On-line detection of root-induced volatiles in Brassica nigra plants infested with Delia radicum L. root fly larvae

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Abstract

Plants emit various volatile organic compounds (VOCs) upon herbivore attack. These VOC emissions often show temporal dynamics which may influence the behavior of natural enemies using these volatiles as cues. This study analyzes on-line VOC emissions by roots of *Brassica nigra* plants under attack by cabbage root fly larvae, *Delia radicum*. Root emitted VOCs were detected using Proton-Transfer-Reaction Mass Spectrometry (PTR-MS) and Gas Chromatography Mass Spectrometry (GC-MS). These analyses showed that several sulfur containing compounds, such as methanethiol, dimethyl sulfide (DMS), dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS) and glucosinolate breakdown products, such as thiocyanates (TC) and isothiocycanates (ITC), were emitted by the roots in response to infestation. The emissions were subdivided into early responses, emerging within one to six hours after infestation, and late responses, evolving only after six to 12 hours. The marker for rapid responses was detected at *m/z* 60. The ion detected at *m/z* 60 was identified as thiocyanic acid, which is also a prominent fragment in some TC or ITC spectra. The emission of *m/z* 60 stopped when the larvae had pupated, which makes it an excellent indicator for actively feeding larvae. Methanethiol, DMS and DMDS levels increased much later in infested roots, indicating that activation of enzymes or genes involved in the production of these compounds may be required. Earlier studies have shown that both early and late responses can play a role in tritrophic interactions associated with *Brassica* species. Moreover, the identification of these root induced responses will help to design non-invasive analytical procedures to assess root infestations.

***Keywords*:** *Brassica nigra*; Brassicaceae; black mustard; root volatile emissions; sulfur compounds; glucosinolate breakdown products; isothiocyanates.

1. Introduction

The induction of volatile organic compounds (VOCs) in plants as a response to herbivore feeding has received considerable attention during the last decades (Vet and Dicke, 1992; Dicke, 1999; Peñuelas and Llusià, 2001; Dicke and Baldwin 2010). It has been well established that these VOC emissions affect the behavior of herbivores as well as their predators and parasitoids (Turlings et al., 1990; van Poecke and Dicke, 2004). To date, most studies have focused on the role of volatiles in aboveground interactions, while induction by belowground feeding herbivores so far has received relatively little attention (van Dam et al., 2003; Erb et al., 2008; Olson et al., 2008). Recently it has been shown that belowground herbivores also induce VOCs that are released by the plant, thereby affecting the behavior of natural enemies associated with root and shoot herbivores (Neveu et al., 2002; Rasmann et al. 2005; Soler et al. 2007; Ferry et al. 2007; Ali et al., 2011). However, the exact nature of VOC emissions induced by root herbivores has not always been studied in depth, and even less is known about the temporal dynamics of root-emitted VOCs. Increasing our knowledge of belowground induced VOCs will not only contribute to a better understanding of plant-insect interactions in wild plant species, but may also contribute to improving biocontrol strategies that reduce the use of synthetic pesticides (Rasmann and Turlings, 2008).

Here, the VOCs released by roots of *Brassica nigra* plants in response to infestation with larvae of the crucifer specialist *Delia radicum* L. (cabbage root fly), a natural root herbivore of both wild and cultivated Brassicaceae (Finch and Ackley 1977), are studied. Brassicaceous plants that are damaged, or treated with signaling hormones such as jasmonic acid, induce a complex bouquet of VOCs which can comprise up to 200 compounds (Geervliet et al. 1997; Rohloff and Bones, 2005; van Dam et al. 2010). Among these volatiles are the breakdown products of glucosinolates. Glucosinolates are sulfur-containing compounds that are typical for the Brassicaceae (Hopkins et al., 2009). Despite the fact that glucosinolates themselves have defensive properties, their major hydrolysis products, such as isothiocyanates and nitriles, are generally more effective defenses than glucosinolates against pathogens and herbivores (Kim and Jander, 2007; Hopkins et al., 2009). In contrast to the non-volatile glucosinolates, the breakdown products can be found as volatiles in the headspace of herbivore-infested *Brassica* plants (Cole, 1976; de Vos et al., 2008; Fig. 1). Upon tissue disruption glucosinolate hydrolysis is catalyzed by the enzyme myrosinase. The initial hydrolysis products include thioglucose, sulfate and an unstable intermediate. This intermediate rearranges spontaneously to produce several degradation products, such as isothiocyanates, nitriles and thiocyanates. Which of these products will be formed depends on the side chain structure and the hydrolysis conditions, such as the pH and the presence of specific modifier proteins in the plant (Halkier and Du, 1997; Wittstock et al., 2003).

Previous studies on *B. nigra* plants infested with *D. radicum* larvae showed that the glucosinolate levels change in roots of infested plants (van Dam and Raaijmakers,

2006; Hopkins et al., 1998). In addition, headspace analyses revealed that root fly-infested plants and turnips emit higher levels of specific volatile sulfides than non-infested plants (Soler et al., 2007; Ferry et al., 2007). Predators and parasitoids of above ground (AG) and belowground (BG) herbivores were found to use these sulfides as cues to locate suitable hosts. The behavioral response of parasitoids and predators, however, was found to be species-specific as well as dose-dependent. Ground-dwelling beetles that are predators of herbivorous cabbage root fly larvae are attracted to dimethyl disulfide (DMDS) in a dose-dependent way; traps baited with 0.2 - 2 µl of pure DMDS contained more predatory beetles than traps with lower or higher amounts (Ferry et al, 2007). AG parasitoids, on the other hand, avoid plants infested with large root fly larvae and elevated sulfide emissions, whereas plants with small root fly larvae were equally attractive as non-infested plants (Soler et al., 2007). These behavioral observations strongly suggest that there may be temporal dynamics in the amounts of sulfides and possibly other VOCs released from root infested *B. nigra* plants, which may have important consequences for the attraction of both AG and BG natural enemies. However, the temporal dynamics of the herbivore-induced volatile emissions underlying the natural enemies' preferences have not been analyzed to date. As it seems likely that the strongest and most reliable cues come from the feeding site of the herbivores, i.e. the roots, this investigation focuses on the temporal profile of sulfur-containing compound emissions from the roots.

Commonly, techniques such as gas chromatography (GC) or gas chromatography mass spectrometry (GC-MS) are used to analyze VOCs released by plants. The sampling and analytical procedures of these methods can be very time-consuming,

and mostly they do not allow the simultaneous, time-resolved monitoring of different classes of compounds. Because of these limitations, these techniques are not optimal to assess the temporal dynamics of VOC emissions caused by biotic stresses. Therefore, Proton-Transfer-Reaction Mass Spectrometry (PTR-MS) was used, a technique allowing rapid, on-line detection of trace gases from various chemical groups in the order of seconds at (sub) parts per billion (ppb) levels (Hansel et al., 1995; Boamfa et al., 2004; Blake et al., 2009). However, PTR-MS only provides information about the molecular weight of the detected volatile, and therefore, the identity of the compound still needs to be confirmed by other methods such as GC-MS (Steeghs et al., 2004). For this reason, on-line PTR-MS measurements and GC-MS analyses were combined to follow the temporal dynamics, and to quantify and identify the volatiles emitted from *B. nigra* roots infested by *D. radicum* larvae.

2. Results

2.1 Temporal dynamics of VOC signals on PTR-MS

In initial experiments three ions, *m/z* 60, *m/z* 63 and *m/z* 95 (see Fig. 2), showed increased signal intensities in infested roots, while these signals remained low in control plants. As can be seen in the Fig. 2, *m/z* 63 and *m/z* 95 were induced between six and 12 hours after the infestation, while *m/z* 60 (Fig. 2) started increasing shortly after infestation (between one and six hours). Those three volatiles increased until they reached a peak value between one and three days after infestation, after which they decreased to initial values. Further replicates, in which volatiles with lower

molecular weights were screened as well, showed that also *m/z* 49 emissions differed between infested and control roots (Fig. 2). The temporal dynamics of *m/z* 49 closely followed that of the compounds detected at *m/z* 63 and *m/z* 95.

It is well known that after wounding, the aerial parts of plants emit C6 wound compounds (hexenal, hexanal and related compounds, Hatanaka, 1993). Infested *B. nigra* roots did not emit these compounds (results not shown). This is in agreement with Steeghs et al. (2004), who did not observe induction of C6 wound compounds in *Arabidopsis* root cultures that were mechanically damaged or infested with root-feeding insects. In contrast to Steeghs et al. (2004), however, we did not observe increased 1,8-cineole levels (*m/z* 155, detected at its main fragment *m/z* 81 with PTR-MS) in our system, neither using PTR-MS nor using GC-MS.

2.2 GC-MS analysis and identification of the sulfides

GC-MS analysis of the volatiles released during infestation confirmed the induction of dimethyl sulfide (DMS; *m/z* 63 in the PTR-MS analysis) and dimethyl disulfide (DMDS; *m/z* 95 in the PTR-MS) in the root headspace of infested plants (Fig. 3). Additionally, GC-MS measurements showed increased levels of dimethyl trisulfide (DMTS) in root infested plants (Fig. 3). The temporal dynamics of DMTS could not be observed with our PTR-MS due to its lack of sensitivity at higher masses and the low emission rate of this compound by the root system.

Because of its low molecular weight and high volatility, the compound represented by *m/z* 49 could not be detected under the specific conditions of the GC-MS system that was used. Therefore, natural isotopic ratios (Danner et al., 2012) were used to identify this compound as methanethiol (CH_3SH). Experimentally, the ratio *m/z* 51/

m/z 49 was equal to 4.46 ± 0.05 % (3 replicate average, R^2 = 0.81). This ratio is close to the most abundant atomic sulfur ratio $^{34}S/^{32}S$ = 4.41 % (NIST). The only candidate for *m/z* 49 that includes a sulfur atom has as molecular formula CH_3SH, with an abundance of: *m/z* 51/ *m/z* 49 = 4.44 %. This value is in very good agreement with the experimental value, confirming the assignment of *m/z* 49 to methanethiol, a compound that has been previously reported in Brassicaceae (Bending and Lincoln, 1999; Attieh et al., 2000).

2.3 Glucosinolate breakdown products and identification of m/z 60

Furthermore, the GC-MS analyses identified the presence of allylisothiocyanate, phenylethylisothiocyanate and benzonitrile (Fig. 4), which are volatile glucosinolate breakdown products and possibly also the origin of the compound detected at *m/z* 60 with PTR-MS. As mentioned above, the major glucosinolate breakdown products are divided into nitriles, thiocyanates and isothiocyanates (see Fig. 1). The compounds in the last two groups contain a CNS group. In the PTR-MS, (iso)thiocyanates may fragment into thiocyanic acid (HCNS), which, after being protonated, would be detected at *m/z* 60. To test this hypothesis, the isotopic ratios of *m/z* 60 emitted during infestation were compared to the experimental isotopic ratios of pure HCNS in our PTR-MS, and these were compared to literature based isotopic ratios. In HCNS the most abundant isotope for ^{32}S is ^{34}S (4.25%) (^{33}S=0.75%), while $^{13}C/^{12}C$ is 1.08%. The $^{15}N/^{14}N$ ratio is 0.36%, and $^{2}H/^{1}H$ ratio is (0.011%) (NIST). Taking this into account, with PTR-MS the most abundant isotope for HCNS would be detected at *m/z* 62. By correlating the signals of *m/z* 60 and *m/z* 62 from root infested plants over time a natural abundance ratio *m/z* 62/ *m/z* 60 of

4.57 +/- 0.05 % was found, with R^2 = 0.90 (Fig. 5). Model calculations predict *m/z* 62/ *m/z* 60 = 4.44%. Pure HCNS (thiocyanic acid) measured with PTR-MS shows a natural isotopic abundance *m/z* 62/ *m/z* 60 = 4.74 +/- 0.02 % with R^2 = 0.988. Therefore, HCNS itself, or a bigger molecule fragmenting into HCNS, is a likely candidate for this mass *m/z* 60.

To further investigate the origin of *m/z* 60, the fragmentation patterns of a number of volatile glucosinolate breakdown products were studied. Based on GC-MS analysis, *B. nigra* root glucosinolate and VOC profiles reported in the literature, the following thiocyanates (TC) and isothiocyanates (ITC) were considered as candidates for the parent mass: methyl TC, methyl ITC, ethyl TC, ethyl ITC, allyl ITC and 2-phenethyl ITC. Pure samples of the above mentioned compounds were measured with PTR-MS in order to obtain their mass spectra and fragmentation patterns. For this, closed vials (5 mL volume) containing the pure compound were used, with a syringe needle penetrating the septum in the cap. These emitters were placed in a 1 L volume cuvette and flushed with air. The combined effect of vapor diffusion through the needle and ventilation of the 1 L cuvette resulted in a diluted gas concentration that did not saturate the PTR-MS detector. Based on these experiments, methyl TC, methyl ITC, and 2-phenethyl ITC could be discarded as candidates for *m/z* 60. Even though *m/z* 60 was found in the spectra of some of these pure compounds, in none of the spectra it was a major fragment. In addition, the main fragments for each of these compounds did not show any significant difference in emission rates between infested and non-infested roots in the experiments.

This leaves three potential glucosinolate breakdown compounds as candidates for the parent mass of *m/z* 60: ethyl TC, ethyl ITC and allyl ITC. These pure compounds

all yielded a main fragment at *m/z* 60, and the natural isotopic ratio *m/z* 62/ *m/z* 60 supports the hypothesis that this *m/z* 60 originates from HCNS. However, neither ethyl TC nor ethyl ITC was detected in the GC-MS analyses. Allyl ITC, on the other hand, was measured with GC-MS. Moreover, the glucosinolate giving rise to allyl ITC, sinigrin, is one of the major glucosinolates in *B. nigra* roots (van Dam and Raaijmakers, 2006). Thus *m/z* 60 most likely originates from allyl ITC, even though the signal at *m/z* 100 (the parent mass) was not observed with PTR-MS in damaged roots, which may be due to low concentration, since the intensity for *m/z* 100 for the pure compound was much lower than for *m/z* 60 (i.e., allyl ITC suffered from strong fragmentation in the PTR-MS drift tube). Alternatively, enzymes such as methyltransferases, which normally catabolize glucosinolate-derived TC and ITC to smaller compounds *in planta*, may play a role in the production of HCNS molecules from (any) ITC (Attieh et al., 2000). These enzymes were not present when measuring pure compounds.

In separate experiments using one single plant (Fig. 6) the main root of a *B. nigra* plants was artificially damaged with a single puncture of a scalpel, 2-5 cm below the soil surface. The emission of *m/z* 60 increased immediately after damage; the response started within seconds and reached a maximum about 10 min after the damage. Two hours after damage, the emissions had returned to control levels, indicating the reaction between myrosinase and the glucosinolates had ended. Renewed damage, however, initiated the reaction again resulting in increased *m/z* 60 emissions (Fig. 6). From this figure it can also be seen that artificial damage has no direct effect on DMS (*m/z* 63) and DMDS (*m/z* 95) signals; methanethiol (*m/z* 49)

showed increased emissions after artificial damage, though much smaller than the one observed for *m/z* 60. The rapid increase of *m/z* 60 supports our hypothesis that the fast *m/z* 60 emissions result from the rapid conversion of glucosinolates by the enzyme myrosinase and possibly other enzymes involved in glucosinolate conversion (Wittstock et al., 2003).

2.4 Influence of larval instar on the temporal profile

In addition, the influence of the larval developmental stage, or instar, on the temporal emission profile or intensity of the emissions was studied. For this, *m/z* 60 (HCNS) was chosen as a marker, because of its high reproducibility, and the quick and strong signal that was measured directly after infestation. Fig. 7 shows *m/z* 60 emissions of roots infested with four small first instar larvae, four large late second instar larvae, and control roots. At the end of the experiments, actively feeding larvae were found in all replicates infested with first instar larvae, on average two in each root. In the experiments with the larger larvae, all larvae had pupated by the end of the experiment. Emissions of *m/z* 60 clearly discriminated between plants in the different treatment groups (Fig. 7). Plants infested with large second instar larvae rapidly emit high levels of this volatile, but these emissions are dropping to control values by the fourth day of the experiment. Most likely, this is the moment that the larvae stopped feeding because of the transition to the pupal stage. Emissions from plants infested with small larvae increased gradually in time as the larvae grew and increasingly did more damage to the roots; six days after the start of the experiment, emission levels were still higher than control values. A similar temporal pattern was observed for sulfide emissions (data not shown). These temporal dynamics match

earlier observations showing that plants damaged with early instar larvae do not elicit strong behavioral responses in AG parasitoids, most likely because of the low concentration of sulfide emissions (Soler et al., 2007).

2.5 Biogenic source of the sulfur containing volatiles

The sulfur compounds detected in our experiments could be either directly emitted by the plant as a result of feeding damage, or have originated from the larvae themselves. In Fig. 8 the mass spectra of volatiles emitted from 20 second instar larvae placed in a 100 mL cuvette filled with plain river sand are shown as compared with plain river sand alone. Both sand and larvae emit equally low amounts of *m/z* 60, which supports the hypothesis that the roots, and not the larvae, are the source of *m/z* 60. For methanethiol (*m/z* 49), DMS (*m/z* 63) and DMDS (*m/z* 95) higher values are observed in the cuvette with the larvae (Fig. 8). Therefore, *D. radicum* larvae themselves, or the plant material and bacteria that can be found in their alimentary tract or frass (Lukwinski et al., 2006) may contribute to the signal detected at those masses during the infestation experiments. However, the emission levels of these compounds (methanethiol, DMS and DMDS) from the cuvettes with the larvae were much lower than expected, if we take into account that in this experiment there were 4 to 5 times more larvae in a much smaller volume than were added to the plant roots. Moreover, sulfide emissions from infested roots follow a pattern similar to that of *m/z* 60, and are decreasing when larvae start pupating (Fig. 2). Therefore, it is likely that the majority of the sulfide signals originate from the roots themselves and not from the insect or associated microorganisms.

3. Discussion

This study shows that root damage, either by root feeding insects or by artificial damage, induces various time-resolved volatile responses in plant roots. When considering these temporal dynamics, the emissions seem to be divided into early and late responses. HCNS, represented by *m/z* 60, showed an immediate response both after infestation and artificial damage. The results presented here show that HCNS likely arises from the two component glucosinolate-myrosinase system that is constitutively present in the plant, and is activated by artificial as well as herbivore damage to the plant (Rask et al., 2000). Allyl ITC was found to be the most likely candidate for being the parent compound of HCNS. The precursor glucosinolate of allyl ITC, allylglucosinolate (sinigrin), is one of the major glucosinolates in *Brassica nigra* roots, constituting about 40-50% of the glucosinolate profile in the main roots (van Dam and Raaijmakers, 2006). As in most *Brassica* species, the aromatic 2-phenylethyl glucosinolate is the other major root glucosinolate in *B. nigra*, constituting 50-60% of the glucosinolate profile (van Dam et al 2009). PTR-MS analyses of its conversion product, PE-ITC, however, did not show a major signal at *m/z* 60. Moreover, root fly feeding was shown to increase the percentage of allylglucosinolate to 57 – 65% of the total glucosinolate main root profile to the expense of 2-phenylethylglucosinolate levels (van Dam and Raaijmakers, 2006). This adds to the evidence that the increase of HCNS emissions from root infested plants results from the conversion of allylglucosinolate into allylITC. In addition to myrosinase, methyltransferases that detoxify ITC *in planta* may also have contributed to the emergence of HCNS from allyITC (Attieh et al., 2000). Because

the PTR-MS analysis was performed with pure compounds, the exact contribution of methyltransferases and various other enzymes involved in glucosinolate conversion in plants (for example epithiospecifier proteins (ESP, Wittstock et al., 2003)) to the enhanced *m/z* 60 emissions from infested roots cannot be specified.

Methanethiol, DMS and DMDS emissions, on the other hand, were much slower and increased only six to 12 hours after infestation. No immediate increase was observed in response to artificial damage other than the slight increases of methanethiol (Figure 6). This points to an induction process which involves the transcription of genes or activation of enzymes necessary for the synthesis of these sulfides in the plant. Methanethiol, DMS and DMDS have been studied in *Brassica* crops because they cause the typical 'cabbage odour' (Jones, 2010). Indeed, several enzymes, mainly transferases and lyases, are present in the aboveground parts of Broccoli and other cultivated cabbages that produce sulfides from the amino acids methionine and cysteine (Derbali et al., 1998, Chin and Lindsay, 1994, Attieh et al., 1995). The activity of these enzymes was found to be required as the production of these sulfides was reduced when enzyme activity was inhibited (Derbali et al., 1998).

In addition to the plant roots, there are other natural sources that may contribute to sulfide emissions. Several soil-dwelling microorganisms are known to produce volatile sulfides, including DMDS (Kai et al., 2009, Kai et al., 2010). The low levels of methanethiol and sulfides emerging from control plants show that the direct contribution - if any - of soil dwelling microorganisms to these emissions is small (Fig. 2, 3 and 4). Alternatively, the interaction between root fly gut microbes and the damaged roots could lead to the production of methanethiol, DMS and DMDS. *D. radicum* larvae contain a highly diverse gut microflora that helps them to infest and

digest the recalcitrant plant material they feed on; several of these gut bacteria are closely related to sulfide producing bacteria found in soils (Lukwinsky et al., 2006). Indeed, larvae on sand emitted more sulfides than pure sand, but the emission levels per larva were relatively low compared to the emissions recorded from infested roots. In addition, there was no immediate increase of sulfide emissions after the larvae were added to the plants, so initially the direct contribution of gut bacteria to these emissions is minimal. However, this does not preclude that the activities of gut microbes have contributed to the emission of sulfides from *D. radicum* infested roots at later time points during active feeding (Fig. 2). Finally, the emission of sulfide compounds decreased when the larvae pupated (Fig. 2); remaining frass and microbes alone therefore can not be responsible for the elevated sulfide emissions. This also precludes that root pathogens entering the roots at the damage sites have a major effect (Soroka et al., 2004), as they would also remain after larval feeding has stopped.

C6 wound compounds were not detected in our analyses, neither after insect damage nor after mechanical wounding. This result is in agreement with *in vitro* experiments with *Arabidopsis* roots (Steeghs et al., 2004). Roots probably do not emit these green-leaf volatiles as the enzymes involved in the production of these VOCs from α-linoleic and linolenic acids are bound to the chloroplasts membrane (Hatanaka, 1993).

Both early and late VOC emissions in roots may have ecological relevance for the plant and its associated insect communities. Insect behavioral studies showed that the parasitic wasp, *Cotesia glomerata,* has the ability to distinguish between plants

with and without *D. radicum* feeding on the root system when searching for their AG host, but also that this ability depended on the developmental stage of the root herbivore (Soler et al., 2007). *C. glomerata* females only avoided plants infested with large root fly larvae, whereas they did not (or could not) distinguish between undamaged plants and plants infested with small root fly larvae. Apparently, the emissions of root-induced volatiles such as sulfides must pass a certain threshold before parasitoids can detect them. This is also in agreement with the dose-dependent response of several ground-dwelling *D. radicum* predators to DMDS (Ferry et al., 2007). The online measurements presented here clearly show that the emissions of glucosinolate conversion products as well as sulfides closely correlate with the developmental progress of the larvae. Therefore, these emissions can be a reliable cue for aboveground and belowground natural enemies searching for hosts with an optimal quality for their offspring (Vet and Dicke, 1992). This also highlights the importance of analyzing temporal dynamics of VOC emission when aiming to understand the role of belowground induced responses in aboveground and belowground multitrophic interactions associated with plants.

4. Conclusions

The results presented here show the power of PTR-MS combined with GC-MS for elucidating the temporal dynamics of VOCs emitted by root infested plants. The PTR-MS analyses showed that infested plants with actively feeding herbivores can clearly be distinguished from uninfested plants within hours after infestation until the feeding had stopped. The temporal analyses also provided valuable information

about the progression of larval feeding and development. PTR-MS analyses thus may provide a valuable tool for breeding companies screening for more resistant crop varieties. Recently, several synthetic pesticides have been banned for use in the European Union, which has incited an increased interest by plant breeding companies to select crop varieties with high natural levels of resistance. On-line PTR-MS analyses may provide a rapid and non-invasive tool to assess levels of infestation and the progression of larval growth in varieties with different levels of resistance. Similarly, PTR-MS analyses may be used to develop a detector which could be used for identifying crops contaminated with this cryptic pest in quarantine facilities. Moreover, the knowledge gained on the temporal dynamics of the volatile emissions may deepen our understanding of the role of root induced volatiles in multitrophic interactions. Further studies will reveal to which extend each of the players in this tripartite interaction between plant, herbivore and gut microorganisms determines the nature and dynamics of root induced VOC emissions in wild and agricultural *Brassica* species.

5. Experimental

5.1 Plants

Brassica nigra seeds from a bulk seed batch of plants collected in a feral population near Wageningen, the Netherlands, in 2008 were germinated and transferred to tall 2.5 l 'Rosepots' (11cm x11 cm x 21 cm). The lower 15 cm of the pots were filled with a peat soil-sand mixture (number 4, Lentse Potgrond, Lent, the Netherlands) which was covered with 5 cm of plain river sand. This facilitated the placement of

the sampling cuvette around the roots, as well as the retrieval of the root fly larvae, which mainly feed on the upper 5 cm of the roots. At the same time, it provided the roots with sufficient nutrients in the lower layer. The pots with the seedlings were placed in a glasshouse at 21 °C during the day and 16 °C, at night. The ambient light conditions were supplied with sodium lamps to maintain the minimum photosynthetically active radiation (PAR) at 225 µmol m^{-2} s^{-1} for at least 16 h per day.

5.2 Addition of the root fly larvae and count of the remaining larvae

Delia radicum L. (Diptera: Anthomyiidae) larvae are specialist root herbivores of many Brassicaceae species. The adult flies live aboveground. Females oviposit at the base of the shoot, on or next to the stem. After hatching, the larvae crawl down beneath the soil and start feeding on the main roots until they pupate in the soil (Finch and Ackely, 1977). Eggs and larvae of *D. radicum* larvae reared on turnips (*B. rapa*) were kindly provided by Anne Marie Cortesero, University of Rennes, France. Just before the infestation with the larvae, the root crowns of all plants selected for analysis were gently moved sideways to create some space next to the main root, which enabled the root fly larvae to crawl down quickly. The larvae used to infest the plants were gently removed from the turnips, cleaned with tap water to remove remaining turnip materials and placed in the space next to the root crown. Larvae that had not crawled down into the sand within 10 minutes (usually less than 10% of the larvae) were removed and replaced. After the PTR-MS measurement had ended, the roots were carefully checked to count the number of living *D. radicum*

larvae and pupae that may have formed. To do so, plants were lifted from their pots and placed in a flat plastic dish. The upper layer of sand surrounding the main roots was gently flushed with tap water to uncover the feeding larvae. To recover the pupae, the sand was washed in a large plastic bowl which easily revealed the floating pupae. It was also noted whether and to which extend the roots were damaged by larval feeding activities, which is evidenced by the presence of brown feeding trenches on the surface of the main roots.

5.3 Experimental set-up

In a typical experiment, the roots of six *B. nigra* plants were monitored by the PTR-MS over time for a period up to seven days. The experiments were performed at a constant temperature of 21°C; ambient light conditions were supplied with sodium lamps, maintaining a photosynthetically active radiation (PAR) of 225 µmol photons m^{-2} s^{-1} during the light period (16 h). Based on the literature, a number of masses, representing compounds expected to be emitted from wounded roots and/or shoots were selected and followed over time (Table 1). Also, to prevent the risk of loosing information due to monitoring of pre-selected masses, full mass scans covering a range from *m/z* 20 to *m/z* 150 were performed at different time points during the infestation. In this way, other masses showing different emission levels in infested roots as compared to control roots were included (Table 1). The plants were six to eight weeks old when three plants were infested with five cabbage root fly larvae each, whereas the other three served as non-infested control plants. Each cuvette was sampled for 20 minutes within a period of two hours. Similar experiments were

repeated six times. The volatiles released by *B. nigra* roots in infested and control/non-infested plants were monitored immediately after the addition of *D. radicum* root-fly larvae.

For the GC-MS analyses, the root headspace was collected in separate experiments. *B. nigra* plants from the same batch as used for the PTR-MS experiments were infested with six to ten second instar *D. radicum* larvae (with the amount of larvae depending on their size in order to ensure damage). Two days later, a 25x40 cm cooking bag (Toppits, Germany) prepared as in Stewart-Jones and Poppy (2006) and cut open at the bottom, was pulled up from the bottom of the pot and fixed with a rubber band around the pot. The open top of the bag was closed at ~5 cm over the soil around the stem of the plant using binder clips, leaving an opening to insert a steel trap. Volatiles were collected by pulling air from the interior of the cooking bag over a steel trap filled with 150 mg Tenax TA and 150 mg Carbopack B (Markes International Ltd., Llantrisant, UK) with a vacuum pump. Flow rates over the traps were set to 100 ml/min using mass flow regulators (Sho rateTM, Brooks Instrument, Hatfield, PA, USA). After 80 min. the traps were removed. Four plants with individual mass flow meters were sampled in parallel. In total three control and three damaged plants were sampled, plus two background controls. After the experiment, infested plants were checked for larval damage. The roots of one of the control plants turned out to be mechanically damaged and the data of this plant were removed from the analysis.

Volatiles were desorbed from the traps into the GC-MS (model Trace, ThermoFinnigan, Austin,TX, USA) using an automated thermodesorption unit (model Unity, Markes International Ltd., Llantrisant, UK) as described in van Dam

et al. (2010). Compounds were identified by their mass spectra using deconvolution software (AMDIS) in combination with NIST 2005 (National Institute of Standards and Technology, USA, http://www.nist.gov) and Wiley 7th edition spectral libraries. Additionally, reference spectra was obtained from several plant volatile compounds (farnesene, +-limonene, methyljasmonate, methylsalicilate, dimethyl disulfide, dimethyltrisulfide, octanal, nonanal, decanal, cis-3-hexen-1-ol, 2-phenylethylalcohol, indole, benzylcyanide and phenylisothiocyanate (Sigma-Aldrich; St. Louis, Il, USA); as well as methyl thiocyanate, methyl isothiocyanate, ethyl thiocyanate, ethyl isothiocyanate and allyl isothiocyanate (Acros Organics BVBA, Geel, Belgium). For identification, linear retention indices (lri) were used, and the lri data for the compounds in the study were confirmed by authentic standards. The integrated signals generated by the AMDIS software from the MS-chromatograms were used for comparison between the treatments. Peak areas in each sample were divided by the total air volume (in ml) that was sampled over the trap to correct for differences sampling times between experiments. Differences in volatile emissions between infested and control plants were assessed using a t-test.

5.4 Artificial wounding of Brassica nigra roots

For the artificial wounding experiments, the main root of non-infested *Brassica nigra* plants was damaged with a single puncture of a scalpel, 2-5 cm below the soil surface. Once the increased emissions coming from the roots after damage decreased back to initial values, the damage was repeated up to three times in total. This experiment was performed with a single non- infested *B. nigra* plant each time, and replicated three times in total with different plants

5.5 Influence of larval instar on volatile emissions

In order to study the influence of the larval instar on the temporal profile of the *B. nigra* roots emissions, two experiments were conducted. For each of them, three *B. nigra* plants were infested with four small first instar larvae per root, one with four large second instar larvae, and one plant was not infested and used as control.

5.6 Pure compounds for PTR-MS analysis

Methyl thiocyanate, methyl isothiocyanate, ethyl thiocyanate, ethyl isothiocyanate, allyl isothiocyanate were purchased from Acros Organics BVBA, (Geel, Belgium). 2-Phenethyl isothiocyanates was purchased from Sigma- Aldrich (St. Louis, Il, USA).

Thiocyanic acid was prepared as described by Bagliano et al. (1966). A Dowex $50H^+$ column was prepared (20-50 mesh, diameter 1.2 cm, length 6 cm), converted to the hydrogen form by washing with 2M HCl and washed with distilled water until the eluent became neutral. Subsequently, 2 mL of a 2M potassiumthiocyanic acid solution was added to the column followed by elution with distilled water. When the pH of the eluent became acid collecting of eluents was started.

HPLC vials (1.5 mL volume) containing 2 µL of compound were prepared for each of the compounds mentioned. For the PTR-MS measurements, a vial with a needle (1.10 x 40 mm, Microlance, Dublin, Ireland) inserted in its cap was placed in a 5 L volume glass cuvette with an inlet and an outlet. The cuvette was flushed with a constant 1.5 L/h airflow free of hydrocarbons. The outlet of the cuvette was connected to PTR-MS.

5.7 Proton Transfer Reaction Mass Spectrometry (PTR-MS)

The volatiles released by *B. nigra* roots were analyzed with a home built PTR-MS. A detailed description of the system can be found in Crespo et al. (2011); here we will give a brief description of the working principles. Proton-transfer reaction is a soft ionization technique to ionize trace gas molecules in air (Lindinger et al., 1998). In PTR-MS, trace gases with a proton affinity higher than that of water are ionized by a proton-transfer reaction with H_3O^+ ions in a drift tube, after which the product ions are mass analyzed and detected with the quadrupole mass spectrometer. The operation conditions of the drift tube are generally described by the E/N (E over N) value expressed in Townsend (1 Td = 10^{-17} V·cm^2) (de Gouw and Warneke, 2007); a value of 120 Td was used in our experiments.

H_3O^+ ions are produced in the ion source by establishing a discharge in water vapor. It should be noted, however, that back diffusion of air from the drift tube into the ion source leads to a percentage of contaminant ions, with NO^+ and O_2^+ as main impurities (de Gouw and Warneke, 2007). It is important to reduce the amount of NO^+ and O_2^+ ions since those ions can undergo charge-transfer reactions with most VOCs (Smith and Španěl, 2004) without adding a proton to the trace gas molecule, thereby making the identification of certain masses more complicated. Experimentally, higher *m/z* 60 levels could be observed at higher levels of the contaminant primary ions NO^+ and O_2^+.

The system was calibrated by mixing a variable flow of air free of hydrocarbons (by passing it through a catalytic converter at 350°C), with a fixed flow of 0.3 L/h of a

gas mixture of methanol, acetaldehyde, acetone, isoprene, benzene, toluene and xylene (molecular weights ranging from 32 amu to 106 amu) in concentrations of 1 ppmv (±5%) (Linde, Dieren, the Netherlands). In this way, calibration factors are obtained for these compounds converting ion intensity (in ncps, normalized counts per seconds) to gas mixing ratios (in ppbv, part per billion volume). From these conversion factors the calibration factors of other compounds at a specific *m/z* ratio were calculated, taking into account the difference in collision rate constant in the drift tube and transmission efficiency factors of the mass spectrometer (de Gouw et al., 2007).

5.8 Gas handling set-up

To maximize the collection of root volatiles, cuvettes such as the one shown in Fig. 9A were employed. Silicon tubing was used to connect the glass inlet and the Teflon tube into the PTR-MS. Care was taken to minimize the contact (< 1mm) of the VOC flow with the silicon because silicon adsorbs VOCs. In Fig. 9B a typical set-up with the root cuvette is shown; the plants were grown with a pot somewhat larger than the diameter of the cuvette to maximize VOC influx, and a synthetic rubber based sealant (Terostat IX) is used between the two halves of the cuvette to avoid major leaks.

The headspace of the cuvettes was continuously flushed with hydrocarbon-free air, which was regulated by mass flow controllers (flow 1 L/h, Brooks Instrument, Ede, the Netherlands). All the sampling lines and connectors were made of Teflon (Polyfluor Plastics, Oosterhout, the Netherlands). Sampling lines and drift-tube were heated to 55^{o}C to reduce memory effects caused by VOC-surface interactions. For

each experiment the roots of six cuvettes were sequentially monitored by switching from cuvette to cuvette using a stream selector valve (Valco cheminert valves, Bester BV, Amstelveen, the Netherlands).

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7. Figure captions

Figure 1: Glucosinolate structure and its major volatile autolysis products, isothiocyanates, nitriles and thiocyanates.

Figure 2: Intensities of m/z 49, m/z 60, m/z 63 and m/z 95 emissions from B. nigra roots under the attack of five second instar D. radicum larvae; average of 3 replicates + standard deviation. Symbols: open squares: control (non-infested) plants; closed squares: infested plants.

Figure 3: Relative peak areas (+ SE) on GC-MS of sulfides emerging from Brassica nigra roots with (n=3 plants) and without (control, n = 2 plants) root fly larvae. DMS = dimethyl sulfide (P =0.065), DMDS = dimethyl disulfide (P =0.008), DMTS = dimethyl trisulfide (P <0.001). P-values of independent t-tests on the square root-transformed data are given between brackets after each compound name.

Figure 4: Relative peak areas (+ SE) on GC-MS of glucosinolate breakdown products emerging from Brassica nigra roots with (n=3 plants) and without (control, n = 2 plants) root fly larvae. ITC = isothiocyanate. P-values of independent t-tests on the square root-transformed data: Allyl ITC, P =0.029; Phenyethyl ITC, P =0.008; Benzonitrile, P >0.9.

Figure 5: Correlation between the emissions at m/z 60 and m/z 62 (isotopes of thiocyanic acid, HCNS) from B. nigra roots infested with the cabbage root fly larvae.

Figure 6: m/z 60 increase by artificial wounding of B. nigra roots at t = 15 min (see arrow); other sulfur volatiles (methanethiol, DMS and DMDS) did not show such immediate increase as a result of artificial wounding. Symbols: closed circles: m/z 60 (thiocyanic acid); open squares: methanethiol; open triangles: dimethyl sulfide; open circles: dimethyl disulfide.

Figure 7: Time profile of the emissions of m/z 60 from roots infested with big larvae (four late second instar larvae; closed triangles), small larvae (four first instar larvae, 3 replicate average plus s.d., closed squares) and from control roots (open circles).

Figure 8: Emissions of methanethiol, HCNS, DMS and DMDS of 20 second instar larvae 5 cm deep in plain river sand, as compared to only river sand.

Figure 9: A) Cuvette for the collection of root volatiles. B) Root cuvette in B. nigra plants. Terostat was used to connect the two halves of the cuvette, thereby avoiding major leaks.

8. Table captions

Table 1: Compounds potentially emitted by roots during infestation. Most of the compounds selected are reported to be emitted by whole plants. The compound name is followed by its related masses detected with PTR-MS, and references are from either Brassicaceae or other plant species ([a]Main fragments, [b]parent mass and main fragments.)

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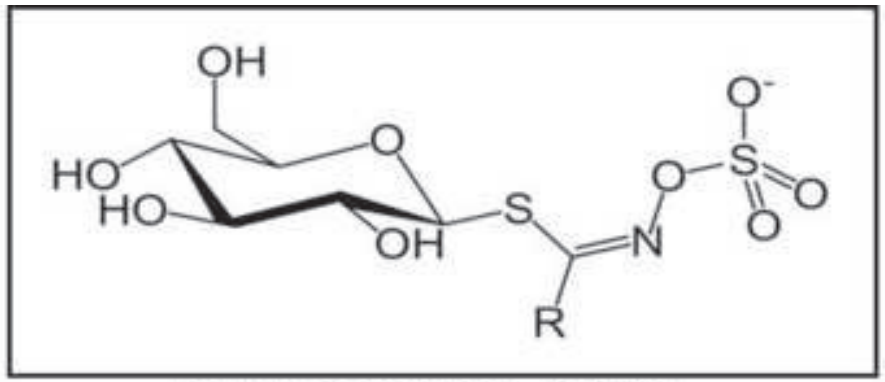

GLUCOSINOLATES

GLUCOSINOLATES
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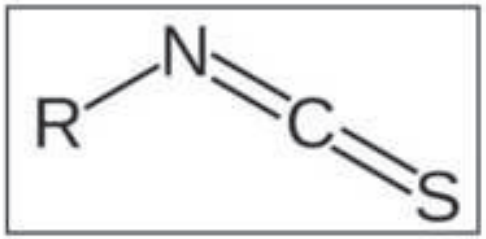

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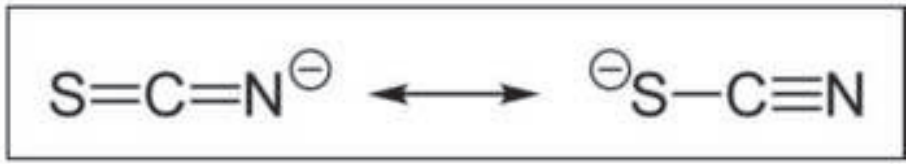

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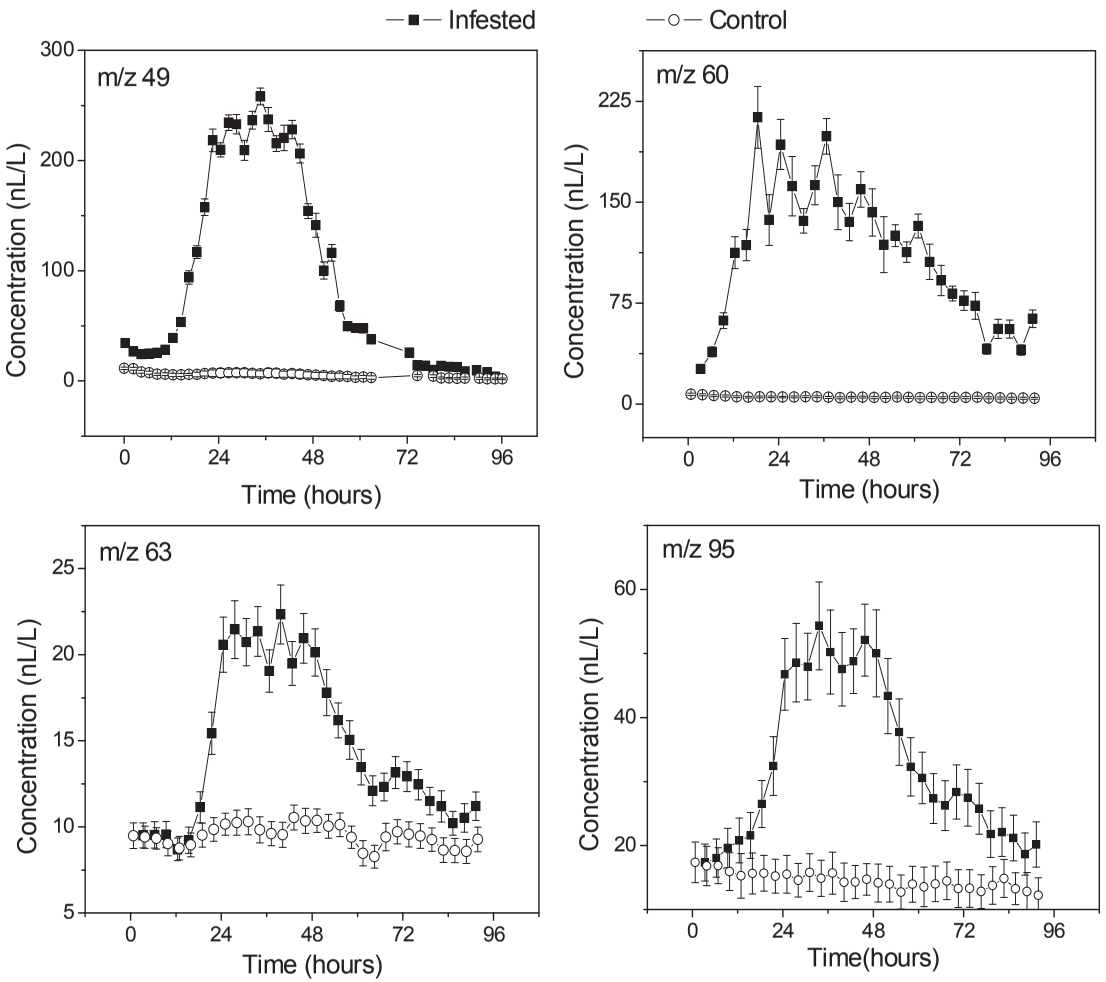
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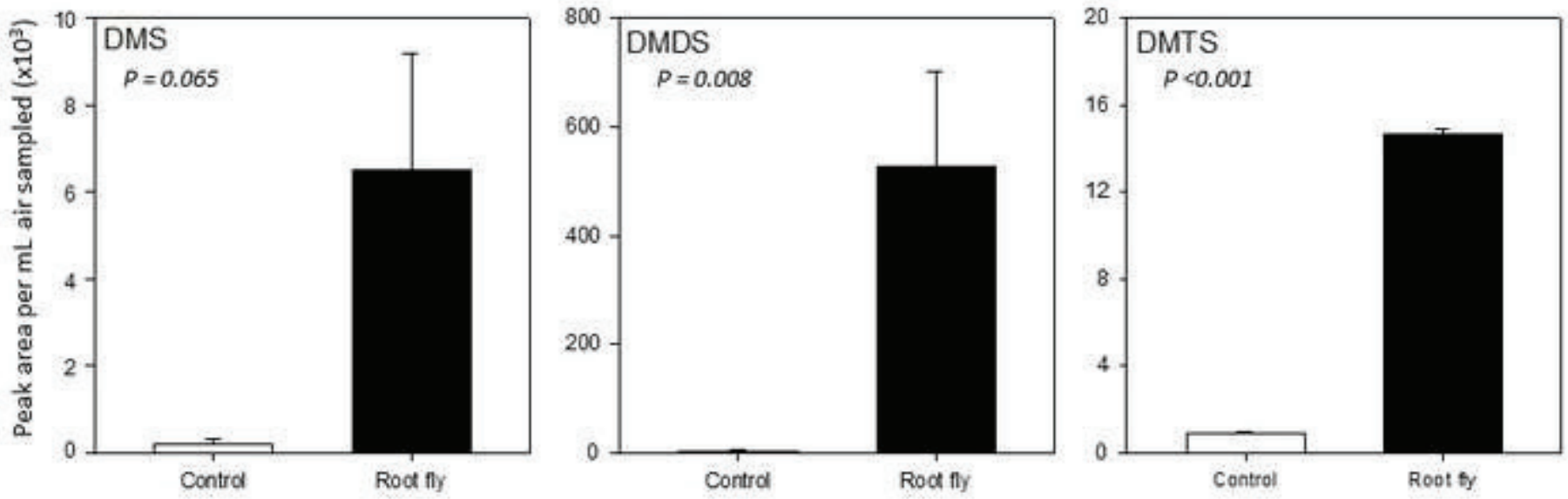

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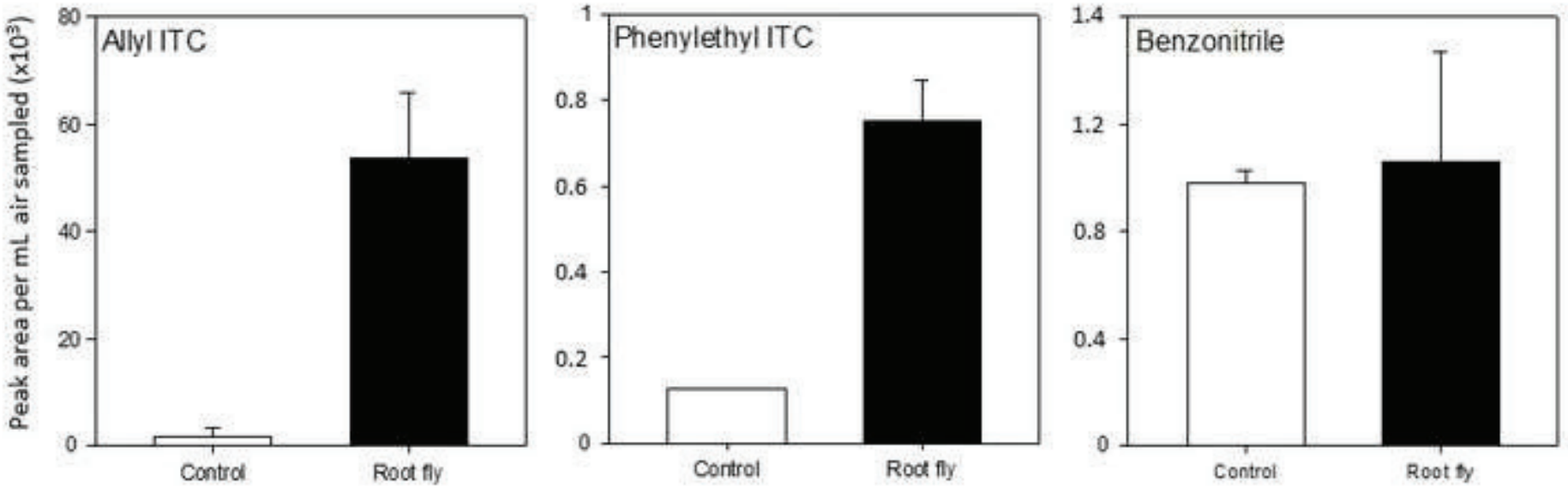

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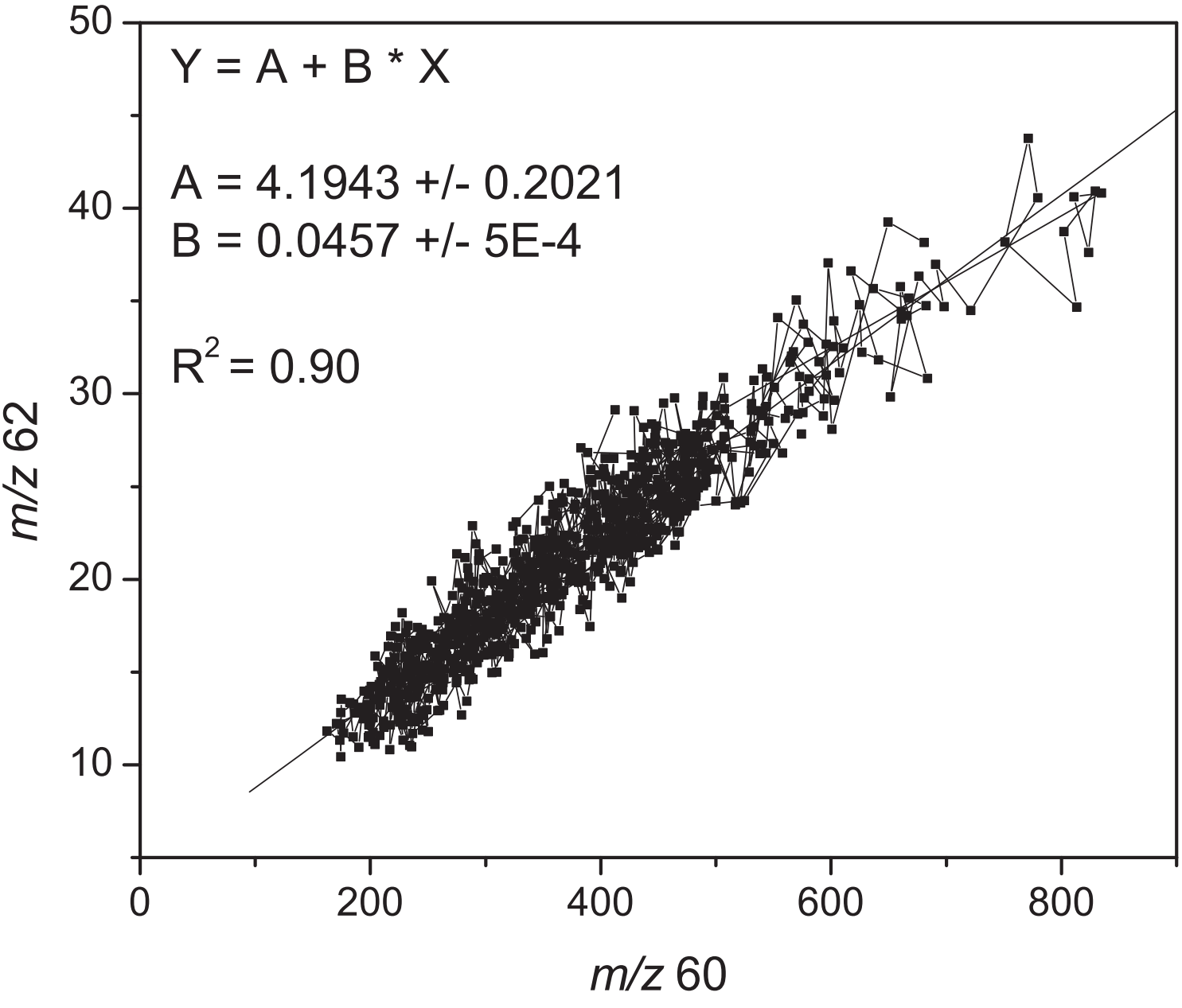
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A = 4.1943 +/- 0.2021
B = 0.0457 +/- 5E-4
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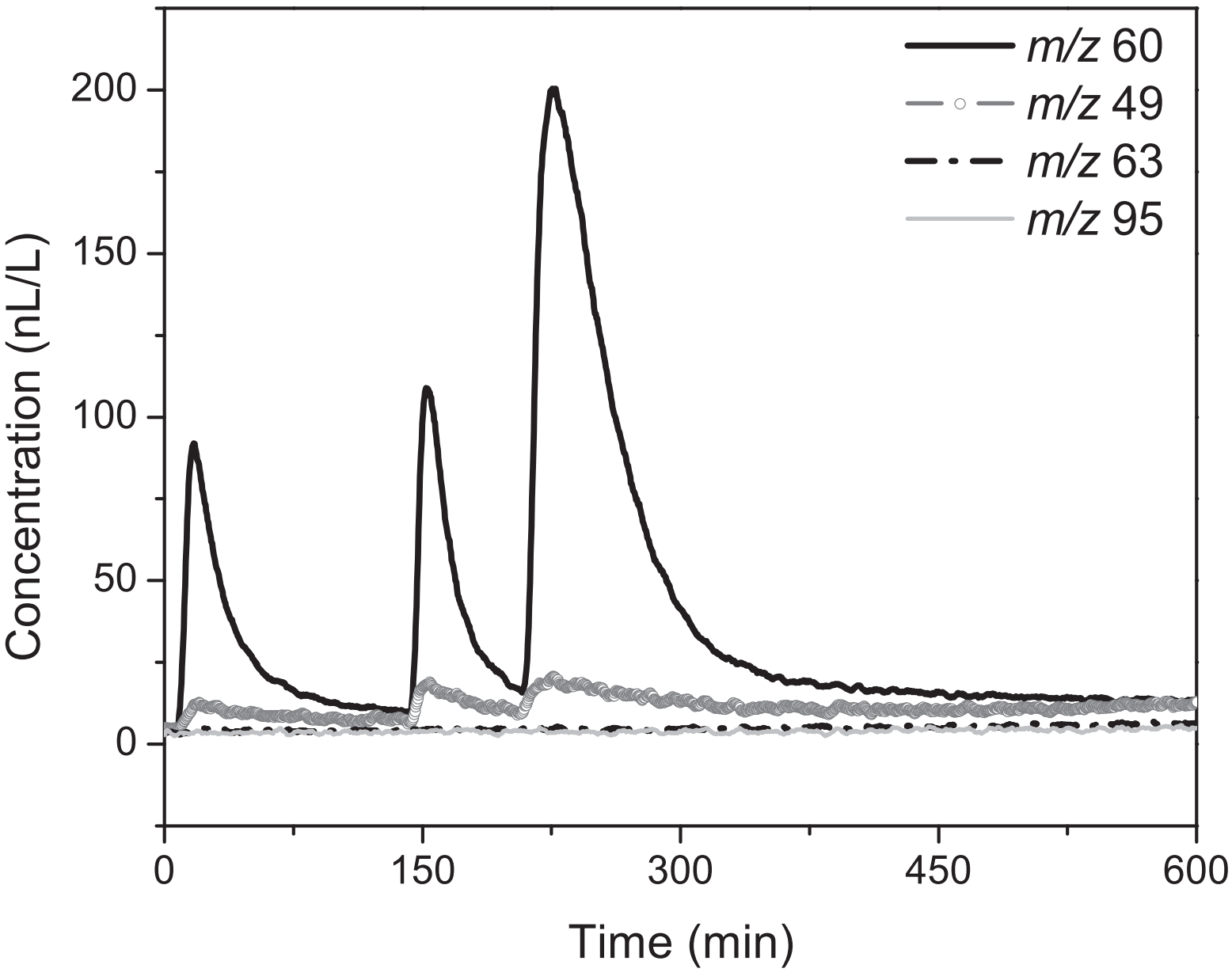
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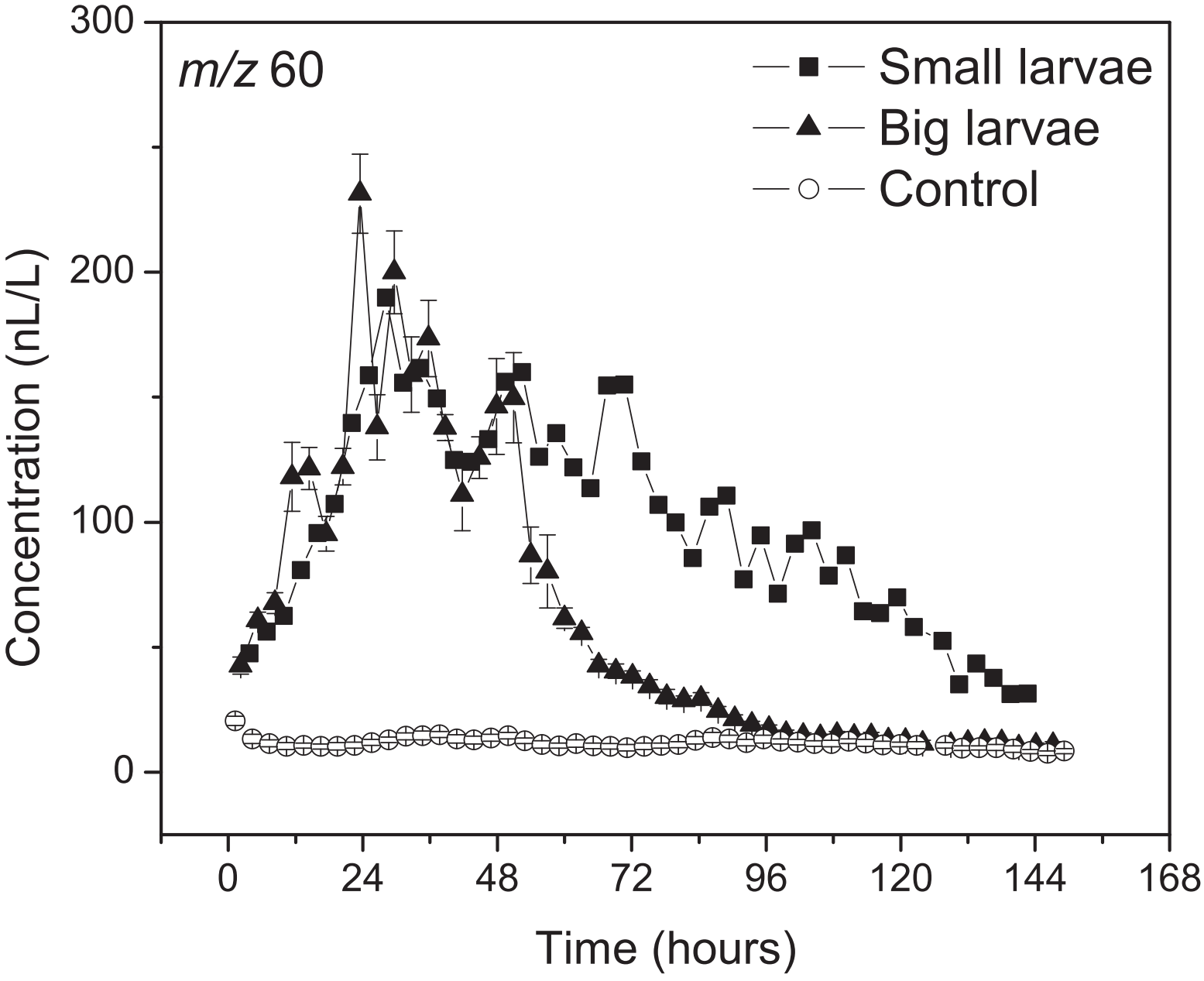
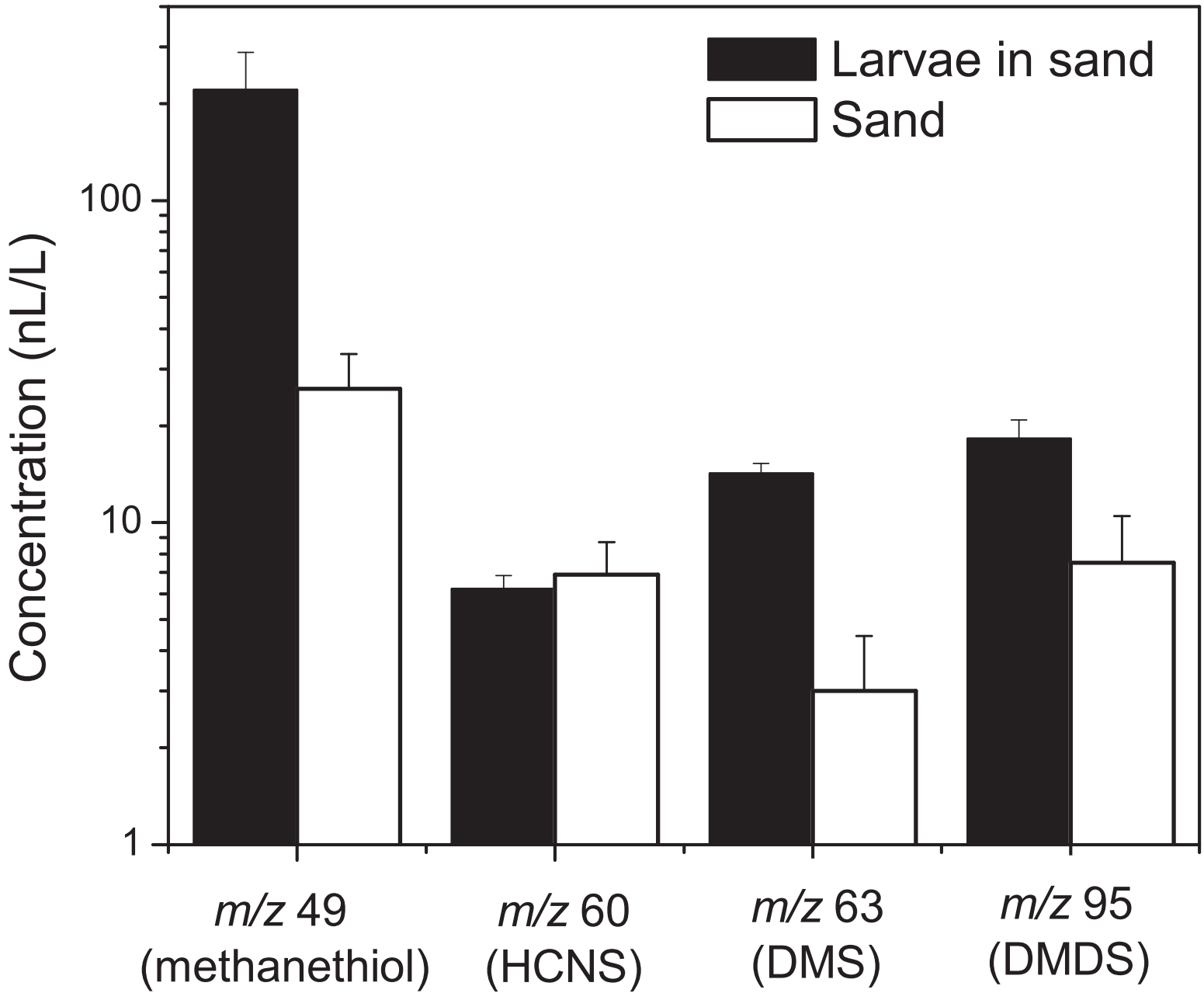
Time (min)

Figure(s)


m/z 60
Small larvae
Big larvae
Control
Concentration (nL/L)
300
200
100
0
0
24
48
72
96
120
144
168
Time (hours)

Figure(s)


Larvae in sand
Sand
Concentration (nL/L)
100
10
1
m/z 49
(methanethiol)
m/z 60
(HCNS)
m/z 63
(DMS)
m/z 95
(DMDS)

Figure(s)
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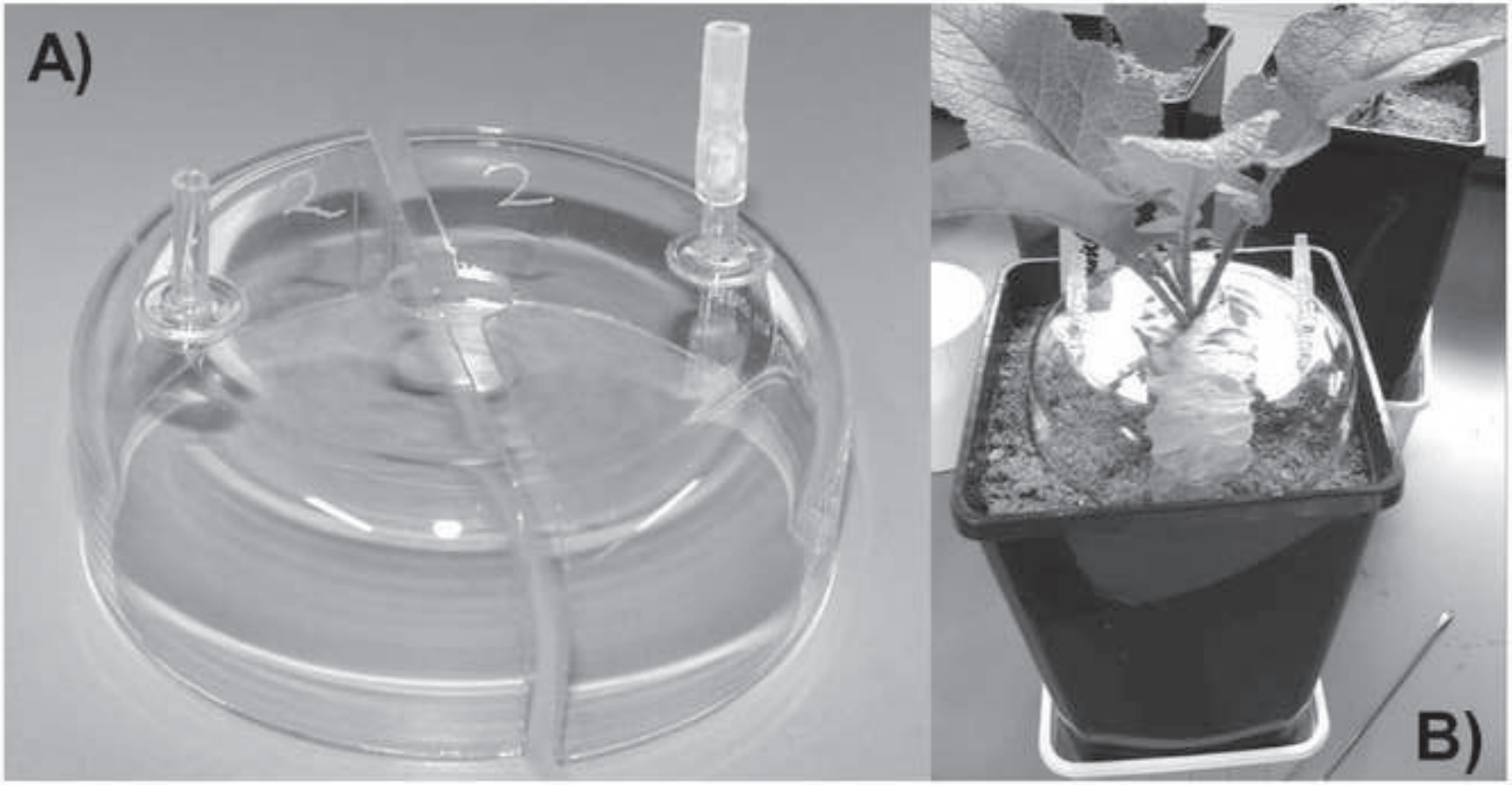

Table(s)

Species	Protonated mass (*m/z*)	Reference
Methanol	33	Day-night rhythm, induction Davison et al., 2009 Loreto et al., 2006 Peñuelas et al., 2005
Acetaldehyde	45, 46 (^{13}C isotope)	Day-night, induction Davison et al., 2009 Loreto et al., 2006
Ethanol	47	Day-night, induction?
Methanethiol	49, 51 (^{34}S isotope)	Bending and Lincoln, 1999
Acetone	59	Day-night Davison et al., 2009 Janson and de Serves, 2001
Acetic acid	61	Day-night, induction Kesselmeier, 2001 Steeghs et al., 2004
Dimethyl sulfide (DMS)	63, 65 (^{34}S isotope)	Bending and Lincoln, 1999
Isoprene	69, 41[b]	Day-night; Davison et al., 2009
1,8-Cineole	81[a]	Induction; Steeghs et al., 2004
Dimethyl disulfide (DMDS)	95, 97 (^{34}S isotope)	Induction; Soler et al., 2007
Indole	118	Induction; Paré and Tumlinson, 1997
Dimethyl trisulfide (DMTS)	127	Induction; Soler et al., 2007
C6 wound compounds		
(*Z*)-3-hexenol,	83, 55[a]	Induction
(*E*)-2-hexenol,	83, 55[a]	Fall et al., 1999
(*E*)-2-hexenylacetate	83, 55[a]	Loreto et al., 2006
(*Z*)-3-hexenyl acetate	83, 55[a]	Peñuelas et al., 2005
(*E*)-2-hexenal	99, 81, 57[b]	Tollsten and Bergstrum, 1988
(*Z*)-3-hexanal	99, 81[b]	Wallbank ,1976
(*E*)-3-hexenol	83, 59[a]	
(*E*)-2-hexenol	83, 55[a]	
n-Hexanal	83, 55[a]	
n-Hexanol	85, 57, 43, 41[a]	
Hexyl acetate	61, 43[a]	
C5 wound compounds		
Isoprene	69, 41[b]	Induction
3-Methylbutanal	87, 69[b]	Fall et al., 2001
2-Methylbutanal	87, 69[b]	Loreto et al., 2006
3-Methyl-2-buten-1-ol	69[a]	
2-Methyl-3-buten-2-ol	87, 69, 41[b]	
3-Methyl-3-buten-1-ol	69[a]	
2(*Z*)-penten-1-ol	69[a]	
2(*E*)-penten-1-ol	69[a]	
1-Penten-3-ol	69[a]	
1-Penten-3-one	85	
Monoterpenes	137, 93, 81[b]	Day-night, induction Paré and Tumlinson, 1997 Peñuelas et al., 2005 Soler et al., 2007. Tollsten and Bergstrum, 1988
Glucosinolate breakdown products		
Thiocyanic acid	60, 62 (^{34}S isotope)	Induction
Butanenitrile	70	Soler et al., 2007
Methylisothiocyanate,	74	Tollsten and Bergstrum 1988
Methylthiocyanate	74	
Ethylisothiocyanate,	88, 60[b]	
Ethylthiocyanate	88, 60[b]	
Allyl isothiocyanate	100, 60[b]	