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Plant chemical signalling affecting aphid-plant interactions

by

Erika Qvarfordt

Fakulteten för landskapsplanering, trädgårds- och jordbruksvetenskap

SLU-Alnarp

Author:
Erika Qvarfordt

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Dr Robert Glinwood

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***R. padi* and *M. persicae* feeding on barley leaf**

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Abstract

Aphid-plant interaction are complex processes where much still remains to be explored. This project tested the capacity of aphid-infested barley, *Hordeum vulgare* (L.), to induce defence-like responses in neighbouring plants via chemical signals, making them less acceptable to the bird cherry-oat aphid *Rhopalosiphum padi* (L.). Chemicals released into the air or the rhizospshere from infested plants can potentially act as defence-inducing signals in neighbouring plants. Results showed that both volatiles and rhizospshere extracts from infested plants can make receiving plants less acceptable to *R. padi*. Despite a range of experiments, the decisive factors behind the rhizospshere interaction could not be identified; however a hypothesis for future study is that soil micro-organisms play a role. Feeding by a different aphid species, *Myzus persicae* (Sulzer), made barley plants more acceptable to *R. padi* in settling tests. Aphids were attracted to the odour of *M. persicae*-infested plants and settled more often on plants that had been exposed to *M. persicae*-infested plants. This between-species interaction in aphids has not been previously reported. Further research is needed to gain a deeper understanding of the importance of plant-plant chemical signalling in aphid ecology, for example responses at the molecular level and effects on trophic interactions. This project has however provided the first step for these investigations in this study system.

Sammanfattning

Samspelet växter — bladlöss är ett komplex ämne där fortfarande mycket återstår att utforska. I detta arbete prövas kornets, *Hordeum vulgare* (L.), förmåga att inducera en försvars liknande respons hos en intillväxande kornplanta och därmed göra den mindre attraktiv som värdväxt för Havrebladlusen, *Rhopalosiphum padi* (L.). Avgivna kemikalier från en bladlusinfesterad planta når via luften eller inom rhizosfären mottagarplantan och fungerar som startsignaler till plantans försvars mekanismer. Förändringen för *R. padis* värdväxt acceptans har prövats genom tvåvals test (preference test), där plantans förändrade ytkemikalier påverkar bladlusens initiala kontakt, och genom olfactometri test. Resultaten visar att bladlusinfesterade kornplantor kan inducera ett försvar i en annan planta både genom volatiler och rotutsöndringar. Trots många olika experiment genomfördes kunde inte de avgörande försvarsinducerande faktorerna särskiljas, en hypotes att studera vidare på är att mikrorganismer i jorden tillsammans med avgivna ämnen från rötterna har en effekt på växtens försvars mekanism.

I studien visas för första gången att en bladlusart kan påverka annan bladlusarts värdacceptans genom att förändra en plantas avgivna kemiska signaler till en intillväxande planta. *R. padi* föredrog i samtliga test de kornplantor som mottagit kemikalier från en *Myzus persicae* (Sulzer) infesterad kornplanta framför obehandlade plantor. Detta samspel mellan olika bladlusarter och deras värdväxter är mycket intressant och fortsatt forskning krävs för att få en djupare förståelse där responsen från bladlössens naturliga fiender vägs ihop med gen-molekulär forskning och kemiska analyser av signalämnen.

Introduction

Plants are challenged by many threats such as competitors, herbivores and pathogens. These interactions have driven plants to evolve different strategies to protect themselves. One such strategy is the induction of biochemical defences against the attacker. However, both insects and other plants have developed the ability to detect and respond to these changes in plant status via chemical signalling. In this study I investigated chemical signalling between organisms in a system consisting of barley plants and insect herbivores, aphids.

Plant induced defence and chemical signalling

Plant defences can be divided into direct defence and indirect defence, and both types can be constitutive or induced. Constitutive direct defence can be thorns and spines or other physical barriers that keep attackers away, also primary and secondary metabolites that are harmful to attacking herbivores (Kessler and Baldwin 2002).

Indirect induced defences often involve attraction or retention of the parasites and predators of the herbivore via emitted volatile organic compounds (VOCs) (Arimura et al. 2000; Dicke and Dijkman 2001; Ninkovic et al. 2001) or increased production of extra floral nectar (EFN) (Heil and Bueno 2006). Induced direct defences can be triggered by an attacker and make the plant resistant or less suitable as a host. Induction of biochemical defences can also be accompanied by production of VOCs that can deter subsequent attackers or function in indirect defence as described above.

VOCs emitted from wounded plant tissue can also function as cues in defence signalling between plant organs or between neighbouring individuals, triggering immediate induction of defence mechanisms in receiving plants (Karban et al. 2000; Chamberlain et al. 2001; Baldwin et al. 2006). However, there is now evidence that herbivore-induced volatiles play a role in within-plant signalling. This action may be divided into two phases; the emitted volatiles from wounded tissue are received by other plant parts and prime the plant, after which vascular signalling confirms the threat and a more substantial defence mechanism is deployed (Heil and Ton 2007).

In those plants that have been studied, VOCs released in response to herbivore damage have often been characterised as blends of green leaf volatiles (C6-alcohols and C6 aldehydes derived from C_{18} fatty acids such as linolenic acid and linoleic acid), mono and sesquiterpenes and methyl salicylate. Emitted blends may include as many as 200 different compounds synthesized from at least three different biochemical pathways (Kessler and Baldwin 2002). The composition of a particular blend depends on several factors such as the type of attack, plant genotype and abiotic conditions. Some VOCs, such as ethylene, methanol and isoprene, are highly volatile and dissipate rapidly in the atmosphere and are therefore suggested to be involved only in signalling within a plant individual's own canopy (Baldwin et al. 2006). Other commonly emitted substances, such as methyl salicylate, methyl jasmonate (MeJa) and green leaf volatiles can be transferred by air currents and be taken up by neighbouring plants (Thaler 1999; Karban et al. 2000). Chemical signalling between plants occurs not only via volatiles in the air; a herbivore-infested plant can release root exudates that induce defence in a receiving undamaged neighbouring plant and make it more attractive for parasitoids and predators (Dicke and Dijkman 2001;Chamberlain et al. 2001).

There is now increased understanding of plant biochemical and molecular responses to herbivore attack (Kessler et al. 2006; Moran and Thompson 2001). Gene expression analyses of plants attacked by herbivores (Zhu-Salzman et al. 2004) or exposed to defence-inducing VOCs (Farag et al. 2005) have revealed defence-related genes, and the identify of several inducing signals has been determined by analytical chemistry. Much work has focussed on understanding the biochemical interaction between plants and chewing herbivores (Walling 2000), and important findings such as identification of elicitors in insect saliva that induce volatile emission (Alborn et al. 1997) and indirect defences that attract parasitoids (Turlings and Benrey 1998) have been followed by studies of gene expression that show the importance of volatiles as signalling substances (e.g. Engelberth et al. 2007). Piercing insects such as aphids cause little visible damage to plants, and much less is known about plant molecular responses to aphid attack. However, studies suggest that aphid feeding stimulates pathways associated with both pathogen infection and herbivore wounding (Moran and Thompson 2001 2007).

Aphids and their interactions with plants

Aphids (*Homoptera*; Aphididae) are pests in many of the world's crops. They weaken plants by sucking sap and decreasing the ability to photosynthesise, which often results in decreased yields. Aphids are also important virus vectors (Alford 1999). There are more than 4000 aphid species, most of them living on one or a few plant species, but some alternate between two often very different plant taxa. In cold climates, host alternating aphids such as *Rhopalosiphum padi* overwinter as eggs on primary hosts, hatch in spring and after a few generation developing winged forms, alatae, that migrate to summer hosts. Winged aphids are also formed if a colony becomes overcrowded or host quality decreases. Although a sexual stage may be present, aphid reproduction is mainly by parthenogenesis giving rise to live nymphs. This results in the formation of genetic clones and allows dramatically fast population growth and rapid adaptation to changes in the environment (Dixon 1998). The most common aphid in Swedish cereals, *R. padi*, can be a serious pest in certain years (Wiktelius et al. 1990) by directly affecting growth and yield, but also by transmitting barley yellow dwarf-virus (BYDV) (Riedell et al. 2003), while rose-grain aphid *Metopolophium dirhodum* and English grain aphid *Sitobion avenae* are sporadic pests.

Aphids are using chemical cues to solve the challenges presented by their often complex lifecycles; mating, population density regulation, location and assessment of host plants and warning of danger are all mediated by emitted or received chemical substances (Pickett and Glinwood 2007). Habitat location and host choice are divided into several steps starting with visual cues which later act together with olfactory cues. Olfactory sensors on the antennae are important in long-range detection of volatile chemical information, while chemoreceptors on the legs and antennae allow aphids to perceive and assess the plant surface after landing. By probing with the mouthparts, aphids then assess the chemical composition and nutrient value of the plant before stylet penetration. Aphid stylet penetration is highly accurate, using the area between the epidermal cells called the anticlinal grooves. The stylet follows the apoplastic pathway and the aphid releases sheath saliva that contains proteins, phospholipids and conjugated carbohydrates (Miles 1999). Before ingesting sap from the phloem, aphids inject watery saliva to prevent plugging of cells in sieve tube elements (Will and Bel 2006). It has been suggested that this watery saliva may function as elicitor of induced plant defence (Smith and Boyko 2006; Walling 2000). Many of the details of what exactly happens at the

plant biochemical and molecular level during an aphid attack still remain to be discovered (Moran and Thompson 2001; Divol et al. 2005).

Hypothesis and Aim of the Study

The majority of published studies reporting plant defence signalling via chemicals have used chewing herbivores as models, but very few studies have addressed whether it also occurs when plants are attacked by a piercing/sucking herbivore such as an aphid. The aim of this study was to experimentally examine the evidence for plant-plant chemical signalling in a system consisting of barley plants and aphids. The following questions were addressed:

1. Are volatiles emitted by a *R. padi*-infested barley plant able to induce defences responses in a neighbouring plant?

2. Can a *R. padi*-infested barley plant induce responses in a neighbouring barley plant via the rhizospshere?

3. Does the identity of the attacking aphid species differently affect the outcome of aphid-plant interaction?

Methods and Materials

Aphids

Aphids used in experiments were wingless *R. padi*, *M. dirhodum* and *Myzus persicae* of mixed instars. *R. padi* and *M. dirhodum* were reared on a mix of barley and oat in glasshouse at 20-22˚c and a 18L:6D photoperiod. *M. persicae* was reared on a mix of pepper and oilseed rape at 21˚c with a 18L:6D photoperiod.

Plants

Barley *Hordeum vulgare* (L.) cultivar Prestige was used for all experiments, unless otherwise stated. Other cultivars and breeding lines used were 28:4 and Lina, which have been characterised as resistant and susceptible respectively to *R. padi* (Delp et al. 2009). Seeds were sown in pots (8×8×6 cm) in potting soil (Hasselfors special). Plants were 4-6 days old (2-leaf stage) at the start of each experiment.

Aphid-infestation

Plants used as chemical emitters were infested with an average of 30 aphids/plant. For pots with one plant, the seedling was covered with a plastic tube (2.3 cm diameter, 12 cm high) and aphids were carefully released into the tube with a fine brush. The tube was enclosed with a net and rubber band. For pots with five plants, the pots were covered with a plastic cylinder with net top (6.8 cm diameter, 28.5cm high) or with a net cylinder (13 cm diameter, 30.5 cm high). Tubes and cylinders were removed after approximately 24, 48 or 72 hours depending on the experiment.

Exposure of plants to plant volatiles

Barley plants were exposed to volatiles from either aphid-infested or uninfested barley plants inside specially-designed two-chamber cages (Pettersson et al. 1996; Ninkovic et al. 2002; Glinwood et al. 2004). The cage was divided into two chambers connected by a 7 cm wide opening covered with net to prevent aphids moving between chambers. Both openings at the top were covered with cellophane. Air flowed from the first chamber (containing the infested or uninfested plant) to the second chamber (containing the receiving plant) and was extracted from the cage and vented outside the greenhouse. The pots were placed in Petri dishes (9 cm)

to avoid root contact. Each treatment was represented by 4-20 separate cages (replicates), which were placed in an alternating pattern within the glasshouse to compensate for any spatial bias in conditions. Exposure time was 4-6 days.

Preference test

Leaves from two plants, one control and one treated, which still were attached to the plant were placed without touching each other on a white paper sheet. For tests with previously infested plants, aphids were carefully removed with a fine brush and plant tissue was rinsed with distilled water and gently wiped with wet paper tissue. Control plants were treated the same way. Ten aphids were released between the two plants, and the area was enclosed in a plastic cylinder (11 cm diameter, 4 cm high) covered with net. To prevent plants drying out, soil still remained on the roots and was covered with wet paper towels. Twenty pairs of plants were placed on a glasshouse bench, with the position of treatment and control plants alternating. Settled aphids were recorded after 2 hours and 4 hours, this being the average time taken for aphids to locate the phloem and begin feeding (Prado and Tjallingii 1997). To minimize the effect of diurnal cycles on volatile emission (Loughrin et al. 1994), all tests were performed at the same time of day.

Olfactometry

A two-way airflow olfactometer (Glinwood et al. 2003) was used to test aphid olfactory responses. This was a standard four-arm device, but with two of the arms closed using silicone rubber inserts, creating a two-way olfactometer consisting of two stimulus zones (arms) directly opposite each other, with a central neutral zone separating them. Air was extracted using a vacuum pump, with a flow rate through each olfactometer of 250 ml/min. A single wingless aphid was introduced into the olfactometer, and its position was recorded every 3 minutes over a 30-minute period. Three minutes was long enough to permit an aphid to move from one end of the arena to the other. If an aphid was inactive in the olfactometer (observed to be stationary in the same position for three consecutive observations) it was removed and the bioassay started with a fresh aphid. Two pots of plants, one control and one treated were placed in separate Perspex exposure cage (described above) or in plastic jars constructed to allow air to enter through the lid via a Teflon tube to the bottom of the jar and via another Teflon tube into the olfactometry arm. A cage or jar containing the treatment was connected to one olfactometer arm and the control to the opposite arm.

Statistical Analysis

Data from experiments were analysed by two-sample t-test.

List of experiments

The following experiments were conducted to test the various aspects of the overall hypothesis. A summary along with experiment codes is also given in Table 1 below.

Interaction via volatiles

*Experiment V 1.*Barley plants were exposed to *R. padi*-infested uninfested barley plants to test the hypothesis that volatiles from an aphid infested plant can affect *R. padi* interaction with a receiving plant. Olfactometry and preference tests were performed.

Experiment V 2. Barley plants were exposed to *M. persicae*-infested uninfested barley plants to test the hypothesis that volatiles from plants infested with a different aphid species can affect *R. padi* interaction with a receiving plant. Olfactometry and preference tests were performed.

Experiments V3.1-3.5 R. padi olfactory preference to aphid infested barley plants was evaluated to test the hypothesis that aphid infestation can influence plant preference via volatile cues. Plants were infested and left for 72 hours covered with net cylinder. In an olfactometer, *R. padi* chose between the odours of infested or uninfested plants, or plants infested with different aphid species. The different aphid species used to infest plants were, in V3.1 *R. padi* chose between odour of *R. padi* and *M. persicae* and in V3.5 between *R. padi* and *M. dirhodum* infested barley plants. In V3.3 and V3.4 *R. padi* chose between odours from infested (V3.3 *M. persicae,* V3.4 *R. padi*) and uninfested plants. In V3.2 *R. padi* chose between odours of plants previously infested with *M. persicae* from which aphids had been removed immediately before the bioassay.

Experiments V4.1-4.3. To test whether constitutive resistance affects a plants capacity to engage in volatile interactions, two barley genotypes were compared; cultivar Lina which is considered susceptible to *R. padi* and the breeding line 28:4 which is considered resistant (Delp et al., 2009). *R. padi* was given a settling choice between plants that had been exposed

to infested plants or uninfested plants of the same cultivar (Lina in V4.1 or 28:4 in V4.2). In V4.3, *R. padi* was given a settling choice between Lina and 28:4 that had been exposed to infested plants of the same cultivar.

Experiments V5.1-5.3. R. padi settling preference on previously aphid infested barley plants was evaluated to test the hypothesis that previous aphid infestation can influence plant preference via host acceptance on contact. Plants were previously infested with *M. persicae* in V5.1, *R. padi* in V5.2 and *M. dirhodum* in V5.3.

Interactions via the rhizosphere

Experiments R1.1-1.4, were preformed to test if root exudates from aphid infested plants could cause a receiving plant to have altered acceptability to *R. padi*. One chamber of the two-chamber cage (described above) was placed on top of the other and fastened with cellophane to form a two-tier cage (Glinwood et al. 2003). A pot containing five barley plants on a Petri dish (9 cm) with a 6 cm opening covered with filter paper was placed on a shelf of inert polythene foam plastic (Plastazote PZ940). The plastic shelf was put into the bottom of the upper cage and cellophane separated the two cages. A funnel was inserted into a hole in the shelf, and protruded through a small hole in the cellophane into the bottom cage. Receiving plants were placed in the lower cage in pots with five plants. Air was extracted through both cages as described above, thus plants interacted via root exudates alone with no exchange of volatiles.

The upper cage plants were watered daily by hand with 75 ml distilled water, which collected root exudates from the provoking plants and dripped through the funnel on the soil around the receiving plants in the lower cage. Eight two-tier cages were placed in a glasshouse, four of which held aphid infested plants in the upper chamber, and four with uninfested plants. In all experiments plants were infested with aphids 24 hour before exposure. Barley cultivars used for the different test were; in R1.1 Lina and in R1.2 28:4, in, R1.3 and R1.4 Prestige. Plants were infested with *R. padi*, apart from R1.3 where aphid was *M. persicae*. Preference tests were performed.

Experiment R 2 was carried out to categorically rule out even minimal exchange of volatiles between plants in the two-tier cages. Instead of allowing water to drip from the upper plants

onto the soil of the receiving plant, water was instead collected in a beaker and then used to treat receiving plants held in separate two-chamber cages. Preference tests were performed.

Experiment R 3 tested whether exposure to root exudates from infested plants can induce release of volatiles from undamaged plants that cause changes in exposed, undamaged plants. The method was identical to R 3, except that a second pot of receiving plants was placed in the second chamber of the twin cage and was thus exposed to volatiles from the exudates-treated plants. Preference tests were performed.

Experiment R4 tested whether *R. padi* acceptance of plants was affected when they shared a rhizospshere environment with neighbouring infested plants. Ten barley plants divided into two groups were sown in a plastic box (19 cm x 25.5 cm, cm 5.5high). When plants reached the 2-leaf stage, two chamber cages (described above) were placed over plants to prevent volatile exchange. One group of plants were infested with 30 *R. padi*/seedling. Plants grew alongside each other for 5 days before a preference test was performed.

Experiment R5 tested whether aphid-produced substances were involved in plant-plant signalling. Receiving plants were watered with a solution containing the fall-off products of an aphid colony; almost exclusively honeydew but also some dead skins. The substances were collected on foil film (7cm x 10 cm), which were placed under infested and uninfested plants (control treatment). Collected substances were rinsed off the foil film daily with 75 ml of distilled water and 5ml of hexane and new piece of foil film placed under the plants. Preference tests were performed.

Experiment R6 further tested the possible involvement of aphid-produced substances in plant-plant signalling by repeating experiment R1 but preventing aphid products from falling onto the soil by placing a piece of plastic film carefully around the base of the plants. Seedlings grew up through the plastic film and no space was left between the plastic film and the stem to avoid aphids to climbing down. The pots were watered carefully under the plastic film. A preference test was performed.

Experiment R7 aimed to test whether an elicitor from an aphid infested barley plant could be transferred in hydroponic solution. Seedlings, approximately 3 days old, were carefully

removed from soil by running tap water and then rinsed in distilled water. Seedlings were placed one and one in test tubes (12 mm diameter, 13.8 cm high) containing 18 ml of Murashige and Skoog basal salt mixture (MS) (Sigma) solution, 4.3 g powder / litre water. Test tubes were wrapped with foil and plastic foam surrounded the plants at the top of the test tubes to keep seedlings in position. Twenty seedlings were infested with 30 *R. padi* /plant and twenty remained uninfested. Test tubes were placed in test tube rack and different treatments were placed in separate chamber cages (18cmx 18cm, 60high) to avoid volatile exchange. After 72 hours all plant were replaced with new uninfested plants and the MS solution in test tubes was topped up if needed. New seedlings were left in the solution for 24 hours before a preference test was performed.

Experiment R8 tested the possible influence of soil micro-organisms on plant-plant signalling. The planting soil was autoclaved for 20 minutes in 120˚ C to sterilise it. The experiment then proceeded in the same way as R1.

Table 1 Experiments

Code	Type of bioassay	Plant material compared
V1	olfactometry preference test	Plants were exposed to *R. padi* infested or uninfested plants
V2	olfactometry preference test	Plants were exposed to *M. persicae* infested plants or uninfested plants

V3.1	Olfactometry	*R. padi* infested plants vs *M. persicae* infested plants
V3.2	Olfactometry	*M. persicae* infested plants vs uninfested plants- aphids removed before test
V3.3	Olfactometry	*M. persicae* infested plants vs uninfested plants
V3.4	Olfactometry	*R. padi* infested plants vs uninfested plants
V3.5	Olfactometry	*R. padi* infested plants vs *M. dirhodum* infested plants
V4.1	preference test	Lina exposed to *R. padi* infested or uninfested Lina
V4.2	preference test	Lina 28:4 was exposed to *R. padi* infested or uninfested 28:4
V4.3	preference test	Lina and 28:4 were exposed to infested plants of the same genotype
V5.1	preference test	*M. persicae* damaged plants and uninfested plants- aphids removed before test
V5.2	preference test	*R. padi* damaged plants vs uninfested plants - aphids removed before test
V5.3	preference test	*M. dirhodum* damaged plants vs uninfested plants- aphids removed before test
R1.1	preference test	Lina received root exudates from infested or uninfested Lina
R1.2	preference test	28:4 received roots exudates from infested or uninfested 28:4
R1.3	preference test	Plants received roots exudates from *M. persicae* infested or uninfested plants
R1.4	preference test	Plants received root exudates from infested or uninfested plants
R2	preference test	Root exudates from infested and uninfested plant were collected in a beaker and administered to receiving plants.
R3	preference test	Receiving plants were exposed to volatiles plants that had received collected root exudates from infested or uninfested plants
R4	preference test	Plants grown in soil together with infested or uninfested plants
R5	preference test	Plants treated with solution of collected aphid honey
R6	preference test	Plants received roots exudates from infested or uninfested plants but aphid products were prevented from reaching the soil
R7	preference test	Plants were grown in hydroponic medium that previously supported infested or uninfested plants
R8	preference test	Plants received roots exudates from infested or uninfested plants growing in sterilised soil

Results

R. padi response to volatile exposed plants

Significantly fewer *R. padi* settled on barley plants that had been exposed to volatiles from *R. padi*-infested plants than on plants exposed to uninfested plants (control) (Table 2). In the olfactometer, *R. padi* did not discriminate between odours from plants exposed to infested or uninfested plants (2.71± 1.45 and 2.29 ±1.49; *t-test* p=0.35) in olfactometry test.

Table 2 *R. padi* settling on barley after 2 and 4 hours in a preference test when offered a choice between barley plants exposed to volatiles from *R. padi*-infested or uninfested plants

		Aphid settled (mean ± SD) [a]		T-test
Experiment, treatment	N[d]	Treated	Control	P
V1 [b] plant volatiles	20	2.7 ±1.56	4.4 ±1.67	0.002
V1 [c] plant volatiles	20	3.1 ±1.45	4.3 ±1.81	0.02

[a] Ten aphids were used in each test [b] Settled aphids after 2 hours
[c] Settled aphids after 4 hours [d] nr individual plants (replicates)

Lina and 28:4

Significantly fewer *R. padi* settled on barley plants of 28:4 that had been exposed to volatiles from *R. padi*-infested 28:4 plants than on plants exposed to uninfested plants (Table 3). No significant effect was found in similar interactions in cultivar Lina, and aphids showed no significant preference when offered a choice of Lina exposed to infested Lina and 28:4 exposed to infested 28:4. When chemical interaction occurred via root exudates, no significant effects on aphid settling were found.

Table 3 *R. padi* settling on barley after 2 and 4 hours in a preference test when offered a choice between barley plants exposed to volatiles or root exudates from *R. padi*-infested or uninfested plants- interactions in aphid-susceptible (Lina) and resistant (28:4) barley genotypes.

	Aphid settled (mean ± SD) [a]	T-test

Experimental treatment	N [d]	Treated	Control	P
Volatile interactions				
V4.1 [b] Lina-Lina	4/20	2.75±1.29	3.20±1.70	0.35
V4.1 [c] Lina-Lina	4/20	2.90±1.62	3.80±1.64	0.09
V4.2 [b] 28:4-28:4	4/20	2.85±1.42	3.90±1.97	0.06
V4.2 [c] 28:4-28:4	4/20	2.80±1.36	4.65±2.28	0.004
V4.3 [b] Lina-Lina v 28:4-28:4	4/20	3.35 ± 1.98^{Lina}	$3.05\pm2.01^{28:4}$	0.64
V4.3 [c] Lina-Lina v 28:4-28:4	4/20	3.85 ± 1.93^{Lina}	$3.30\pm1.69^{28:4}$	0.34
Rhizosphere interactions				
R1.1[b] Lina-Lina	4/20	3.25±1.02	3.9±1.92	0.19
R1.1[c] Lina-Lina	4/20	3.05±1.23	3.75±1.55	0.12
R1.2[b] 28:4-28:4	4/20	2.9±1.37	2.85±1.35	0.91
R1.2[c] 28:4-28:4	4/20	3.2±1.28	3.3±1.42	0.73

[a] Ten aphids were used in each test [b] Settled aphids after 2 hours
[c] Settled aphids after 4 hours [d] nr of pots (blocks)/individual plants (replicates)

R. padi response to root exudates-exposed plants

R. padi settled significantly less on barley that had been treated with root exudates from *R. padi*-infested plants than with those from uninfested plants (Table 4, R1:4). The same was true when exudates were collected from infested plants and administered to receiving plants isolated in separate cages (Table 4, R2), ruling out involvement of volatile signals. Aphid settling was unaffected when aphid products were prevented from reaching the soil of emitting plants (Table 4, R6), however aphid honey itself did not cause treated plants to have reduced aphid settling (Table 4, R5). No effects on aphid settling were found when receiving plants shared either soil or hydroponic medium with infested plants (Table 4, R4 and R7). When the soil of emitting plants was sterilised, no effect of exudates from infested plants on aphid settling was observed (Table 4, R8). Aphid settling was unaffected when plants were exposed to volatiles produced by plants that had been treated with exudates from aphid-infested plants (Table 4, R3).

Table 4 *R. padi* settling on barley leaf after 2 and 4 hours when offered a choice of barley plant received roots exudates from treated or plant received roots exudates from untreated plant

	Proportion of aphid settling [a] (Mean ± SD)			T-Test
Experiment, treatment	N [d]	Treated	Control	P
R1.4 [b] Exudates from *Rp*-infested plant	4/20	3.30±1.13	4.55 ±1.36	0.003
R1.4 [c] Exudates from *Rp*-infested plant	4/20	3.55±1.39	4.65 ±1.50	0.02
R2 [b] Exudates transferred via beaker	4/20	2.35±1.04	3.6 ±1.57	0.005
R2 [c] Exudates transferred via beaker	4/20	2.65±1.39	3.95 ±2.04	0.02
R3 [b] Volatiles from exudate-treated plant	4/20	2.95±2.21	3.8±1.44	0.16
R3 [c] Volatiles from exudate-treated plant	4/20	3.60±2.62	4.1±1.71	0.41
R4 [b] Sharing soil with infested plant	4/20	4.05±1.9	3.45±1.57	0.28
R4 [c] Sharing soil with infested plant	4/20	4.25±1.9	3.35±1.50	0.09
R5 [b] Treated with aphid honeydew	4/20	3.4±1.23	4.1±1.29	0.08
R5 [c] Treated with aphid honeydew	4/20	3.55±1.57	3.75±1.33	0.67
R6 [b] Soil of infested plant covered	4/20	3.55±1.90	3.95±2.01	0.52
R6 [c] Soil of infested plant covered	4/20	2.65±1.22	4.40 ±1.67	0.11
R7 [b] Sharing medium with infested plant	20	2.54±1.06	2.70 ±2.07	0.78
R7 [c] Sharing medium with infested plant	20	2.88±1.48	3.04±2.03	0.75
R8 [b] Infested plant in sterile soil	4/19	2.90±1.49	3.63 ±1.67	0.16
R8 [c] Infested plant in sterile soil	4/19	3.53±1.95	3.74±1.74	0.71

[a] Ten aphids were used in each test [b] Settled aphids after 2 hours
[c] Settled aphids after 4 hours [d] nr of pots (blocks)/individual plants (replicates)

R. padi response to *M. persicae* damaged plants

R. padi had significantly higher settling on barley plants that had been exposed to volatiles from *M. persicae*-infested barley than on unexposed plants (Table 5, V2), and was attracted/arrested by the odour of exposed plants in the olfactometer (Table 6, V2). *R. padi* had significantly higher settling on barley plants that had been previously infested with *M.*

persicae than on uninfested plants (Table 5, V5.1), and preferred the odour of *M. persicae*-infested plants to that of *R. padi*-infested plants in the olfactometer (Table 6, V3.1). *R. padi* however did not show olfactory attraction to the odour of plants previously infested with *M. persicae* (aphids removed before the test) (Table 6, V3.2) or to plants infested with *M. persicae* (Table 6, V3.3). Aphid settling was unaffected when plants were treated with root exudates from *M. persicae*-infested plants (Table 5, R1.3).

Table 5 *R. padi* settling on barley after 2 and 4 hours in preference tests when offered a choice of barley plants exposed to volatiles from *M. persicae (Mp)*-infested plants, or previously infested with *M. persicae*.

		Aphid settled (mean ± SD) [a]		T-test
Experiment, treatment	N[d]	Treated	Control	P
V2[b] Plant exposed to *Mp*-infested plant	20	3.8 ±1.94	2.75 ±1.65	0.073
V2[c] Plant exposed to *Mp*-infested plant	20	4.0 ±1.45	2.6 ±1.60	0.0062
V5.1[b]Previously *Mp*-infested plant	20	4.15 ±1.53	2.5 ±1.15	0.0005
V5.1[c] Previously *Mp*-infested plant	20	4.25 ±1.62	2.7 ±1.45	0.003
R1.3[b] Exudates from *Mp*-infested plant	4/20	3.05±1.85	2.65±1.57	0.46
R1.3[c] Exudates from *Mp*-infested plant	4/20	3.40±1.73	3.00±1.56	0.45

[a] Ten aphids were used in each test [b] Settled aphids after 2 hours
[c] Settled aphids after 4 hours [d] nr of pots (blocks)/individual plants (replicates)

Table 6 *R. padi* visits in olfactometer arms with odours of barley plants that had been exposed to *M. persicae* infested plants, infested with *M. persicae* (*Mp*) or *R. padi* (*Rp*).

		Aphid visits (mean ± SD)		T-test
Experiment, treatment	N[d]	Treated arm	Control arm	P
V2 Exposed to *Mp*-infested plant	22	3.09 ± 1.95	1.82 ±1.10	0.01

V3.1 *Mp* vs *Rp*-infested plants	20	2.75 ±1.68 [M.p]	1.65 ± 1.22 [R.p]	0.02
V3.2 Previously *Mp*-infested plant	20	2.28±1.65	2.38±2.03	0.87
V3.3 *Mp* infested plants	20	3.45 ±1.88	2.4 ± 1.79	0.08

[a] number of individually tested aphids [M.p] *M. persicae* [R.p] *R. padi*

R. padi response to *R. padi* and *M. dirhodum* damaged plants

R. padi did not discriminate between odour of *R. padi*-infested and uninfested plants in the olfactometer (Table 7, V3:4), nor between odour of *R. padi* and *M. dirhodum*-infested plants (Table 7, V3:5). In preference test with *R. padi* (Table 8, *V5.2*) and *M. dirhodum* (Table 8, *V5.3*) preinfested plants and untreated plants, *R. padi* showed no difference in settling.

Table 7 *R. padi* visits in olfactometer arms with odours of infested or uninfested barley plants with the aphid *R. padi* (*Rp*) and *M. dirhodum* (*Md*).

		Aphid visits (mean ± SD)		T-test
Experimental treatment	N[a]	Treated arm	Control arm	P
V3:4 *Rp*-infested plant	24	2.54±1.61	2.12 ± 1.62	0.38
V3:5 *Rp* and *Md*- infested plant	19	2.16 ±1.71 [M.d]	2.84 ± 1.64 [R.p]	0.22

[a]nr of individually tested aphids

Table 8 *R. padi* settling on barley leaf after 2 and 4 hours when offered a choice of barley plant preinfested or uninfested plant.

		Aphid settled (mean ± SD) [a]		T-test
Experimental treatment	N[d]	Treated	Control	P
V5.2[b] Previously *Rp*-infested plant	4/20	3.5 ±1.57	3.75 ±1.55	0.62
V5.2[c] Previously *Rp*-infested plant	4/20	.00 ±1.72	3.85 ±1.81	0.80

V5.3[b] Previously *Md*-infested plant	20	3.2 ±1.75	3.25 ±1.78	0.91
V5.3 [c]Previously *Md*-infested plant	20	3.05 ±1.39	3.30 ±1.34	0.57

[a] Ten aphids were used in each test [b] Settled aphids after 2 hours
[c] Settled aphids after 4 hours [d] nr of pots (blocks)/individual plants (replicates)

Discussion

In the present study there were induced changes in *R. padi* host preference that suggest changes in host quality in barley plants that had received chemicals from an infested plant. These indicate induction of defences, although no direct evidence for this was obtained in the current study. The main findings of interest were (i) that *R. padi* preferred to settle on untreated plants rather than on plants that had received volatiles or rhizosphere exudates from an infested plant and (ii) that *R. padi* was attracted to odours from plants that were infested or preinfested with *M. persicae* and had greater settling on such plants. This indicates that the barley plant chemical and volatile composition is altered depending on the species of attacking aphid, and this affects interaction with *R. padi*.

Several studies have demonstrated that volatiles released by plants in response to herbivore feeding can induce defence responses in neighbouring, exposed plants. VOCs, such as green leaf volatiles, mono and sesquiterpenes, cis- jasmine, methyl salicylate and other substances derived from the shikimate pathway can up-regulate defence genes (Zhu-Salzman et al. 2004; Farag et al. 2005; Moraes et al. 2007). Recent interesting findings indicate that volatile compounds emitted from damaged plants are also able to prime neighbour plants to provide a better and faster defence response when attacks appear (Ton et al. 2007). Nearly all the work on herbivore-induced signalling has been done with chewing insects such as Lepidoptera, so the current results are interesting since they suggest that aphids, relatively 'stealthy' phloem feeders, may also trigger these volatile interactions.

To compare induced defence response in barley plants with different susceptibilities to aphids the susceptible cultivar Lina and the more resistant breeding line 28:4 were used. The molecular responses of these genotypes have recently been profiled, with 28:4 characterised as more resistant than Lina (Delp et al. 2009). In the current study, evidence for volatile

defence signalling between plants was stronger for 28:4 than for Lina, suggesting that this type of plant behaviour may be linked to aphid-resistance. Defence response to pathogens has been found to be induced faster and stronger in plants with resistance, compared to susceptible plants (Conrath et al. 2001), and in cereals attacked by aphids, the resistant barley genotype CI 16145 emitted chitinease faster after aphid infestations than a more susceptible genotype (Forslund et al. 2000). There are many different cultivars and breeding lines of barley available, so it would be interesting to characterise these populations in terms of plant-plant volatile signalling.

R. padi settled less on barley plants that had been exposed to solution collected from the rhizospshere of infested plants. It can be assumed that this solution contained root exudates from the plants, but probably also contained other substances associated with the rhizospshere, including soil micro-organisms and their products. The effect was shown in plants where no volatile exchanged occurred; root exudates were collected in beakers and poured to a receiving plant, suggesting that an elicitor released from roots is able to change the chemical composition of the receiving plants. The evidence for chemical signalling via the rhizospshere was not overwhelming- the effect was found only with cultivar Prestige and with *R. padi* infestation, and did not seem to occur when plants shared the same soil. However, the results encourage further investigation of this interaction, and are in line with previous studies in an aphid-plant system showing induction of indirect defences via rhizospshere signalling (Chamberlain et al. 2001; Guerrieri et al. 2002).

It was interesting that the effect was not found when the soil around the emitting plant was sterilised. This suggests that involvement of soil micro-organisms in plant-plant interactions should be considered in future studies. Interaction between aphids and micro-organisms has been reported in barley (Vestergård et al. 2004), and plants with arbuscular mycorrhizal symbiosis are more attractive for parasitoids but appear to have a negative influence on aphid development and reproduction (Guerrieri et al. 2004). However, some studies report the opposite, i.e. plants should be a better host because of the advantage from interaction between nitrogen fixing bacteria (reviewed by Dixon 1998). This is also supported by Gange (1994) who found that arbuscular mycorrhizal symbionts reduce the number of chewing herbivores but phloem feeding insects perform better on those plants.

To evaluate if honeydew, which contains various sugars and protein (Dixon 1998), could interact with micro-organisms and affect rhizospshere exudates, two different experiments were conducted; one with honeydew present and one there honeydew were prevented to fall into soil. There was no evidence that honeydew (and other aphid products) could directly affect the quality of treated plants for *R. padi*. However, its role in the plant-plant interaction cannot be ruled out since the effect was not apparent when it was prevented from reaching the rhizospshere surface.

Chemical interaction between uninfested plants via the rhizospshere has been shown to have a similar negative effect on *R. padi* in barley exposed to allelochemicals from couch grass *Elytrigia repens* (Glinwood et al. 2003). There are also studies showing that rhizospshere interactions between infested and uninfested plants affect herbivores natural enemies by making the receiving plants more attractive (Chamberlain et al. 2001; Dicke and Dijkman 2001). An interesting further experiment should of course be to test aphid natural enemy response to treated barley plants. Underground signalling between plants, particularly in connection with defence signalling, is still a relatively unexplored area but in coming years will attract increased attention.

R. padi did not show any preference choosing between *M. dirhodum* and *R. padi* preinfested or infested and uninfested plants which is in line with previous studies made by Johansson et al. (1997) where *R. padi* did not show any odour recognition of heterospecific cereal aphids. This might be explained by that *M. dirhodum* and *R. padi* actually do not compete in field situation due to different feeding sites of the plant and also different arrival times (Jarošik et al. 2003). However for *R. padi* not to recognise *M. dirhodum* suggests feeding by the latter does not have a meaningful impact on host plant quality for the former.

Feeding by *M. persicae* appeared to have several effects on *R. padi* (a) settling was increased on plants that had been previously infested, (b) odour of previously infested plants was attractive, (c) settling was increased on plants that had been exposed to volatiles from infested plants. While (a) has been shown previously with other combinations of aphid species, findings (b) and (c) are reported here for the first time. The results suggest that that plant chemical compositions and volatile profiles can be altered as a response to an aphid attack, and become more attractive to following heterospecific aphid damage. It is known that aphids

affect plants during an attack in different ways (Ni et al. 2006), for exemple change the amino acid content (Petersen and Sandström 2001). By injecting watery saliva aphids are believed to alter the phloem chemical and nutrient content to their advantage (Sandström 2000). For some aphids species, a conspecific preinfestation is proved to be beneficial but this has not been demonstrated for *R. padi* (Prado and Tjallingii 1997).

Even though *M. persicae* is a generalist feeding on several plant families and is not commonly found on grasses, it can apparently adapt to the physical and chemical aspects and infest and reproduce successfully on barley plants (E Qvarfordt personal observation). An aphid attack causes minimal damage to its host by inserting its stylet very carefully, and is suspected to activate plant defence systems more commonly associated with pathogen attacks (Zhu-Salzman et al. *2004)*. One explanation of the attraction of *R. padi* to *M. persicae* damaged plants may be that *M. persicae* alters barley plant chemical compositions by causing the plant to release higher amounts of secondary compound that *R. padi* uses as host recognition cues. Alternatively, *M. persicae* might suppress plant defences by damaging barley in a more substantial way than *R. padi,* being less well adapted to the plant, resulting in suppression between different defence signalling pathways (Bostock et al. 2005). This crosstalk between pathways has been seen in tomato and *Arabidopsis,* where salicylic acid can inhibit wounding responses (reviewed by Maleck and Dietrich 2000).

Much work has been done on plant responses to attack by pathogens and chewing insects (Paul et al. 2000), but more studies are needed with piercing/sucking insects, and on the effects of co-existence of different species on host plants, particularly at the molecular level. To determine whether *R. padi* actually performs better *M. persicae* damaged plants, population development tests should be carried out. The results might also have been different in response to infestations of different aphids if winged aphids were used, since abilities to detect plant cues can vary between different aphid morphs (Park et al. 2000).
In this study defence response in barely plants were defined by *R. padi* olfactory response and plant acceptance. Both of these behaviours are critical for aphid population development, since initial plant colonisation has a major impact on the final population due to the exponential growth shown by aphid colonies. However, a more advanced technique could explain the induced defence effect in more detail. Development tests could have added more information on how an *R. padi* population actually performs on treated plants but on the other

hand an aphid initial acceptance of a plant has a big effect on later population growth. Many studies have presented evidence for an interaction between plants that received emitted volatiles or root exudates from infested plants and parasitoids and predators. Therefore it would have been interesting to examine the response from ladybirds, one of the predators of *R. padi,* in several of the conducted experiments. Ultimately identification of the changes in barley volatile profile induced by aphid feeding, and profiling of plant molecular responses are required to complete this study. Although this was outside the scope of the current project, a suitable model system as now been established upon which to apply these techniques.

Conclusion

The aim with this study was to get greater knowledge in plant-plant chemical signalling with aphids as the inducing herbivore, as opposed to chewing insects with which most of the current knowledge has been obtained. I found that volatile emission from an infested barley plant could induce defence-like responses in neighbouring plants that affect *R. padi* host acceptance. *R. padi* were more attracted to plants infested with *M. persicae* but there was no response of *R. padi* to *M. dirhodum* preinfested plants which indicate that different aphid species affect barley plant chemical signalling and thus plant-aphid interaction, in different ways. Root exudates released from infested plants can induce defence-like response in barley but it is not clear which components are involved in the effect, a suggestion is that it could be an interaction of root exudates and soil micro-organisms.

Although this study does not offer conclusive evidence for chemical defence signalling in an aphid-plant system, the results do suggest that such a mechanism may exist and merits further investigation. The signalling mechanism appears to be less obvious than that reported for chewing herbivores, perhaps reflecting the idea that aphid are ‘stealthy’ feeders that avoid induced plant defences to a great extent (Zhu-Salzman et al. 2004). The cross-species affects between *M. persicae* and *R. padi* are also of great interest and further investigation of these could provide fundamental knowledge of aphid-plant interaction. Clearly this type of interaction in aphid – plants systems needs a lot more study for us to completely understand all mechanisms involved, but doing so will shed new light on the behaviour and adaptations of these important insect pests.

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