Development and validation of a SPME-GC-MS method for in situ passive sampling of root volatiles from glasshouse-grown broccoli plants undergoing below-ground herbivory by larvae of cabbage root fly, Delia radicum L

Deasy, W; Shepherd, T; Alexander, CJ; Birch, ANE; Evans, KA

Published in:
Phytochemical Analysis

DOI:
10.1002/pca.2637

First published: 30/09/2016

Document Version
Peer reviewed version

Development and validation of a SPME-GC-MS method for *in situ* passive sampling of root volatiles from glasshouse-grown broccoli plants undergoing below-ground herbivory by larvae of cabbage root fly, *Delia radicum L.*

William Deasy,[a, c, d] Tom Shepherd,[a]* Colin J. Alexander,[b] A. Nicholas E. Birch,[a] K. Andrew Evans[c]

* Correspondence to: Tom Shepherd, The James Hutton Institute, Invergowrie, Dundee DD2 5DA, United Kingdom. Email: tom.shepherd@hutton.ac.uk

[a] The James Hutton Institute, Invergowrie, Dundee DD2 5DA, United Kingdom

[b] Biomathematics and Statistics Scotland, Invergowrie, Dundee DD2 5DA, United Kingdom

[c] Scotland's Rural College, Nicholas Kemmer Road, Edinburgh EH9 3FH, United Kingdom

[d] School of Biological Sciences, The University of Edinburgh, Darwin Building, The King's Buildings, Max Born Crescent, Edinburgh, EH9 3BF, United Kingdom

Development and validation of a SPME method for *in situ* passive sampling of root volatiles from glasshouse-grown broccoli plants undergoing below-ground herbivory by larvae of cabbage root fly, *Delia radicum L*

ABSTRACT:

Introduction - Research on plant root chemical ecology has benefited greatly from recent developments in analytical chemistry. Numerous reports document techniques for sampling root volatiles, although only a limited number describe *in situ* collection.

Objectives - To demonstrate a new method for non-invasive *in situ* passive sampling using solid phase micro extraction (SPME), from the immediate vicinity of growing roots.

Methods – SPME fibres inserted into polyfluorotetrafluoroethylene (PTFE) sampling tubes located *in situ* which were either perforated, covered with stainless steel mesh or with microporous PTFE tubing, were used for non-invasive sub-surface sampling of root volatiles from glasshouse-grown broccoli. Sampling methods were compared with above surface headspace collection using Tenax TA. The roots were either mechanically damaged or infested with *Delia radicum* larvae. Principal component analysis (PCA) was used to investigate the effect of damage on the composition of volatiles released by broccoli roots.

Results - Analyses by gas chromatography-mass spectrometry (GC-MS) with SPME and automated thermal desorption (ATD) confirmed that sulfur compounds, showing characteristic temporal emission patterns, were the principal volatiles released by roots following insect larval damage. Use of SPME with *in situ* perforated PTFE sampling tubes was the most robust method for out-of-lab sampling.

Conclusion - This study describes a new method for non-invasive passive sampling of volatiles *in situ* from intact and insect damaged roots using SPME. The method is highly suitable for remote sampling and has potential for wide application in chemical ecology/root/soil research.

Keywords: Chemical ecology; *in situ* root volatiles analysis; *Brassica*; *Delia radicum*; SPME-GC-MS

Introduction

Introduction

Plants emit volatile compounds from their roots that play critical roles in interactions with their environment. Research on root chemical ecology and the function of root volatiles as signals in interactions between plants, insect herbivores and their natural enemies has greatly benefitted from recent advances in sampling and analytical methods (D'Alessandro and Turlings 2006; Rasmann *et al.,* 2012; Campos-Herrera *et al.,* 2013; Peñuelas *et al.,* 2014; van Dam 2014).

Overcoming the methodological difficulties associated with non-invasively investigating volatiles emissions from plant roots poses challenges for researchers studying below-ground interactions (D'Alessandro and Turlings 2006; Rasmann *et al.,* 2012; Campos-Herrera *et al.,* 2013; van Dam 2014). Whilst techniques for sampling volatiles from the headspace above growing roots have been successfully used to detect changes in volatiles released in response to root herbivory (Soler *et al.,* 2007; Pierre *et al.,* 2011; Crespo *et al.,* 2012; Danner *et al.,* 2012; van Dam *et al.,* 2012), very few methods have been developed for *in situ* collection of root volatiles (Mohney *et al.,* 2009; Weidenhamer *et al.,* 2009; Ali *et al.,* 2012, Eilers et al., 2015).

In previous experiments measuring the diffusion of volatile compounds injected into sand and soil, Rasmann *et al.,* (2005) and Hiltpold and Turlings (2008) demonstrated that solid phase micro extraction (SPME) can be used to sample volatiles below-ground by exposing fibres in pre-made holes. This approach was subsequently used to study attraction of cockchafer larvae to damaged roots of young oak trees (Weissteiner et al., 2012) and the release of phytotoxic volatiles from sagebrush roots (Jassbi et al., 2010). However, in these studies, the sampling holes were made adjacent to the stems of established growing plants and therefore root damage may have been caused during the sampling process.

Delia radicum L. (Diptera: Anthomyiidae), the cabbage root fly, is an important insect pest of *Brassica* crops. Adult females lay their eggs in the soil near suitable host plants and, after hatching, the larvae crawl down to feed on the roots before pupating in the surrounding soil. Thus far, a number of invasive, non-invasive, passive and dynamic headspace sampling approaches have been used to study volatile emissions from *Brassica* roots infested with *D. radicum* larvae (Ferry *et al.,* 2007; Soler *et al.,* 2007; van Dam *et al.,* 2010; Danner *et al.,* 2012).

As part of our studies to develop alternative measures for control of *Delia radicum* during commercial cultivation of broccoli (*Brassica oleracea* L) we identified a requirement for *in situ* collection of volatiles from broccoli roots in undisturbed soil using SPME under field conditions. We hypothesised that development of an accessorial tool to create a pre-positioned headspace in the soil/substrate next to growing roots using variously perforated polytetrafluoroethene (PTFE) tubes would protect the fragile SPME fibre assembly from damage or fouling during sampling by avoiding direct contact with the soil/substrate/roots. This could also support the SPME holder in position during sampling. We further hypothesised that this technique would facilitate repeated non-destructive temporal and location-specific collection of root volatiles pre- and post-damage to roots, enabling time course studies of volatile signal dynamics. Here, we present the development and validation of a novel SPME-based method for below-ground passive sampling of volatiles *in situ* from intact roots of glasshouse-grown broccoli (*Brassica oleracea* L. convar. *botrytis* L. Alef. var. *cymosa* Duchesne 'Parthenon') and those damaged mechanically or by feeding *D. radicum* larvae.

Experimental

Plants. Broccoli 'Parthenon' seeds from Sakata UK Ltd., Boston, UK were germinated in 345 module trays (Westhorpe Plants Ltd., Boston, UK) containing Levington M2 compost for experiments using SPME or in 12 cm deep Rootrainers (Ronaash Ltd., Kelso, UK) using Levington M2 compost for experiments using ATD-GC-MS.

Insects. *Delia. radicum* first instar larvae used for plant infestation and root volatiles induction were obtained from a continuously reared culture maintained at The James Hutton Institute**.**

Reagents. Reference compounds were obtained from Sigma-Aldrich UK Ltd.

Sampling of root volatiles *in situ* using SPME and analysis of volatiles by GC-MS

Plant growth. At the 3-4 true leaf stage plants were transplanted to custom-modified containers (described below) for growing on and for collection of root derived volatiles *in situ* using SPME. Transplantation in this way is the standard practice for commercial growth of broccoli in the UK. Since we ultimately wish to evaluate our *in situ* sampling methodology using field-grown plants this practice was adopted here. Glasshouse conditions were maintained at 21°C:16°C (day:night) temperature and 16:8 hours (light:dark) photoperiod throughout. Natural daylight was supplemented when required by artificial lighting (MASTER SON-T PIA Green Power; Philips, Guildford, UK) to maintain irradiance >200 W m^{-2}. Containers were watered daily to beyond field capacity and allowed to drain freely before the next irrigation event.

Perforated PTFE sampling tubes. Each sampling tube (Figs 1a and 1d.2) consisted of a 19 cm length of PTFE tubing (Fig. 1d.1; 5 mm internal diameter [Ø], 1 mm wall thickness; Radleys, Saffron Walden, UK, catalogue no. S1810-46) manually perforated 1,400 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 and stopped 2.5 cm from the bottom.

Stainless steel mesh covered PTFE sampling tubes. To create the sampling tubes (Figs 1b, 1d.3 and 1d.4) a 4.8 cm x 4 mm slot was cut and removed 4.5 cm from the top end of a 20 cm

length of PTFE tube using a fixed blade utility knife (Stanley, Slough, UK). A single piece (5.1 cm x 2.3 cm) of 165/1400 stainless steel mesh (Nisjemetall AS, Røyken, Norway) with a pore size of 20 μm was wrapped around the tube to cover the opening and held in place with PTFE tape (RS Components Ltd., Corby, UK).

Microporous PTFE tubing covered PTFE sampling tubes. Initial construction of sampling tubes (Figs 1c and 1d.5) was similar to that described above for use with stainless steel mesh. However, in place of the mesh, microporous PTFE tubing (5.1 cm long piece of 5 mm internal Ø, 1 mm wall thickness; Aeos ePTFE tubing; Zeus, Letterkenny, Ireland) was slid over the opening made in a PTFE tube and held in place with PTFE tape.

Sampling containers. Sampling containers, shown in cross section (Fig. 2a) and during sampling with a plant *in situ* (Fig. 2b), were constructed as follows. Holes (6 mm Ø) were drilled in the base of a 3 L container (SM, 19 cm Ø x 15 cm; Soparco, 61110 Condé-sur-Huisne, France), 2.5 cm apart, to hold the sampling tubes in position. Sampling tubes were inserted such that approximately 2.5 cm of the lower part of their length protruded below the pot. A ¼" PTFE ferrule (Thames Restek UK Ltd., Saunderton, UK, catalogue no. 21128) was placed around the base of each tube on the inside of the container, and a silicone rubber ring (Fisher Scientific UK Ltd., Loughborough, UK, catalogue no. QAK-165-Y) was placed on the outside, to support and seal the tubes in position. Sampling tubes covered with stainless steel mesh or microporous PTFE tubing were positioned such that the slots cut in the PTFE tube faced toward the centre of the pot. PTFE push-fit end caps (Perkin Elmer, Cambridge, UK, catalogue no. L4270122) were fitted to both ends of each tube and only removed during sampling (upper cap) and when inspecting the inside of the tube (both caps). When assembled, the 3 L container with the tubes attached (Fig. 1) was placed within a taller 4 L container (AKG Hortiproducts, Vroomshoop, The Netherlands) (Figs 2a and 2b). This enabled the sampling container to stand upright as normal, without the tube ends contacting the base of the 4 L container. Transplantation of the module grown broccoli plant involved filling the 3 L container with Levington M2 compost and positioning the plant between the sampling tubes which were gently positioned against either side of the module or "plug" of growing medium and root, with the top of the root plug at surface level. Controls were containers with sampling tubes in compost but without plants.

Experimental design. Each sampling container, with or without a plant, incorporated two sampling tubes. Experiments conducted with perforated PTFE and stainless steel mesh covered PTFE sampling tubes each had three replicates with a plant and one control without a plant. Experiments using PTFE sampling tubes covered with microporous PTFE had two replicates with a plant and one control without a plant. Sampling containers, including controls, were arranged in the glasshouse using a completely randomised design.

Induction of root volatiles. At the 8-10 true leaf stage plants in containers with the perforated PTFE and stainless steel mesh covered PTFE sampling tubes were infested with 150 freshly laid *Delia. radicum* eggs. Eggs suspended in water were carefully injected onto the growing medium next to the plant stem using a 60 mL plastic syringe and drinking straw. Controls without plants were also infested with the same number of *D. radicum* eggs. Plants in containers with PTFE sampling tubes covered with microporous PTFE tubing were mechanically damaged using a stainless steel spatula. The number of larvae which actually fed on roots is unknown. Whilst the eggs were viable at the point of infesting plants, 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.

Sampling of root volatiles. Each system was sampled once using SPME immediately prior to initiation of root damage. Systems using perforated PTFE sampling tubes and stainless steel mesh covered PTFE sampling tubes were sampled simultaneously at two further time points, 24 hours and one week (8 days), after estimated egg hatch and induction of volatiles by larval feeding damage, 24 hours after infestation with *Delia. radicum* eggs. Systems using PTFE sampling tubes covered with microporous PTFE tubing were sampled once immediately following mechanical damage to roots. SPME fibre exposure and volatiles entrainment time for all collections was 24 hours, under glasshouse conditions. Sampling schedules for experiments conducted with perforated tubes, stainless steel mesh and microporous tubing are given in Supporting Information, Tables S2 and S3.

Root volatiles were collected using a single fibre type, 65 μm Polydimethylsiloxane/-Divinylbenzene (PDMS/DVB) with a 23 gauge needle (Supelco, Sigma-Aldrich, UK, catalogue no. 57293-U). Fibres were conditioned at 250°C for 30 min in a flow of dry nitrogen according to manufacturer's guidelines before collections using a fibre conditioning

station (see SPME-GC-MS section below). For sampling, the fibre was attached to a SPME fibre holder for use with CTC autosamplers (Supelco, Sigma-Aldrich, UK, catalogue no. 57347-U). Prior to inserting the SPME fibre holder and fibre within its protective needle into the PTFE sampling tubes, the O-ring insert from a Merlin Microseal™ High Pressure Septum (Fig. 2d; Merlin Instrument Company, USA) was slid over the needle 10 mm from its end. This was followed by a 50 mm length of 5 mm Ø drinking straw (Morrisons, UK) which was pushed onto the end of the fibre assembly to keep the microseal spacer in place (Fig. 2c). Once inserted into the top of the PTFE sampling tube both served to create a seal between the inserted fibre holder and inner wall of the tube whilst keeping the fibre holder upright independent of any further support requirements. This also kept the needle straight and centred, ensuring the exposed fibre avoided contact with the internal wall of the tube during entrainment. When exposed, by fully depressing the fibre holder plunger, the fibre was situated approximately 5 cm below surface level. On completion of volatiles collection, the SPME fibre was retracted and the fibre and holder assembly was removed from the sampling tube. The microseal spacer and straw were then removed and the fibre assembly was detached from the fibre holder and transferred to a screw cap glass Pyrex® culture tube, purged with dry nitrogen, for storage. 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), fitted with a CTC CombiPAL autosampler configured for SPME with an attached SPME fibre conditioning station supplied with nitrogen purge gas at a fixed flow of 6 mL/min. (CTC Analytics, Switzerland). The volatiles were desorbed at 250°C by exposure of the fibre for 2 min within a programmed temperature vaporising (PTV) injector operating in constant temperature splitless mode and fitted with a Merlin Microseal™ High Pressure Septum and a Siltek™ deactivated metal PTV liner (120 mm x 1 mm internal Ø x 2.75 mm external Ø, Thermo Scientific, UK). On completion of desorption the fibre was reconditioned automatically within the fibre conditioning attachment. Separation of volatiles was achieved on a DB-1701 GC column (30 m x 0.25 mm internal Ø x 0.25 µm film thickness; Agilent Technologies, UK) using helium carrier gas at a flow rate of 1.5 mL/min in constant flow mode. The GC temperature programme was 40°C for 2 min, 10°C/min to 240°C then

isothermal at 240°C for 1 min. The GC-MS interface temperature was 250°C. After a 1 min delay, mass spectra were acquired at 6.5 scans/sec over the mass range of 25-400 u under electron ionisation (EI) conditions at 70 eV, with a source temperature of 200°C. The GC-MS was tuned daily using perfluorotertiarybutyl amine (PFTBA) with the instrument's autotune function.

Data were acquired and analysed using Xcalibur™ 2.0.7 (Thermo Electron Corporation, Hemel Hempstead, UK). Specific ions characteristic of each compound in the samples were selected by examination of the mass spectrum of each component in the total ion chromatogram (TIC) of several raw data files, representative of each stage of the experiments, using Xcalibur™ (Table 1). These ions, to be used for compound identification and measurement of raw abundance, were selected on the basis that they should have a high relative abundance, should be unique to the compound and/or the compound should be well resolved chromatographically from other compounds with ions with the same *m*/*z* (Cognat *et al.,* 2012). A defined time window centred on the chromatographic peak apex, along with the selected characteristic ions were used for compound detection and abundance measurement in a processing method created in Xcalibur™. A summed selected ion chromatogram (SIC) for all of the chosen ions within the appropriate time window was then generated and integrated. This value constituted the raw abundance of each compound. Processed data were checked for correct peak assignment and adjusted where necessary. Compounds were identified by comparison of their mass spectra and retention indices (Table 1) with those of reference standards where indicated in Table 1. Tentative identifications 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 Supporting Information, Table S1, for a list of references).

Sampling of root volatiles in headspace using Tenax TA and automated thermal desorption-gas chromatography-mass spectrometry (ATD-GC-MS)

Plant growth. At the 2-3 true leaf stage plants were transplanted, one per vessel, to the centre of 2 L Quickfit® flanged culture vessels (FV2L) lined with oven bags (25 cm x 38 cm; Tesco, UK) containing a 3:1 Levington M2 compost:sand mixture. Glasshouse conditions were as described for experiments using SPME. Vessels were watered when required.

Sampling vessels. Following transplanting, a five socket Quickfit® lid (MAF2/2) was placed on each vessel (Figs 3a and 3b) by gently passing the plant leaves through the centre socket. The flanged surface was covered with a thin layer of PTFE tape to provide a seal between the vessel and lid. The vessels were then placed in a 4 L container and watered immediately through the lid's side sockets, and then only when required thereafter. Plants were grown until 6-8 true leaves were established before preparing the vessel for headspace volatiles trapping using two adsorbent tubes per vessel. Controls were vessels containing compost without a plant, from which headspace samplings were similarly made using two adsorbent tubes per vessel.

Experimental design. Sampling vessels containing plants had four replicates. Control vessels without plants were replicated twice. Vessels, including controls, were arranged in the glasshouse using a completely randomised design.

Induction of root volatiles. At the 6-8 true leaf stage plants in sampling vessels were infested with 150 freshly laid *Delia. radicum* eggs as described above for sampling by SPME. Vessels without plants were similarly infested with 150 *D. radicum* eggs.

Root volatiles sampling. Volatiles were collected at two time points, once 12 days before infestation with *Delia. radicum* and once immediately upon commencement of larval feeding, 24 hours after infestation (see supplementary information, Table S4). Collections were for 48 hours under glasshouse conditions. Passive headspace sampling (Fig. 3b) was carried out using Silcosteel™ coated stainless steel absorbent tubes (89 mm x 6 mm), packed with 200 mg of Tenax TA (2.6-diphenylene oxide polymer resin, 60-80 mesh, surface area 35 m^2/g; Markes International, UK). The tubes were preconditioned by passing a stream of dry helium through them at 20 mL/min for 18 h (overnight) at 240°C, a procedure used successfully in our laboratories over many years (Robertson *et al.,* 1993). Conditioning activated the adsorbent phase and reduced the chemical background to an acceptable level. The inward-pointing ends of the adsorbent tubes positioned in the headspace were capped with diffusion caps with a sampling membrane (Perkin Elmer, Cambridge, UK) to restrict uptake of water by the adsorbent. DiffLok™ caps (Markes International, UK) were attached to the outward-pointing ends of tubes to prevent inward diffusion from the ambient environment. Immediately prior to volatiles collection, the contact joint between the vessel and lid flanges

was fastened with a metal clip to secure the two pieces of glassware together. The space between the plant stem and inside wall of the centre socket was filled with cotton wool. Prior to collection of volatiles from plants or controls, the headspace in each vessel was flushed with air, cleaned by passage through a Puritube column of activated charcoal, at a rate of 200 mL/min for 30 min. Two of the culture vessel lid's side sockets were sealed with stoppers while the remaining pair were closed off with cone/screw-thread adaptors (Quickfit® ST 52/13) coupled to adsorbent tubes. In both cases, PTFE tape was wrapped around the sealing surface. Following collection of volatiles, the adsorbent tubes were loaded onto an ATD autosampler for analysis of trapped volatiles by GC-MS.

ATD-GC-MS. Root volatiles were analysed using a UNITY™ thermal desorber with an UltrA TD™ autosampler (Markes International, UK) coupled to an Agilent 5975B GC-MS system (Agilent Technologies, UK). Sample tubes were heated at 240°C for 5 min in the primary desorption step to transfer the trapped compounds from the Tenax tube to a cryofocussing trap, also containing Tenax, maintained at −10°C. Subsequently, during secondary desorption, the cold trap was rapidly heated from −10°C to 240°C to transfer the volatiles onto a DB-1701 GC column (60 m x 0.25 mm x 1 µm film thickness; Agilent Technologies, UK) through a transfer line heated at 150°C. The oven temperature was initially 40°C increasing to 240°C at 5°C/min, and was then maintained at 240°C for 20 min. Helium carrier gas flow through both the ATD system and GC-MS was controlled by the ATD pneumatics at a constant pressure of 20 psi, which was equivalent to a flow rate in the analytical column of approximately 0.5 mL/min at 240°C. After a 2 min solvent delay, EI (70 eV) mass spectra were acquired at 1.33 scans/sec over the mass range 20-300 u with a source temperature of 230°C. Data were acquired using the MSD Chemstation software (G1710DA, Rev. D.03.00; Agilent Technologies, UK). Chemstation raw data files were converted to the Xcalibur™ format before processing. Specific characteristic ions for each compound were selected for compound detection and measurement of raw abundance in a processing method created in Xcalibur™, as described for the SPME-GC-MS data (Table 1). Where compounds were common with those in the SPME-GC-MS Xcalibur™ processing method, the same ions were used. The basis for compound identification etc. was as previously described. Variation between the two techniques in the calculated relative retention index (RRI) values for the same compounds are a consequence of the differences in

the sample introduction techniques, the use of different carrier gas flow modes and also to a lesser extent the lengths of GC column and stationary phase film thickness used.

Statistical analysis

A summary table showing mean abundances and standard errors (SE) for all volatiles for all experiments is shown in Supporting Information Table S5, and the full data sets are given in Tables S6 – S9.

Processed (raw abundance) data for volatiles detected in experiments using SPME for *in situ* sampling of volatiles within perforated PTFE sampling tubes and stainless steel mesh covered PTFE sampling tubes was investigated using principal component analysis (PCA) of the combined data sets using GenStat 16th Edition (VSN International Ltd., UK). PCA identifies the largest sources of variation amongst the samples and the volatiles which drive these. PCA can be carried out using either the sample variance–covariance matrix or the sample correlation matrix: the former focuses on the most abundant metabolites, whereas the latter standardises each metabolite, dividing by the standard deviation. All PCAs were performed on the correlation matrix. Data acquired from experiments using passive headspace sampling with Tenax were similarly subject to PCA. Given the limited number of datasets produced from the experiments and the dependency structure of plant replicatess over time (repeated analyses from the same plant), and among sampling wells within plant replicatess, it was not possible to construct a robust statistical model for testing even with a mixed model approach. For many of the volatiles there were also samples in which their abundances were below the level of detection pre-damage, further reducing the ability of restricted maximum likelihood methods (REML) to estimate parameters.

Results and discussion

Method development

The general experimental design described in the Experimental section was selected following a series of preliminary experiments using both perforated tubes and those covered with stainless steel mesh. In its initial form the sampling system used four sampling tubes in which two pairs of SPME fibres of different chemistries could be used simultaneously in a cross arrangement with each pair positioned at opposite sides of the plant to account for the heterogeneous distribution of roots in substrate/soil (Hinsinger *et al.,* 2005; Weidenhamer *et al.,* 2009).

The sampling concept was first evaluated by mechanically damaging the plant roots. From a visual inspection of the unprocessed data (not shown) it was found that a wide range of induced root-derived volatiles could be detected, which were similar in composition to those previously reported to arise from root damage to *Brassica* plants (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). This established the general feasibility of the *in situ* sampling approach using a pre-positioned sampling well, and also provided a potential yardstick by which to assess the effectiveness of subsequent experiments using *Delia. radicum* larvae to induce root volatiles associated with root damage.

Further experiments showed that when plants were infested with eggs of *Delia. radicum* many of the same volatiles detected following mechanical damage were subsequently detected in the sampling wells at considerably enhanced levels in comparison with samples taken pre-infestation. This suggested that herbivory-induced root volatile production following egg hatch was being detected *in situ* and that the sampling system was sufficiently sensitive to do so. Furthermore, by collecting samples at repeated intervals from the same sampling wells it was possible to show the dynamic progress of volatile signal production following infestation. This is illustrated by the temporal abundance profiles for several key volatiles trapped simultaneously from a single plant over a 20 hour period at approximately weekly intervals on fibres with the PDMS/DVB and Carboxen/polydimethylsiloxane (CAR/PDMS) chemistries in a dual use experiment (Fig. 4). The sampling schedule and full dataset including all volatiles detected are shown in Supporting Information, Tables S10 and S11. This was one of several 'look see' dual-use

experiments where different sampling regimes (timing and duration) were evaluated. The data from these other experiments were reviewed visually and not processed. The low background level of damage induced volatiles observed before induction of damage probably arose from the effects of normal root growth through the growth medium. It was found that addition of sand to the compost growth medium (1 to 3 ratio) increased the observed backround level of these compounds, but not to the levels seen following induced damage. Although CAR/PDMS fibres appeared to trap larger quantities of volatiles, both fibre types were similarly able to detect and characterise the dynamic changes following infestation (Fig 4). However, PDMS/DVB fibres were found to give better chromatographic resolution during GC-MS analysis. Furthermore the shorter fibre conditioning period of 30 min, as opposed to 60 min for CAR/PDMS, integrated more effectively with the analysis when using the fibre conditioning station, increasing sample throughput. Since combined use of two fibres did not offer any advantage, it was decided to use PDMS/DVB fibres in subsequent development and validation of the method.

From our preliminary methods development experiments where several samples were taken over extended periods prior to infestation with larvae or mechanical damage (e.g. Fig. 4), we never observed the type of dramatic increase in volatiles production we found following damage. Indeed some compounds were not detected at all pre-damage. From this we concluded that although volatiles production might increase with time due to plant growth, this would likely be small compared to the changes post-damage. Consequently we considered that the pre-damage volatiles profile would serve as a damage-free control and in the subsequent validation experiments, samples taken immediately pre-damage served this purpose. Plant-free pots were used as controls to determine the chemical background due to the growth medium. However, it is possible that processes such as root maturation and senescence contributed to the increase in volatiles production following infestation with larvae or mechanical damage.

Method validation

Sampling of root volatiles in situ using SPME

Our results demonstrate that porous tubes *in situ* next to growing plant roots can support an SPME fibre holder in position while protecting the fragile fibre and protective fibre sheath during sampling of root volatiles. Sampling approaches using SPME with both perforated and mesh covered PTFE sampling tubes were found to have comparable sensitivity for

detecting volatiles released by broccoli roots. In addition, each method simililarly detected temporal changes in broccoli root volatiles emissions following damage by larvae of *Delia. radicum*.

Excluding contaminants related to fibre chemistry and impurities such as plasticizers, over the course of the experiments a maximum of around 120 compounds were detected in the root volatiles profiles from samples entrained using SPME within each of the different designs of PTFE sampling tube from damaged and undamaged plants (Table 1). Sulphur compounds including alkyl sulphides and isothiocyanates (24-26 examples) were most numerous followed by hydrocarbons including C_6-C_{17} *n*-alkanes (12-14 examples). Lesser amounts of aldehydes (7) including *n*-homologues (C_6-C_{11}), ketones (6-8) and terpenes (5-6) were present with only a few examples each of alcohols (2), acids and esters (3), alkyl furans (1-2) , nitriles (1), and other components (3-4). In addition there were a substantial number (18-19) of unknown compounds.

Comparison of chromatographic profiles from intact roots with roots damaged by larval feeding showed that injured root tissue was particularly associated with an increase in the number and abundance of compounds detected (Figs 5a, 5b; Supporting Information, Figs S1, S2 for expanded annotated chromatograms; Table 1).

Principal among these were the sulfur containing volatiles such as the alkyl sulphides methanethiol (**3**), dimethyl sulphide (**6**) dimethyl disulphide (DMDS) (**25**), dimethyl trisulphide (DMTS) (**60**) and, 2,4-dithiapentane (2,4-DTP) (**43**), and the isothiocyanates butyl isothiocyanate and 2-butyl isothiocyanate (**53** and **54**) which are characteristic of plants in the Brassicaceae family (Stoewsand 1995; Edmands *et al.,* 2013). Bar charts showing raw abundance measurements + standard errors for these and several other related compounds before and after damage are shown in Fig 6. These findings agree with those reported from previous studies of the effect of *Delia. radicum* larval infestation on emission of induced volatiles from *Brassica* roots (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).

Principal components analysis (PCA) further revealed that for both methods samples collected from undamaged roots (date 1) and 24 hours following infestation with *Delia. radicum* (date 2) were compositionally similar. However, samples collected one week post-infestation (date 3), while still similar for the two methods, were very different from those collected earlier due to the presence of induced volatiles related to larval feeding damage (Fig. 7a). A plot of PC 1 versus PC 2 shows separation of samples from collection date 3 from samples from collection dates 1 and 2 mainly on PC score 2. PC 1 and PC 2 accounted

for 17.5 and 15.8% of the total variance, respectively. Compounds characteristic of post infestation date 3 have high negative loadings for PC 2 and consisted primarily of sulphur compounds including those mentioned above. A selected list of loadings for PC2 is shown in Table 2 and the full list is given in Supporting Information, Table S12). Compounds with high positive loadings on PC2 were predomonantly *n*-alkanes in the range C_6-C_{14} and *n*-aldehydes in the range C_6-C_{12}. These were present in samples and controls before and after inoculation with larvae at broadly similar levels of abundance, although in some instances their abundance was greater pre-inoculation. Taken together, these components constitute the chemical background of the system, possibly associated with the growth matrix (compost). There may also be a contribution from brief exposure of fibres to general laboratory and glasshouse atmosphere during sample handling.

Although the profiles of root volatiles detected using both methods were similar, PCA did reveal differences relating to tube type. PC 1 versus PC 4 shows separation of samples and controls collected in perforated tubes from those collected in mesh covered tubes on PC score 4 (Fig. 7b). PC 1 and PC 4 accounted for 17.5 and 8.5% of the total variance, respectively. However, there was no clear evidence in these results of tube type compound selectivity or discrimination based on compound class, molecular weight, vapour pressure, or polarity. Volatiles that mainly contributed to the observed difference were from a number of classes and are shown in the loadings for PC 4 (selected list in Table 2, full list in Supporting Information, Table S12). The separation hints that the perforated tubing may in some way favour trapping of the longer alkanes and aldehydes (high positive loadings), whereas the mesh covered tubes may favour trapping of shorter homologs (high negative loadings).

A pilot experiment using microporous PTFE tubing as an alternative to stainless steel mesh for sampling *in situ* using SPME, where roots were only damaged mechanically, showed that broccoli root volatiles could be detected pre- and post-mechanical damage, using this type of commercially available porous tubing (Fig. 5c; Supporting Information, Fig. S3a). Overall, the range of compounds identified was similar to those found using the other tube designs for sampling *in situ* (Table 1). PCA showed that there was some overlap among root volatiles samples and controls collected pre-infestation and post-infestation. A plot of PC 1 versus PC 2 (Fig. 8) shows separation of three post-damage samples, two from plant 2 (P2) and one from plant 1 (P1) from three of the pre-damage samples (P1, P1, P2), the pre-damage controls (C) and to a lesser extent the post-damage controls (C) on PC score 1. The pre- and post-damage controls also separate on PC 1. In addition, a single post-damage sample from plant 1, sampling well 2 (P1) is separated from the other samples along PC score

2. PC 1 and PC 2 accounted for 29.4 and 24.9% of the total variance, respectively. Compounds that contributed the most to the differences were predominantly sulphur containing damage-induced volatiles with high negative loadings on PC1 and PC2 (selected list in Table 3, full list in Supporting Information, Table S13). Among those driving separation along PC1, as shown in (Fig 5c, Supporting Information,Fig. S3a), were dimethyl disulphide (**25**), dimethyl trisulphide (**60**) and S-methyl methanethiosulphonate (**100**). Separation along PC2, shown in Fig. S3b for sample P1, sampling well 2, was largely driven by a different group of compounds, primarily isothiocyanates, including 4-methylthiobutyl isothiocyanate (**117**) and 2-phenylethyl isothiocyanate (**118**). Some compounds such as dimethyl disulphide (**25**), 2-methybutyl isothiocyanate (**79**) and 3-methylbutyl isothiocyanate (**80**) contributed to separation along both PC scores but to differing extents. Mean raw abundance measurements + standard errors for these compounds before and after damage are shown in Fig. 6. Differences between the post-damage samples from plant 1, which were collected from sampling wells on opposite sides of the plant, may reflect non-uniformity in the extent of root mechanical root damage. In the initial stages of method development we found that release of large amounts of isothiocyanates was a characteristic of severe mechanical damage to roots.

Mean abundances and standard errors for individual compounds entrained using SPME within perforated PTFE tubing, mesh covered PTFE tubing and PTFE tubing covered with micropourous PTFE are shown in Supporting Information, Table S5. Raw data sets for experiments conducted using the three sampling techniques are listed in Supporting Information, Tables S6, S7 and S8 respectively.

Sampling of root volatiles in headspace using Tenax TA

During method development the range of compounds detected using our passive *in situ* sampling approach with SPME-GC-MS, was compared with that detected using an alternative sampling method in which the headspace above the growing substrate of similar plants was sampled passively using Tenax TA and analysed by ATD-GC-MS. Brassica root volatiles have been sampled previously in above ground headspace (Crespo *et al.,* 2012; Danner *et al.,* 2012 and van Dam *et al.,* 2012). However, their method differed substantially in that volatiles were sampled actively and analysed on-line using proton-transfer-reaction mass spectrometry (PTR-MS). Overall the distribution by compound classes trapped on Tenax was similar to those collected using SPME, however fewer compounds (57) were present (Table 1). The major difference was a reduction in the number of sulphur compounds

(16), ketones (4) and terpenes (1) and unknowns (2) detected within the headspace samples. Marked differences were seen in the abundance of volatiles detected before and after feeding damage by *Delia. radicum* larvae using ATD-GC-MS (Fig. 5d; Fig S4). In particular, elevated levels of dimethyl sulphide (**6**) dimethyl disulphide (DMDS) (**25**), methyl thiocyanate (**29**), dimethyl trisulphide (DMTS) (**60**), 2,4-dithiapentane (2,4-DTP) (**43**), butyl isothiocyanate (**53)** and 2-butyl isothiocyanate (**54**) were detected following damage (Fig. 6).

PCA showed distinct separation of post- and pre-infestation samples as seen in a plot of PC 1 versus PC 3 (Fig. 9). PC 1 and PC 3 accounted for 46.6 and 10.2% of the total variance, respectively. Compounds that contributed to the separation observed have high positive loadings for PC 1 (selected list in Table 4; full list in Supporting Information, Table S14), and were composed largely of volatiles that are typically enhanced following *Delia. radicum* root feeding including those mentioned above. Although fewer sulphur volatiles (headspace) were trapped on Tenax following feeding damage than was the case when using SPME during the *in situ* sampling methods, the sulphur compounds showing the greatest enhancement were the same.

Mean abundances and standard errors for individual compounds trapped on Tenax are shown in Supporting Information, Table S5, and the raw data set is listed in Table S9.

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

Distinct from methods that sample root volatiles from plants removed from their growing container or substrate (Rasmann *et al.,* 2011; Robert *et al.,* 2012; Ghimire *et al.,* 2013) or excised roots (Rasmann *et al.,* 2005; Ferry *et al.,* 2007; Gfeller *et al.,* 2013), *in situ* SPME sampling methods such as the one developed in this study permit non-invasive collection of root volatiles. Crucially, this avoids disturbing the root system, which could potentially misrepresent the profile of volatiles emitted by the roots due to inadvertent damage. Since our method involved transplantation of young plants into pre-prepared growth vessels, sufficient time was allowed between transplantation and volatiles collection for the root tips and root hairs to recover from the traumatic replanting event. The pattern of volatiles collected would then reflect normal growth and the subsequent effects of induced damage and not be a carry-over from damage caused during the transplantation process. Although SPME has previously been used for collection of volatiles below ground (Jassbi *et al*., 2010; Weissteiner *et al*., 2012), this involved excavation of sampling recesses 10 cm from the stems of growing plants, and it is unclear how damage was avoided.

Different from headspace entrainment of root volatiles either trapped from whole plants in an enclosure (Soler *et al.,* 2007; Pierre *et al.,* 2011; van Dam *et al.,* 2010) or at/just above soil level (Crespo *et al.,* 2012; Danner *et al.,* 2012; van Dam *et al.,* 2012), our *in situ* SPME sampling methods facilitate collection from the immediate vicinity of growing roots. In the method using SPME described by Jassbi *et al*., 2010 a relatively wide hole (3 x 10 cm) was excavated adjacent (10 cm) to the stem of a growing plant and a polypropylene sampling enclosure (50 mL volume) was partly inserted in the hole such that the SPME fibre was exposed above the hole. The total volume of the headspace (hole + enclosure) was approximately 100 – 120 mL. Our pre-positioned device has an internal volume of 3.4 mL, consequently the fibre is located much closer to the source of volatiles, furthermore the design allows for root growth around the sampling enclosure. On disassembly of the sampling apparatus after the completion of experiments it was found that root systems were intimately associated with the sampling tubes, with lateral roots extending beyond the immediate sampling region. The diffusion of root derived volatiles below-ground is influenced by the chemical properties of the volatile, the physicochemical properties of the growing substrate and the activity of subterranean microorganisms (Insam and Seewald 2010; Effmert *et al.,* 2012). Differences in concentrations of volatiles can, therefore, occur over short distances below-ground (D'Alessandro and Turlings 2006; Hiltpold and Turlings 2008). Because *in situ* SPME sampling allows volatiles sampling next to roots, loss of signal due to diffusion, degradation or adsorption within the surrounding substrate is likely to be minimised. This may be a contributing factor to the reduction in the number of volatiles, particularly sulphur compounds, trapped in the above surface headspace using Tenax in our experiments.

A soil probe developed by Ali *et al.,* (2012) for *in situ* analysis of herbivore induced volatiles from citrus roots (*Citrus paradisi* Macf. x *Poncirus trifoliata* L. Raf.) employed flow-through dynamic sampling via a vacuum pump and adsorbent traps for entrainment and solvent elution for compound isolation. Eilers *et al.* (2015) recently described a three chamber mesocosm in which volatile root-derived compounds were sampled passively *in situ* by adsorption onto polydimethysiloxane (PDMS) tubes. However, the sampling tubes were inserted into the growth matrix once plants had become established, and were subsequently removed prior to analysis of volatiles by thermal desorption and GC-MS. In contrast, our *in situ* SPME methods use passive non-invasive sampling and solvent free extraction in combination with a relatively simple design for ease of assembly, portability and use. These features make them highly suitable for remote out-of-lab sampling (e.g. glasshouse or field).

Single point in time collection, and repeated sampling from the same spatial location to investigate temporal patterns in root volatiles is possible using these methods (Fig. 4). The option of repeated sampling, in particular, is of key relevance for dynamic root-insect interactions, where changing below-ground signal concentrations which are spatially distinct can cause important behavioural effects on below-ground herbivores (in preparation). The use of the sampling system to detect and follow the dynamics of volatiles production from roots undergoing *Delia. radicum* induced herbivory, has the potential to be used to study such interactions in a full field trial over extended periods of time. An evaluation of the practicality of using the *in situ* method within a field situation will be described in a subsequent paper (Deasy *et al*., 2016).

From a functionality perspective, the perforated PTFE tubes were easier to construct, more robust during use and suitable for cleaning and re-use compared to the stainless steel mesh covered PTFE tubes. The aim of testing the microporous PTFE tubing was to determine whether manually perforated tubes could be replaced with a prefabricated version. It was envisaged, ideally, that microporous PTFE tubing should have similar dimensions and structural properties to the PTFE tubing used for making the perforated tubes. However, we were unable to source tubing with these properties. The tubing that was used was quite flaccid and unable to support itself. Therefore the microporous PTFE tubing was supported over a pre cut window in a PTFE tube, similar to how the stainless steel mesh covered tubes were assembled. However, in use these sampling tubes were less effective than either the perforated or mesh-covered tubes, consequently we discontinued their evaluation prior to testing with root fly larvae. Future research could continue to explore the potential of using more rigid microporous PTFE tubing to create a more refined method for *in situ* root volatiles collection. Although separate undamaged plants were not used as controls during evaluation of this methodology, use of such controls is highly recommended for future application of this technique.

Quantification of volatiles by SPME is possible by adding appropriate standard mixtures for calibration into the sample (Shirey 2006), but can be both difficult and impractical to achieve (Tholl *et al.,* 2006). *In situ* SPME experiments here employed qualitative measurements using raw signal levels without internal standards. This approach was sufficient for the fulfilment of the aims of these experiments, that is, the non-invasive sampling and identification of volatile compounds emitted by broccoli roots and the detection of time-dependent changes pre- and post-root damage. Although quantification was beyond the scope of this work, development of an applicable quantification method is planned for

future studies. Possible methods might include addition of a standard/mixture of standards into the growing substrate (Higashikawa *et al.,* 2013) or into an *in situ* sampling tube immediately prior to exposing the fibre for collection. Alternatively, it may be possible to briefly expose the pre-loaded fibre in a headspace vial containing a standards mixture just before desorption. Key considerations would be identifying the correct volume of standard compound to apply to avoid overloading the fibre, and reproducibility. It should also be noted that the characteristics of the compounds and growing substrate are likely to impact recovery of volatiles.

Since we ultimately wanted to trial the method with field grown plants, we concluded that passive sampling with SPME would be simpler to manage. The method was considered to have the benefit of relatively low cost, simplicity, and did not require the use of additional specialised equipment such as an ATD sampler, pumps and filters for active entrainment. However, SPME does have drawbacks. Passive trapping of volatiles on an SPME fibre is not a particularly sensitive technique since it involves establishment of an equilibrium between volatiles in the the gas phase (sampling device) and those in the liquid phase (fibre). Consequently only a small proportion of the volatiles present in the sampling volume will be collected on the fibre, in comparison with active entrainment on a porous polymer such as tenax, and their composition may differ from that in the surrounding volume. Use of a low sampling volume, as in our device, may increase the proportion of trapped volatiles and accelerate attainment of equilibrium. In our experiments, a high loading of insects was used to maximise the extent of damage. We have not tested the method yet with lower numbers of insects. It might be necessary to further develop the sampling system to increase the capture of volatiles by increasing the number of perforations or to re-evaluate the use and design of sampling tubes employing stainless steel mesh. In our preliminary dual use experiments using DVB/PDMS and carboxen/PDMS fibres, there was some indication that the latter may trap more volatiles. Thus use of carboxen/PDMS or a tri adsorbent SPME fibre combination such as carboxen/PDMS/DVB might increase trapping efficiency and should be investigated further. It is likely also that use of a more sensitive mass spectrometer than the quadrupole used in this investigation, such as a GC-Time of Flight (TOF) MS instrument, might increase the overall sensitivity of the method. We had previously found in studies of the development of off flavours in food, that use of passive headspace sampling using SPME in combination with a GC-(TOF) MS instrument provided levels of sensitivity comparable to use of active entrainment on Tenax and ATD-GC-MS employing a quadruple mass analyser (Cognat *et al*., 2012). The variously perforated ¼ inch PTFE tubes used in our sampling system can be

directly coupled to standard ¼ inch diameter sorbent tubes using appropriate fittings as an alternative approach for passive or active sampling *in-situ*. Although we did not persue this, evaluation of the sampling device for active entrainment could be investigated. Manual aspiration of a small volume of air (5-10 mL) from the PTFE tube through a sorption tube containing Tenax or other sorbant might be practical using a syringe as an alternative to a powered pumping system. There may also be scope for development of the device as a sampling probe. This would require provision of a robust permanent seal at one end of the PTFE tubing to prevent coring on insertion into the growth medium, since the end caps used in our method to seal the open tubing would not be adequate for this purpose.

Overall, each of the tube types tested combined with SPME-GC-MS similarly characterised the chemical differences between the profiles of volatiles emitted by intact and damaged roots. The main distinguishing feature between the methods was robustness. For this reason, perforated PTFE tubes were selected as the most suitable sampling method for use in subsequent field studies (Deasy *et al*., 2016). This technique has potential for wide application in chemical ecology/root/soil research.

Acknowledgements

This research project was funded by the Horticultural Development Company (HDC; Studentship FV 364). We also thank Jim McNicol (BioSS) and Susan Verrall (The James Hutton Institute) for statistics and data analysis advice, Geir Kjølberg Knudsen (Bioforsk, Norway) for providing stainless steel mesh, Brian Clarke (Zeus Ltd, Ireland) for supplying Aeos ePTFE tubing and Sakata UK Ltd. for contributing seeds.

References

Ali JG, Alborn HT, Campos-Herrera R, Kaplan F, Duncan LW, Rodriguez-Saona C, Koppenhöfer AM, Stelinski LL. 2012. Subterranean, herbivore-induced plant volatile increases biological control activity of multiple beneficial nematode species in distinct habitats. *PloS One 7(6)*, DOI:10.1371/journal.pone.0038146.

Campos-Herrera R, Ali JG, Diaz BM, Duncan LW. 2013. Analyzing spatial patterns linked to the ecology of herbivores and their natural enemies in the soil. *Front Plant Sci* **4**: 1-18.

Cognat C, Shepherd T, Verrall SR, Stewart D. 2012. Comparison of two headspace sampling techniques for the analysis of off-flavour volatiles from oat based products. *Food Chem* **134**: 1592-1600.

Crespo E, Hordijk CA, de Graaf RM, Samudrala D, Cristescu SM, Harren FJ, van Dam NM. 2012. On-line detection of root-induced volatiles in *Brassica nigra* plants infested with *Delia radicum* L. root fly larvae. *Phytochemistry* **84**: 68-77.

D'Alessandro M, Turlings TCJ. 2006. Advances and challenges in the identification of volatiles that mediate interactions among plants and arthropods. *Analyst* **131**: 24-32.

Danner H, Samudrala D, Cristescu SM, Van Dam NM. 2012. Tracing hidden herbivores: time-resolved non-invasive analysis of belowground volatiles by proton-transfer-reaction mass spectrometry (PTR-MS). *J Chem Ecol* **38**: 785-94.

Deasy W, Shepherd T, Alexander CJ, Birch ANE, Evans KA. 2016. Field-based evaluation of a novel SPME-GC-MS method for investigation of below ground interaction between brassica roots and larvae of cabbage root fly, *Delia radicum*. *Phytochem Anal*, following paper.

Edmands WMB, Gooderham NJ, Holmes E, Mitchell SC. 2013. S-Methyl-L-cysteine sulphoxide: the Cinderella phytochemical? *Toxicol Res* **2**: 11-22.

Effmert U, Kalderás J, Warnke R, Piechulla B. 2012. Volatile mediated interactions between bacteria and fungi in the soil. *J Chem Ecol* **38**: 665-703.

Eilers EJ, Pauls G, Rillig MC, Hansson, BS, Hilker M, Reinecke A. Novel set-up for low disturbance sampling of volatile and non-volatile compounds from plant roots. 2015. *J Chem Ecol* **41** 253-66.

Ferry A, Dugravot S, Delattre T, Christides JP, Auger J, Bagneres AG, Poinsot D, Cortesero AM. 2007. Identification of a widespread monomolecular odor differentially attractive to several *Delia radicum* ground-dwelling predators in the field. *J Chem Ecol* **33**: 2064-77.

Gfeller A, Laloux M, Barsics F, Kati DE, Haubruge E, du Jardin P, Verheggen FJ, Lognay G, Wathelet JP, Fauconnier ML. 2013. Characterization of volatile organic compounds emitted by barley (*Hordeum vulgare* L.) roots and their attractiveness to wireworms. *J Chem Ecol* **39**: 1129-39.

Ghimire RP, Markkanen JM, Kivimäenpää M, Lyytikäinen-Saarenmaa P, Holopainen JK. 2013. Needle removal by pine sawfly larvae increases branch-level VOC emissions and reduces below-ground emissions of Scots pine. *Environ Sci Technol* **47**: 4325-32.

Higashikawa FS, Cayuela ML, Roig A, Silva CA, Sánchez-Monedero MA. 2013. Matrix effect on the performance of headspace solid phase microextraction method for the analysis of target volatile organic compounds (VOCs) in environmental samples. *Chemosphere* **93**: 2311-18.

Hiltpold I, Turlings TCJ. 2008. Belowground chemical signaling in maize: When simplicity rhymes with efficiency. *J Chem Ecology* **34**: 628-35.

Hinsinger P, Gobran GR, Gregory PJ, Wenzel WW. 2005. Rhizosphere geometry and heterogeneity arising from root-mediated physical and chemical processes. *New Phytol* **168**: 293-303.

Insam H, Seewald MSA. 2010. Volatile organic compounds (VOCs) in soils. *Biol Fertil of Soils* **46**: 199-213.

Jassbi AR, Zamanizadehnajari S, Baldwin IT. 2010. Phytotoxic volatiles in the roots and shoots of *Artemisia tridentata* as detected by headspace solid-phase microextraction and gas chromatographic-mass spectrometry analysis. *J Chem Ecol* **36**: 1398 -1407.

Mohney BK, Matz T, Lamoreaux J, Wilcox DS, Gimsing AL, Mayer P, Weidenhamer JD. 2009. *In situ* silicone tube microextraction: a new method for undisturbed sampling of root-exuded thiophenes from marigold (*Tagetes erecta* L.) in soil. *J Chem Ecol* **35**: 1279-87.

Peñuelas J, Asensio D, Tholl D, Wenke K, Rosenkranz M, Piechulla B, Schnitzler JP. 2014. Biogenic volatile emissions from the soil. *Plant, Cell Environ* **37**: 1866-91.

Pierre PS, Jansen JJ, Hordijk CA, van Dam NM, Cortesero AM, Dugravot S. 2011. Differences in volatile profiles of turnip plants subjected to single and dual herbivory above- and belowground. *J Chem Ecol* **37**: 368-77.

Rasmann S, Erwin AC, Halitschke R, Agrawal AA. 2011. Direct and indirect root defences of milkweed (*Asclepias syriaca*): trophic cascades, trade-offs and novel methods for studying subterranean herbivory. *J Ecol* **99**: 16-25.

Rasmann S, Hiltpold I, Ali J. 2012. The role of root-produced volatile secondary metabolites in mediating soil interactions. In *Advances in Selected Plant Physiology Aspects*. Montanaro G and Bartolomeo D (Eds.). Tech Open Access Publisher,: Rijeka, 269-90.

Rasmann S, Kollner TG, Degenhardt J, Hiltpold I, Toepfer S, Kuhlmann U, Gershenzon J, Turlings TCJ. 2005. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature* **434**: 732-7.

Robert CA, Erb M, Duployer M, Zwahlen C, Doyen GR, Turlings TCJ. 2012. Herbivore-induced plant volatiles mediate host selection by a root herbivore. *New Phytol* **194**: 1061-9.

Robertson GW, Griffiths DW, Woodford JAT, Birch ANE, Picket JA, Wadhams LJ. 1993. A comparison of the flower volatiles from hawthorn and four raspberry cultivars. *Phytochemistry* **33**: 1047-53.

Schomburg G. (1990) *Gas Chromatography. A Practical Course.* VCH: Weinheim; 293.

Shirey R. 2006. Developing a quantitative SPME method. *The Reporter Europe* **22**: 13-14. http://www.sigmaaldrich.com/technical-documents/articles/reporter-eu/developing-a-quantitative.html [Accessed 17 March 2014].

Soler R, Harvey JA, Kamp AFD, Vet LEM, Van der Putten WH, Van Dam NM, Stuefer JF, Gols R, Hordijk CA, Martijn Bezemer T. 2007. Root herbivores influence the behaviour of an aboveground parasitoid through changes in plant-volatile signals. *Oikos* **116**: 367-76.

Stoewsand GS. 1995. Bioactive organosulfur phytochemicals in *Brassica oleracea* vegetables-A review. *Food Chem Toxicol* **33**: 537-43.

Tholl D, Boland W, Hansel A, Loreto F, Rose US, Schnitzler JP. 2006. Practical approaches to plant volatile analysis. *Plant J* **45**: 540-60.

van Dam NM. 2014. Plant chemical ecology finally gets to its root(s). *J Chem Ecol* **40**: 220-1.

van Dam NM, Qiu BL, Hordijk CA, Vet LE, Jansen JJ. 2010. Identification of biologically relevant compounds in aboveground and belowground induced volatile blends. *J Chem Ecol* **36**: 1006-16.

van Dam NM, Samudrala D, Harren FJM, Cristescu SM. 2012. Real-time analysis of sulfur-containing volatiles in *Brassica* plants infested with root-feeding *Delia radicum* larvae using proton-transfer reaction mass spectrometry. *AoB Plants.* pls021. DOI:10.1093/aobpla/pls021.

Weidenhamer JD, Boes PD, Wilcox DS. 2009. Solid-phase root zone extraction (SPRE): A new methodology for measurement of allelochemical dynamics in soil. *Plant and Soil* **322**: 177-86.

Weissteiner S, Huetteroth W, Kollman M, Weißbecker B, Romani R, Schachtner J, Schütz S. 2012. Cockchafer larvae smell host root scents in soil. *PLoS One* 7, e45827. DOI:10.1371/journal.pone.0045827.

Table 1. Compounds identified in broccoli root volatiles samples by SPME-GC-MS and ATD-GC-MS before and after larvae-induced or mechanical damage. Samples were trapped *in situ* within (1) perforated PTFE tubes, (2) stainless steel mesh covered PTFE tubes, (3) microporous PTFE tubing covered PTFE sampling tubes, and (4) within above ground headspace using Tenax TA.

				SPME		ATD					
No.	Compound	MW	Selected Ions	t_R	RRI	t_R	RRI	Collection method			
1	Sulphur dioxide	64	48, 64	1.17	556	4.66	452	1	2	3	4
2	Acetaldehyde	44	41, 42, 43, 44, 45, 46			5.12	480	*nd*	*nd*	*nd*	4
3	Methanethiol	48	44, 45, 46, 47, 48, 49, 50	1.23	564	5.14	481	1	2	3	4
4	1-Propanethiol	76	76	1.43	592			1	2	*nd*	*nd*
5	Pentane[a]	72	43, 57, 72			5.44	500	*nd*	*nd*	*nd*	4
6	Dimethyl sulphide[a]	62	47, 61, 62	1.43	592	6.54	568	1	2	3	4
7	Carbon disulphide[a]	76	32, 38, 44, 64, 76, 78, 80			5.56	569	*nd*	*nd*	*nd*	4
8	Hexane[a]	86	43, 56, 57, 71, 86	1.49	600	7.06	600	1	2	3	4
9	Acetone[a]	58	58	1.50	601	6.89	590	1	2	3	4
10	3-Methylfuran	82	39, 50, 51, 53, 54, 81, 82			8.69	661	*nd*	*nd*	*nd*	4
11	2,3-Dimethyl-2-butene	84	69, 84	1.71	630			1	*nd*	3	*nd*
12	2-Methylfuran	82	39, 50, 51, 53, 54, 81, 82	1.90	656			*nd*	*nd*	3	*nd*
13	2-Butanone[a]	72	72	2.10	684	9.50	691	1	2	3	4
14	2,3-Butanedione[a]	86	86			9.55	693	*nd*	*nd*	*nd*	4
15	Heptane[a]	100	43, 56, 57, 71, 100	2.22	700	9.74	700	1	2	3	4
16	Unknown 718		41, 42, 43, 45, 55, 56, 57, 70			10.39	718	*nd*	*nd*	*nd*	4
17	2-Methyl-1-Propanol	74	31, 33, 39, 41, 42, 43, 74			10.82	731	*nd*	*nd*	*nd*	4
18	S-Methyl thioacetate	90	42, 43, 44, 45, 46, 47, 48, 75, 90	3.03	765			1	*nd*	*nd*	*nd*
19	Isobutyronitrile[a]	69	39, 41, 42, 52, 53, 54, 68			11.56	752	*nd*	*nd*	*nd*	4
20	Acetic acid 758[a]	60	43, 45, 60			11.77	758	*nd*	*nd*	*nd*	4
21	Acetic acid 775[a]	60	43, 45, 60	3.16	775			1	*nd*	3	*nd*
22	3-Pentanone[a]	86	39, 42, 56, 57, 58, 86	3.23	781			1	2	3	*nd*
23	2,4-Dimethylfuran	96	39, 41, 43, 53, 65, 67, 81, 95, 96			12.12	768	*nd*	*nd*	*nd*	4

No.	Compound	MW	Selected Ions	SPME t_R	SPME RRI	ATD t_R	ATD RRI	Collection method			
24	Octane[a]	114	43, 57, 71, 85	3.47	800	13.26	800	1	2	*nd*	4
25	Dimethyl disulphide[a]	94	45, 46, 47, 61, 64, 79, 94, 96	3.60	808	14.24	824	1	2	3	4
26	Acetic acid 808[a]	60	43, 45, 60	3.61	808			*nd*	2	*nd*	*nd*
27	3-Methyl-1-butanol	88	39, 41, 42, 43, 55, 57, 70			14.94	841	*nd*	*nd*	*nd*	4
28	3-Methylbutyronitrile	83	39, 41, 42, 43, 44, 55, 68			15.42	853	*nd*	*nd*	*nd*	4
29	Methyl thiocyanate[a]	73	45, 46, 47, 58, 72, 73	4.30	850	16.04	868	1	2	3	4
30	Acetic acid 862[a]	60	43, 45, 60	4.50	862			*nd*	*nd*	3	*nd*
31	Butyl acetate	116	41, 43, 55, 56, 57, 61, 73			16.41	877	*nd*	*nd*	*nd*	4
32	1,3-Dithiethane	92	45, 77, 92, 94	4.80	881			1	*nd*	*nd*	*nd*
33	Hexanal[a]	100	44, 56, 57, 72, 82, 100	4.81	881	16.92	889	1	2	3	4
34	Nonane[a]	128	43, 56, 57, 71, 85	5.12	900	17.35	900	1	2	3	4
35	Methyl ethyl disulphide	108	45, 46, 47, 64, 79, 80, 108			17.96	916	*nd*	*nd*	*nd*	4
36	Unknown 929		65, 77, 79, 91, 92, 93, 105, 121, 136			18.47	929	*nd*	*nd*	*nd*	4
37	1-Nonene	126	43, 56, 69, 84, 97	5.14	901			1	2	*nd*	*nd*
38	Isopropyl isothiocyanate[a]	101	39, 41, 42, 43, 59, 60, 86, 101			19.08	944	*nd*	*nd*	*nd*	4
39	Acetic acid 905[a]	60	43, 45, 60	5.21	905			*nd*	2	*nd*	*nd*
40	Unknown 947		65, 77, 79, 91, 92, 93, 105, 121, 136	5.92	947			1	2	3	*nd*
41	Methyl isopropyl disulphide	122	79, 80, 122	6.00	952			1	2	3	*nd*
42	2-Methylcyclopentyl acetate	142	72, 84, 100	6.20	964			1	2	3	*nd*
43	2,4-Dithiapentane[a]	108	45, 46, 47, 61, 63, 108, 110	6.21	965	20.78	988	1	2	3	4
44	Camphene[a]	136	41, 77, 79, 91, 93, 107, 121, 136	6.28	969			1	2	3	*nd*
45	Unknown 972		105, 120	6.34	972			1	2	3	*nd*
46	Heptanal[a]	114	43, 44, 55, 57, 70, 81, 86, 114	6.57	986	21.08	995	1	2	3	4
47	2-(Methylthio)ethanol	92	61, 92	6.59	987			1	*nd*	3	*nd*
48	Decane[a]	142	43, 57, 71, 85, 99, 113	6.81	1000	21.27	1000	1	2	3	4
49	Unknown 1004		91, 120	6.87	1004			1	2	3	*nd*
50	Unknown 1009		55, 69, 82, 98	6.96	1009			1	2	3	*nd*
51	Unknown 1018		105, 120	7.11	1018			1	2	3	*nd*

No.	Compound	MW	Selected Ions	SPME t_R	SPME RRI	ATD t_R	ATD RRI	Collection method			
52	β-Myrcene[a]	136	69, 93, 121, 136	7.15	1021			1	2	3	*nd*
53	Butyl isothiocyanate[a]	115	115	7.27	1028	22.95	1045	1	2	3	4
54	2-Butyl isothiocyanate	115	86	7.27	1028	23.00	1046	1	2	3	4
55	4-Isopropoxy-2-butanone	130	43, 45, 55, 71, 87	7.29	1029			1	2	3	*nd*
56	Unknown 1030		91, 93, 105, 121, 136	7.31	1030			1	2	3	*nd*
57	2-Ethylhexanal	128	41, 57, 72	7.33	1032			1	2	3	*nd*
58	2-Pentylfuran	138	53, 81, 82, 138	7.37	1034	22.77	1040	1	2	3	4
59	Methylstyrene	118	77, 78, 103, 117, 118	7.54	1045			1	*nd*	3	*nd*
60	Dimethyl trisulphide[a]	126	45, 46, 47, 64, 79, 80, 111, 126, 128	7.54	1045	24.21	1078	1	2	3	4
61	Unknown 1048		105, 120	7.59	1048			1	2	3	*nd*
62	Limonene[a]	136	67, 68, 79, 93, 107, 121, 136	7.71	1055	23.95	1071	1	2	3	4
63	Isobutyl isothiocyanate	115	57, 72, 73, 86, 100, 115	7.72	1055			1	2	3	*nd*
64	S,S-Dimethyl dithiocarbonate[a]	122	47, 75, 94, 122	7.76	1058			1	2	*nd*	*nd*
65	1-Octen-3-one	126	39, 41, 42, 43, 55, 70, 83	7.94	1069			1	2	*nd*	*nd*
66	3-Octanone	128	43, 57, 71, 72, 99	8.00	1073			1	*nd*	3	*nd*
67	Cymene[a]	134	119, 134	8.00	1073			1	2	3	*nd*
68	Unknown 1079		57, 83, 84	8.10	1079			*nd*	2	3	*nd*
69	Benzaldehyde[a]	106	50, 51, 77, 105, 106	8.14	1081	25.43	1111	1	2	3	4
70	6-Methyl-5-hepten-2-one	126	55, 58, 69, 71, 108, 111, 126	8.19	1084			1	2	*nd*	*nd*
71	Octanal[a]	128	43, 44, 55, 56, 57, 67, 69, 81, 82, 84, 100	8.27	1089	25.02	1100	1	2	3	4
72	Unknown 1090		193, 209	8.28	1090			1	2	3	*nd*
73	E-Conophthorin	156	84, 87	8.43	1099			1	2	3	*nd*
74	Undecane[a]	156	43, 57, 71, 127, 141	8.45	1100	25.02	1100	1	2	3	4
75	Methoxy-phenyl-oxime	151	105, 133, 151	8.75	1120	24.43	1084	1	2	3	4
76	4-Hydroxybutanoic acid	86	86	8.88	1128	27.01	1154	1	2	3	4
77	2-Ethyl-1-hexanol	130	55, 57, 70, 83, 98, 112	8.94	1132	26.18	1132	1	2	3	4
78	Hexanoic acid[a]	116	36, 41, 43, 45, 55, 60, 73			26.36	1137	*nd*	*nd*	*nd*	4
79	2-Methylbutyl isothiocyanate	129	41, 43, 57, 71, 72, 73, 100, 114, 129	9.43	1164			1	2	3	*nd*

No.	Compound	MW	Selected Ions	SPME t_R	SPME RRI	ATD t_R	ATD RRI	Collection method			
80	3-Methylbutyl isothiocyanate	129	41, 43, 55, 72, 101, 114, 129	9.43	1164			1	2	3	*nd*
81	2-Methylbutyl isothiocyanate 1166	129	100	9.45	1166			1	2	3	*nd*
82	3-Methylbutyl isothiocyanate 1168	129	114	9.49	1168			1	2	3	*nd*
83	1-Octanol	130	41, 42, 43, 55, 56, 69, 70, 84	9.60	1176			1	2	3	*nd*
84	Unknown 1176		71, 85, 100	9.60	1176			1	2	3	*nd*
85	Unknown 1177		43, 67, 80, 95, 101, 123, 138	9.62	1177			1	2	3	*nd*
86	2-Nonanone	142	43, 58, 71	9.76	1186			1	2	3	*nd*
87	Nonanal[a]	142	43, 57, 70, 82, 98, 114	9.86	1193	28.71	1201	1	2	3	4
88	Acetophenone[a]	120	51, 77, 105, 120	9.96	1199	29.52	1227	1	2	3	4
89	Dodecane[a]	170	43, 55, 56, 57, 71, 85, 99	9.97	1200	28.68	1200	1	2	3	4
90	Pentyl isothiocyanate	129	39, 41, 42, 43, 55, 72, 101, 129	10.10	1209			1	2	3	*nd*
91	Phenol[a]	94	39, 66, 94	10.20	1216	29.10	1213	1	2	3	4
92	Unknown 1230		43, 57, 69, 71, 83, 98	10.39	1230			1	2	3	*nd*
93	Methyl methylthiomethyl disulphide	140	35, 45, 46, 47, 61, 63, 79, 93, 140, 142	10.54	1240	31.13	1278	1	2	*nd*	4
94	Unknown 1244		43, 57, 67, 69, 79, 93, 107	10.60	1244			1	2	3	*nd*
95	Camphor	152	41, 55, 69, 81, 95, 108, 109, 152	10.92	1267			1	2	3	*nd*
96	4-Methylpentyl isothiocyanate	143	39, 41, 42, 43, 55, 56, 69, 72, 128, 143	11.03	1275			1	2	3	*nd*
97	Borneol	139	95, 110, 121, 139	11.25	1290			1	*nd*	*nd*	*nd*
98	Unknown 1294		81, 110	11.30	1294			1	2	3	*nd*
99	Decanal[a]	156	43, 55, 57, 68, 69, 81, 82, 83, 95, 96, 112, 138	11.34	1296	32.12	1310	1	2	3	4
100	S-Methyl methanethiosulphonate	126	40, 45, 46, 47, 63, 64, 95, 110	11.37	1299	32.81	1332	*nd*	*nd*	3	4
101	Tridecane[a]	184	43, 57, 71, 85, 99, 113, 127, 141	11.39	1300	31.81	1300	1	2	3	4
102	Hexyl isothiocyanate	143	110, 115	11.57	1313			1	2	3	*nd*
103	Dimethyl tetrasulphide	158	45, 46, 47, 48, 64, 79, 80, 94, 158, 160	11.69	1322			1	2	*nd*	*nd*
104	Unknown 1334		61, 99, 145	11.85	1334			1	2	3	*nd*
105	Benzoisothiazole or Benzothiazole	135	91, 108, 135	12.21	1361	35.27	1413	1	2	3	4
106	Unknown 1370	74	81, 95, 123, 138	12.33	1370			1	2	3	*nd*
107	Heptyl isothiocyanate	157	115, 124	12.44	1378			*nd*	*nd*	3	*nd*

No.	Compound	MW	Selected Ions	SPME t_R	SPME RRI	ATD t_R	ATD RRI	Collection method			
108	Unknown 1398		41, 55, 69, 70, 83, 97, 111	12.70	1398			1	2	3	*nd*
109	Tetradecane[a]	198	43, 57, 71, 85, 99, 113, 127, 141	12.73	1400	34.92	1400	1	2	3	4
110	Undecanal[a]	170	55, 67, 68, 82, 96, 110, 126			35.31	1414	*nd*	*nd*	*nd*	4
111	5-(Methylthio)pentanenitrile	129	39, 41, 45, 47, 48, 54, 55, 61, 82, 129	12.92	1415			1	2	3	*nd*
112	3-Phenylpropionitrile	131	39, 50, 51, 63, 65, 77, 89, 91, 92, 131	13.40	1452			1	2	3	*nd*
113	3-(Methylthio)propyl isothiocyanate	147	27, 31, 41, 45, 47, 61, 72, 101, 147	13.85	1488			*nd*	2	3	*nd*
114	Pentadecane[a]	212	43, 57, 71, 85, 99, 113, 127, 141	14.01	1500	37.72	1500	1	2	3	4
115	Unknown 1503		41, 43, 55, 56, 57, 69, 70, 71, 83, 97	14.05	1503			1	2	3	*nd*
116	Hexadecane[a]	226	57, 71, 85, 99, 113, 127, 141, 155	15.20	1600	40.41	1600	1	2	3	4
117	4-(Methylthio)butyl isothiocyanate	161	61, 72, 85, 115, 146, 147, 148, 161, 162, 163	15.47	1624			*nd*	2	3	*nd*
118	2-Phenylethyl isothiocyanate[a]	163	39, 51, 63, 65, 72, 77, 91, 92, 105, 163	15.78	1651			1	2	3	*nd*
119	Heptadecane[a]	240	43, 57, 71, 85, 99, 113, 127, 141	16.34	1700	43.24	1700	1	2	3	4
120	Unknown 1713		55, 57, 85, 91, 93, 105, 119, 120, 161, 189, 204	16.48	1713			1	2	3	*nd*

Number, order of the compounds in the Xcalibur™ data processing method and also in the example chromatograms (Fig. 5); Compound, [a]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 Supporting Information, 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; *nd*, not detected.
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 selected compounds on PC 2 and PC 4 from a PCA of broccoli root volatiles sampled on three dates, one pre-and two post-infestation with *Delia radicum* larvae (Fig.7). Samples were trapped *in situ* using SPME either in perforated PTFE sampling tubes or PTFE tubes covered with stainless steel mesh. Compounds and numbers listed correspond with those in Table 1. The complete list of loadings can be found in Supporting Information, Table S12.

PC 2			PC 4		
No.	Compound	Loading	No.	Compound	Loading
89	Dodecane	0.20	119	Heptadecane	0.26
101	Tridecane	0.19	55	4-Isopropoxy-2-butanone	0.18
71	Octanal	0.17	86	2-Nonanone	0.18
109	Tetradecane	0.16	119	Hexadecane	0.17
87	Nonanal	0.15	95	Camphor	0.16
99	Decanal	0.15	77	2-Ethyl-1-hexanol	0.14
74	Undecane	0.15	83	1-Octanol	0.11
8	Hexane	0.14	99	Decanal	0.11
114	Pentadecane	0.12	75	Methoxy-phenyl-oxime	0.10
116	Hexadecane	0.11	57	2-Ethylhexanal	0.10
119	Heptadecane	0.11	58	2-Pentylfuran	0.10
46	Heptanal	0.08	8	Hexane	0.09
34	Nonane	0.05	109	Tetradecane	0.09
33	Hexanal	0.02	70	6-Methyl-5-hepten-2-one	0.09
24	Octane	0.02	87	Nonanal	0.05
15	Heptane	-0.05	114	Pentadecane	0.04
25	Dimethyl disulphide	-0.10	101	Tridecane	0.02
118	2-Phenylethyl isothiocyanate	-0.10	46	Heptanal	−0.06
48	Decane	-0.14	71	Octanal	−0.08
6	Dimethyl sulphide	-0.17	74	Undecane	−0.08
43	2,4-Dithiapentane	-0.17	22	3-Pentanone	−0.09
81	2-Methylbutyl isothiocyanate 1166	-0.21	91	Phenol	−0.11
3	Methanethiol	-0.22	25	Dimethyl disulphide	−0.13
64	S,S-Dimethyl dithiocarbonate	-0.22	33	Hexanal	−0.15
60	Dimethyl trisulphide	-0.22	24	Octane	−0.16
53	Butyl isothiocyanate	-0.22	69	Benzaldehyde	−0.17
54	2-Butyl isothiocyanate	-0.23	13	2-Butanone	−0.19

Table 3. Loadings for for selected compounds on PC 1 and PC 2 from a PCA of broccoli root volatiles sampled pre-and post-mechanical damage (Fig. 8). Samples were trapped *in situ* using SPME in PTFE sampling tubes covered with microporous PTFE tubing. Compounds and numbers listed correspond with those in Table 1. The complete list of loadings can be found in Supporting Information, Table S13.

PC 1			PC 2		
No.	Compound	Loading	No.	Compound	Loading
114	Pentadecane	0.18	33	Hexanal	0.10
74	Undecane	0.15	71	Octanal	0.09
101	Tridecane	0.15	69	Benzaldehyde	0.09
116	Hexadecane	0.15	8	Hexane	0.09
46	Heptanal	0.14	22	3-Pentanone	0.07
109	Tetradecane	0.14	34	Nonane	0.05
71	Octanal	0.13	15	Heptane	0.05
87	Nonanal	0.13	89	Dodecane	0.04
119	Heptadecane	0.13	74	Undecane	0.03
89	Dodecane	0.12	119	Heptadecane	0.03
34	Nonane	0.12	63	Isobutyl isothiocyanate	−0.009
69	Benzaldehyde	0.09	100	S-Methyl methanethiosulphonate	−0.01
113	3-(Methylthio)propyl isothiocyanate	0.07	25	Dimethyl disulphide	−0.02
117	4-(Methylthio)butyl isothiocyanate	0.07	54	2-Butyl isothiocyanate	−0.02
118	2-Phenylethyl isothiocyanate	0.07	53	Butyl isothiocyanate	−0.02
79	2-Methylbutyl isothiocyanate	0.06	6	Dimethyl sulphide	−0.02
80	3-Methylbutyl isothiocyanate	0.06	116	Hexadecane	−0.03
29	Methyl thiocyanate	0.04	3	Methanethiol	−0.05
43	2,4-Dithiapentane	0.06	60	Dimethyl trisulphide	−0.15
60	Dimethyl trisulphide	-0.04	113	3-(Methylthio)propyl isothiocyanate	−0.20
3	Methanethiol	-0.07	96	4-Methylpentyl isothiocyanate	−0.20
63	Isobutyl isothiocyanate	-0.07	90	Pentyl isothiocyanate	−0.20
41	Methyl isopropyl disulphide	-0.07	117	4-(Methylthio)butyl isothiocyanate	−0.20
100	S-Methyl methanethiosulphonate	-0.08	118	2-Phenylethyl isothiocyanate	−0.20
25	Dimethyl disulphide	-0.10	79	2-Methylbutyl isothiocyanate	−0.20
6	Dimethyl sulphide	-0.10	80	3-Methylbutyl isothiocyanate	−0.20
54	2-Butyl isothiocyanate	-0.10	43	2,4-Dithiapentane	−0.20
53	Butyl isothiocyanate	-0.11	29	Methyl thiocyanate	−0.20

Table 4. Loadings for selected compounds on PC 1 from a PCA of broccoli root volatiles trapped in above ground headspace pre-and post-infestation with *Delia radicum* larvae using Tenax TA (Fig. 9). Compounds and numbers listed correspond with those in Table 1. The complete list of loadings can be found in Supporting Information, Table S14.

No.	Compound	Loading	No	Compound	Loading
3	Methanethiol	0.18	38	Isopropyl isothiocyanate	0.15
15	Heptane	0.18	93	Methyl methylthiomethyl disulfide	0.15
60	Dimethyl trisulfide	0.17	35	Methyl ethyl disulfide	0.15
71	Octanal	0.17	46	Heptanal	0.15
99	Decanal	0.17	101	Tridecane	0.13
29	Methyl thiocyanate	0.17	24	Octane	0.12
43	2,4-Dithiapentane	0.17	17	2-Methyl-1-Propanol	0.12
100	S-Methyl methanethiosulphonate	0.16	14	2,3-Butanedione	0.11
87	Nonanal	0.16	88	Acetophenone	0.11
25	Dimethyl disulfide	0.16	34	Nonane	0.11
110	Undecanal	0.16	33	Hexanal	0.10
6	Dimethyl sulfide	0.16	48	Decane	0.10
8	Hexane	0.16	69	Benzaldehyde	0.10
53	Butyl isothiocyanate	0.16	109	Tetradecane	0.05
28	3-Methylbutyronitrile	0.16	114	Pentadecane	0.03
54	2-Butyl isothiocyanate	0.16	116	Hexadecane	0.01
31	Butyl acetate	0.16	119	Heptadecane	−0.03

Figure Legends

Figure 1. (a) Perforated PTFE sampling tubes. (b) Stainless steel mesh covered PTFE sampling tubes. (c) Microporous PTFE tubing covered PTFE sampling tubes. (d) Construction of sample tubes showing: 1, Length of PTFE tubing; 2, Perforated region; 3, Slot cut in tube; 4, Mesh covering slot held in place with PTFE tape; 5, Microporous PTFE tubing covering slot held in place with PTFE tape.

Figure 2. (a) Schematic diagram of SPME fibre inserted into an *in situ* sampling tube. 1, PTFE collection tube; 2, 3 L collection container; 3, ¼" PTFE ferrule; 4, silicone rubber ring; 5, PTFE end cap; 6, 4 L container; 7, SPME fibre assembly attached to a fibre holder; 8, O-ring insert of a Merlin Microseal™ Septum; 9, 50 mm length of 5 mm Ø drinking straw. (b) Sampling of root volatiles *in situ* using SPME with two sampling tubes. (c) SPME fibre holder with fibre and custom-made *in situ* root volatiles sampling attachments (O-ring insert of a Merlin Microseal™ Septum and 50 mm length of 5 mm Ø drinking straw) assembled. (d) The upper O-ring assembly (1) of a used (worn) Merlin Microseal™ (2) is separated from the main body and trimmed at the position shown to create the O-ring (3) used in the sampling attachment. A suitably pierced and trimmed chemically inert rubber disk could be used in place of the microseal septum, but it must grip the SPME fibre protective needle as shown in the upper image.

Figure 3. (a) Schematic diagram of vessel for headspace sampling of root volatiles using sample tubes containing Tenax TA. 1, 2 L Quickfit® culture vessel; 2, Oven bag; 3, Five socket Quickfit® lid; 4, 4 L container; 5, Adsorbent tube in screw thread adaptor; 6, Diffusion cap with membrane; 7, DiffLok™ cap. (b) Headspace sampling of root volatiles with two Tenax TA sampling tubes.

Figure 4. Time course plots for **s**elected sulfur containing compounds detected following *Delia radicum* larval feeding damage to broccoli roots using SPME fibres in stainless steel mesh covered PTFE sampling tubes located *in situ* during pilot studies. Sampling began at the 5-6 true leaf stage of plant growth. Volatiles were collected simultaneously from a single plant using DVB/PDMS and Carboxen/PDMS SPME fibres at elapsed times of 1, 8, 14, 20, 27, 34 and 41 days. Plant-free control samples were also collected using DVB/PDMS and

Carboxen/PDMS fibres at 1, 14, 20 and 41 days elapsed time. Plants were infested with *Delia radicum* eggs, due to hatch within 24 hours, at the 16-18 true leaf stage (day 18, indicated by the vertical line and arrow). A sampling schedule and full raw data sets can be found in Supporting Information, Tables S10 and S11. Numbering of compounds corresponds to those in Table1.

Figure 5. Example GC-MS total ion chromatograms (TIC) for broccoli root volatiles. Samples were collected *in situ* for 24 hours using a PDMS/DVB SPME fibre within PTFE sampling tubes that were (a) perforated or covered with (b) stainless steel mesh or (c) microporous PTFE tubing. Samples were collected (a, b) 24 hours pre-damage and 24 hours and 8 days post-damage by *Delia radicum* larvae or (c) 24 hours pre- and post-mechanical damage. Samples were also collected over 48 hours in above surface headspace using Tenax TA tubes (d) 12 days pre- and 48 hours post- damage by *Delia radicum* larvae. Sulfur containing compounds showing particular enhancement following damage are numbered as in Table 1. Expanded versions of these chromatograms can be found in Supporting Information, Figs S1-S4.

Figure 6. Sulfur containing volatiles trapped over 24 h *in situ* from roots of broccoli using SPME within perforated PTFE tubing, stainless steel mesh-covered PTFE tubing or PTFE tubing covered with micropourous PTFE. Volatiles were also trapped over 48 h from above ground headspace on Tenax. Collections were made at the indicated times (in days) before (UD) and post damage (PD) by *Delia radicum* larvae, or following mechanical damage (micropourous tubes only). Larval damage was taken to commence 24 h after infestation of plants with *D. radicum* eggs.

Figure 7. Principal component analysis (PCA) scores plots of broccoli root volatiles (n = 3) and controls (n = 1) entrained *in situ* using SPME within two PTFE collection tubes per sampling container. Sampling tubes were perforated (P) or covered with stainless steel mesh (M). PC1 versus PC2 (a) shows separation of samples collected from roots and controls 24 hours pre-infestion (black) and 24 hours (red) and 8 days (green) post-infestation with *Delia*

radicum larvae (collection dates 1, 2 and 3). PC1 versus PC 4 (b) shows separation of samples based on sampling tube type.

Figure 8. PCA score plot of PC1 versus PC2 for broccoli root volatiles sampled *in situ* within PTFE sampling tubes covered with microporous PTFE tubing using SPME pre-and post-mechanical damage. Pre-damage collections were made from two plants P1, P2 and a control C using two tubes per sampling container immediately prior to damaging roots. Post-damage collections from the same plants P1, P2 and control C, were made 24 h following mechanical damage to roots.

Figure 9. PCA score plot of PC1 versus PC3 for broccoli root volatiles sampled 12 days pre- and 24 hours post-infestation with *Delia radicum* larvae using Tenax TA. Pre-damage collections were made from four plants denoted as P1-P4 and two controls C1, C2 using two Tenax TA tubes per sampling vessel prior to infesting roots (mean of two tubes used for PCA). Post-damage collections were from the same plants denoted P1-P4 and controls C1, C2 immediately upon commencement of larval feeding

Table Titles and footnotes

Table 1. Compounds identified in broccoli root volatiles samples by SPME-GC-MS and ATD-GC-MS before and after larvae-induced or mechanical damage. Samples were trapped *in situ* within (a) perforated PTFE tubes, (b) stainless steel mesh covered PTFE tubes, (c) microporous PTFE tubing covered PTFE sampling tubes, and (d) within above ground headspace using Tenax TA.

Footnotes

Number, order of the compounds in the Xcalibur™ data processing method and also in the example chromatograms (Fig. 5); Compound, [a]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; *nd*, not detected.

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).

Table2. Loadings for selected compounds on PC 2 and PC 4 from a PCA of broccoli root volatiles sampled on three dates, one pre-and two post-infestation with *Delia radicum* larvae (Fig.7). Samples were trapped *in situ* using SPME either in perforated PTFE sampling tubes or PTFE tubes covered with stainless steel mesh. Compounds and numbers listed correspond with those in Table 1. The complete list of loadings can be found in Supporting Information, Table S12.

Table3. Loadings for for selected compounds on PC 1 and PC 2 from a PCA of broccoli root volatiles sampled pre-and post-mechanical damage (Fig. 8). Samples were trapped *in situ* using SPME in PTFE sampling tubes covered with microporous PTFE tubing. Compounds and numbers listed correspond with those in Table 1. The complete list of loadings can be found in Supporting Information, Table S13.

Table4. Loadings for selected compounds on PC 1 from a PCA of broccoli root volatiles trapped in above ground headspace pre-and post-infestation with *Delia radicum* larvae using Tenax TA (Fig. 9). Compounds and numbers listed correspond with those in Table 1. The complete list of loadings can be found in Supporting Information, Table S14.

Supporting Information

Figures

Figures S1-S4 are in the word file 'Root volatiles method lab & glasshouse Supplementary Figures'.

Figure S1. Example Total Ion Chromatograms (TIC) for broccoli root volatiles collected pre-damage and 24 hours and 8 days post-damage by *Delia radicum* larvae using a PDMS/DVB SPME fibre in perforated PTFE sampling tubes located *in situ* and analysed by GC-MS. I denotes impurity, peaks denoted F (fibre) are non-sample derived. Compounds are numbered as in Table 1. Data is for plant 3 sampling well 2.

Figure S2. Example Total Ion Chromatograms (TIC) for broccoli root volatiles collected pre-damage and 24 hours and 8 days post-damage by *Delia radicum* larvae using a PDMS/DVB SPME fibre in stainless steel mesh covered PTFE sampling tubes located *in situ* and analysed by GC-MS. I denotes impurity, peaks denoted F (fibre) are non-sample derived. Compounds are numbered as in Table 1. Data is for plant 3 sampling well 2.

Figure S3a. Example Total Ion Chromatograms (TIC) for broccoli root volatiles collected pre- and immediately post-mechanical damage using a PDMS/DVB SPME fibre in microporous PTFE tubing covered PTFE sampling tubes located *in situ* and analysed by GC-MS. I denotes impurity, peaks denoted F (fibre) are non-sample derived. Compounds are numbered as in Table 1. Data shown is for plant 2, sampling well 1.

Figure S3b. Example Total Ion Chromatograms (TIC) for broccoli root volatiles collected pre- and immediately post-mechanical damage using a PDMS/DVB SPME fibre in microporous PTFE tubing covered PTFE sampling tubes located *in situ* and analysed by GC-MS. I denotes impurity, peaks denoted F (fibre) are non-sample derived. Compounds are numbered as in Table 1. Data shown is for plant 1, sampling well 2.

Figure S4. Example Total Ion Chromatograms (TIC) for broccoli root volatiles collected in above surface headspace pre- and 48 hours post-commencement of *Delia radicum* larval

feeding using Tenax TA tubes and analysed by GC-MS. I denotes impurity. Compounds are numbered as in Table 1. Data shown is for plant 4, sampling tube B.

Tables

Table S1 is in the word file 'Supplementary material published refrerences for compound identification'. Tables S2 to S11 are included in Excel workbook 'Supplementary material table of abundance and SE_Glasshouse.xlsx'. Individual tables are located in the specific spreadsheets indicated after the table title. Tables S12 to S14 are in the word file Root volatiles method lab & glasshouse supplementary tables'.

Table S1. References from published data on Brassicaceae volatile compounds used for the identification of broccoli root volatiles.

Table S2. Broccoli root volatiles collection, pre- and post-damage by *Delia radicum* larvae, using in situ perforated PTFE tubes or stainless steel mesh covered PTFE tubes analysed by SPME-GC-MS. (p) denotes plant, (c) denotes control (no plant). *In sheet 'Sampling Schedules'.*

Table S3. Broccoli root volatiles collection, pre- and post-mechanical damage, using *in situ* microporous PTFE tubing covered PTFE tubes analysed by SPME-GC-MS. (p) denotes plant, (c) denotes control (no plant). *In sheet 'Sampling Schedules'.*

Table S4. Headspace collection of broccoli root volatiles pre- and post-damage by *Delia radicum* larvae using adsorbent tubes packed with Tenax TA analysed by ATD-GC-MS. (p) denotes plant (vessel with plant), (c) denotes control (vessel without plant). *In sheet 'Sampling Schedules'*

Table S5. Means and SE for abundance of compounds in broccoli root volatiles. Samples were entrained *in situ* using SPME from undamaged and damaged roots using different sampling methods. *In sheet 'Abundance & SE'*.

Table S6. Raw abundance data for broccoli root volatiles entrained *in situ* on PDMS/DVB SPME fibres within perforated PTFE tubing. *In sheet 'Perforated tubes raw data'*.

Table S7. Raw abundance data for broccoli root volatiles entrained *in situ* on PDMS/DVB SPME fibres within PTFE tubing covered in stanless steel mesh. *In sheet 'Stainless mesh tubes raw data'*.

Table S8. Raw abundance data for broccoli root volatiles entrained *in situ* on PDMS/DVB SPME fibres within PTFE tubing covered in microporous PTFE. *In sheet 'Microporous tubes raw data'*.

Table S9. Raw abundance data for broccoli root volatiles entrained on Tenax TA from above-ground headspace. *In sheet 'Tenax TA tubes raw data'*.

Table S10. Preliminary time course experiment. Broccoli root volatiles collection, pre- and post-damage by *Delia radicum* larvae, using in situ stainless steel. *In sheet 'Time course sampling schedule'*.

Table S11. Time course experiment. Raw abundance data for broccoli root volatiles entrained in situ on PDMS/DVB and Carboxen/PDMS SPME fibres within PTFE tubingcovered in stanless steel mesh. *In sheet 'Time course raw data'*.

Table S12. Loadings for PC 2 and PC 4 from a PCA of broccoli root volatiles sampled on three dates, one pre-and two post-infestation with *Delia radicum* larvae (Fig. 7). Samples were entrained *in situ* using SPME either in perforated PTFE sampling tubes or PTFE tubes

covered with stainless steel mesh. Compounds and numbers listed correspond with those in Table 1.

Table S13. Loadings for PC 1 and PC 2 from a PCA of broccoli root volatiles sampled pre- and post-mechanical damage (Fig. 8). Samples were entrained *in situ* using SPME in PTFE sampling tubes covered with microporous PTFE tubing. Compounds and numbers listed correspond with those in Table 1.

Table S14. PC 1 loadings from a PCA of broccoli root volatiles entrained in above ground headspace pre-and post-infestation with *Delia radicum* larvae using Tenax TA (Fig. 9). Compounds and numbers listed correspond with those in Table 1.

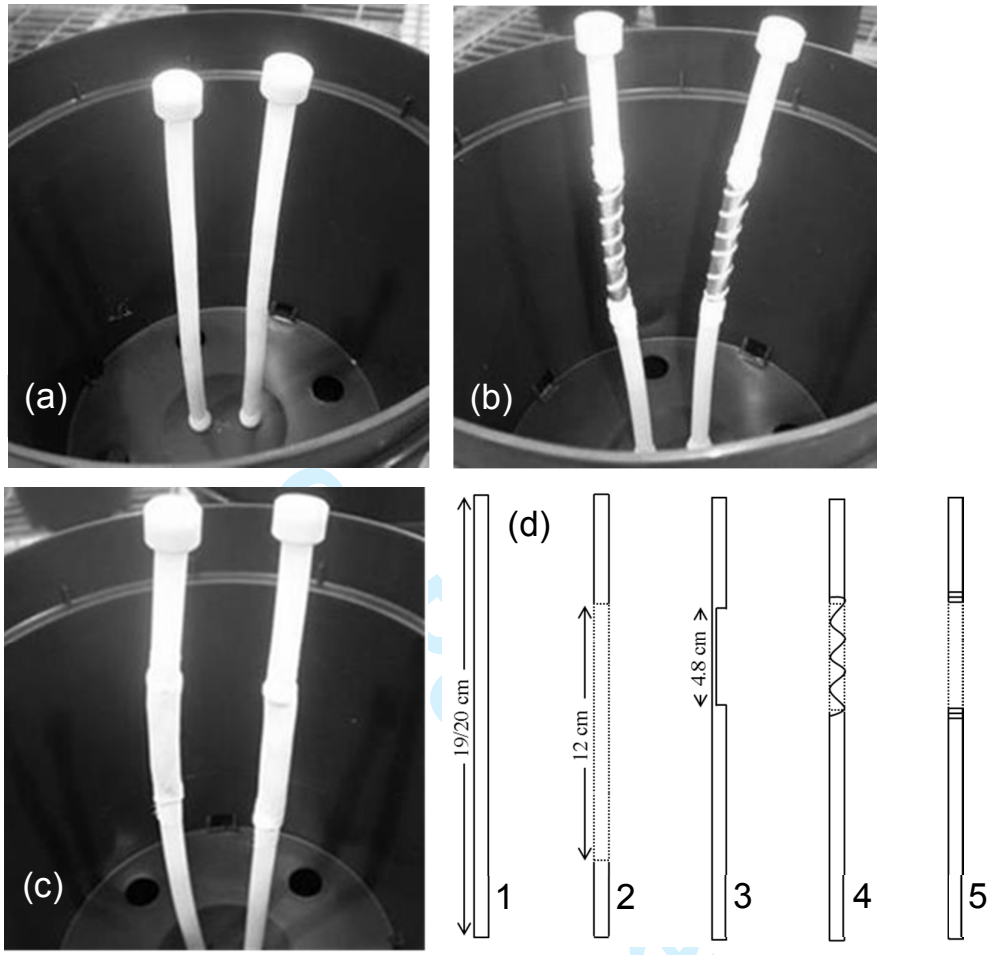


Figure 1. (a) Perforated PTFE sampling tubes. (b) Stainless steel mesh covered PTFE sampling tubes. (c) Microporous PTFE tubing covered PTFE sampling tubes. (d) Construction of sample tubes showing: 1, Length of PTFE tubing; 2, Perforated region; 3, Slot cut in tube; 4, Mesh covering slot held in place with PTFE tape; 5, Microporous PTFE tubing covering slot held in place with PTFE tape.

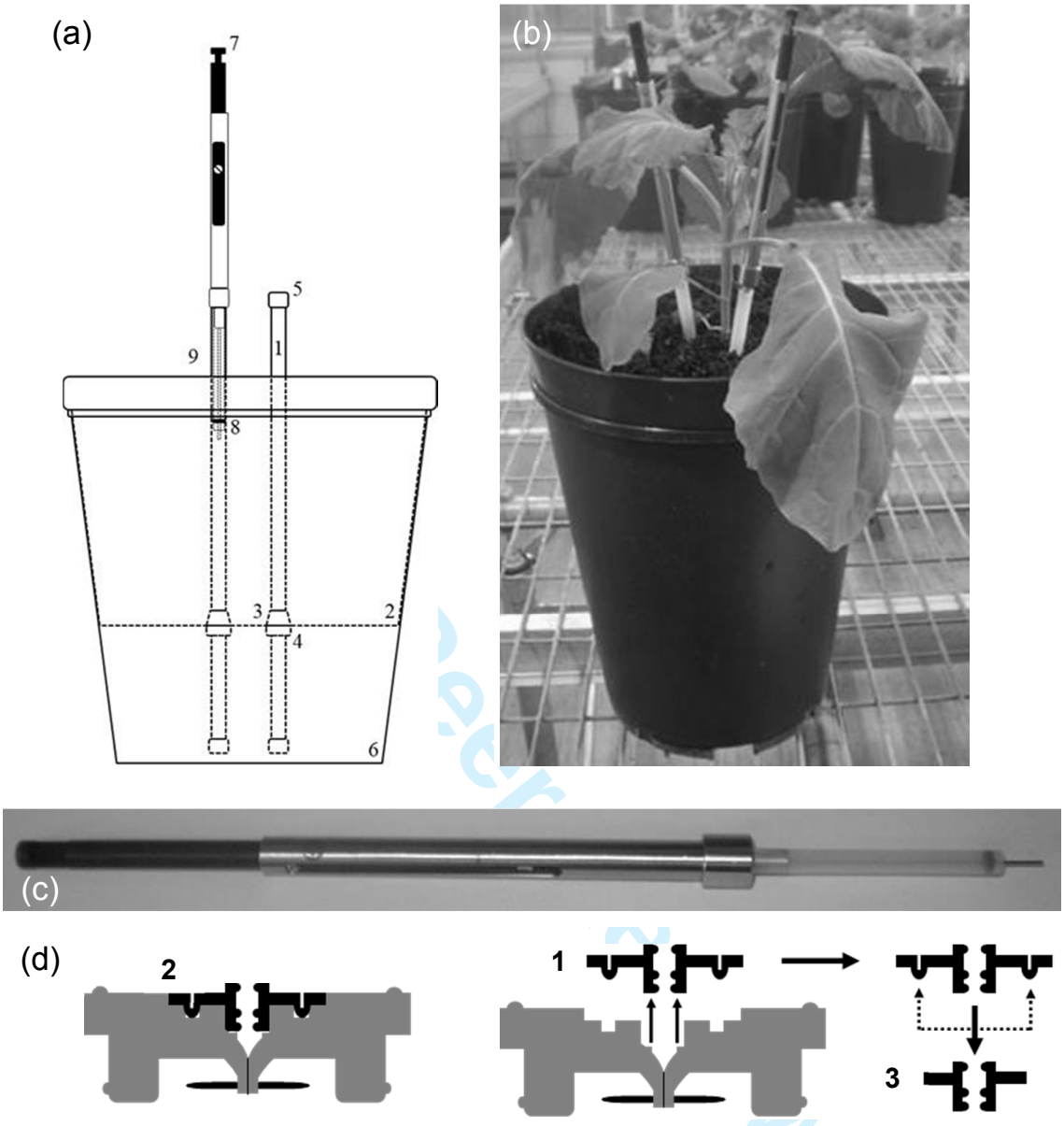


Figure 1. (a) Schematic diagram of SPME fibre inserted into an *in situ* sampling tube. 1, PTFE collection tube; 2, 3 L collection container; 3, ¼" PTFE ferrule; 4, silicone rubber ring; 5, PTFE end cap; 6, 4 L container; 7, SPME fibre assembly attached to a fibre holder; 8, O-ring insert of a Merlin Microseal™ Septum; 9, 50 mm length of 5 mm Ø drinking straw. (b) Sampling of root volatiles *in situ* using SPME with two sampling tubes. (c) SPME fibre holder with fibre and custom-made *in situ* root volatiles sampling attachments (O-ring insert of a Merlin Microseal™ Septum and 50 mm length of 5 mm Ø drinking straw) assembled. (d) The upper O-ring assembly (1) of a used (worn) Merlin Microseal™ (2) is separated from the main body and trimmed at the position shown to create the O-ring (3) used in the sampling attachment. A suitably pierced and trimmed chemically inert rubber disk could be used in place of the microseal septum, but it must grip the SPME fibre protective needle as shown in the upper image.

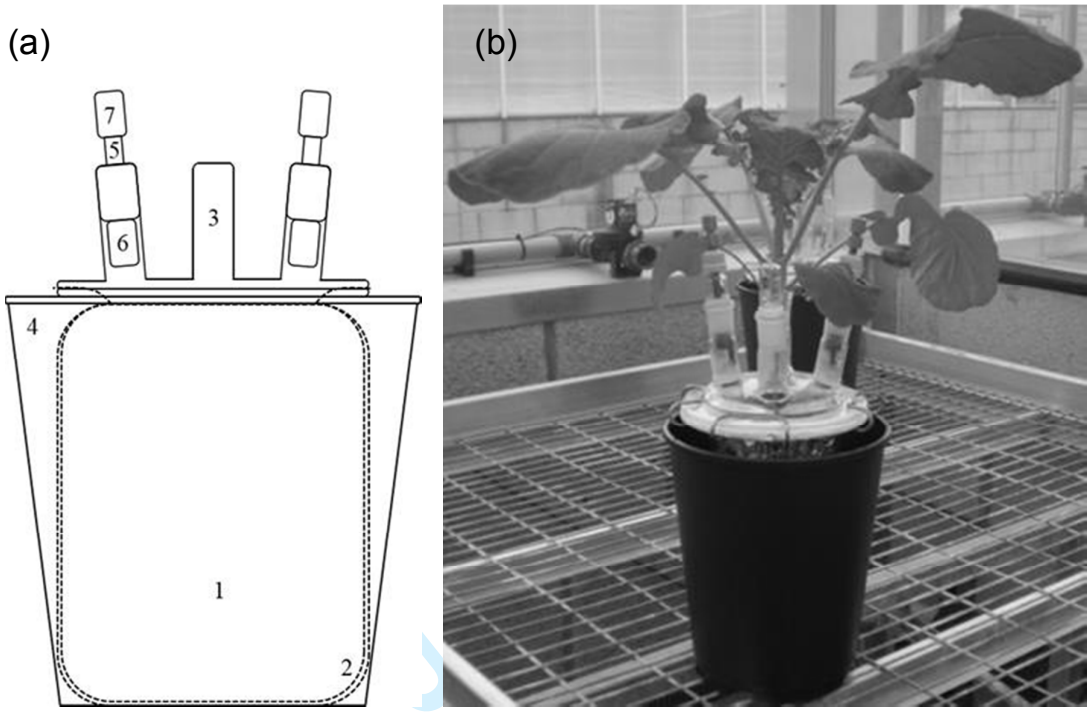


Figure 1. (a) Schematic diagram of vessel for headspace sampling of root volatiles using sample tubes containing Tenax TA. 1, 2 L Quickfit® culture vessel; 2, Oven bag; 3, Five socket Quickfit® lid; 4, 4 L container; 5, Adsorbent tube in screw thread adaptor; 6, Diffusion cap with membrane; 7, DiffLok™ cap. (b) Headspace sampling of root volatiles with two Tenax TA sampling tubes.

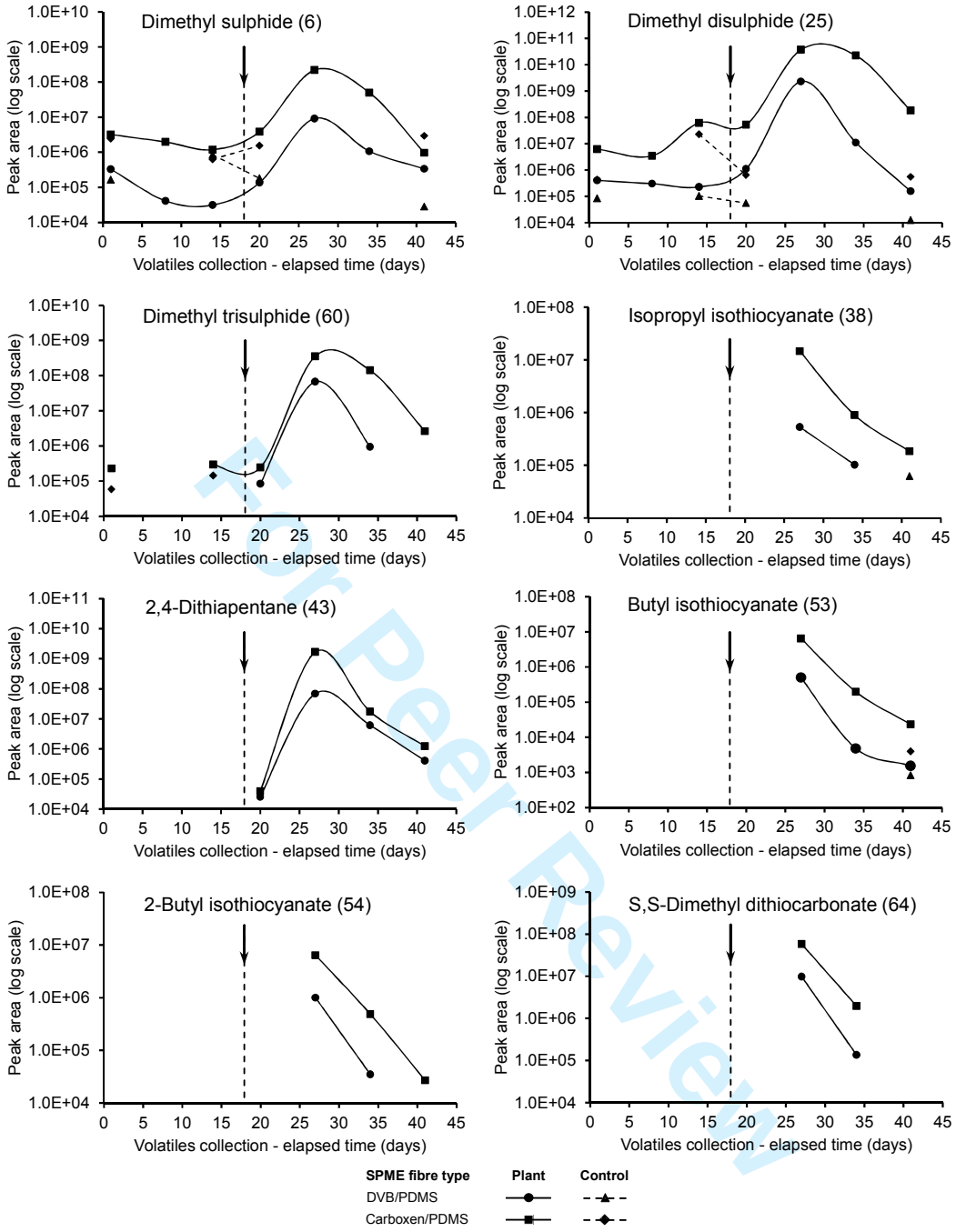


Figure 4. Time course plots for **s**elected sulfur containing compounds detected following *Delia radicum* larval feeding damage to broccoli roots using SPME fibres in stainless steel mesh covered PTFE sampling tubes located *in situ* during pilot studies. Sampling began at the 5-6 true leaf stage of plant growth. Volatiles were collected simultaneously from a single plant using DVB/PDMS and Carboxen/PDMS SPME fibres at elapsed times of 1, 8, 14, 20, 27, 34 and 41 days. Plant-free control samples were also collected using DVB/PDMS and Carboxen/PDMS fibres at 1, 14, 20 and 41 days elapsed time. Plants were infested with *Delia radicum* eggs, due to hatch within 24 hours, at the 16-18 true leaf stage (day 18, indicated by the vertical line and arrow). A sampling schedule and full raw data sets can be found in Supporting Information, Tables S10 and S11. Numbering of compounds corresponds to those in Table1.

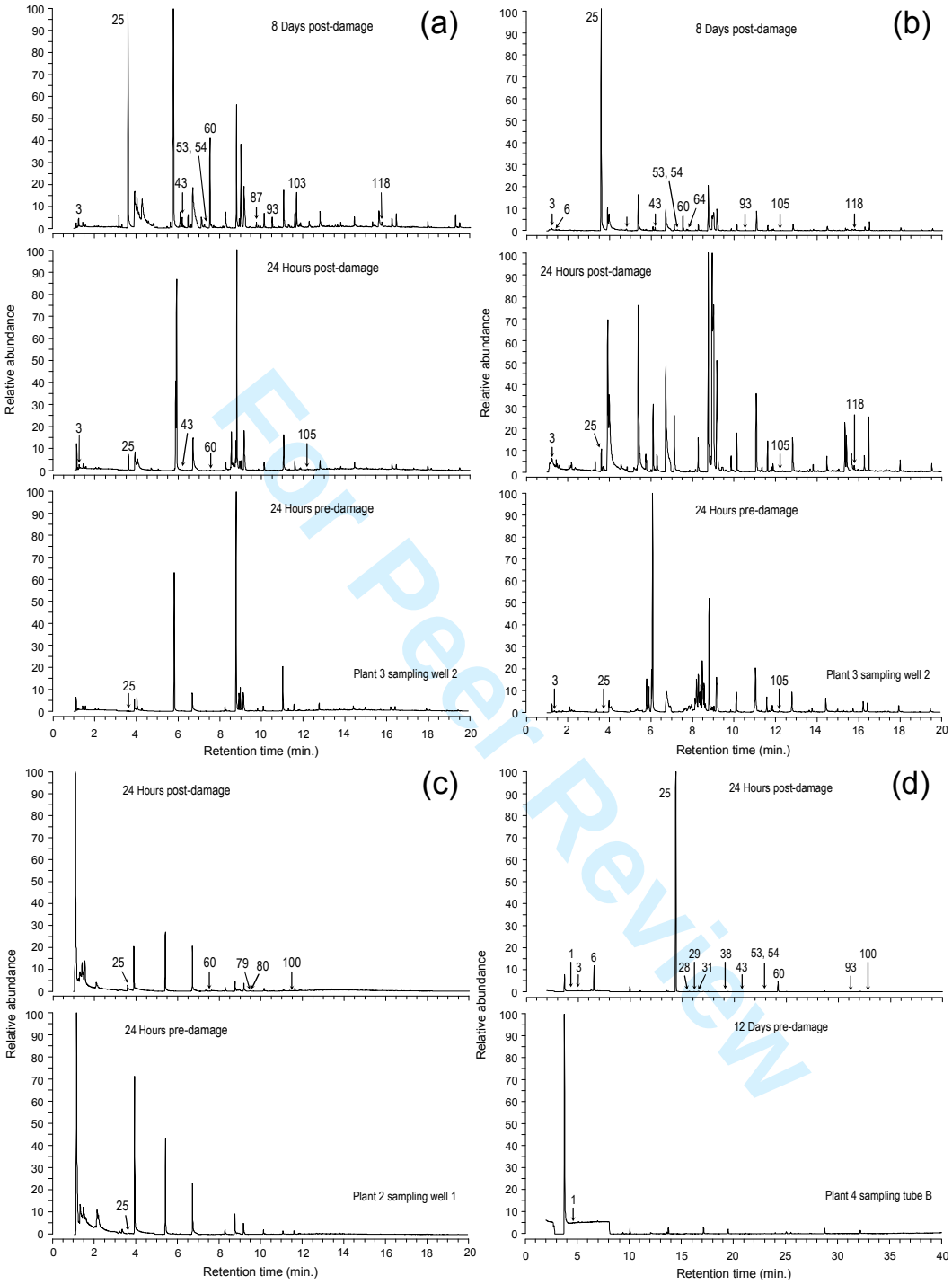


Figure 5. Example GC-MS total ion chromatograms (TIC) for broccoli root volatiles. Samples were collected *in situ* for 24 hours using a PDMS/DVB SPME fibre within PTFE sampling tubes that were (a) perforated or covered with (b) stainless steel mesh or (c) microporous PTFE tubing. Samples were collected (a, b) 24 hours pre-damage and 24 hours and 8 days post-damage by *Delia radicum* larvae or (c) 24 hours pre- and post-mechanical damage. Samples were also collected over 48 hours in above surface headspace using Tenax TA tubes (d) 12 days pre- and 48 hours post- damage by *Delia radicum* larvae. Sulfur containing compounds showing particular enhancement following damage are numbered as in Table 1. Expanded versions of these chromatograms can be found in Supporting Information, Figs S1-S4.

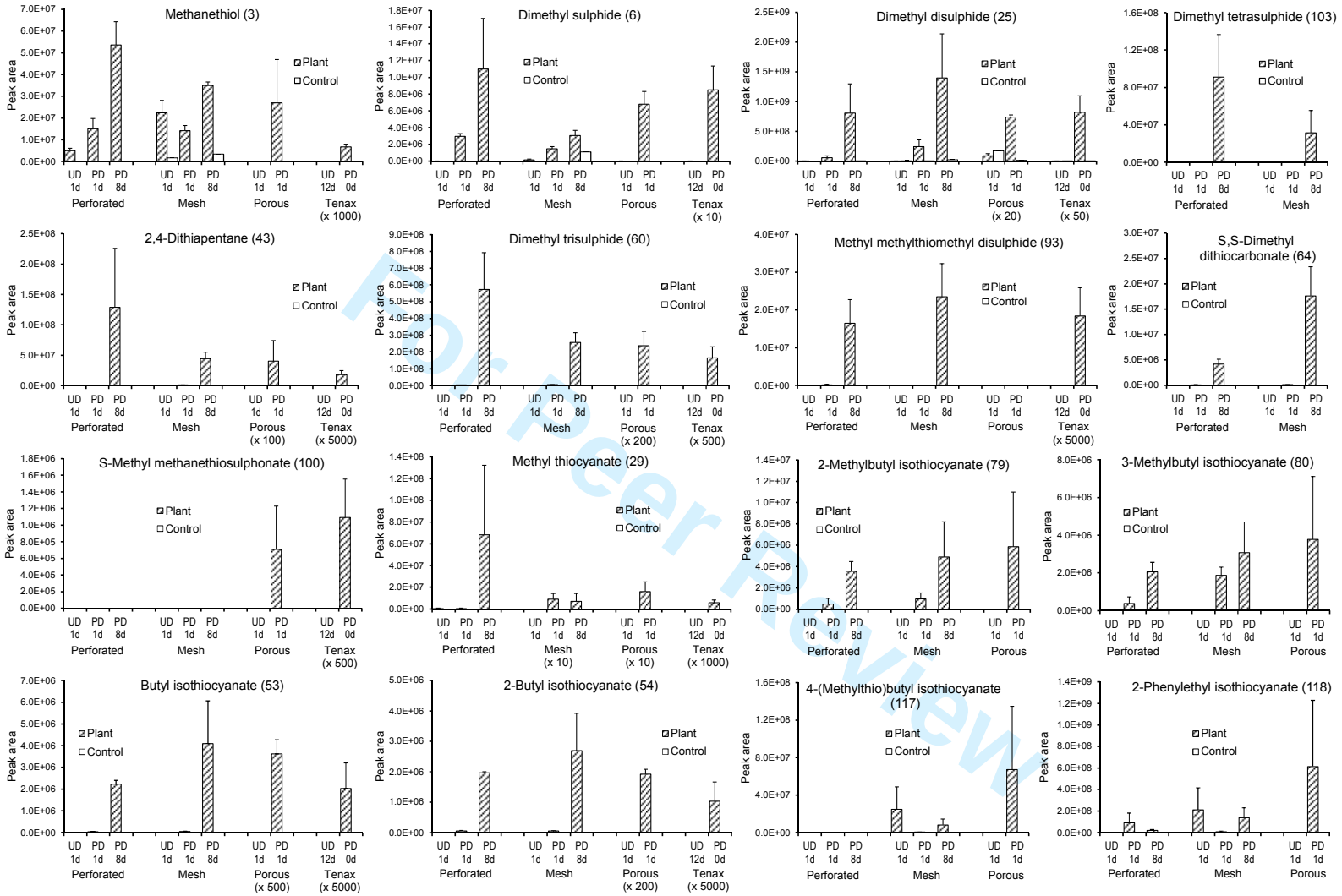


Figure 6. Sulfur containing volatiles trapped over 24 h *in situ* from roots of broccoli using SPME within perforated PTFE tubing, stainless steel mesh-covered PTFE tubing or PTFE tubing covered with micropourous PTFE. Volatiles were also trapped over 48 h from above ground headspace on Tenax. Collections were made at the indicated times (in days) before (UD) and post damage (PD) by *Delia radicum* larvae, or following mechanical damage (micropourous tubes only). Larval damage was taken to commence 24 h after infestation of plants with *D. radicum* eggs.

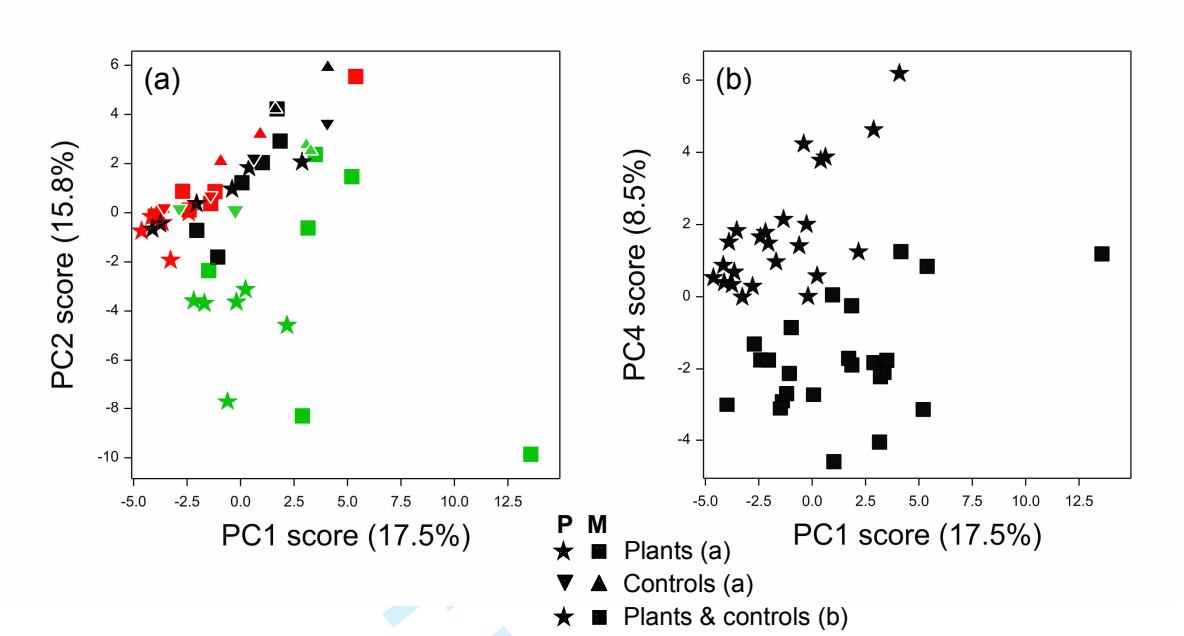


Figure 7. Principal component analysis (PCA) scores plots of broccoli root volatiles (n = 3) and controls (n = 1) entrained *in situ* using SPME within two PTFE collection tubes per sampling container. Sampling tubes were perforated (P) or covered with stainless steel mesh (M). PC1 versus PC2 (a) shows separation of samples collected from roots and controls 24 hours pre-infestion (black) and 24 hours (red) and 8 days (green) post-infestation with *Delia radicum* larvae (collection dates 1, 2 and 3). PC1 versus PC 4 (b) shows separation of samples based on sampling tube type.

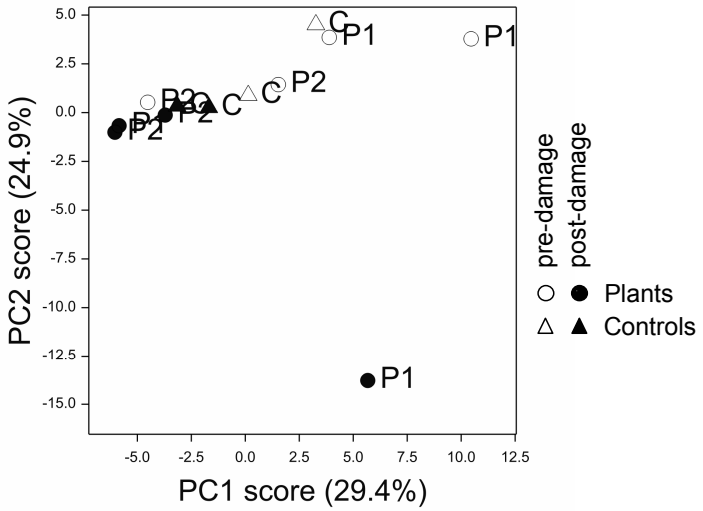
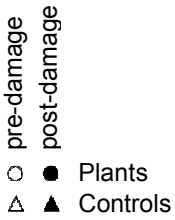


Figure 8. PCA score plot of PC1 versus PC2 for broccoli root volatiles sampled *in situ* within PTFE sampling tubes covered with microporous PTFE tubing using SPME pre-and post-mechanical damage. Pre-damage collections were made from two plants P1, P2 and a control C using two tubes per sampling container immediately prior to damaging roots. Post-damage collections from the same plants P1, P2 and control C, were made 24 h following mechanical damage to roots.



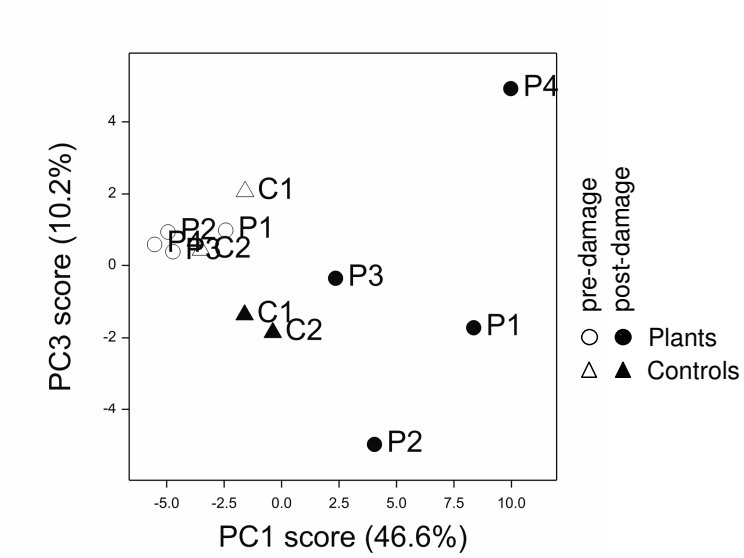


Figure 9. PCA score plot of PC1 versus PC3 for broccoli root volatiles sampled 12 days pre- and 24 hours post-infestation with *Delia radicum* larvae using Tenax TA. Pre-damage collections were made from four plants denoted as P1-P4 and two controls C1, C2 using two Tenax TA tubes per sampling vessel prior to infesting roots (mean of two tubes used for PCA). Post-damage collections were from the same plants denoted P1-P4 and controls C1, C2 immediately upon commencement of larval feeding.

GTOC abstract:

A non-invasive method is described for *in situ* passive sampling of volatiles below-ground from roots of glasshouse-grown plants using SPME fibres located in pre-positioned sampling devices, consisting of perforated PTFE tubes or tubes covered with steel mesh. When both designs of sampling device were evaluated with broccoli plants, similar temporal changes were observed in the production of sulphur containing root volatiles before and after induction of root damage by larvae of cabbage root fly, *Delia radicum*.