Project title:	Novel approaches for the management of cabbage root fly
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Project leader:	Andy Evans, SAC
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Key staff:	William Deasy (PhD student)
Location of project:	SAC Edinburgh, James Hutton Institute Dundee
Industry Representative:	Richard Haacker, East of Scotland Growers
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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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CONTENTS

GROWER SUMMARY

Headline

Research is ongoing to identify the chemicals that cabbage root fly larvae use to find calabrese roots. These chemicals could be used to divert or prevent colonization of roots by cabbage root fly larvae. Sugars may also be able to 'switch on' natural defences against cabbage root fly.

Background

Cabbage root fly, *Delia radicum* L. (Diptera: Anthomyiidae), is an economically important specialist insect pest of plants in the Brassicaceae family. Damage is caused by below-ground larvae feeding on plant roots. Plants can be attacked at any growth stage but the most serious damage is caused to young transplants soon after planting in the field. Crop protection relies almost predominantly on synthetic insecticides, covers and plant resistance. Current pesticide legislation is placing a greater emphasis on Integrated Pest Management (IPM) and alternative control strategies.

Female flies lay eggs at the base of the shoot or in the soil near the roots. Larvae that emerge from eggs move through the soil to locate host-plant roots to feed on in order to survive. While only limited information exists about how *D. radicum* larvae detect and find roots, the consensus is that chemical cues released in Brassica plant root exudates, either as volatiles or in solution, play a key role in root location. Through a combination of techniques, including choice-test bioassays, chemical analysis of root exudates, and detailed behavioural observations, this project aims to identify compounds in root exudates that larvae exploit to locate roots to feed on. This will facilitate testing and development of potential control methods, using attractant and/or repellent compounds, to disrupt normal orientation behavior by larvae for use as part of a sustainable IPM programme.

Plants protect themselves against insect attack using many defense strategies, such as secondary compounds that are toxic, repellent or anti-digestive, or morphological traits, which can negatively affect the performance of the herbivore. Elicitors are compounds that characterise attack and whose perception by the plant can induce a defensive response both locally in herbivore-attacked regions and systemically in undamaged parts. Sugar sensing and signalling pathways interact with plant hormone signalling mechanisms to

control metabolism, growth and stress responses. It has recently been hypothesised that sugars occurring outside their normal compartment within plant cells, indicate a disrupted or damaged plant cell, triggering hormone-mediated defense responses within plants. The aims of this work are to investigate how sugar sensing affects Brassica plants' defense system and growth, and whether foliar and root applications of sugars can mimic and elicit inducible resistance against *D. radicum*.

Gucosinolate-containing plants in the Brassicaceae family, incorporated into soil as biofumigants, represent a potential source for pest, disease and weed control. Isothiocyanates, products of glucosinolate-myrosinase hydrolysis, are unpalatable and toxic to many generalist and specialist insects. Despite the fact that several specialist insects including *Delia* spp. have evolved mechanisms to cope with the toxicity of these compounds, beyond certain levels even these insects can be repelled and/or deterred. Using glasshouse pot tests and field trials, this work aims to evaluate the effect of an isothiocyanate-containing liquid biofumigant formulation ('Caliente' mustard), applied as a root drench, on *D. radicum* oviposition, egg survival, and larvae, along with resulting crop yields.

Cabbage root fly *(Delia radicum)* control in the UK is currently reliant almost predominantly on pest forecasting (e.g. the HDC Pest Bulletin), pre-planting application of an organophosphorus insecticide (chlorpyrifos), use of crop covers (where applicable), and plant resistance. Current pesticide legislation is placing a greater emphasis on Integrated Pest Management (IPM) programmes. Under an IPM system, growers are encouraged to employ a combination of available chemical, cultural, and biological control methods in order to minimise the harmful side effects that can result from exclusive use of chemical insecticides (Kogan, 1998; Finch & Collier, 2000; Khan *et al*., 2008). The ongoing review and withdrawal of several pesticides as a result of environmental, food safety and operator health concerns, means that growers are faced with fewer chemical control options to utilise while alternatives are being researched and developed.

This project aims to evaluate several alternatives approaches for the management of cabbage root fly, initially using calabrese as a model crop.

Summary of the results and main conclusions

This research aims to utilise the chemicals present in root and plant exudates that newly hatched cabbage root fly larvae use to locate roots to feed on, to disrupt their behaviour and

reduce the larval colonisation of calabrese plants. There are plant-derived extracts marketed as plant stimulants that have the potential for activity against cabbage root fly if applied to the soil as a drench at the time of egg hatch. These are likely to contain chemicals that will affect the behaviour of cabbage root fly larvae.

Sugar sensing in plants has recently been discovered to be involved in triggering inducible and systemic resistance to insects, nematodes and fungi. This project will determine whether the application of sugars to foliage and/or seed can induce defence mechanisms in calabrese plants that can protect roots from cabbage root fly damage.

The most effective treatments will be utilised in a novel system of cabbage root fly pest management that disrupts host-plant location by the larvae. This will be evaluated in field trials. The delivery of these treatments will be in the form of incorporation into soil-applied slow-release granular formulations, seed coatings, foliar /soil sprays and/or treated plugs for transplants.

Financial benefits

At this stage in the project (end of Year 1 out of 3) we are not at a stage to be able to give an accurate estimate of financial benefits to growers. The financial benefits will become clearer once data from field trials in Years 2 & 3 have been obtained.

Action points for growers

At this point trials are underway to determine the optimal approaches for the application of these alternative treatments to reduce cabbage root fly damage, so it is too early to offer growers specific action points to achieve significant benefits for cabbage root fly management.

SCIENCE SECTION

Introduction

Cabbage root fly (Delia radicum) larval responses to host-plant root exudates and attractant/repellent chemicals

The period between egg hatch and location of roots is known to be a particularly vulnerable stage of a soil-dwelling insect's life cycle (Johnson *et al*., 2006). With only limited mobility and time, *Delia radicum* larvae not only need to locate roots, but also potentially distinguish unsuitable host-plant roots. Investigations on the orientation behaviour and responses of neonate *D. radicum* larvae have been undertaken previously (Finch, 1974; 1977; 1978; Koštál, 1992; Ross & Anderson, 1992; den Ouden *et al*., 1996). Several isothiocyantes have been identified as an important factor in larval olfactory responses (Finch, 1974; 1977; 1978; Koštál, 1992; Ross & Anderson, 1992). For example, larvae were shown to be attracted to low concentrations of allyl isothiocyanate but repelled at higher concentrations (Koštál, 1992; Ross & Anderson, 1992). Glucosinolate-myrosinase hydrolysis products are known to mediate interactions between insects and plants in the Brassicaceae (Hopkins *et al*., 2009). Despite these findings, the exact cues involved in larval host-plant location still remain elusive. Compounds released into the rhizosphere by growing host-plant roots are hypothesised to be centrally important in influencing larval responses when searching for host-plant roots in the soil. Understanding how larvae orient towards their food may provide means for controlling these root feeding insects through behavioural manipulation methods, such as directing them away from crops using attractants/repellents (Foster and & Harris, 1997; Cook *et al*., 2006).

The main aims of this work were to: test the hypothesis that *D. radicum* larvae exploit chemicals released in Brassica host-plant root exudates, to locate and select roots in the soil to feed on; identify compounds in exudates that elicit a response in larval orientation behaviour and; validate and optimise a suitable choice-test bioassay using attractants/repellents identified from the literature.

Field trial 2011

Insect attack on cultivated crops can reduce yields and cause economic losses. Exploitation of constitutive and inducible plant resistance is considered a major component of Integrated Pest Management. Resistance represents the ability of a certain plant variety to produce a larger crop of better quality than other varieties grown under the same level of insect infestation and environmental conditions (Panda & Khush, 1995). Constitutive defences are

always present, while inducible defences come into action after attack. Resistance is generally categorised as antixenosis, antibiosis, or tolerance (Painter, 1951; Kogan & Ortman, 1978). Antixenosis describes the inability of a plant to serve as a host, and is influenced by plant morphological and chemical characteristics that alter insect behaviour, resulting in non-preference or total avoidance of the plant. Antibiosis includes the adverse effects that occur on the biology of an insect when a resistant plant is consumed, and is influenced by factors such as root hardness, dry matter content and concentrations of primary and secondary plant metabolites. Tolerance can be described as the ability of a resistant plant to grow and reproduce, or to repair injury in the presence of a pest population that would otherwise damage a susceptible host. In *D. radicum*, perception and acceptance of a host plant by gravid female flies dictates the food source for larval feeding (Johnson *et al*., 2006). This interaction is strongly influenced by antixenosis (Birch, 1988), which might also be an important factor in host-plant root location by larvae (Koštál, 1992; Ross & Anderson, 1992). Subsequent development depends on the quality of the host (antibiosis) (Ellis *et al*., 1999; Gols *et al*., 2008), or the plant may respond to compensate for damage (tolerance).

The plant hormone jasmonic acid (JA) plays a central role as a signal for tissue damage and induced responses against herbivores (Browse & Howe, 2008; Howe & Jander, 2008). Methyl jasmonate, a volatile methylester of JA has been shown to induce glucosinolates and volatile organic compounds (VOCs) in Brassica plants (Loivamäki *et al*., 2004). Several insect-derived elicitors that trigger plant defense responses have been identified (Walters, 2011). Heil (2009) has proposed that the damaged plant cell itself can serve as a source of plant defense elicitors, and that VOCs and extracellular sugars released by cell disruption are perceived by receptors that monitor extracellular chemistry. Thus, information on the presence of these compounds in the apoplast is transported into the inner compartments of intact, metabolically active cells, which respond by synthesising systemic signals and defense compounds.

There is increasing interest in the use of naturally-derived isothiocyanates in soil biofumigation (Matthiessen & Kirkegaard, 2006). The biocidal properties of glucosinolate-containing plants, particularly their isothiocyanate hydrolysis products, released into the soil following crop rotation or incorporation have been shown to have significant potential for suppression of soil borne pathogens, pests and weeds (Brown *et al*., 1991; Bjorkman *et al*., 2011). The effectiveness of biofumigants can be attributed to specific glucosinolates in leaf or root tissue (Kirkegaard & Sarwar, 1998; van Dam *et al*., 2009) and can be increased by selecting appropriate species and cultivars with elevated glucosinolate content (Kirkegaard

et al., 2001). Other factors in efficacy include the stage of plant development, method of tissue maceration, method and speed of incorporation of tissue into soil, soil type, temperature and moisture (Zasada *et al*., 2010).

Field trials beginning mid June 2011 will determine elicitor (D-fructose, methyl jasmonate) induced resistance responses in *Brassica oleracea* convar. *botrytis* var. c*ymosa* 'Parthenon' (Broccoli/Calabrese) against *D .radicum* and the efficacy of an isothiocyanate-containing biofumigant root drench, 'Caliente' liquid mustard.

Materials and methods

Cabbage root fly (Delia radicum) larval responses to host-plant root exudates and attractant/repellent chemicals

Validation and optimisation of choice-test bioassay technique

To date, discovery of compounds that elicit responses in *Delia* spp. larvae have typically been investigated using a bioassay approach (Finch, 1974; Finch, 1977; Finch, 1978; Soni & Finch, 1979; Koštál, 1992; Ross & Anderson, 1992; den Ouden *et al*., 1996). The bioassay methodology employed in the present study was modified after (Ross & Anderson 1992; Marriott & Evans 2003). To validate the test, an identified *D. radicum* larval attractant and repellent (Koštál, 1992; Ross & Anderson, 1992) were presented to larvae in arenas as outlined below.

Chemicals tested

All chemicals used other than 'Caliente' liquid mustard (Plant Health Care Ltd., UK) were sourced from Sigma-Aldrich, UK. Diethyl ether (solvent contol), allyl isothiocyanate (95%) (attractant/repellent), sterile distilled water (control), 2-chlorophenyl isothiocyanate (98%) (repellent), 'Caliente' mustard (3.7% allyl isothiocyanate) (biofumigant).

Choice-test bioassay

Serial dilutions of chemicals (see Table 1 for full list) were presented to larvae in arenas that consisted of 150 x 20 mm diameter plastic Petri dishes (Sarstedt Ltd., UK) lined with 90 mm diameter black filter paper (Whatman®, Fischer Scientific, UK) moistened with 1 ml of sterile distilled water. 30 µl of the test compound was applied to 6 mm absorbent assay discs (Fischer Scientific, UK) before being placed on the filter paper, 20 mm from the arena centre. An assay disc placed on the opposite side of the arena centre containing the same volume of sterile distilled water was the control (Figure 1). Arenas were placed in the dark at

18 ± 3°C for one hour before tests commenced. Using a fine soft brush, an individual larva, no older than 12 hours, was placed in the arena centre, covered and returned to darkness for 20 minutes. After which time, the final resting position of the larva was recorded using a ChemiImager™ (Alfa Innotech Corporation). Each treatment was replicated 24 times.

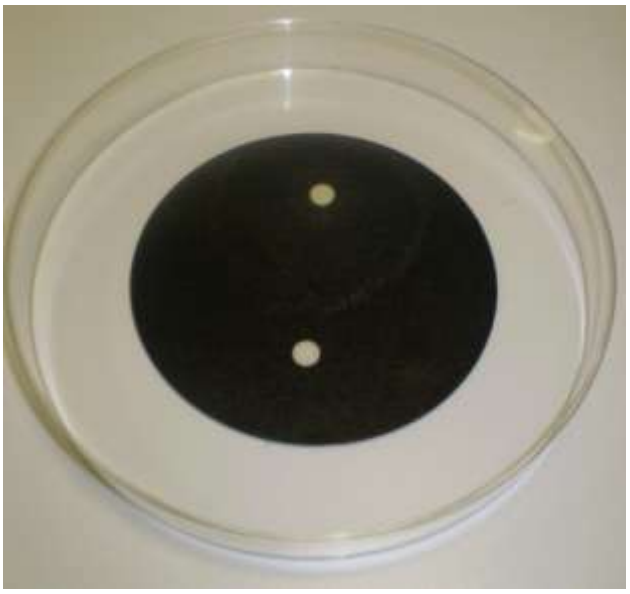
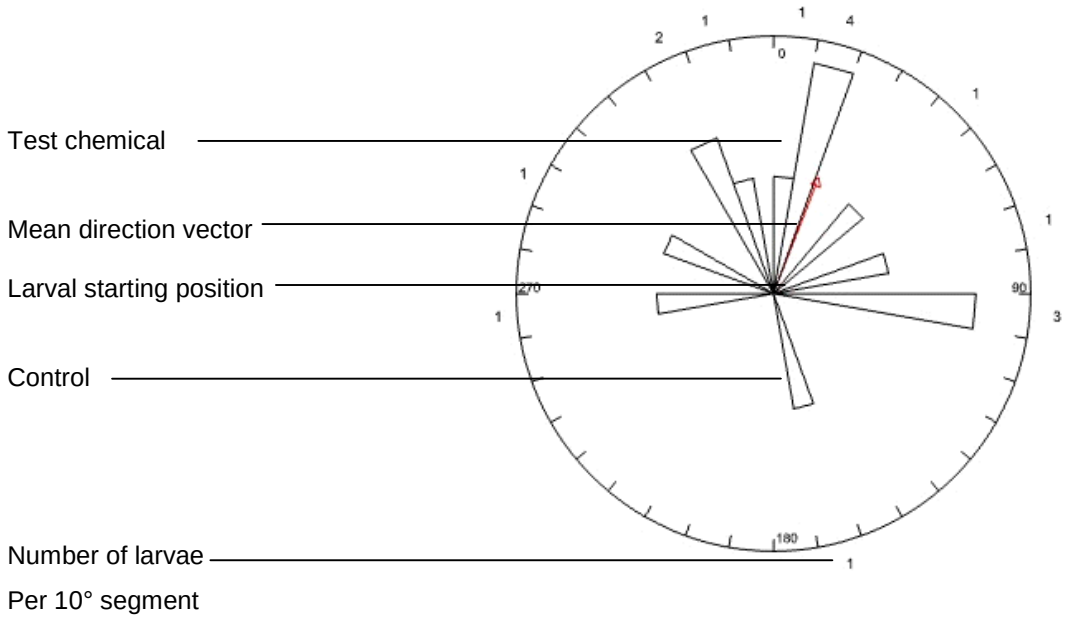


Figure 1. Choice-test bioassay arena

Statistical analysis

Larval final resting position was analysed using the Rayleigh test of uniformity against unimodal direction 0° (root exudates) giving a mean direction vector strength (r) using Genstat 11th edition.

An example of the bioassay arena area and notation used in the results section is given below.



Root exudates bioassay tests and chemical analysis

Brassica oleracea convar. *botrytis* var. c*ymosa* 'Parthenon' (Broccoli/Calabrese), and *B*. p*ekinensis* 'Richi' (Chinese cabbage) were used.

Seeds were surface sterilised with 3% v/v sodium hypochlorite (Sigma-Aldrich, UK) for 2 minutes before being germinated on 0.6% w/v Technical Agar No. 3 (Oxoid) and full strength MS basal medium (Sigma-Aldrich, UK) at 21°C, 16L/8D photoperiod for 3 days. After 7 days, seedlings were transferred aseptically to 6-well plates (Sarstedt Ltd., UK) containing 5 ml Ruakura nutrient solution (increased to 10 ml after the first sampling) before being returned to controlled environment conditions. pH was monitored throughout. The experiment was a randomised complete block design with sterile distilled water and Ruakura nutrient solution as controls (Figure 2).

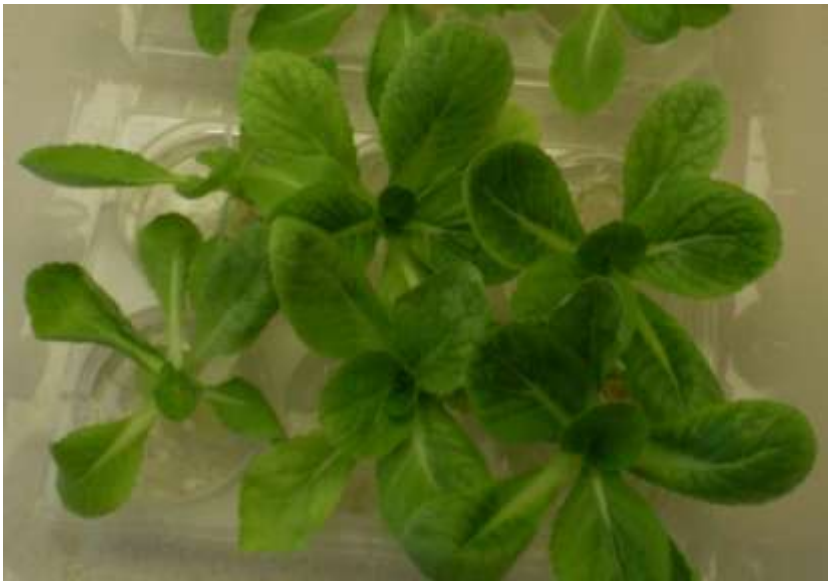


Figure 2. Plant growth for root exudates collection

Root exudates collection

The first root exudates collection was conducted on 18 day old plants and weekly thereafter for a further 30 days at which point roots were destructively sampled for bioassay tests. To collect the exudates, plants were removed from the original wells containing Ruakura nutrient solution (retained for bioassay), rinsed, and transferred to new 6-well plates with sterile distilled water. Following a 72 hour exudation period, plants were placed in new 6-well plates containing fresh nutrient solution for further growth and sampling. 250 µl subsamples of each treatment were collected from individual exudates-containing wells along with controls and pooled together separately for immediate use in choice-test bioassays. The remaining root exudates solution was transferred to 100 ml Duran glass bottles and stored at -22 ±2°C for freeze drying and subsequent GC-MS. On the final sampling date, in addition to collecting exudates and damaging/wounding roots, myrosinase (Sigma-Aldrich, UK) was added to a subsample for testing in bioassays.

Choice-test bioassay

Arenas consisted of 150 x 20 mm diameter plastic Petri dishes (Sarstedt Ltd., UK) lined with 125 mm black filter paper (Whatman®, Fischer Scientific, UK) moistened with 2 ml of sterile distilled water. 40 µl of root exudates in solution was applied to 6 mm absorbent assay discs (Fischer Scientific, UK) before being placed on the filter paper, 20 mm from the arena centre. An assay disc placed on the opposite side of the arena centre containing the same volume of sterile distilled water was the control. Arenas were placed in the dark at 18 ± 3°C for one hour before tests commenced. Using a fine soft brush, an individual larva, no older

than 12 hours, was placed in the arena centre, covered and returned to darkness for 20 minutes. After which time, the final resting position of the larva was recorded using a ChemiImager™ (Alfa Innotech Corporation). Each treatment was replicated 30 times (20 replicates on the final sampling date due to the number of treatments).

Chemical analysis (compound identification)

Gas chromatography-mass spectrometry (GC-MS) was used to measure the metabolite composition of the root exudates. Extraction and derivatization was conducted using similar methods to Shepherd *et al*., (2007).

Results

Attractant/Repellent bioassay tests

Larvae displayed a significant directed response away from allyl isothiocyanate at both 10^{-1} (Figure 3, Table 1) and 10^{-2} dilutions (Figure 4, Table 1), 10^{-2} dilution of 2-chlorophenyl isothiocyanate (Figure 5, Table 1) and 10^{-1} dilution of 'Caliente' mustard (Figure 6, Table 1). Larval responses to all other doses did not differ from random uniformity (Table 1).

Table 1. Summary of Rayleigh test of uniformity *P*-values for larval responses to concentrations of test chemicals

Treatment	**Rayleigh Test *P*-value**				
Serial dilution	*10^{-4}*	*10^{-3}*	*10^{-2}*	*10^{-1}*	*10^{0}*
Allyl isothiocyanate	0.301	0.960	0.033*	0.025*	0.081
2-chlorophenyl isothiocyanate	0.555	0.077	0.005*	0.452	0.066
'Caliente' liquid mustard		0.709	0.606	0.005*	0.195

*$P \leq 0.05$ - repellency

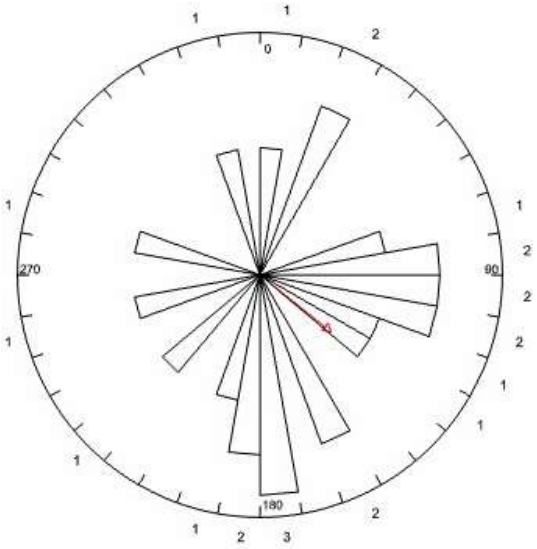


Figure 3. Larval directional response to 10^{-1} dilution of allyl isothiocyanate (*p*=0.025)

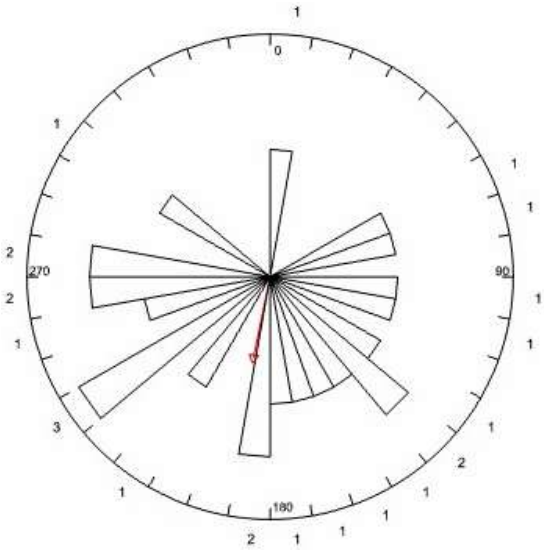


Figure 4. Larval directional response to 10^{-2} dilution of allyl isothiocyanate (*p*=0.033)

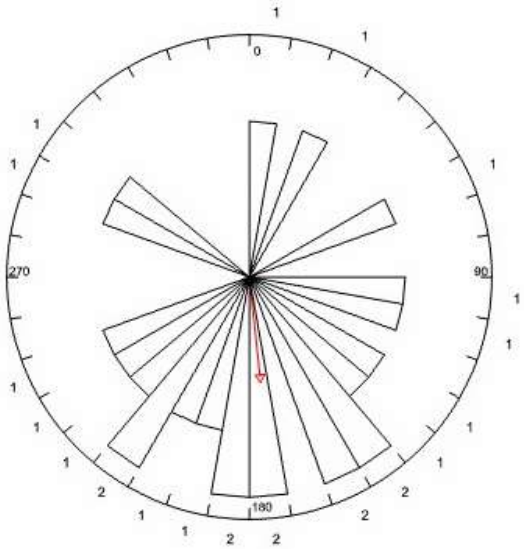


Figure 5. Larval directional response to 10^{-2} dilution of 2-chlorophenyl isothiocyanate *(p*=0.005)

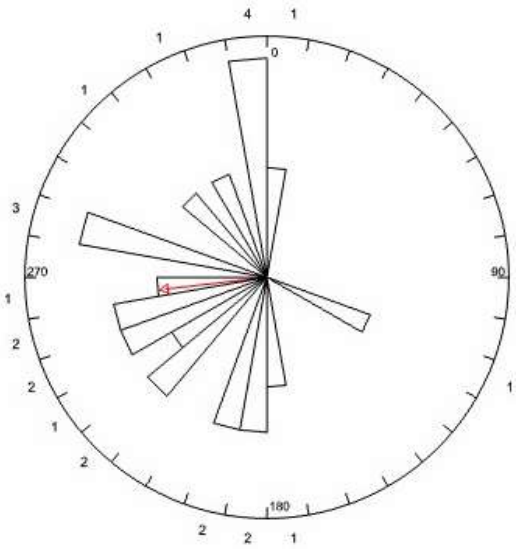


Figure 6. Larval directional response to 10^{-1} dilution of 'Caliente' mustard (*p*=0.005)

Root exudates bioassay tests and chemical analysis

Preliminary data from root exudates collection and bioassay pilot studies was used to guide subsequent experiments (outlined above). Statistical analysis of larval responses in these tests and chemical analysis (gas chromatography-mass spectrometry) is currently ongoing.

Discussion

Attractant/Repellent bioassay tests

Results from the present study agree with findings from previous work that larvae are repelled by 2-chlorophenyl isothiocyanate and high concentrations of allyl isothiocyanate (Koštál, 1992; Ross & Anderson, 1992). While the studies of Koštál (1992) and Ross & Anderson (1992) have shown larvae to be attracted to low concentrations of allyl isothiocyanate, further bioassays will be required here to determine the concentration required to produce a positive directed response.

Root exudates bioassay tests and chemical analysis

Experiments and analysis are ongoing.

Conclusions

- Methods have been developed for bioassay tests and growing Brassica plants *in vitro* to collect root exudates.
- Analysis of results from bioassays are ongoing.
- Chemical analysis (GC-MS) of root exudates are ongoing.
- Field trial to start in June 2011 to look at induced resistance in broccoli plants and the efficacy of a soil biofumigant product against *Delia radicum*.
- See Appendix 1 for future work plan and milestones.

Knowledge and Technology Transfer

Event description	Date
Seminar: Presented to Plant Propagators Ltd.	6/10/10
Seminar: Presented at CSS Seminar Series SAC	16/03/11
Conference: Presented at SAC Postgraduate Conference	6 & 7/04/11
Seminar: Presented at James Hutton Institute EPI Forum 1st Year PhD	19/05/11

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APPENDICES - *Appendix 1 Future work & milestones*

Future work

Solid-phase microextraction (SPME)

SPME is an extraction procedure in which compounds are ad/absorbed by a thin polymer film or by a porous carbonaceous material bonded to a fused-silica fibre. The fibre is attached within a modified syringe and volatiles can be sampled by inserting the needle through a septum and pushing the plunger to expose the fibre. When equilibrium has been reached between the fiber and the volatile sample, it can be retracted and transferred to a GC-MS for analysis (Prosen & Zupancic-Kralj, 1999; Tholl *et al*., 2006). Several studies have already utilised this sampling technique to collect head-space volatiles from plants in the Brassicaceae (Vaughn & Boydston, 1997; de Pinho *et al*., 2009). Rasmann *et al*., (2011) recently published methodology to collect head-space volatiles from living and intact harvested roots of *Asclepias syriaca*. I aim to develop a method to collect and identify volatile compounds emitted from actively growing plant roots in soil using SPME and GC-MS. This method will also facilitate investigation of larval feeding and elicitor induced defense responses in roots and leaves as well as the identification of isothiocyanates released from 'Caliente' mustard incorporated into soil.

Sugar inducible defenses

Using exogenous applications of different concentrations of sugar analogues, I aim to examine elicited effects on inducible plant defenses and growth through a combination of chemical and phenotypic analysis combining glasshouse pot tests and field trials with metabolomic analysis (SPME, GC-MS).

Behavioural experiments

The Observer® XT/Ethovision® XT will be employed to study the behaviour of larvae in more detail (Noldus *et al*., 2001; Noldus *et al*., 2002). Host plant searching responses such as velocity of linear movement and rate of turning (localised and/or ranging behaviour) will be examined (Bernklau *et al*., 2009).

Behavioural manipulation experiments

Development of formulations and artificial diets containing identified attractant/repellent compounds to influence larval host-plant location and feeding behaviour will be undertaken and compared with conventional control treatments.

SPME/GC-MS and Delia radicum responses to application of biochar to plant growth medium

Biochar, a carbon-rich material formed by incomplete combustion of biomass, can adsorb organic compounds released from growing roots when applied to soil (Joseph *et al*., 2010). This work will investigate the potential of using soil incorporated biochar to reduce the activity of chemical released in host-plant root exudates, used by cabbage root fly larvae (*Delia radicum* L. Diptera: Anthomyiidae) to locate roots to feed on.

Milestones	*Month*
Field trial (monitoring and assessment)	June-September 2011
Presentation-HDC Protecting Your Field Vegetable Crop Event 2011	
GC-MS analysis of root exudates	
Data analysis (bioassay and GC-MS results)	
SPME root volatiles collection protocol development	
Seminar-HDC Studentship and Fellowship Conference 2011	
SPME root volatiles collection experiments	
GC-MS analysis of root volatiles	
Graduate School Poster Day 2011	
The Observer® XT/Ethovision® XT training	October-May 2011-2012
Bioassay and behavioural analysis using GC-MS/SPME identified compounds	
Glasshouse tests (sugar inducible resistance, 'Caliente' mustard, methyl jasmonate)	
SPME experiments-induced responses (feeding, elicitors)	
Formulation development based on GC-MS/SPME/bioassay results	
Field trial	June-September 2012
Continuation of experiments outlined above	October-May 2012-2013
Laboratory (artificial diet development experiments)	
Biochar glasshouse pot and SPME/GC-MS experiments	

