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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 isothiocyanates (ITC), were emitted by the roots in response to infestation. The emissions were subdivided into early responses, emerging within 1–6 h after infestation, and late responses, evolving only after 6–12 h. 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.

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. Glucosino-

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lates 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.

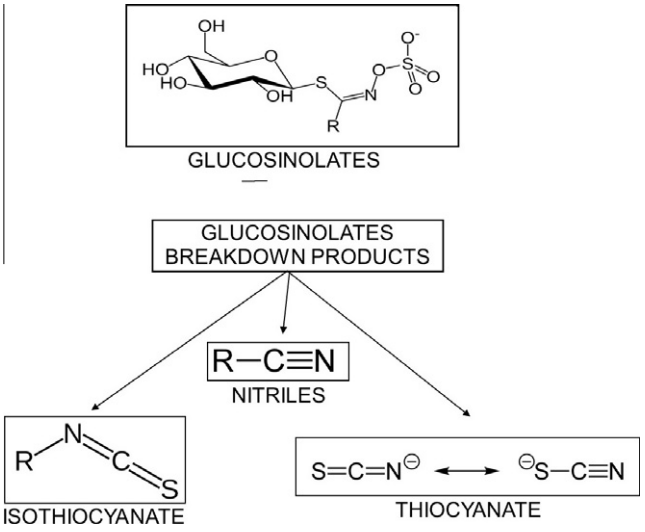

Fig. 1. Glucosinolate structure and its major volatile autolysis products, isothiocyanates, nitriles and thiocyanates.

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 h after the infestation, while *m*/*z* 60 (Fig. 2) started increasing shortly after infestation (between 1 and 6 h). Those three volatiles increased until they reached a peak value between 1 and 3 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