores may also affect predators and parasitoids negatively. When predators or parasitoids are attracted more to plants that are suboptimal in terms of its own fitness, a conflict may arise (Gols 2008). In this study, *D. rapae* performs equally well on the different cultivars of *B. oleracea* and is equally attracted to the volatiles emitted by these plants. Therefore there is no conflict between the direct and indirect defenses of *B. oleracea* for *D. rapae* in the system studied here. However, there is a possibility that the architecture of Badger Shipper negatively affects the ability of *D. rapae* to find its host, although no clear conclusions can be drawn from the available results. The parasitoid parasitized aphids on Badger Shipper less than on Christmas Drumhead which may implicate that it has a harder time finding aphids on the leaves of Badger Shipper. This may also negatively affect the fitness of *D. rapae*, because the fitness of a parasitoid is closely related with its ability to find its host (Gols 2008). However, even if *D. rapae* has a harder time finding aphids on Badger Shipper, no real conflict would arise because the wasp is not attracted more to this cultivar compared to the other cultivars. Additionally, it remains uncertain if the parasitoid is unaffected by differences in glucosinolate content of the cultivars used in this study or that these differences are simply too small to make a difference in the performance of *D. rapae* and no general conclusion can be drawn from these results in relation to the occurrence of a conflict between direct and indirect plant defenses in other systems based on the plant's glucosinolate content.

Further recommendations

Parasitoids are the most commonly used natural enemy for the control of aphids on economically important crops (Emden and Harrington 2007). Manipulation of direct and indirect plant defenses may provide a means of improving performance of *D. rapae* on *Brassica* crops and making the crops more attractive to this parasitoid. This study was performed to find out which chemicals are most attractive to the wasp and what affects its performance. However, little variation existed between the cultivars used. Direct and indirect defenses of crops are often affected by years of cultivation for traits such as taste and yield. The plant mediated effects of wild plant species on the performance and behaviour of parasitoids are likely to be much more pronounced. A system made up of wild and cultivated brassicaceous plant species will provide the variation needed to study plant mediated effects on the performance and behaviour of *D. rapae.* Therefore, further research should focus on the comparison of wild and cultivated brassicaceous plant species.

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Appendix
Protocol glucosinolate extraction and detection in aphids and phloem (EDTA solution) (Provided by NIOO)

Prepare

- 70% MeOH in water (MillliQ)
- 20 mM NaOAC (pH = 5.5.)
- (= 0.82 g NAOAC or 1.36 g NaOAC.3H2O in 500 ml water, adjust pH with HCL. Keep in fridge!!)
- sulfatase solution (freezer, see below)
- DEAE Sephadex A25 in water (mix 10g with 125 ml MilliQ water and put in fridge)
- Sinigrin reference curve (5 concentrations between 0.1 – 10 mM)

Prepare column

- Prepare column by putting a small piece of glass wool in a Pasteur pipet. Use a sateh-stick to push the glass wool down.
- Pipette 0.5 ml of DEAE-Sephadex A25 in water (shake before pipetting) on column.
- Wash column with 1 ml MilliQ water

Extraction

1. Weigh in dried and grinded aphid material 50-100 mg dry weight in 2 ml eppendorf tubes with safety caps. (Extraction volume = 1 ml) Make two pinpricks in each cap for ventilation or take 1 ml of EDTA solution
2. Add 1 ml of 70% MeOH with the dispensor and vortex.
3. Place tubes for 6 minutes in hot water bath (90 degrees) to boil the 70% MeOH.
4. Place tubes in ultrasonic bath for 15 minutes.
5. Centrifuge aphid samples (skip steps 5 to 9 for EDTA solution) at 4500 rpm for 10 minutes.
6. Add extract to column (before adding see preparation column)
7. Add a second time 1 ml of 70% MeOH with the dispensor and vortex.
8. Place tubes in ultrasonic bath for 15 minutes.
9. Centrifuge aphid samples at 4500 rpm for 10 minutes.
10. Add extract from aphid samples and EDTA solution to column.
11. Wash column with 2 x 1ml 70% MeOH.
12. Wash column with 1ml MilliQ water
13. Wash column with 2 x 1ml 20 mM NaOAC buffer.
14. Add 20 µl sulfatase solution.
15. Flush sulfatase down into column with 50 µl NaOAC buffer.
16. Cover collomns with aluminum foil and let stand overnight.
17. Elute, the day after, desulfoglucosinolates with 2 x 1ml MilliQ water
18. Cap the tubes (make sure that there are holes in the caps) and freeze
19. Place frozen samples in freeze-dryer (at least one night).
20. Redissolve residue in exact volume (1ml of less) of MilliQ water.
21. Filter sample over 0.2 µm nylon syringe filter and put in a HPLC vial. You can use 1 filter for 10 samples. Flush in between samples with water and air.
22. Place sample in fridge for up till two weeks or freezer (-20 degrees) for up till one year.
23. Place frozen samples in freeze-dryer (at least one night).
24. Redissolve in 100 µl MilliQ water
25. Inject 70 µl on HPLC

Sulfatase solution

- Dissolve 700 mg of aryl sulfatase (Sigma type H-1 of Helix pomatia) in 30 ml of deionized water ad add 30 ml of absolute EtOH and put in 250 centrifuge container.
- Mix well and centrifuge at 4000 rpm for 20 minutes.
- Add additional 90 ml of EtOH to supernatant.
- Centrifuge at 2500 rpm for 15 minutes
- Dissolve pellet in 25 ml deionised water and store in 1ml aliquots in –20℃. Those will keep for at least one year.