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Systemic root signalling in a belowground, volatile-mediated tritrophic interaction

IVAN HILTPOLD, MATTHIAS ERB, CHRISTELLE A. M. ROBERT & TED C. J. TURLINGS
FARCE Laboratory, University of Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland

ABSTRACT

Plants attacked by leaf herbivores release volatile organic compounds (VOCs) both locally from the wounded site and systemically from non-attacked tissues. These volatiles serve as attractants for predators and parasitoids. This phenomenon is well described for plant leaves, but systemic induction of VOCs in the roots has remained unstudied. We assessed the spatial and temporal activation of the synthesis and release of (*E*)-β-caryophyllene (Eβ C) in maize roots upon feeding by larvae of *Diabrotica virgifera virgifera*, as well as the importance of systemically produced Eβ C for the attraction of the entomopathogenic nematode *Heterorhabditis megidis*. The production of Eβ C was found to be significantly stronger at the site of attack than in non-attacked tissues. A weak, but significant, increase in transcriptional activity of the Eβ C synthase gene *tps23* and a corresponding increase in Eβ C content were observed in the roots above the feeding site and in adjacent roots, demonstrating for the first time that herbivory triggers systemic production of a volatile within root systems. In belowground olfactometers, the nematodes were significantly more attracted towards local feeding sites than systemically induced roots. The possible advantages and disadvantages of systemic volatile signalling in roots are discussed.

Key-words: *Diabrotica virgifera virgifera*; *Heterorhabditis megidis*; belowground tritrophic interaction; entomopathogenic nematode; induced plant defence; systemic root response; volatile emission; Western corn rootworm.

INTRODUCTION

Plants can adopt different strategies to reduce damage and loss of vital tissue upon herbivore attack (Howe & Jander 2008). One apparent strategy is the release of volatile organic compounds (VOCs), which increases the plant's attractiveness to the natural enemies of herbivores (Dicke & Sabelis 1988; Turlings *et al*. 1991; De Moraes *et al*. 1998; Rasmann *et al*. 2005), thus providing protection from herbivory (Kessler & Baldwin 2001; Degenhardt *et al*. 2009). In aboveground tissues, it has been demonstrated that the induction of VOC does not only happen locally at the wounded site, but also systemically in non-attacked leaves (Turlings & Tumlinson 1992; Heil 2008; Rostas & Eggert 2008; Champigny & Cameron 2009). This systemic response may boost the plant's signalling capacity to attract natural enemies: parasitoids, in particular, forage over a spatial scale of meters (Puente *et al*. 2008) up to several kilometres (Thies, Roschewitz & Tscharntke 2005), and long range cues may therefore help to increase their recruitment (Geervliet *et al*. 1998).

An entirely different situation can be found in the rhizosphere. Soil provides a complex and small-scaled matrix, but also offers an environment that protects substances from degradation by oxygen and light, making belowground VOC signals more stable and possibly more reliable than aboveground. In this particular ecosphere, many beneficial organisms, including entomopathogenic nematodes (EPNs), can only travel shorter distances at much lower velocities than the aboveground enemies of herbivores. EPNs might therefore benefit more from local host location cues rather than signals that are emitted systemically from attacked roots. Conversely, if plants would benefit from attracting EPNs, they may have evolved inducible volatiles that are predominantly emitted from the site of attack in order to provide the necessary short-range stimuli. Neveu *et al*. (2002) conducted the only behavioural study that provides solid evidence for systemic emissions of volatiles after root herbivory on turnip. They found that a parasitoid that attacks a belowground herbivore uses signals that are systemically emitted aboveground to locate host-infested plants. Other *Brassica* appear not to respond systematically to herbivory (van Dam & Raaijmakers 2006), implying that differences in vascular anatomy may play an important role in systemic plant response (Bledsoe & Orians 2006).

It is surprising that so little is known about the systemic induction of root defences, given the fact that roots are frequently subjected to direct attacks (Blossey & Hunt-Joshi 2003). Soil-dwelling herbivores influence plant development and survival in nature, as well as crop yield in agriculture (Rasmann & Agrawal 2008). For instance, the Western corn rootworm (WCR) (*Diabrotica virgifera virgifera* LeConte, Coleoptera: Chrysomelidae) is an economically important root pest of *Zea mays* L. in the USA, as well as in Europe (Krysan & Miller 1986; Vidal, Kuhlmann & Edwards 2005). By feeding on maize roots, the larvae can cause losses of over a billion US dollars per

Correspondence: T. C. J. Turlings. Fax: +41 32 7183001; e-mail: ted.turlings@unine.ch

year in the USA (Krysan & Miller 1986), and estimations for future costs in Europe go as high as 0.6 billion € (Wesseler & Fall 2010). Upon attack by WCR larvae, the roots of many European maize varieties produce the sesquiterpene (*E*)-*β*-caryophyllene (E*β*C) (Rasmann *et al*. 2005; Köllner *et al*. 2008). E*β*C is a particularly potent rhizosphere signal that diffuses readily through soil (Hiltpold & Turlings 2008) and is attractive to several EPN species (Rasmann *et al*. 2005; Rasmann & Turlings 2008; Hiltpold *et al*. 2010b). EPNs can be effective in controlling most larval instars of the pest (Kurzt *et al*. 2009; Pilz *et al*. 2009), but is significantly more effective when the roots emit E*β*C (Hiltpold *et al*. 2010a,b). Interestingly, root damage by WCR can also profoundly alter the direct and indirect defensive state of maize aboveground, as feeding by WCR belowground increases the resistance against the leaf herbivore *Spodoptera littoralis* (Boisduval) and the fungal pathogen *Setosphaeria turcica* (Luttr.) (Erb *et al*. 2009a), while the attractiveness of the plant to parasitoids may decrease (Rasmann & Turlings 2007). The vertical response of the plant in this case does not follow a classical pattern of wound induction, but is mainly caused by hydraulic changes and abscisic acid (ABA) signalling (Erb *et al*. 2009a,b, 2010). No induced VOC emission or increased volatile synthase activity is detected in the leaves of root herbivore-damaged plants (Köllner *et al*. 2008; Rasmann & Turlings 2008), suggesting that the E*β*C response is mainly local. Yet, it is not clear if this pattern also holds for non-attacked parts of the roots, or if they follow the typical systemic wound induction pattern documented for plant leaves (Baldwin, Schmelz & Ohnmeiss 1994; Glauser *et al*. 2008; Howe & Jander 2008), which involves a combination of vascular transport through the xylem and phloem, as well as non-vascular induction via cell-to-cell signalling and volatile diffusion (Orians 2005).

To better understand the extent to which maize roots systemically respond after a WCR attack, we assessed the spatial and temporal activity of the E*β*C *Zm-TPS23* synthase gene (Köllner *et al*. 2008) and the production of E*β*C of different parts of the root system. We also studied the impact of the systemic response on the third trophic level by assessing the attractiveness of different parts of the root system to EPN. To confirm an emission of E*β*C by damaged roots, we also performed an *in vivo* VOC sampling experiment.

MATERIALS AND METHODS

Vertical systemic response

To distinguish between local and systemic root responses above the feeding site, maize plants (*Z. mays*, variety Delprim) were sown in plastic tubes (4 cm diameter, 11 cm depth) in a multilayer environment. The lowest 1 cm of the tube was filled with standard potting soil (Ricoter Aussaaterde, Aarberg, Switzerland). A fine nylon mesh (0.25 mm, stretchable) penetrable by roots, but not by second-instar WCR larvae, was then placed on the layer, and a second plastic tube (3.8 cm diameter, 1 cm depth) was pushed into the outer plastic pot and filled with sand up to 3 cm from the top. The seeds were placed on this sand layer and covered with 3 cm of standard potting soil. Tubes were wrapped in aluminium foils and placed in a phytotron (25 °C, 8:16 h dark : light photoperiod, 400 μmol m^{-2} s^{-1}, CLF Plant Climatics, Percival, DE). In total, 72 plants (*Zea mays*, variety Delprim, DSP SA, Switzerland) were grown and fertilized twice with standard mineral fertilizer (Mio-Plant Gemüse- und Kräuterdünger; Migros Deutschland GmbH, Lörrach, Germany) after 4 and 8 d of growth. Ten days after planting, 36 seedlings were infested with eight second-instar WCR larvae by releasing them into the lower compartment formed by the two pots via four holes drilled in the bottom of the pots that had been sealed with aluminium foil. After introducing the larvae, the holes were covered again with aluminium foil to prevent the larvae from escaping. The 36 control plants did not receive any WCR larvae. At different time points after infestation (0.5, 1, 2, 4, 24 and 48 h), six WCR-damaged root systems and six controls were harvested. The roots from the harvested plants were carefully rinsed with water, excised from the shoots, individually wrapped in aluminium foil and immediately frozen in liquid nitrogen. For each plant, roots were separated into local tissue (root parts that had been in direct contact with WCR) and systemic tissue (roots above the nylon mesh that had not been directly fed upon by herbivores).

Horizontal systemic response

To assess the systemic response of roots adjacent to the feeding site, 72 maize plants (*Z. mays*, variety Delprim) were grown in plastic tubes (4 cm diameter, 11 cm depth). The bottom 9 cm of each tube was filled with sand. A maize seed was placed on top of the sand and covered with 2 cm of standard potting soil. Tubes were wrapped in aluminium foil and stored as described above. After 4 d of germination, seedlings were transplanted in plastic tubes that were vertically cut in two equal parts. Then, a layer of cardboard covered with aluminium foil was placed between the two halves, and they were held together with a rubber band to obtain a two-compartment tube. The plants were then grown for 6 additional days before half of the plants were infested with eight second-instar WCR larvae. The larvae were dropped in 5-cm-deep holes drilled in one compartment of the modified pots. None of the 36 control plants were infested with WCR larvae. At different time points after infestation (1, 2, 4, 24 and 48 h), six WCR-damaged root systems and six controls were harvested. The roots from the harvested plants were carefully rinsed with water, excised, wrapped in aluminium foil and immediately frozen in liquid nitrogen. For each plant, roots were separated into local tissue (root parts that had been in direct contact with WCR) and systemic tissue (roots in the adjacent compartment that had not been directly fed upon by herbivores).

EPN response in split root belowground olfactometers

To measure the attractiveness of systemically induced tissue compared to locally WCR-damaged tissue, we carried out a belowground olfactometer experiment. For this purpose, plants were sown in plastic pots (8 cm diameter, 4.5 cm depth). The bottom 3 cm of the pots was filled with sand. On top of this first layer, a single seed was placed in each pot and covered with 1.5 cm of standard potting soil. The pots were placed in a phytotron (23 ± 1 °C, 8:16 h dark : light photoperiod, CLF Plant Climatics, Percival, DE). The plants were fertilized twice with standard mineral fertilizer (Mio-Plant Gemüse- und Kräuterdünger; Migros) after 4 and 8 d of growth. After 10 d of growth, the plants were transplanted in belowground six-arm olfactometers. The olfactometers were assembled following the methodology developed by Rasmann *et al*. (2005), but to test the possible effects of systemic responses, the root system of the three plants per olfactometer was split into two glass pots. One pot was connected to the olfactometer and the other pot was not (Fig. 1). For each trial, 2000 nematodes of the species *Heterorhabditis megidis* (obtained from Andermatt Biocontrol, Grossdietwil, CH) were released in the central chamber of the olfactometer. The nematodes had the choice between four different treatments: (1) a plant induced with five second-instar WCR larvae in the connected pot; (2) a plant induced with five second-instar WCR larvae in the unconnected adjacent pot; (3) a plant without WCR induction; and (4) three control pots filled only with wet sand. Twenty-four hours after EPN release, the olfactometers were disassembled, the sand from each of the six connectors was placed in a Baermann extractor (Baermann 1917), and the next day, the number of nematodes extracted from each arm was recorded.

After the olfactometers were disassembled, the roots from each pot were carefully rinsed with water, excised from the shoots, wrapped in aluminium foil and immediately frozen in liquid nitrogen. Volatiles were collected and analysed as described below.

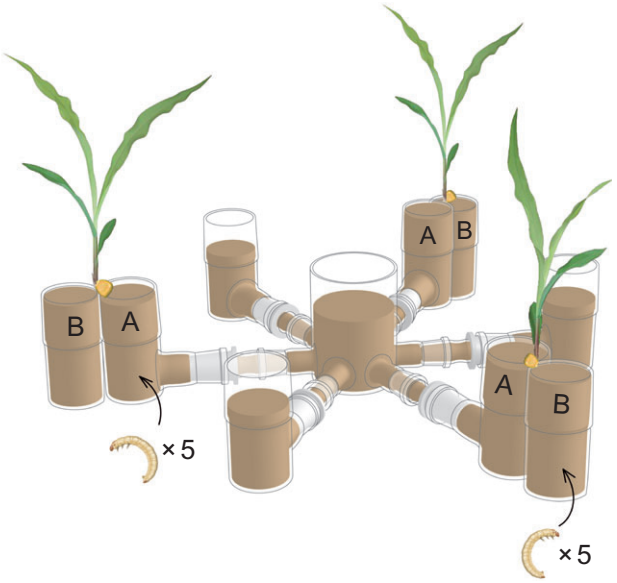

Figure 1. Drawing of the six-arm belowground olfactometer used for behavioural experiments. The root system of each 10-day-old maize plant was divided between the two sand-filled glass pots. It resulted in having half of each root system being connected to the central chamber of the olfactometer (pot A in the figure) and the other half being isolated from this device (pot B in the figure). Of the three plants, only two received five Western corn rootworm (WCR) larvae. In one case, these larvae were placed in the pot connected to the central glass chamber, whereas in the other case they were placed in the adjacent unconnected pot. The third plant served as an uninfested control. As negative controls, three pots were filled with sand only. Entomopathogenic nematodes (EPNs) were released in the central pot of the olfactometer and their choice was determined the next day. (Modified after an original drawing by T. Degen, http://www.thomas-degen.ch).

Measurements of root volatile production and quantification of *Zm-tps23* expression

Root material from the systemic vertical response and the split-root systemic response described in the previous sections was used to measure the induced production of EβC and expression of *Zm-tps23*, the EβC-synthase gene of maize. Roots of each time point were pooled before they were ground in liquid nitrogen. To determine the relative amounts of induced volatiles, 0.3 g of ground root material was transferred to a glass vial sealed with a Teflon-coated septum (Agilent Technologies SA, Basel, Switzerland). A 100 μm polydimethylsiloxane SPME (Supelco, Sigma-Aldrich Chemie SA, Buchs, Switzerland) fibre was inserted through the septum and exposed for 60 min at 40 °C. The compounds adsorbed on the fibre were injected in an Agilent 6890 Series GC system G1530A (Palo Alto, CA, USA) coupled to a quadrupole-type mass-selective detector (Agilent 5973; transfer line 230 °C, source 230 °C, ionization potential 70 eV). The fibre was inserted into the injector port (230 °C), desorbed and chromatographed on an apolar column (HP1-MS; 30 m, 0.25 mm internal diameter, 0.25 mm film thickness; J&W Scientific, Agilent Technologies SA, Basel, Switzerland). Helium at a constant pressure of 18.55 lb in^{-2} (127.9 kPa) was used as carrier gas flow. After fibre insertion, the column temperature was maintained at 50 °C for 3 min, and then increased to 180 °C at 5 °C min^{-1} followed by a final stage of 3 min at 250 °C. Chromatograms were analysed with ChemStation E.02.00.4983 software (Agilent Technologies SA, Basel, Switzerland). The rest of the root material was used for the quantification of *Zm-tps23* expression. Total RNA was extracted using Qiagen RNA-Easy extraction kits (Quiagen AG, Hilden, Germany) following the manufacturer's instructions. The quality of the RNA was assessed by photometry and gel electrophoresis. To remove contaminant genomic DNA, all samples were treated with Ambion DNAse (Austin, TX,

USA) following the standard protocol. cDNA was then synthesized using Invitrogen Super-Script III reverse transcriptase (Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative reverse transcriptase real-time PCRs (q-PCRs) were then carried out using *Zm-tps23*-specific primers (L: tctggatgatgggagtcttctttg; R: gcgttgccttcctctgtgg). The q-PCR mix consisted of 5 μL Quantace Sensimix containing Sybr Green I (Biolone GmbH, Luckenwalde, Germany), 3.4 μL H_2O, 100 nmol of each primer (2×0.3 μL H_2O) and 1 μL of cDNA sample. Then, q-PCR was carried out using 45 cycles with the following temperature curve: 10 s, 95 °C; 20 s, 60 °C; 15 s, 72 °C. The final melt curve was obtained by ramping from 68 to 98 °C in 1 °C steps every 5 s. To determine primer efficiencies and optimal quantification thresholds, a dilution series of a cDNA mix consisting of 4 μL solution from every sample was created. Six 10-fold dilution steps were carried out, and the standard curve was included into every q-PCR run. The final obtained C_t values (using the automated threshold determination feature of the Rotor-Gene 6000 software, Quiagen AG, Hilden, Germany) were corrected for the housekeeping gene *Zm-gapc*, and normalized to average control levels to obtain fold changes of treated plants. Root material sampled from the plants used for the olfactometer experiments was analysed using the same method as described earlier.

In vivo collection of EβC emitted from WCR-damaged roots

To evaluate the actual emission of EβC by maize damaged roots, six maize plants (*Z. mays*, variety Delprim) were grown in plastic tubes (4 cm diameter, 11 cm depth). The bottom 9 cm of each tube was filled with sand. A maize seed was placed on top of the sand and covered with 2 cm of standard potting soil. Tubes were wrapped in aluminium foil and stored as described earlier. After 10 d, each plant was transplanted in sand in a spherical glass pot (7 cm diameter). Three pots received five second-instar WCR larvae. The three remaining uninfested pots served as controls. After 48 h of feeding by the larvae, volatiles emitted from the roots were sampled following the procedure modified after Turlings *et al*. (1991). Briefly, pure and humidified air was blown through the sand via ports in the spherical glass pots and pulled out at a rate of 900 mL min^{-1} through a filter containing 25 mg of Super-Q adsorbent (Alltech Assoc., Deerfield, IL, USA). After a collection of 6 h, the Super-Q filters were removed and volatiles were desorbed with 150 μL of dichloromethane. Two internal standards (*n*-octane and *n*-nonyl-acetate, 20 ng mL^{-1}) in 10 μL of dichloromethane were added. Of the extracts, 2.5 μL was injected in an Agilent 6890 Series GC system G1530A coupled to a quadrupole-type mass-selective detector (Agilent 5973; transfer line 230 °C, source 230 °C, ionization potential 70 eV). The aliquot was injected in the injector port (230 °C) and chromatographed on an apolar column (HP1-MS, 30 m, 0.25 mm internal diameter, 0.25 mm film thickness; J&W Scientific, Agilent Technologies SA, Basel, Switzerland). Helium at a constant pressure of 18.55 lb in^{-2} (127.9 kPa) was used as carrier gas. After injection, the column temperature was maintained at 60 °C for 3 min, and then increased to 220 °C at 10 °C min^{-1}, held for 6 min, and then increased again for 5 min to 250 °C. Chromatograms were processed with ChemStation E.02.00.4983 software and relative quantities for EβC were calculated based on the peak areas of the internal standard.

Statistical analyses

Data for volatile emissions and gene activity over time were analysed using an analysis of covariance (ANCOVA). Treatment (with or without WCR larvae), time (time of infestation) and treatment × time were considered as independent co-variables, and volatile production or *Zm-tps23* activity as dependent variable.

The nematode behavioural responses in the six-arm olfactometer were analysed with a log-linear model. The entity computing a repetition in the statistical analysis corresponds to the response of a group of 2000 nematodes released, which was shown to follow a multinomial distribution. As the data did not conform to simple variance assumptions implied in using the multinomial distribution, we used quasi-likelihood functions to compensate for the over-dispersion of nematodes within the olfactometer (Turlings, Davison & Tamò 2004). The model was fitted by maximum quasi-likelihood estimation in the software package R (http://www.R-project.org), and its adequacy was assessed through likelihood ratio statistics and examination of residuals (Turlings *et al*. 2004). Differences between EβC contents were tested using an analysis of variance (ANOVA). The differences between treatments were analysed with Tukey post-hoc tests. Differences in *in vivo* root emission of EβC were assessed using the non-parametric Mann–Whitney *U*-test.

RESULTS

Systemic vertical and split-root responses (Figs 2 & 3)

EβC, the dominant VOC emitted by maize roots upon WCR attack, was strongly induced after 4 h of infestation at the site of WCR feeding (Fig. 2b ANCOVA, $F_{\text{treatment}1,8} = 296.2$, $P < 0.001$; $F_{\text{time}1,8} = 601.2$, $P < 0.001$; $F_{\text{treatment*time}1,8} = 638.02$, $P < 0.001$, and Fig. 3b ANCOVA, $F_{\text{treatment}1,6} = 19.4$, $P = 0.005$; $F_{\text{time}1,6} = 42.5$, $P < 0.001$; $F_{\text{treatment*time}1,6} = 41.9$, $P < 0.001$). The production was systemic, but EβC synthesis distant from the site of damage occurred later (after 24 h in vertical induction experiment and 48 h in the split-root induction experiment) and in smaller quantities (Fig. 2a ANCOVA, $F_{\text{treatment}1,8} = 2.0$, $P = 0.195$; $F_{\text{time}1,8} = 0.4$, $P = 0.563$; $F_{\text{treatment*time}1,8} = 0.005$, $P = 0.947$ and Fig. 3a ANCOVA, $F_{\text{treatment}1,6} = 0.03$, $P = 0.864$; $F_{\text{time}1,6} = 1.04$, $P = 0.336$; $F_{\text{treatment*time}1,6} = 0.02$, $P < 0.883$). The EβC-synthase gene *Zm-TPS23* was induced locally at the site of damage

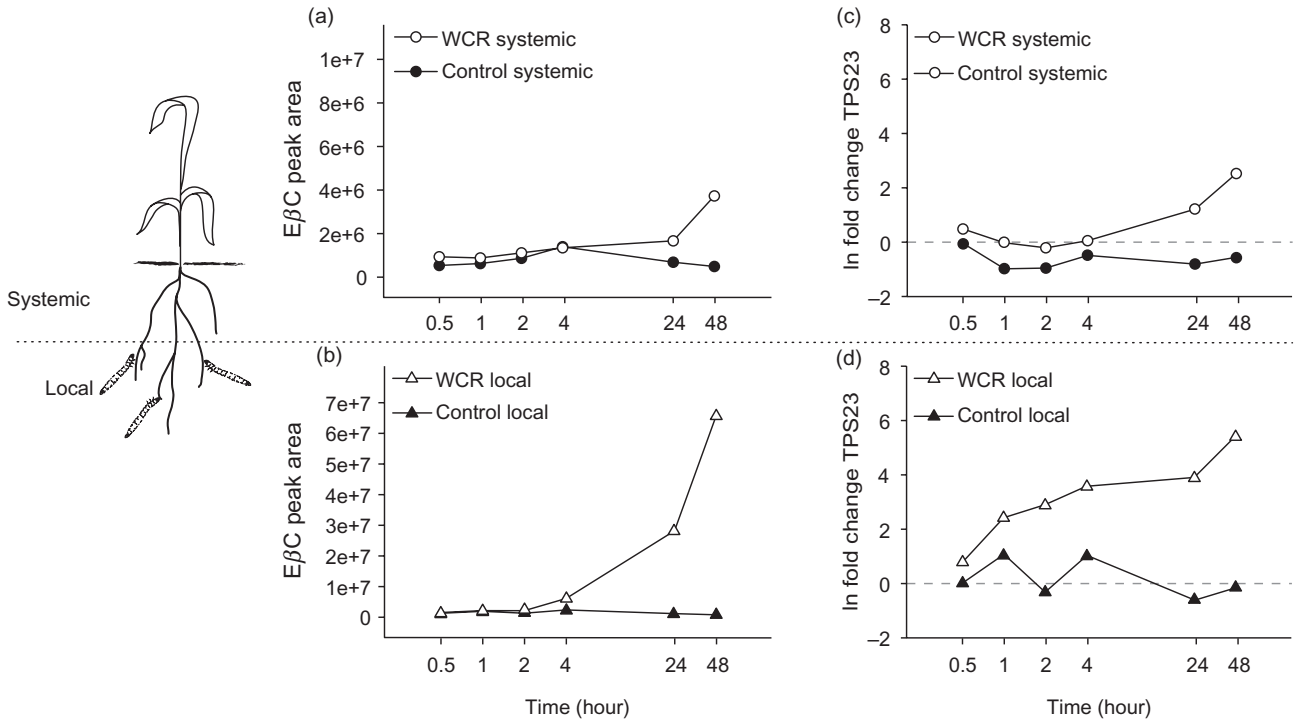

Figure 2. Vertical systemic response of maize roots. Production of (*E*)-*β*-caryophyllene (E*β*C) in control (filled shapes) and Western corn rootworm (WCR)-attacked roots (empty shapes) in systemic tissues above the feeding site (circles; a) and the locally attacked tissue (triangles; b) at different time points is shown. The corresponding systemic (circles; c) and local (triangles; d) increases in *Zm-tps23* expression levels corrected for controls are depicted.

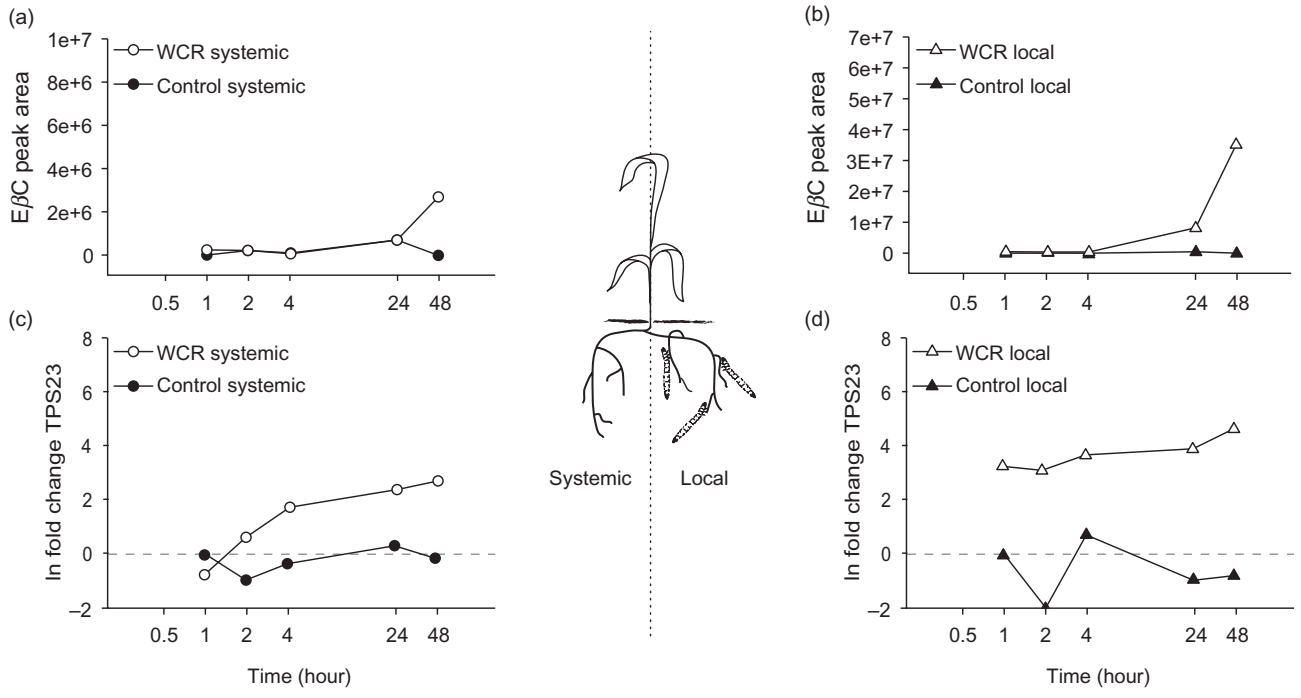

Figure 3. Horizontal systemic response of maize roots. Production of (*E*)-*β*-caryophyllene (E*β*C) in control (filled shapes) and roots attacked by Western corn rootworm (WCR) larvae (empty shapes) in systemic tissues (circles; a) and the locally attacked tissue (triangles; b) at different time points. The corresponding systemic (circles; c) and local (triangles; d) increases in *Zm-tps23* expression levels corrected for controls are depicted.

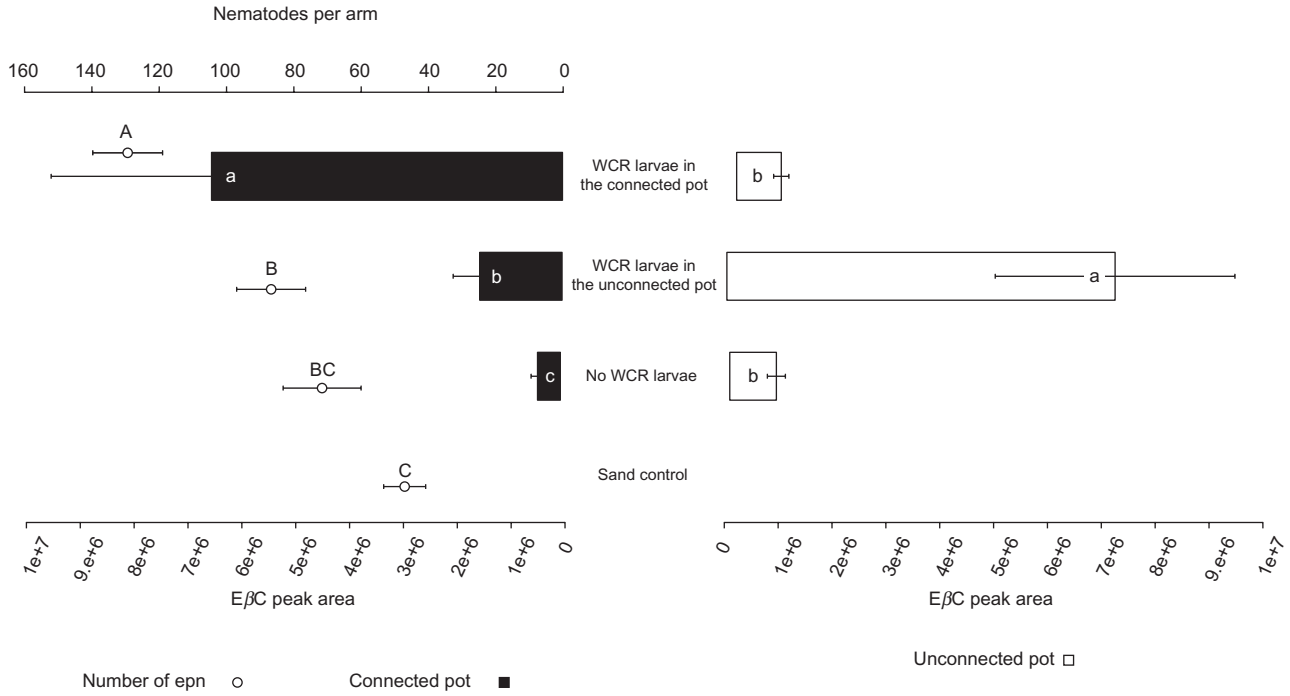

Figure 4. Nematode attraction to locally and systemically induced maize roots. The bars indicate the average emission (±SE) of (*E*)-*β*-caryophyllene (E*β*C) in different tissues of maize roots. Filled bars represent the amount of E*β*C detected in roots sampled from pots connected to the olfactometer. Open bars represent the average amount of E*β*C detected from the other half of the roots sampled from the pots that were not connected to the olfactometer. The average number of entomopathogenic nematodes (EPNs) retrieved from each olfactometer arm (±SE) is indicated by open circles. Nematodes retrieved from the three empty arms were pooled. Different letters indicate significant differences between treatments ($P > 0.05$).

already 1 h after infestation and was also up-regulated systemically after 24 h in the vertical induction experiment (Fig. 2c,d ANCOVA, $F_{treatment1,8} = 33.08$, $P < 0.001$; $F_{time1,8} = 24.7$, $P = 0.001$; $F_{treatment*time1,8} = 0.2$, $P = 0.66$) and 4 h in the split-root induction experiment (Fig. 3c,d ANCOVA, $F_{treatment1,6} = 69.08$, $P < 0.001$; $F_{time1,6} = 0.4$, $P = 0.539$; $F_{treatment*time1,6} = 1.5$, $P = 274$).

EPN response in a split-root belowground olfactometer (Fig. 4)

Similar to the previous experiment, there was a strong local induction of E*β*C, while a much weaker response was observed in the systemic root part (ANOVA, $F_{5,12} = 8.64$, $P = 0.001$). This was also reflected in EPN attraction ($F_{3,44} = 12.02$, $P < 0.001$): *Heterorhabditis megidis* preferred the local WCR-damaged roots over the systemically induced parts ($Delprim_{connected}$ + WCR versus $Delprim_{adjacent}$ + WCR, $P = 0.005$). Systemic induction did not result in a higher attraction than uninfested control plants ($Delprim_{adjacent}$ + WCR versus Delprim-WCR, $P = 0.08$). While both local and systemic induction resulted in a significant EPN response compared to sand controls ($Delprim_{connected}$ + WCR versus control, $P < 0.001$; $Delprim_{adjacent}$ + WCR versus control, $P < 0.001$), *H. megidis* was not more attracted towards healthy plants than sand only (Delprim-WCR versus control, $P = 0.06$).

In vivo collection of E*β*C emitted from WCR-damaged roots (Fig. 5)

The emission of E*β*C was significantly higher for maize roots that had been fed upon by five second-instar WCR larvae (Mann–Whitney *U*-test, $U = 0.000$, $P = 0.05$). E*β*C

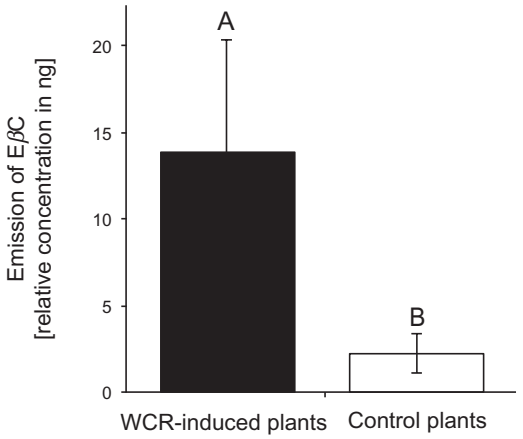

Figure 5. Western corn rootworm (WCR)-damaged maize roots emit significantly more (*E*)-*β*-caryophyllene (E*β*C) than undamaged roots. *In vivo* emissions of E*β*C were sevenfold higher when WCR larvae had fed on the roots than when the root system was intact. Bars indicate standard error of the mean. Letters indicate statistical differences.

relative quantity was sevenfold higher in pots with WCR-damaged roots than in pots without herbivory.

DISCUSSION

Systemic induction of EβC production in maize roots after herbivore attack

Our results show that volatile production in maize roots is induced systemically upon herbivore attack. It has been proposed that a systemic volatile release could be the result of either passive diffusion of the VOCs or active signalling resulting in an increase of biosynthetic activity distal to the wounding site (Erb *et al*. 2008). The data suggest the latter, as the gene responsible for EβC production (*Zm-tps23*) increases its activity in both local and systemic tissues. The expression of *Zm-tps23* has been shown to be the regulative element in EβC production (Köllner *et al*. 2008). In accordance with this, the time-dependent measurements show that *Zm-tps23* is locally induced 1 h after WCR attack, and an increase in EβC emissions can be measured 3 h later. Systemic induction of *Zm-tps23* occurs later still (between 4 and 24 h), and EβC emissions in non-attacked root parts increase between 24 and 48 h post-infestation. From this, we conclude that upon root attack, a signal is induced that moves to distal root parts and induces volatile production systemically. The nature of this signal remains to be elucidated.

Systemic signalling in plant defences is commonly thought to occur via two routes: internal signalling, involving the translocation of signals within the plant tissue, and airborne signalling, mediated by plant volatiles (Erb *et al*. 2008; Heil & Ton 2008). Here, it is shown for the first time that internal signalling, induced by insect herbivory, is sufficient to induce systemic root parts. In the olfactometer experiments, EβC production was induced systemically, even though the roots were separated in two unconnected glass pots, thereby impairing volatile root-to-root signalling. Further studies could aim at investigating if possible volatile communication acts in synergy with an internal inducer. Interestingly, the adjacent roots are not directly connected via the same vascular bundles and an internal signal would therefore have to cross cell membranes to reach these root parts or to travel via the xylem to the shoot and back to the roots via the phloem. However, because shoots of WCR-damaged plants do not produce EβC (Rasmann & Turlings 2007), this plant vascular route is highly unlikely. Thus, the systemic root induction demonstrated here seems to be an active process that takes place in the roots rather than the result of passively diffusing signals or metabolites.

Nematodes use local cues to locate their hosts

In a behavioral study, Neveu *et al*. (2002) found that the larval parasitoid *Trybliographa rapae* W. is attracted to turnip roots infested with its host, larvae of the fly *Delia radicum* L. This attraction was still evident when the root tissue with visible *D. radicum* damage was removed, leading the authors to logically conclude that volatile production must have occurred systemically within the root system (Neveu *et al*. 2002). In the case of the parasitoid, such a systemic emission of an attractant is likely to facilitate the location of infested plants. This may be different for organisms that forage in the complexity of the soil matrix. In the relatively small functional spatial scale belowground, it can be expected that root volatiles act as highly localized short-range cues. While aboveground parasitoids can, from a certain distance, refer to visual cues for host location, a belowground organism has few alternative cues and probably must fully rely on induced plant volatiles (Boff, Zoon & Smits 2001; van Tol *et al*. 2001; Rasmann *et al*. 2005; Ali, Alborn & Stelinski 2010; Hiltpold *et al*. 2010b), exudates and/or vibrations (Torr, Heritage & Wilson 2004). A 0.4–1.0 mm nematode (Poinar, Jakson & Klein 1987) would indeed need a reliable signal from a relatively confined source to find its hosts (second-instar WCR larvae that measure around 0.5 cm) in a typical volume of a corn root mass of 500 cm^3. In accordance with this prediction, our experiments demonstrate that, although EβC is slightly induced systemically, the local response of the root is 7 to 20 times stronger. In the olfactometer, from a distance of about 18 cm, this reaction resulted in a significant attraction of EPN, whereas the systemic induction did not markedly increase the response of the nematodes compared to uninfested plants (Fig. 4). We thus conclude that EβC production at the site of attack is sufficiently dominant over the systemic induction to successfully guide EPN to the sites where potential hosts are damaging the roots.

Possible benefits of systemic induction

If a local signal is sufficient and effective in attracting EPN, why would maize plants increase EβC production in adjacent roots? Here, we propose three hypotheses that may explain this seemingly contradictory response to root herbivory. Firstly, it is possible that the systemic induction of EβC is a secondary effect of the overall response of the plant to root herbivory. In barley, for example, induction with JA of half the root system results in a rapid reduction of labelled carbon in the induced roots, whereas untreated roots from the same plant show an increase in carbon content (Henkes *et al*. 2008). Carbon re-allocation within the root system possibly helps plants to tolerate herbivory, and the mild systemic increase in EβC production may be a side effect of the overall tolerance and defence response of the plant rather than a direct adaptation to attract EPN. Given the fact that the role of plant volatiles as an indirect defence has not yet been unambiguously demonstrated, such explanations should be considered as well. Secondly, it could be that, despite the relatively small scale at which EPN forage, systemic induction boosts the capacity of the plant to recruit these and other natural enemies of herbivores. In the olfactometer assays, the EPNs were more attracted to systemically infested roots than to sand controls, whereas they did not distinguish between sand and uninfested plants (Fig. 4). It could therefore be that

inducing a systemic signal increases the influx of EPNs from the surrounding soil into the rhizosphere of the plant, from where they would move to the strongly attractive local sites where they find their host. This two-step attraction has been proposed in the context of plant–plant signalling, where uninfested neighbours of infested plants also emit volatiles and thereby serve as guideposts for parasitoids (Kobayashi & Yamamura 2007). Thirdly, it has been found that a mild defence induction in non-attacked plant parts helps to prime plants to respond faster and stronger to future attack (Heil & Ton 2008). Priming has also enhanced the induced production of leaf terpenoids in maize leafs (Ton *et al*. 2007). Similarly, following its low activation in response to the signal, the systemically induced roots might respond faster to a secondary attack, thereby helping the plant to fend off WCR or secondary colonizers that challenge the plant after the first attack. Further experiments will be required to assess if such a priming mechanism takes place in roots after induction by the herbivore.

CONCLUSIONS

This study provides the first evidence for the systemic production of root volatiles upon herbivore attack. Although the local reaction is significantly stronger, the systemic response is still expected to contribute to the recruitment of EPNs towards a root mass that is under WCR attack. The internal plant signals that are involved, as well as the ecological relevance of the systemic induction for the plant and the tritrophic system, remain to be determined.

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