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Field-based evaluation of a novel SPME-GC-MS method for investigation below ground interaction between brassica roots and larvae of cabbage root fly, *Delia radicum L.*

Abstract

Introduction – Collection of volatiles from plant roots poses technical challenges due to difficulties accessing the soil environment without damaging the roots.

Objectives – To validate a new non-invasive method for passive sampling of root volatiles *in situ,* from plants grown under field conditions, using solid phase microextraction (SPME).

Methods – SPME fibres were inserted into perforated polytetrafluoroethene (PTFE) tubes positioned in the soil next to broccoli plants for collection of root volatiles pre- and post-infestation with *Delia radicum* larvae. After sample analysis by gas chromatography-mass spectrometry (GC-MS), principal component analysis (PCA) was applied to determine differences in the profiles of volatiles between samples.

Results – GC-MS analysis revealed that this method can detect temporal changes in root volatiles emitted before and after *D. radicum* damage. PCA showed that samples collected pre- and post-infestation were compositionally different due to the presence of root volatiles induced by *D. radicum* feeding. Sulfur containing compounds, in particular, accounted for the differences observed. Root volatiles emission patterns post-infestation are thought to follow the feeding and developmental progress of larvae.

Conclusion - This study shows that volatiles released by broccoli roots can be collected *in situ* using SPME fibres within perforated PTFE tubes under field conditions. Plants damaged by *D. radicum* larvae could be distinguished from plants sampled pre-infestation and soil controls on the basis of larval feeding-induced sulphur-containing volatiles. These results show that this new method is a powerful tool for non-invasive sampling of root volatiles below-ground.

Keywords: Field and soil; *in situ* root volatiles analysis; SPME-GC-MS: *Brassica*; *Delia radicum*

Introduction

Recent advances in analytical chemistry sampling and analysis techniques have contributed to understanding the formation and function of plant volatiles (D'Alessandro and Turlings 2006; Tholl *et al.,* 2006; Campos-Herrera *et al.,* 2013). For instance, studies on the chemical ecology of plant-insect interactions have shown that plants attacked by insects emit herbivore induced plant volatiles (HIPVs) that have a multifunctional ecological role across multiple trophic levels, and potential for manipulation to enhance crop protection (Dicke and Baldwin 2010; Kergunteuil *et al.,* 2012; Turlings *et al.,* 2012; Ali *et al.,* 2013; Pierre *et al.,* 2013). Despite this progress, research on the chemical ecology of roots has been hindered by methodological challenges associated with studying the soil environment, roots and below-ground herbivores *in situ* under ecologically realistic conditions (Rasmann *et al.,* 2012; van Dam 2014).

Thus far, methods for sampling root derived volatiles have most commonly been conducted under laboratory/glasshouse conditions using headspace techniques at/just above soil level or from whole plants in an enclosure (Soler *et al.,* 2007; Danner *et al.,* 2012), from plants removed from their container/growing substrate (Rasmann *et al.,* 2011; Robert *et al.,* 2012) or from excised roots (Rasmann *et al.,* 2005; Ferry *et al.,* 2007). Relatively few approaches have been developed for the collection of volatiles *in situ* from intact growing roots (Mohney *et al.,* 2009; Weidenhamer *et al.,* 2009; Ali *et al.,* 2012; Eilers *et al*., 2015), particularly those that can be readily adapted to laboratory and out-of-lab sampling in the glasshouse and field.

In agricultural and natural ecosystems, plants are constantly exposed to environmental stresses and a multitude of herbivorous organisms (van Dam and Heil 2011; Ponzio *et al.,* 2013). Field grown plants of the Brassicaceae for example, are exposed to multiple herbivores (Ahuja *et al.,* 2010). In contrast, under laboratory conditions abiotic and biotic stresses are carefully controlled (Kigathi *et al.,* 2009; Vandegehuchte *et al.,* 2010). It is widely recognised that laboratory and field studies can each provide essential information to elucidate the physiological and ecological functions, as well as the crop pest control potential of HIPVs, and should therefore be closely integrated (Dicke *et al.,* 2009; Beck 2012; Hiltpold and Turlings 2012; Soler *et al.,* 2013). We recently reported the development and validation of a new solid phase micro extraction (SPME)-based method for non-invasive *in situ* sampling of root volatiles from glasshouse-grown broccoli (*Brassica oleracea* L. convar. *botrytis* L. Alef. var. *cymosa* Duchesne 'Parthenon') plants pre- and post-damage to Brassica

roots by feeding larvae of *Delia radicum* L. (Diptera: Anthomyiidae) (Deasy *et al*., 2016, preceding paper). In this study, a field experiment was carried out to validate the technique in the face of increased abiotic and biotic variation over the course of a commercial broccoli crop growing season in Scotland, UK. By "...moving the laboratory to the field..." (Beck 2012), we have characterised a root volatiles profile representative of what *D. radicum*, and other organisms, encounter in an agroecological environment.

Experimental

Plants

Broccoli plants (*Brassica oleracea* L. convar. *botrytis* L. Alef. var. *cymosa* Duchesne 'Parthenon') were obtained from Westhorpe Plants Ltd., UK and transplanted to experimental plots located in a commercial crop in Fife, Scotland (NO 40157 24978 UK Grid Reference) as part of a larger field study evaluating treatments for controlling *D. radicum*.

Insects

D. radicum first instar larvae used for plant infestation and root volatiles induction were obtained from our own continuously reared culture at The James Hutton Institute.

Experimental design

A randomised complete block design was used for the main field study consisting of six blocks. Each block comprised 10 randomised treatment plots with 36 plants in a plot. Treatments were a range of applied chemicals under evaluation for control of root fly or no applied chemicals. Root volatiles were sampled from three independent plants randomly selected from the untreated plots within three of the blocks. Three control samples were collected from bare soil adjacent to each block sampled. Samples were not collected from any of the treatment plots and the nature and significance of the treatments are not considered further here.

Sampling of root volatiles *in situ* using SPME

Preparation and installation of perforated polytetrafluoroethene (PTFE) sampling tubes.

Each sampling tube consisted of a 19 cm length of PTFE tubing (5 mm internal diameter [Ø],

1 mm wall thickness, 7 mm outside Ø; Radleys, Saffron Walden, UK, catalogue no. S1810-46) manually perforated 2,200 times using a sewing needle (500 µm Ø; Korbond Industries Ltd., Grantham, UK). Perforations started 4.5 cm from the top end of the tube (Fig. 1a). At planting, a 14-15 cm deep hole with a radius of 10 cm was dug using a hand trowel for the two collection tubes and the broccoli transplant (Fig. 1b). Once the tubes were positioned 25 mm apart, the hole was partially filled with soil to enable placement of the broccoli root plug (transplant) between the two tubes before filling the remainder of the hole to cover the roots to soil level. A 4.5 cm length of unperforated tube was subsequently allowed to protrude above soil level to facilitate attachment of the SPME fibre holder. Tubes were sealed with PTFE end caps outside of collections (Fig. 1c). In addition, felt traps (Ateliers Olbis, Switzerland) placed around plant stems and sampling tubes, were used to prevent oviposition by natural populations of *D. radicum* before infestation for induction of volatiles (Figs 1d and 1e). Traps consisted of lengths of velcro™-backed felt (5 mm thick x 2 cm wide) which were wound in a tight spiral around the plant stem and sampling tubes. An inner layer of expanded foam provided protection for the stem. The spacing between plants in the beds was approximately 33 cm. Plants received rainfall only. Control tubes placed in bare soil were positioned in the centre of the same beds at a distance of 5 m from the nearest broccoli plant.

Induction of root volatiles. Plants and controls (bare soil) were manually infested with 150 freshly laid *D. radicum* eggs on two dates. A suspension of the eggs in water was injected carefully onto the soil next to the plant stem, using a 60 mL plastic syringe and drinking straw. The first infestation on day 29 of the experiment was immediately followed by heavy rainfall which resulted in some of the eggs getting washed from the soil surrounding the plant stems where they were placed. Consequently, a second infestation was carried out on day 49. The number of larvae which actually fed on roots is unknown. Whilst the eggs were viable at the point of infestation, it is possible that larvae may not have emerged from some, for example, due to damage or desiccation. Pupae were not retrieved and counted after the experiment to equate with the number of eggs used at infestation, and roots were not harvested for assessment of damage.

Sampling of root volatiles. Root volatiles were sampled from each plant using two perforated PTFE sampling tubes (Figs. 1f and 1g). Bare soil control samples were sampled using one sampling tube (Fig. 1h). Sampling (day 1) began one week after planting. This corresponded to 16 collection dates, the first five of which (days 1, 8, 15, 22 and 29) were

conducted before infesting plants with *D. radicum* for induction of root volatiles due to larval feeding damage. Subsequently, further samples were taken on days 35, 37, 40, 42, 44, 49, 51, 56, 58, 63 and 77. Sampling schedules are given in supplementary information, Table S2. Root volatiles samples were not taken from separate plants which were in an uninfested state throughout the duration of the experiment due to time constraints as this work was additional to a larger field trial. Sampling was conducted using a single fibre type, polydimethylsiloxane/divinylbenzene (PDMS/DVB). Fibres were conditioned before sampling according to supplier (Supelco, Sigma-Aldrich, UK) guidelines. The procedure used for preparation and insertion of SPME fibres attached to fibre holders into the sampling tubes was as described previously (Deasy *et al.*, 2016). When exposed, the fibre was situated approximately 5 cm below soil level. During collection, SPME fibres were exposed in the perforated tubes for 24 hours overnight. On completion of volatiles collection, the SPME fibre was retracted and the fibre and holder assembly was removed from the sampling tube. The fibre was detached from the fibre holder and transferred to a screw cap glass Pyrex® culture tube, pre-purged with dry nitrogen, for transport from the field and overnight storage in the laboratory. Subsequently, the fibre was reattached to a fibre holder and installed in the gas chromatography-mass spectrometry (GC-MS) autosampler for desorption and analysis by GC-MS.

SPME-GC-MS

Volatiles were analysed by GC-MS using a Trace DSQ™ II Series Quadrupole system (Thermo Electron Corporation, Hemel Hempstead, UK). Further details of the instrumentation and the analytical conditions used were as described previously for glasshouse-grown plants (Deasy *et al*. 2016). Data were acquired and analysed using Xcalibur™ 2.0.7 (Thermo Electron Corporation, Hemel Hempstead, UK). Parameters used for characterisation, identification and abundance measurement of volatiles trapped in the field were the same as those for analysis of volatiles from collections made in the glasshouse. Minor modifications were made to the list of componds in the data processing method created using Xcalibur™, by exclusion of 40 compounds not detected in the field samples and addition of 8 only found in the field. Compounds included in the processing method are listed in Table 1 in elution order. Compounds were identified by comparison of their mass spectra and retention indices with those of reference standards where indicated in Table 1. Tentative identification of the remaining compounds was made by comparison with entries in MS libraries (Palisade 600k, Palisade Corporation, USA; NIST05, National Institute of

Standards, USA) and by reference to published data (see supplementary material, Table S1, for a list of references).

Statistical analysis

A summary table showing mean abundances and sample standard errors (SE) for all volatiles for all experiments is shown in supplementary information Table S3, and the full data sets are given in Tables S4 and S5. Abundance measurements from the duplicate sampling wells per plant were averaged prior to calculation of mean and SE from 3 plants. Plant-free control data was not used to subtract a contribution from the chemical background.

Processed (raw abundance) data for volatiles detected *in situ* using SPME within perforated PTFE sampling tubes were $\log_{10}$ transformed before further statistical analysis. This provided a more normal distribution of values for each metabolite. Data from all 6 plant samples and all 3 control samples were then analysed with principal component analysis (PCA) using GenStat 16th Edition (VSN International Ltd., UK). PCA identifies the largest sources of variation amongst the samples over all the volatiles. PCA was performed using the sample correlation matrix which is equivalent to dividing each volatile's measurements by their sample standard deviation. This has the effect of standardising the volatiles and, in addition to the initial $\log_{10}$ transformation, ensures that the analysis is not dominated by those which are particularly abundant.

Results and discussion

In situ sampling and profiling of root volatiles using SPME-GC-MS

General composition of root-zone volatiles

Excluding known impurities and components relating specifically to SPME fibre chemistry, 82 compounds were detected in the volatile profiles, of which 38 were positively identified by analysis of authentic standards, 26 were tentatively identified and 18 were unknown. These consisted primarily of various sulfur containing compounds including alkyl sulfides and isothiocyanates, *n*-hydrocarbons (C_6-C_{17}), *n*- aldehydes (C_6-C_{11}), ketones, acids, esters and terpenes. Representative SPME-GC-MS chromatograms for samples collected pre-infestation with larvae of *D. Radicum* (day 29) and at two subsequent times post- infestation

2 (days 58 and 77) are shown in Fig. 2 (see also Fig S1 for expanded and annotated versions of the chromatograms). The numbering of peaks in Fig. 2 coresponds to those of the compounds listed in Table 1. Comparison of chromatographic profiles from intact roots with those from roots damaged by *D. radicum* larvae revealed that temporal changes occurred in the patterns of volatiles emitted before and after root damage. This is evident in Fig. 2, particularly for dimethyl disulfide (**11**) and dimethyl trisulfide (**33**), where enhanced levels of these volatiles were seen post-infestation 2 on day 58 in comparison with pre-infestation on day 29 and later post-infestation 2 on day 77.

Principal component analysis

Principal component analysis (PCA) of log transformed data was used to further investigate the effect of *D. radicum* larval feeding damage on the composition of volatiles emitted by broccoli roots. A plot of the first two principal components scores from the PCA is shown in Fig. 3, loadings for scores 1 and 2 for selected volatiles are listed in Table 2, and the full sets of loadings for all detected volatiles are listed in supplementary information Table S6. Progressive separation of samples by date is evident throughout the time course of the experiment, exemplified by the solid lines connecting centroid values for volatiles collected at each date from plants and plant-free controls.

Pre-infestation (day 1-29) samples show a general trend with scores becoming more negative in PC1 and more positive in PC 2 with time, except at day 15, for which there was a reversal of the trend. During this period there was no clear separation of samples from plant-free controls which show essentially similar behaviour. Following infestation 1 (days 35-49) scores initially become more positive in PC2 then more positive in PC1 (days 35-40) then the trend changes with scores becoming more negative in PC 1 and more positive in PC 2 (days 40-49). After infestation 2 (days 51-77) scores in PC2 then become more positive up to a maximum at day 58, then more negative by day 77 while scores on PC 1 become more positive to a maximum at day 77. In the period following infestations 1 and 2, samples are clearly separated in PC 2 from plant-free controls which form alignments paralleling those of the plant samples on PC1, their loadings becoming increasingly more positive in PC1 with time over days 51-77.

Loadings for principal component 2 (Table 2, Table S6) showed that roots sampled on days 51, 56, 58 and 63 had distinctly different profiles of volatiles to those collected pre-damage and following the first infestation with *D. radicum*. Compounds that contributed most to the differences had high positive loading scores on PC 2 and were principally sulfur

containing which were elevated in samples from these days. These included: methanethiol (**2**), dimethyl sulfide (**3**), dimethyl disulfide (**11**), methyl thiocyanate (**12**), 2,4-dithiapentane (**18**), butyl isothiocyanate (**27**), 2-butyl isothiocyanate (**28**), dimethyl trisulfide (**33),** isobutyl isothiocyanate (**36**), S,S-dimethyl dithiocarbonate (**37**), 2-methylbutyl isothiocyanate (**53**) and , 3-methylbutyl isothiocyanate (**55**).. These compounds were absent from plant-free controls, or were present at very low levels. Time course plots of the abundance of the aforementioned compounds based on peak areas for combined integrated single ion chromatogram (SIC) traces + standard errors are shown in Fig. 4 for plant samples and plant-free controls (where detected).

Although a direct assessment of larval development was not included in our study, root volatiles emission patterns post- second infestation on day 49, were considered to follow the likely developmental progress of *D. radicum* larvae. According to Hughes and Salter (1959) first instar larvae feed for approximately 4 days, second instar for about 6 days and third instar for 10-20 days. This is consistent with the pattern observed in our study following the second infestation. Release of larval feeding induced volatiles increased as larval development progressed from first to third instar, peaking around day 58 to 63 as larvae passed from second to third instar, before declining as larval feeding activity decreased during the third instar stage. These temporal patterns were similar to those observed by Crespo *et al*. (2012), Danner et al (2012), and van Dam et al (2012) who analysed real-time volatile emissions by roots of *Brassica* plants infested with *D. radicum* larvae using on-line proton transfer reaction mass spectrometry (PTR-MS) and GC-MS. The increase in the levels of some volatiles observed in our study between the first and second infestations may therefore also be indicative of feeding by first to third instar larvae following the first infestation on day 29.

These results are in good agreement with our earlier glasshouse studies (Deasy *et al*., 2016) and those previously reported from studies on induced volatiles emissions from *Brassica* roots infested with *D. radicum* larvae (Ferry *et al.,* 2007; Soler *et al.,* 2007; Pierre *et al.,* 2011; Crespo *et al.,* 2012; Danner *et al.,* 2012; van Dam *et al.,* 2012). The similarity of results in comparison with our earlier glasshouse studies serves to validate our method since there was no existing direct field-based equivalent method for comparison.

Selected compounds that contributed to the separation along PC 1of samples and controls collected on days 63 and 77 from those collected earlier and separation along PC 2 of day 15 samples and controls from those collected at other dates (Fig. 3) are listed in Table 2 (full list in Table S6). Time course plots for selected compounds belonging to the above

groupings collected from plants and plant-free controls are shown in Figs 5 and 6. Compounds showing an initial peak in abundance at day 15 then a large increase in abundance to a maximum over the period days 58-77, and which have high positive loadings on PC score 1, include 2-pentylfuran (**31**), 2-ethylhexanal (**30**), 2-ethyl-1-hexanol (**51**) and *n*-aldehydes in the range C_6-C_{11}, known products of lipid oxidation (Fig 5). Higher negative loadings on PC score 2 were particularly associated with acetone (**5**), the aromatic compounds benzaldehyde (**43**) and methylstyrene (**32**), the terpenes cymene (**39**) and camphene (**19**) and several unidentified compounds which displayed peak abundances on days 1 and 15 (Fig 6). Uncertainty exists as to why these differences were observed, but similarities between the time course profiles for root volatiles samples and soil control samples suggested they may relate to background microbial or physical processes that were occurring in the soil at the time of collection. The compound methoxy-phenyl-oxime (49) was the most abundant component present in all samples. Its abundance profiles (Fig. 6), having some similarity to the others shown, characterise it a being a member of the chemical background. It has been variously reported as a constituent in the volatile profiles from plants and fungi, or as an impurity, and its origin is uncertain.

In our study volatiles were not collected from wholly uninfested plants, therefore it is not possible to accurately assess the contribution of natural growth processes to the observed abundance profiles for the various sulfur compounds. Whereas there was evidence for a low background level of some of these compounds, mainly alkyl sulfides, from early on in plant growth, most were not detected before the surges in production of volatiles which only occurred following the first infestation with root fly eggs on day 29 and to a greater extent the second infestation on day 49 (Fig 4). As mentioned, these temporal profiles appear consistent with the temporal feeding and development patterns of root fly larvae. However, it is possible that processes such as root maturation and senescence and variation in moisture and nutrient availability may also have contributed to the observed profiles. Irrespective of the absolute contributions of larval-induced damage and other natural processes, the experimental technique was effective in detecting dynamic changes in root derived volatiles *in situ* within a field location.

A new method for *in situ* field sampling of plant root volatiles

In practice, this new *in situ* method has a number of key advantageous features over other techniques: 1. Non-invasive. Tubes were positioned in the soil next to transplants during planting so no further disturbance to roots occurred thereafter. This avoided any potential

damage effects misrepresenting the profile of volatiles. In addition, the *in situ* tubes were independent of subsequent experimental treatments, for example root infestation with *D. radicum*. 2. Passive trapping of volatiles. This circumvents the need to set up more elaborate systems that are associated with dynamic collection of root volatiles. 3. Simplicity. Each component of the technique was prepared in advance and carried to the field in a hand held container for setting up the method *in situ* and subsequent sampling. 4. Time efficient sampling. SPME fibre holders were inserted into the tubes *in situ* and the fibres exposed for collection in minutes, the same following completion of volatiles collecton when fibres were retracted and placed in a vial for storage until desorption in the laboratory. 5. Repeated sampling. Whilst the present study generally sampled volatiles from roots weekly and every few days around the time of infestation with *D. radicum*, this method has the potential to facilitate more regular sampling. 6. Robust. Tubes in the soil retained structural integrity over the duration of the sampling period. Strong winds and heavy rain did not dislodge the SPME fibre holder when positioned in the tube during sampling. Furthermore, there was no evidence of water/moisture building up inside the tubes or that the pores in the tube wall became blocked or sealed with soil particles/roots. 7. Reusable. Tubes are readily washable, for example in methanol and water with ultrasonic treatment, and can be dried in an oven or air for reuse.

The main noteworthy practical limitation of the method observed under field conditions, which was not apparent in the earlier laboratory/glasshouse experiments, was indirectly related to rainfall. On occasions when heavy rain occurred during sampling, drops hitting the soil surrounding the *in situ* SPME assembly resulted in fine soil particles being splashed onto the SPME device and entering the space between the plunger and the internal walls of the stainless steel fibre holder, which at times made it difficult to smoothly retract the plunger and fibre after volatiles collection. Field studies conducted in locations prone to rain might consider making the SPME device more weather/splash proof. In addition, users should factor in plant growth habit when deciding the position of sampling tubes. Foliage that grows later, which may be more relevant in longer term experiments, can cover the top of the *in situ* tubes making it difficult to insert the SPME fibre holder for sampling. Although we were unable to include plant free controls in our study for practical reasons, use of such controls is recommended and should be factored into the design of experiments using any variation of our sampling procedure.

Use of SPME for passive sampling of volatiles as used in this study has some limitations, which have been highlighted previously with regard to sampling from

greenhouse-grown plants (Deasy et al., 2016). In summary, these are relative insensitivity compared to active entrainment on porous polymers, and use of high infestation levels with the insect pest to demonstrate the effectiveness of the method. Approaches to improving sensitivity using SPME, for application under realistic infestation levels, could include use of different fibre types with greater trapping capacity, modification to the sampling well design to increase porosity or use of more sensitive GC-MS instrumentation such as those using a time of flight (TOF)-mass analyser (Deasy et al., 2016). Alternatively, the PTFE sampling wells can be directly coupled to industry-standard ¼ inch sorbent tubes using appropriate fittings. In such a scenario for field sampling, the SPME fibre and holder assembly could be replaced by a sorbent tube fitted with a diffusion restricted end cap allowing for passive trapping over a sampling period such as 24 hours as used in this study. Then the remaining untrapped volatiles within the 3.4 mL volume of the PTFE sampling tube could be entrained on the sorbent tube by withdrawal of 5-10 mL of air using a syringe, prior to subsequent analysis by automated thermal desorption (ATD) and GC-MS (Deasy et al., 2016).

This study has demonstrated that non-invasive, passive collection of root volatiles *in situ* using SPME within perforated PTFE sampling tubes can be successfully applied under variable field conditions to collect volatiles released by broccoli roots. These results show that this new method, which has potential for wide application in chemical ecology/root/soil research, is a powerful tool for non-invasive sampling of below-ground root volatiles that are critical for interactions between trophic levels and important above-below ground signalling chemicals.

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Figure Legends

Figure 1. (a) Schematic diagram of perforated PTFE sampling tubes and a SPME fibre holder with a fibre attached inserted into the sampling tube. (b) Sampling tubes and module grown broccoli transplant at planting. (c) Sampling tubes *in situ* with PTFE end caps. (d) and (e) Felt trap wound around plant stem and sampling tubes to prevent oviposition by natural populations of *D. radicum* before infestation with *D. radicum* eggs. (f) and (g) Simultaneous SPME sampling from two sampling tubes *in situ*. (h) Sampling bare soil (control).

Figure 2. Chromatograms (TIC, Total Ion Chromatogram) of GC-MS analysis of broccoli root volatiles collected *in situ* within a perforated PTFE tube using a PDMS/DVB SPME fibre. Samples were collected pre-damage by *Delia radicum* larvae (day 29) and post-damage (days 58 and 77). See Table 1 for list of compounds. An expanded and annotated version can be found in supplementary information, Fig. S1.

Figure 3. Principal components scores plot for $\log_{10}$-transformed data showing the first two PC scores of broccoli root volatiles sampled *in situ* using SPME within perforated PTFE tubes pre-and post-infestation with *Delia radicum* larvae. Each coloured symbol represents a different collection day. Sampling of root volatiles and controls started on day 1 and ended on day 77. Centroid values for root volatiles and controls collected each day are each connected by a solid line. All control samples are located below the dotted line on PC 2. Plant roots were infested with *D. radicum* on day 29 (Inf 1), and again on day 49 (Inf 2). PC 1 and PC 2 accounted for 31.9 and 15.6% of the total variance, respectively. Compounds that contributed to the separation observed are shown in the loadings for PC 1 and PC 2 (selected compounds inTable 2, full list in supplementary information Table S6).

Figure 4. Abundance time course (sampling day against combined SIC peak area) of compounds identified from positive loadings for principal component 2 (Table 2), which contributed to the separation of broccoli root volatiles samples collected post-infestation 2 from those collected earlier (see Fig. 3). Plants + standard error (n = 3) and soil controls (where detected) – standard error (n = 3) were infested with *D. radicum* eggs on day 29 (inf 1) and day 49 (inf 2).

Figure 5. Abundance time course (sampling day against combined SIC peak area) of compounds identified from positive loadings for principal component 1 (Table 2), which contributed to the separation of volatiles samples collected on days 63 and 77 from those collected earlier (see Fig. 3 Plants + standard error (n = 3) and soil controls (where detected) – standard error (n = 3) were infested with *D. radicum* eggs on day 29 (inf 1) and day 49 (inf 2).

Figure 6. Abundance time course (sampling day against combined SIC peak area) of compounds identified from negative loading loadings for principal component 2 (Table 2), which contributed to the separation of volatiles samples collected on day 15 (see Fig. 3). Plants + standard error (n = 3) and soil controls (where detected) – standard error (n = 3) were infested with *D. radicum* eggs on day 29 (inf 1) and day 49 (inf 2).

Table Titles and footnotes

Table 1. Compounds detected in the broccoli root volatiles trapped *in situ* within perforated PTFE tubes using SPME and analysed by SPME-GC-MS.

Footnotes

Number, order of the compounds in the Xcalibur™ data processing method and also in the example chromatograms (Fig. 2); Compounds, [1]these compounds were identified by comparison of their mass spectra and retention indices with those of pure standards. Tentative identification of the remaining compounds was made by comparison with entries in the Palisade 600k and NIST05 mass spectral databases, and by comparison with published data (see supplementary material, Table S1, for list of references); MW, molecular weight; Selected Ions, ions used for automated compound identification and measurement of raw abundance using Xcalibur™; t_R, retention time (minutes); RRI, relative retention index. RRI values were calculated by comparing retention times to *n*-alkanes (C_5-C_{17}). Each alkane carbon number C_n was assigned a RRI value 100*n*. The RRI value for a compound was calculated by linear interpolation of the spacing of its retention time between two nearest adjacent retention index marker compounds. This corresponds to the linear retention index formula for linearly temperature programmed GC separations (Schomburg, 1990).

Table 2. Loadings for PC 1 and PC 2 from a PCA of log-transformed abundances of broccoli root volatiles sampled *in situ* using SPME in perforated PTFE tubes pre-and post-infestation with *Delia radicum* larvae. Loadings furthest from zero in either positive or negative direction provide information on the compounds that contributed to the separation in the PCA score plot (Fig. 3). Compounds and numbers listed correspond with those in Table 1.

Supplementary Information

Figures

Figure S1 is in the word file 'Supplementary Material Root volatiles method field sampling Fig S1'.

Figure S1. Chromatograms (TIC, Total Ion Chromatogram) of GC-MS analysis of broccoli root volatiles collected *in situ* within a perforated PTFE tube using a PDMS/DVB SPME fibre. Samples were collected pre-damage by *Delia radicum* larvae (day 29) and post-damage (days 58 and 77). I denotes impurity, peaks denoted F (fibre) are non-sample derived. See Table 1 for list of compounds.

Tables

Table S1 is in the word file 'Supplementary material published refrerences for compound identification'. Tables S2 to S5 are included in Excel workbook 'Supplementary material table of abundance and SE_Field.xlsx'. Individual tables are located in the specific spreadsheets indicated after the table title. Tables S6 is in the word file 'Root volatiles method Field sampling Supplementary Information Table S6'.

Table S1. References from published data on Brassicaceae volatile compounds used for the identification of broccoli root volatiles. Compounds are listed in order of elution. PTR-MS denotes proton transfer reaction mass spectrometry, SPME denotes solid phase micro extraction, SD denotes steam distillation, and * denotes references therein.

Table S2. Broccoli root volatiles collection, pre- and post-damage by *Delia radicum* larvae, sampled *in situ* within perforated PTFE tubes using SPME. (p) denotes plant, (c) denotes control (no plant). *In sheet 'Sampling Schedule'.*

Table S3. Means and SE for abundance of compounds in root volatiles from field-grown broccoli . Samples were entrained in situ within perforated PTFE sampling tubes using SPME from undamaged and damaged roots. *In sheet 'Abundance & SE'.*

Table S4. Raw abundance data and calculation of means and SE for broccoli root volatiles entrained in situ on PDMS/DVB SPME fibres within perforated PTFE tubing. *In sheet 'Calc Abund & SE'.*

Table S5. Raw abundance data for broccoli root volatiles entrained in situ on PDMS/DVB SPME fibres within perforated PTFE tubing. (p) denotes plant, (c) denotes control (no plant). *In sheet 'Raw data'.*

Table S6. Loadings for PC 1 and PC 2 from a PCA of log-transformed abundances of broccoli root volatiles sampled *in situ* using SPME in perforated PTFE tubes pre-and post-infestation with *Delia radicum* larvae. Loadings furthest from zero in either positive or negative direction provide information on the compounds that contributed to the separation in the PCA score plot (Fig. 3). Compounds and numbers listed correspond with those in Table 1.

Table 1. Compounds detected in the broccoli root volatiles trapped *in situ* within perforated PTFE tubes using SPME and analysed by SPME-GC-MS.

No.	Compound	MW	Ions used in processing method	t_R	RRI
1	Sulfur dioxide	64	48, 64	1.17	556
2	Methanethiol	48	44, 45, 46, 47, 48, 49, 50	1.26	568
3	Dimethyl sulfide[1]	62	47, 61, 62	1.44	593
4	Hexane[1]	86	43, 56, 57, 71, 86	1.49	600
5	Acetone[1]	58	58	1.50	601
6	2-Butanone[1]	72	72	2.10	684
7	Heptane[1]	100	43, 56, 57, 71, 100	2.22	700
8	Acetic acid 779[1]	60	43, 45, 60	3.21	779
9	3-Pentanone[1]	86	39, 42, 56, 57, 58, 86	3.23	781
10	Octane[1]	114	43, 57, 71, 85	3.47	800
11	Dimethyl disulfide[1]	94	45, 46, 47, 61, 64, 79, 94, 96	3.60	808
12	Methyl thiocyanate	73	45, 46, 47, 58, 72, 73	4.30	850
13	Hexanal[1]	100	44, 56, 57, 72, 82, 100	4.81	881
14	Nonane[1]	128	43, 56, 57, 71, 85	5.12	900
15	1-Nonene	126	43, 56, 69, 84, 97	5.14	901
16	Unknown 947		65, 77, 79, 91, 92, 93, 105, 121, 136	5.92	947
17	2-Methylcyclopentyl acetate	142	72, 84, 100	6.18	963
18	2,4-Dithiapentane[1]	108	45, 46, 47, 61, 63, 108, 110	6.20	964
19	Camphene[1]	136	41, 77, 79, 91, 93, 107, 121, 136	6.28	969
20	Unknown 972		105, 120	6.34	972
21	Heptanal[1]	114	43, 44, 55, 57, 70, 81, 86, 114	6.57	986
22	Decane[1]	142	43, 57, 71, 85, 99, 113	6.81	1000
23	Unknown 1004		91, 120	6.87	1004
24	Unknown 1009		55, 69, 82, 98	6.96	1009
25	Unknown 1018		105, 120	7.11	1018
26	β-Myrcene[1]	136	69, 93, 121, 136	7.14	1020

No.	Compound	MW	Ions used in processing method	t_R	RRI
27	Butyl isothiocyanate[1]	115	115	7.27	1028
28	2-Butyl isothiocyanate	115	86	7.27	1028
29	Unknown 1030		91, 93, 105, 121, 136	7.31	1030
30	2-Ethylhexanal	128	41, 57, 72	7.33	1032
31	2-Pentylfuran	138	53, 81, 82, 138	7.37	1034
32	Methylstyrene	118	77, 78, 103, 117, 118	7.53	1044
33	Dimethyl trisulfide[1]	126	45, 46, 47, 64, 79, 80, 111, 126, 128	7.53	1044
34	Unknown 1048		105, 120	7.59	1048
35	Limonene[1]	136	67, 68, 79, 93, 107, 121, 136	7.70	1054
36	Isobutyl isothiocyanate	115	57, 72, 73, 86, 100, 115	7.73	1056
37	S,S-Dimethyl dithiocarbonate[1]	122	47, 75, 94, 122	7.77	1059
38	1-Octen-3-one	126	39, 41, 42, 43, 55, 70, 83	7.91	1067
39	Cymene[1]	134	119, 134	7.99	1072
40	3-Octanone	128	43, 57, 71, 72, 99	8.00	1073
41	Eucalyptol[1]	154	43, 55, 71, 81, 84, 111, 139, 154	8.03	1074
42	Unknown 1079		57, 83, 84	8.10	1079
43	Benzaldehyde[1]	106	50, 51, 77, 105, 106	8.14	1081
44	6-Methyl-5-hepten-2-one	126	55, 58, 69, 71, 108, 111, 126	8.18	1084
45	Unknown 1088		193, 209	8.26	1088
46	Octanal[1]	128	43, 44, 55, 56, 57, 67, 69, 81, 82, 84, 100	8.26	1088
47	E-Conophthorin	156	84, 87	8.43	1099
48	Undecane[1]	156	43, 57, 71, 127, 141	8.45	1100
49	Methoxy-phenyl-oxime	151	105, 133, 151	8.75	1120
50	4-Hydroxybutanoic acid	86	86	8.85	1126
51	2-Ethyl-1-hexanol	130	55, 57, 70, 83, 98, 112	8.94	1132
52	Unknown 1155		41, 43, 57, 67, 81, 97	9.29	1155
53	2-Methylbutyl isothiocyanate	129	41, 43, 57, 71, 72, 73, 100, 114, 129	9.43	1164
54	2-Methylbutyl isothiocyanate 1164	129	100	9.43	1164

No.	Compound	MW	Ions used in processing method	t_R	RRI
55	3-Methylbutyl isothiocyanate	129	41, 43, 55, 72, 101, 114, 129	9.43	1164
56	Unknown 1174		71, 85, 100	9.58	1174
57	1-Octanol	130	41, 42, 43, 55, 56, 69, 70, 84	9.60	1176
58	2-Nonanone	142	43, 58, 71	9.76	1186
59	3,5-dimethyldihydro-2(3H)-furanone	114	41, 42, 55, 70, 99	9.77	1187
60	Nonanal[1]	142	43, 57, 70, 82, 98, 114	9.85	1192
61	Ectocarpene	148	91, 105	9.86	1193
62	Acetophenone[1]	120	51, 77, 105, 120	9.95	1199
63	Dodecane[1]	170	43, 55, 56, 57, 71, 85, 99	9.97	1200
64	Phenol[1]	94	39, 66, 94	10.19	1215
65	Unknown 1230		43, 57, 69, 71, 83, 98	10.39	1230
66	1-Methyl-2-pyrrolidinone	99	44, 98, 99	10.45	1234
67	Camphor	152	41, 55, 69, 81, 95, 108, 109, 152	10.85	1262
68	Unknown 1285		67, 77, 79, 91, 93, 95, 107, 108, 135	11.18	1285
69	Unknown 1296		81, 110	11.34	1296
70	Decanal[1]	156	43, 55, 57, 68, 69, 81, 82, 83, 95, 96, 112, 138	11.34	1296
71	Tridecane[1]	184	43, 57, 71, 85, 99, 113, 127, 141	11.39	1300
72	Unknown 1330		55, 69, 97, 111	11.82	1330
73	Benzoisothiazole or Benzothiazole	135	91, 108, 135	12.20	1360
74	Unknown 1371	74	81, 95, 123, 138	12.34	1371
75	Bornyl acetate	196	93, 121, 136, 154	12.54	1386
76	Tetradecane[1]	198	43, 57, 71, 85, 99, 113, 127, 141	12.73	1400
77	Undecanal[1]	170	55, 67, 68, 82, 96, 110, 126	12.73	1400
78	Pentadecane[1]	212	43, 57, 71, 85, 99, 113, 127, 141	14.01	1500
79	Unknown 1502		41, 43, 55, 56, 57, 69, 70, 71, 83, 97	14.03	1502
80	Hexadecane[1]	226	57, 71, 85, 99, 113, 127, 141, 155	15.20	1600
81	2-Phenylethyl isothiocyanate[1]	163	39, 51, 63, 65, 72, 77, 91, 92, 105, 163	15.77	1650
82	Heptadecane[1]	240	43, 57, 71, 85, 99, 113, 127, 141	16.34	1700

No.	Compound	MW	Ions used in processing method	t_R	RRI
83	Unknown 1713		55, 57, 85, 91, 93, 105, 119, 120, 161, 189, 204	16.46	1713

Number, order of the compounds in the Xcalibur™ data processing method and also in the example chromatograms (Fig. 2); Compound, [1]these compounds were identified by comparison of their mass spectra and retention indices with those of pure standards. Tentative identification of the remaining compounds was made by comparison with entries in the Palisade 600k and NIST05 mass spectral databases, and by comparison with published data (see Supplementary Material, Table S1, for list of references); MW, molecular weight; Selected Ions, ions used for automated compound identification and measurement of raw abundance using Xcalibur™; t_R, retention time (minutes); RRI, relative retention index. RRI values were calculated by comparing retention times to *n*-alkanes (C_5-C_{17}). Each alkane carbon number C_n was assigned a RRI value 100*n*. The RRI value for a compound was calculated by linear interpolation of the spacing of its retention time between two nearest adjacent retention index marker compounds. This corresponds to the linear retention index formula for linearly temperature programmed GC separations (Schomburg, 1990).

Table 2. Loadings for PC 1 and PC 2 from a PCA of log-transformed abundances of broccoli root volatiles sampled *in situ* using SPME in perforated PTFE tubes pre-and post-infestation with *Delia radicum* larvae. Loadings furthest from zero in either positive or negative direction provide information on the compounds that contributed to the separation in the PCA score plot (Fig. 3). Compounds and numbers listed correspond with those in Table 1.

PC 1			PC 2		
No.	Compound	Loading	No.	Compound	Loading
60	Nonanal	0.18	27	Butyl isothiocyanate	0.22
70	Decanal	0.18	3	Dimethyl sulfide	0.20
51	2-Ethyl-1-hexanol	0.18	33	Dimethyl trisulfide	0.20
30	2-Ethylhexanal	0.18	28	2-Butyl isothiocyanate	0.20
21	Heptanal	0.18	18	2,4-Dithiapentane	0.20
31	2-Pentylfuran	0.18	12	Methyl thiocyanate	0.19
44	6-Methyl-5-hepten-2-one	0.17	37	S,S-Dimethyl dithiocarbonate	0.19
46	Octanal	0.16	2	Methanethiol	0.18
43	Benzaldehyde	0.15	11	Dimethyl disulfide	0.18
45	Unknown 1088	0.14	54	2-Methylbutyl isothiocyanate 1164	0.13
42	Unknown 1079	0.13	81	2-Phenylethyl isothiocyanate	0.10
39	Cymene	0.12	53	2-Methylbutyl isothiocyanate	0.10
20	Unknown 972	0.10	55	3-Methylbutyl isothiocyanate	0.09
29	Unknown 1030	0.10	36	Isobutylisothiocyanate	0.08
5	Acetone	0.10	42	Unknown 1079	0.08
1	Sulfur dioxide	0.10	51	2-Ethyl-1-hexanol	0.03
49	Methoxy-phenyl-oxime	0.10	21	Heptanal	-0.005
32	Methylstyrene	0.08	1	Sulfur dioxide	-0.007
11	Dimethyl disulfide	0.07	31	2-Pentylfuran	-0.01
81	2-Phenylethyl isothiocyanate	0.07	30	2-Ethylhexanal	-0.02
28	2-Butyl isothiocyanate	0.07	46	Octanal	-0.03
19	Camphene	0.06	44	6-Methyl-5-hepten-2-one	-0.03
27	Butyl isothiocyanate	0.05	60	Nonanal	-0.04
33	Dimethyl trisulfide	0.04	70	Decanal	-0.05
2	Methanethiol	0.04	29	Unknown 1030	-0.07
36	Isobutylisothiocyanate	0.03	49	Methoxy-phenyl-oxime	-0.10

PC 1			PC 2		
No.	Compound	Loading	No.	Compound	Loading
55	3-Methylbutyl isothiocyanate	0.03	5	Acetone	-0.12
53	2-Methylbutyl isothiocyanate	0.03	43	Benzaldehyde	-0.13
18	2,4-Dithiapentane	0.03	20	Unknown 972	-0.13
54	2-Methylbutyl isothiocyanate 1164	0.02	45	Unknown 1088	-0.14
37	Carbonodithioic acid S,S dimethyl ester	0.02	19	Camphene	-0.15
63	S,S-Dimethyl dithiocarbonate	0.02	39	Cymene	-0.16
12	Methyl thiocyanate	0.02	4	Hexane	-0.18
3	Dimethyl sulfide	0.01	32	Methylstyrene	-0.19

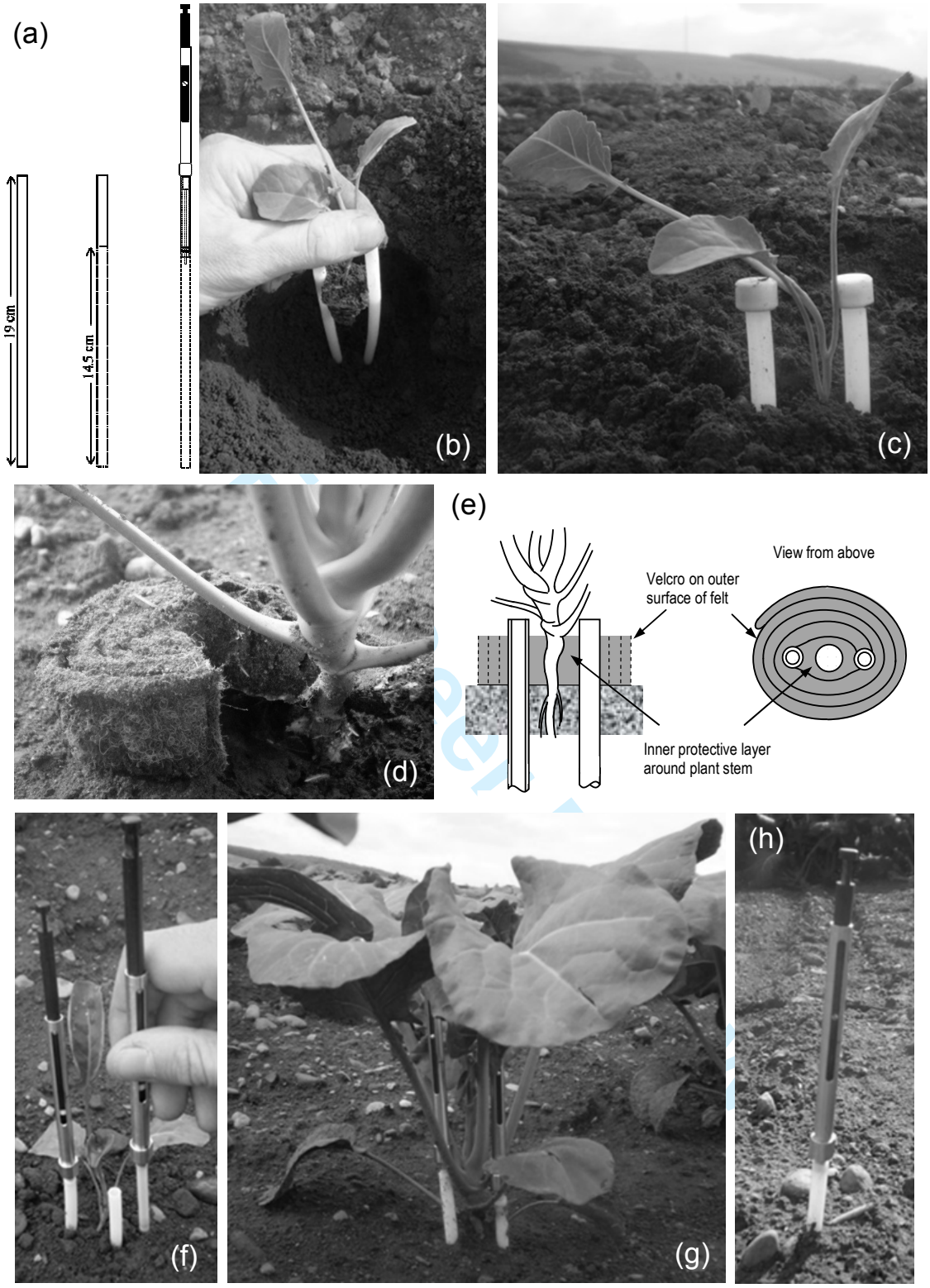


Figure 1. (a) Schematic diagram of perforated PTFE sampling tubes and a SPME fibre holder with a fibre attached inserted into the sampling tube. (b) Sampling tubes and module grown broccoli transplant at planting. (c) Sampling tubes *in situ* with PTFE end caps. (d) and (e) Felt trap wound around plant stem and sampling tubes to prevent oviposition by natural populations of *D. radicum* before infestation with *D. radicum* eggs. (f) and (g) Simultaneous SPME sampling from two sampling tubes *in situ*. (h) Sampling bare soil (control).

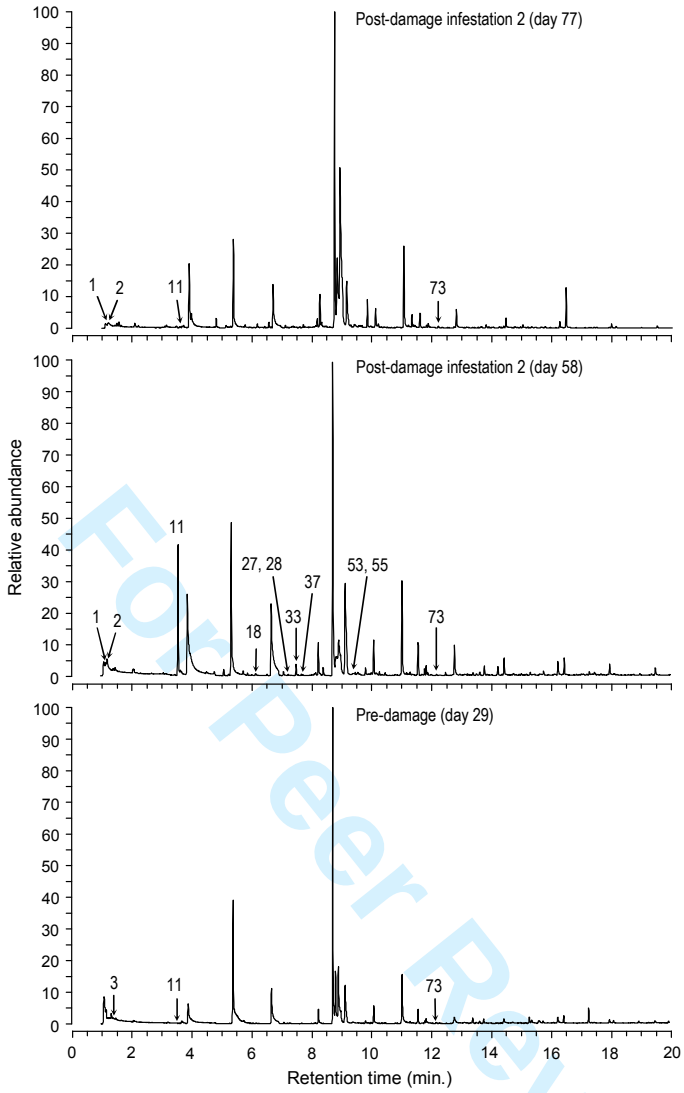
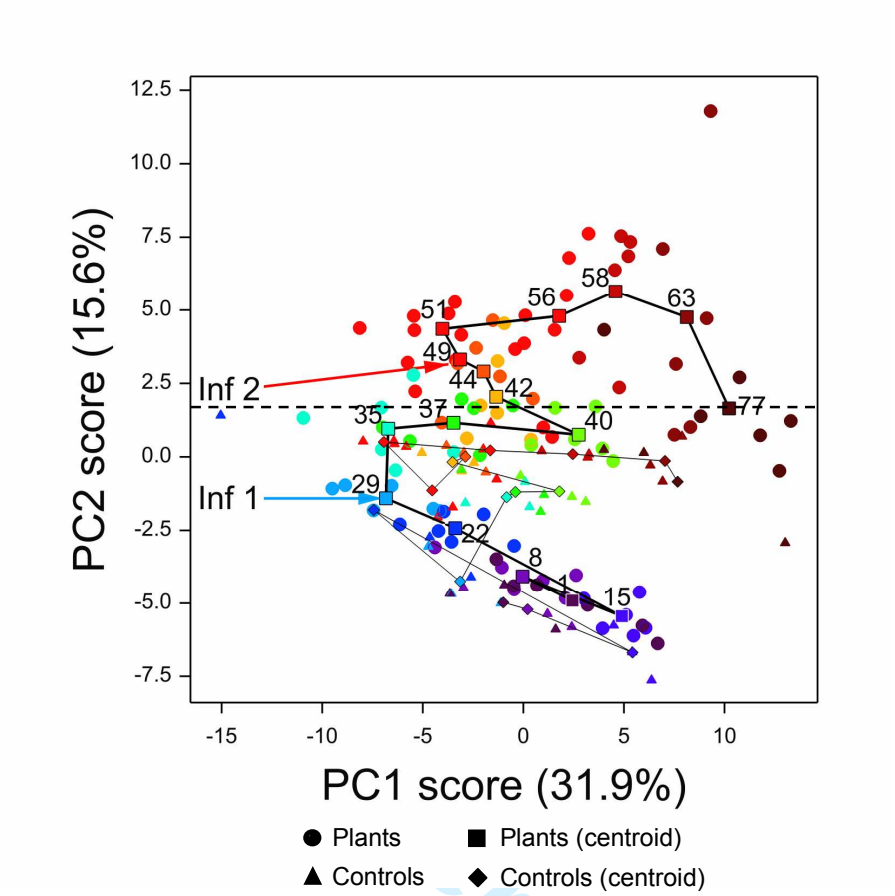


Figure 2. Chromatograms (TIC, Total Ion Chromatogram) of GC-MS analysis of field-grown broccoli root volatiles collected *in situ* within a perforated PTFE tube using a PDMS/DVB SPME fibre. Samples were collected pre-damage by *Delia radicum* larvae (day 29) and post-damage (days 58 and 77). See Table 1 for list of compounds. An expanded and annotated version can be found in supplementary information, Fig. S1.



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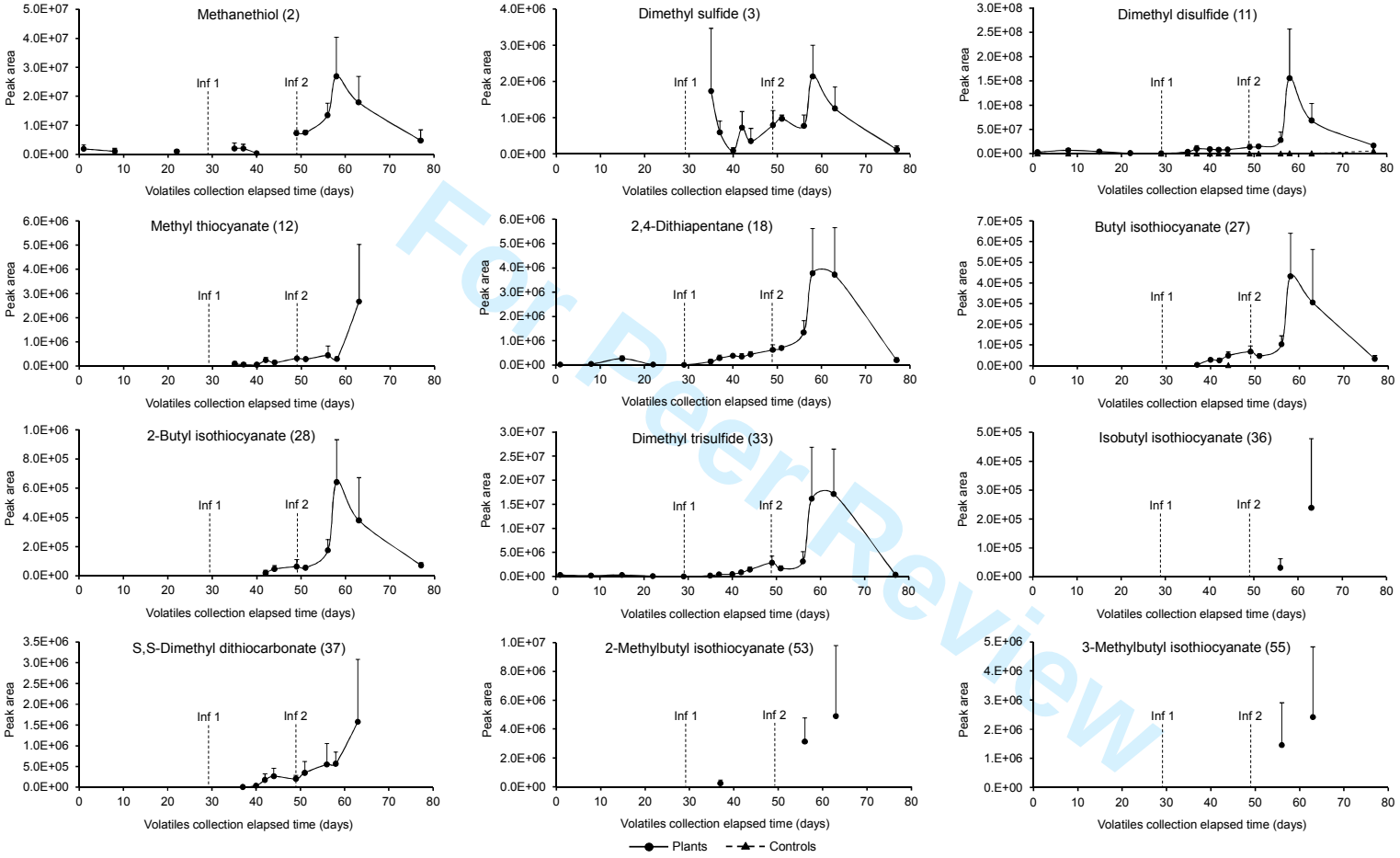

Figure 4. Abundance time course (sampling day against combined SIC peak area) of compounds identified from positive loadings for principal component 2 (Table 2), which contributed to the separation of broccoli root volatiles samples collected post-infestation 2 from those collected earlier (see Fig. 3). Plants + standard error (n = 3) and soil controls (where detected) – standard error (n = 3) were infested with *D. radicum* eggs on day 29 (inf 1) and day 49 (inf 2).

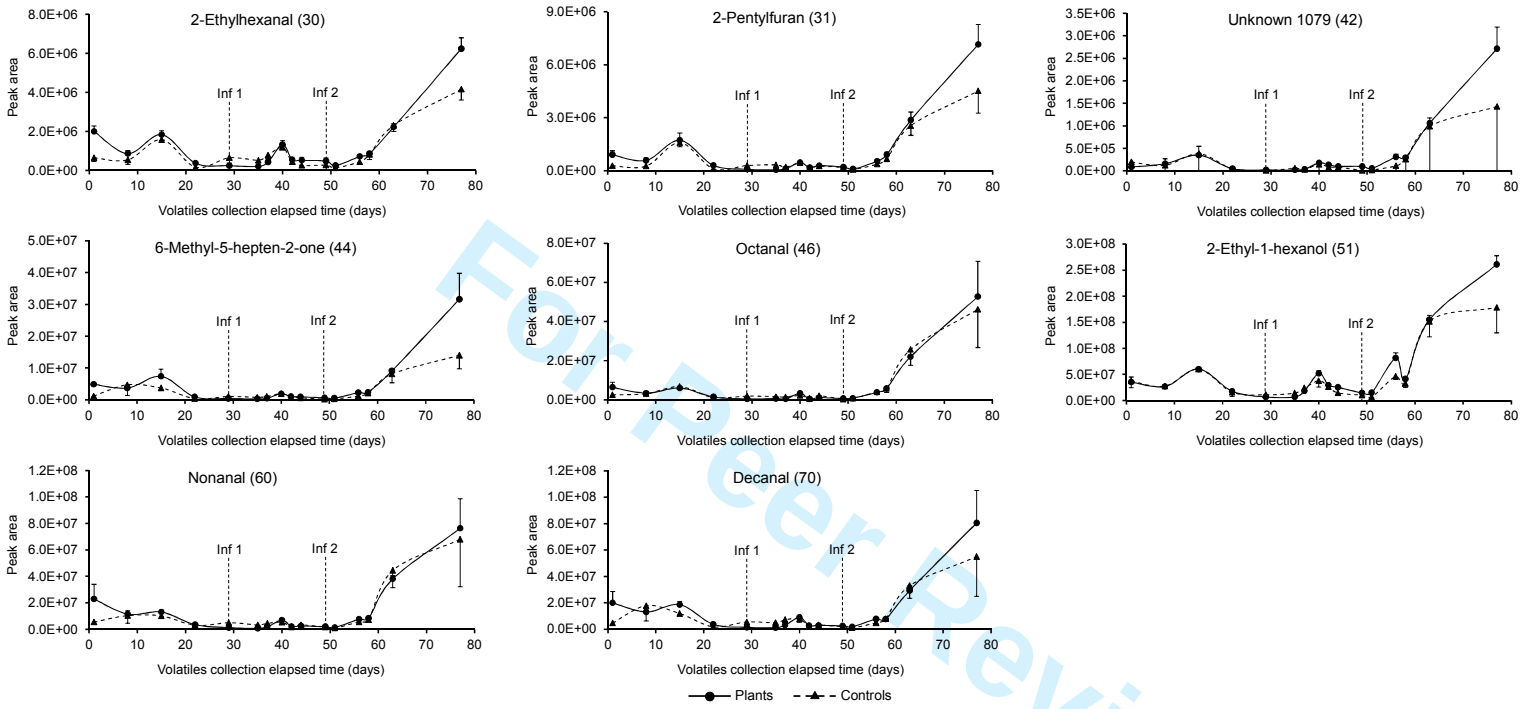

Figure 5. Abundance time course (sampling day against combined SIC peak area) of compounds identified from positive loadings for principal component 1 (Table 2), which contributed to the separation of volatiles samples collected on days 63 and 77 from those collected earlier (see Fig. 3). Plants + standard error (n = 3) and soil controls (where detected) – standard error (n = 3) were infested with *D. radicum* eggs on day 29 (inf 1) and day 49 (inf 2).

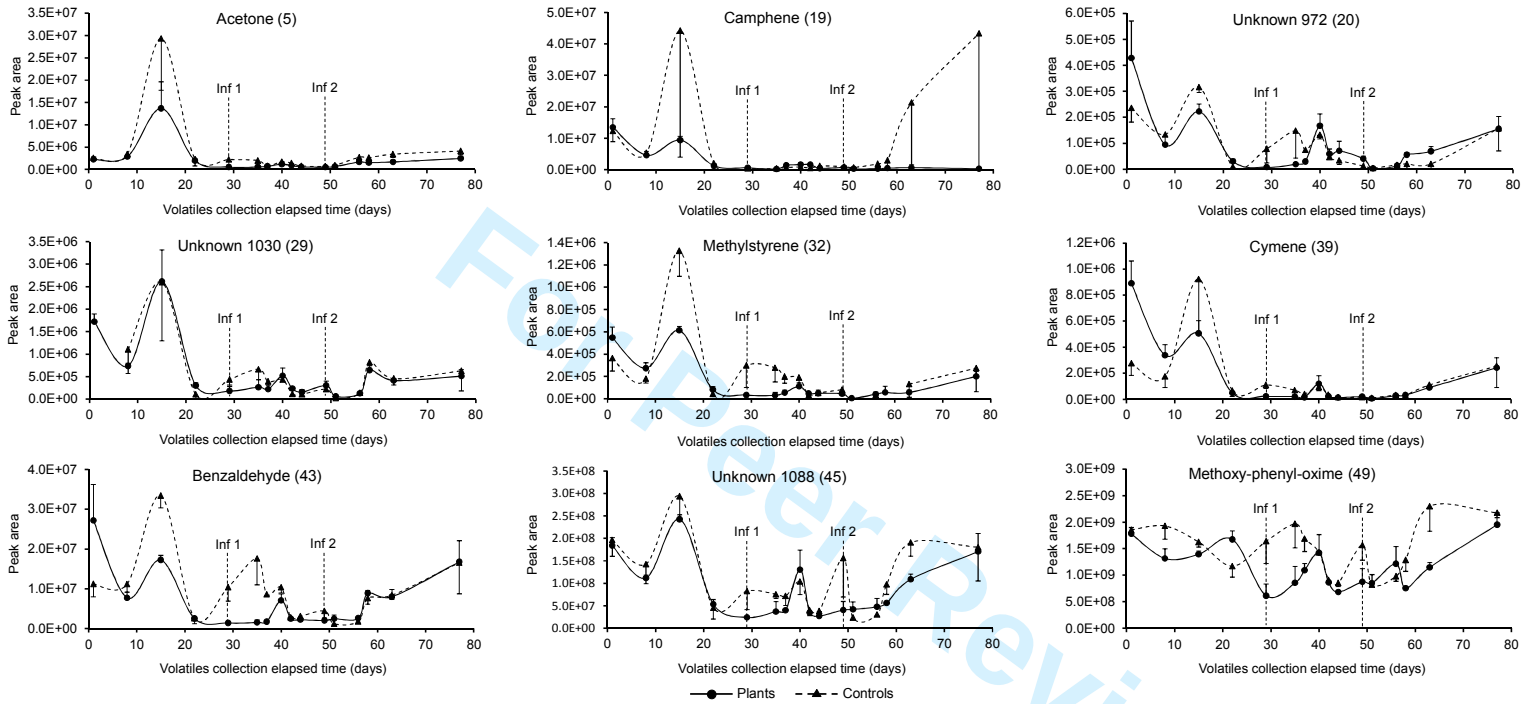

Figure 6. Abundance time course (sampling day against combined SIC peak area) of compounds identified from negative loading loadings for principal component 2 (Table 2), which contributed to the separation of volatiles samples collected on day 15 (see Fig. 3). Plants + standard error (n = 3) and soil controls (where detected) – standard error (n = 3) were infested with *D. radicum* eggs on day 29 (inf 1) and day 49 (inf 2).