J Chem Ecol (2013) 39:1129–1139
DOI 10.1007/s10886-013-0302-3

Characterization of Volatile Organic Compounds Emitted by Barley (*Hordeum vulgare* L.) Roots and Their Attractiveness to Wireworms

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Received: 6 April 2012 / Revised: 28 March 2013 / Accepted: 5 May 2013 / Published online: 21 June 2013
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Abstract Root volatile organic compounds (VOCs), their chemistry and ecological functions have garnered less attention than aboveground emitted plant VOCs. We report here on the identification of VOCs emitted by barley roots (*Hordeum vulgare* L.). Twenty nine VOCs were identified from isolated 21-d-old roots. The detection was dependent on the medium used for root cultivation. We identified 24 VOCs from 7-d-old roots when plants were cultivated on sterile Hoagland gelified medium, 33 when grown on sterile vermiculite, and 34 on non-sterile vermiculite. The major VOCs were fatty acid derived compounds, including hexanal, methyl hexanoate, *(E)*-hex-2-enal, 2-pentylfuran, pentan-1-ol, (*Z*)-2-(pentenyl)-furan, *(Z)*-pent-2-en-1-ol, hexan-1-ol, *(Z)*-hex-3-en-1-ol, *(E)*-hex-2-en-1-ol, oct-1-en-3-ol, 2-ethylhexan-1-ol (likely a contaminant), *(E)*-non-2-enal, octan-1-ol, (*2E,6Z*)-nona-2,6-dienal, methyl *(E)*-non-2-enoate, nonan-1-ol, *(Z)*-non-3-en-1-ol, *(E)*-non-2-en-1-ol, nona-3,6-dien-1-ol, and nona-2,6-dien-1-ol. In an olfactometer assay, wireworms (larvae of *Agriotes sordidus* Illiger, Coleoptera: Elateridae) were attracted to cues emanating from barley seedlings. We discuss the role of individual root volatiles or a blend of the root volatiles detected here and their interaction with CO_2 for wireworm attraction.

Keywords Volatile organic compound · Barley · Roots · Solid-phase-micro-extraction · *Agriotes sordidus* · Root - insect interactions

Introduction

A complex blend of volatile organic compounds (VOCs) is emitted by plants, ranging from fatty acid derivatives, terpenoids, and sulfur compounds to phenylpropanoids (Qualley and Dudareva 2009). The emission can be constitutive and/or induced by environmental or physiological stresses (Maffei 2010). Depending on the stress type (wounding, herbivory, pathogen attack, dehydration, (UV) light, heat, *etc.*), the composition and amounts of released VOCs can vary (Ferry et al. 2004; Filella et al. 2009; Jansen et al. 2011; Kuhn et al. 2004).

Plant VOCs are emitted by various organs (seeds, flowers, leaves, stems, and roots). The rhizosphere provides a nutrient-rich environment for many organisms, as up to 20 % of the photosynthetically fixed carbon is released by roots (Barber and Martin 1976). Vertebrates, invertebrates, plants, fungi, and bacteria all share the same underground space in which VOC-mediated interactions can take place and even affect

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Electronic supplementary material The online version of this article (doi:10.1007/s10886-013-0302-3) contains supplementary material, which is available to authorized users.

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aboveground plant insect interactions (Effmert et al. 2012; Johnson et al. 2009; Soler et al. 2012; Wenke et al. 2010); however, the belowground VOCs potentially responsible for such interactions have to date been partially neglected, due to technical limitations. The release of root VOCs can mediate various interactions: direct or indirect defense of roots against herbivores (Ali et al. 2011; Rasmann et al. 2005, 2012a), plant – plant competition (Ens et al. 2009; Jassbi et al. 2010; Viles and Reese 1996), resistance of roots against pathogens (Cobb et al. 1968; Kalemba et al. 2002; Vilela et al. 2009), and symbiotic interactions (Asensio et al. 2012; Paavolainen et al. 1998). Root derived compounds can also attract herbivores (Wenke et al. 2010), and their emission tends to decrease in unattacked conditions (Piesik et al. 2011b), thus limiting the energy costs incurred by their synthesis (Herms and Mattson 1992; Rasmann et al. 2012a, b). Carbon dioxide gradients are an ubiquitous belowground herbivore attractant, but other volatile and non-volatile semiochemicals also are involved in directing herbivores towards roots (Johnson and Nielsen 2012; Reinecke et al. 2008; Weissteiner et al. 2012). Several studies have shown the attractive role of root-emitted VOCs towards arthropods. For example, di- and trisulfides produced by *Allium cepa* are potent attractants of the larvae of the fly *Delia antiqua* (Matsumoto 1970); VOCs released by damaged oak roots are perceived by the larvae of the forest cockchafer *Melalontha hippocastani* and attract the larvae in natural soil (Weissteiner et al. 2012); volatiles of ryegrass roots attracted the larvae of *Costelytra zealandica* (Sutherland and Hillier 1972). Arthropods can differentiate between root VOCs released from plants that differ with respect to physical or physiological traits (Aratchige et al. 2004; Tapia et al. 2007; Witcosky et al. 1987); they also can differentiate between root VOCs released by different varieties of plants (Guerin and Ryan 1984).

In this study, we characterized VOCs emitted by isolated barley roots. Root emissions from barley cultivated under sterile and non-sterile conditions were compared in order to characterize root emission in the absence of microorganisms. Moreover, the development of an orientation test with the larvae of the click beetle *Agriotes sordidus* Illiger (Coleoptera: Elateridae) led us to investigate the potential semiochemical role of the VOC blend emitted by isolated barley roots. The general working questions were: (i) How complex is the volatile blend of isolated barley roots? (ii) What is the impact of microorganisms on the volatile blend released by barley roots? (iii) How attractive is this volatile blend of barley roots to wireworms?

Larvae of *Agriotes* are polyphagous wireworms that feed on the roots of a variety of crops, including cereals (Johnson et al. 2009; van Herk and Vernon 2013). Baits based on germinating wheat and barley seeds have been proven to be efficient (Parker 1996). However, few studies of wireworm - barley interactions are available, although barley is the second most important crop in Europe (production, 2010, FAOstat). As for most of the root herbivores, the CO_2 gradient is the general search trigger for wireworms. (Doane et al. 1975; Johnson and Nielsen 2012). Plant-derived VOCs can affect the behavior of root-feeding insects, while their identity and role in wireworms' chemical ecology still has to be revealed (Barsics et al. 2013; Johnson and Nielsen 2012). Since such signals could be potent wireworm attractants or repellents, we focused on the release of VOCs from barley roots and tested the attraction of wireworms to barley roots.

Methods and Materials

Plant Material

Growth Conditions Barley plants (var. Quench, Jorion, Belgium) were grown at 22 °C under LED light (95 µmol m^{-2}-sec^{-1}) with a 20/4 h L/D photoperiod and 65 % RH.

Cultivation of 21-d-Old Plants Caryopses were sown at a density of 10 plants per pot (7 l) in vermiculite (Sibli, Belgium). Plants were watered daily and fertilized × 3 per week with aqueous Hoagland's solution (Hoagland's NO.2 basal salt mixture, Sigma, Belgium).

Aseptical Cultivation of 7-d-Old Plants Barley caryopses (28 g) were sterilized as described by Lanoue et al. (2010). Briefly, caryopses were incubated in 50 ml H_2SO_4 (50 % v/v) for 1 h and washed x 5 in 150 ml sterile bidistilled water. Caryopses then were shaken for 20 min in 80 ml $AgNO_3$ (1 % w/v) and washed successively with 150 ml sterile NaCl (1 %, w/v), 150 ml sterile bidistilled water, 150 ml sterile NaCl (1 %, w/v) and × 5 with 150 ml sterile bidistilled water, before sowing: (a) on 124 cm^2 Petri dishes filled with Hoagland medium (Hoagland's NO.2 basal salt mixture, Sigma, Belgium), solidified with 0.8 % agar (w/v; Plant agar, Duchefa Biochemie, Belgium) or (b) on vermiculite with Hoagland solution (Hoagland's NO.2 basal salt mixture, Sigma, Belgium).

(a) Sterile caryopses were placed on Hoagland's medium with the ventral furrow underneath and left to grow for 7 d vertically in a growth chamber. (b) Sterile caryopses were sown aseptically in 2 l jars (le Parfait, Villeurbanne, France), filled with 600 ml sterile vermiculite humidified with 300 ml sterile Hoagland solution. Jars were closed and sealed with plastic film and left for 7 d in a growth chamber. On the sampling day, vermiculite isolated in the vicinity of the roots was incubated on tryptic soy agar (Fluka, Belgium) for 1 wk at 37 °C to check sterility. All glass, media and jars were sterilized.

As controls, non-sterile plants were grown for 7 d in 600 ml vermiculite humidified with 300 ml Hoagland solution in 2 l open jars.

Analyses of Volatile Organic Compounds

Head-Space Solid-Phase-Microextraction (HS-SPME) Roots were isolated from the substrate by shaking the plant gently and were separated from the upper part of the plant by cutting just below the caryopsis. Then 3 g±0.1 g of fresh entire roots were placed in 20 ml SPME vials (Filter Service, Belgium) fitted with a sealed cap (white silicone/blue PTFE, Filter Service). Roots were not cut into pieces, and the sampling conditions were the same for all samples. An internal standard (1 µl of a methanolic solution of butyl benzene (≥ 99 %, Sigma-Aldrich (S.-A.), Belgium) at 0.86 mgl^{-1}) was added on the surface of the vial without touching the roots. The fiber (divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS (50/30 µm); S.-A.) was the same for all repetitions of the same experiment. The fiber was conditioned before first use at 270 °C for 1 h. After equilibration of the vial for 15 min at 30 °C, the fibre was inserted into the headspace for 30 min at the same temperature.

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis After extraction, the volatile compounds were desorbed in pulsed splitless mode for 10 min at 250 °C. GC-MS analyses were performed on an Agilent Technologies 7890A GC System coupled to an Agilent Technologies 5975C Mass Spectrometer equipped with Wax factor four (Agilent technologies USA; 30 m x 0.250 mm I.D, 0.25 µm film thickness). Helium was used as the carrier gas at a flow rate of 1.5 ml/min. The inlet temperature was 250 °C. Pulsed splitless injection mode was used in a 1.5 mm HS-liner (injection pulse pressure of 30 psi for 1 min). The following temperature program was used: 40 °C for 4 min; 15 °C/min to 160 °C; 20 °C/min to 250 °C; and 250 °C for 5 min; 30 °C/min to 300 °C; and final hold at 300 °C for 15 min.

The MS was carried out in EI mode at 70 eV; source temperature, 230 °C; quadrupole temperature, 150 °C; scanned mass range: from 20 to 350 amu, threshold of 150 amu; scan speed, 4.27 scans/s.

Chemical Identification Components were identified by comparing recorded mass spectra with the NIST and Wiley spectral databases. Further identification was carried out by calculating non-isothermal Kovats retention indices by injecting saturated n-alkane standard solution (C_7-C_{30} 1,000 µg/ml in hexane, Supelco, Belgium) under the same chromatographic conditions, using the definition of Van den Dool and Kratz (1963).

Whenever possible, identifications were confirmed by injection of available commercial standards. References of commercial standards are listed in the supplemental text. As the same chromatographic conditions with the same column were used for the analyses of the standards, identification of the detected compounds in the headspace of barley roots was confirmed by comparing their retention data and mass spectra with those of the commercially available reference compounds.

Peaks which showed a signal/noise ratio of three compared to the blank controls were identified and integrated manually with the Agilent MSD Chemstation. The relative area of a target compound was calculated by dividing the peak area of this compound by the total peak area of the sample. Statistical analysis was performed on the relative area with a *two-tailed paired t-test* after having checked that the data were normally distributed with *Kolmogorov-Smirnov test*.

Concentration Estimation Samples were extracted using an autosampler (MPS2, Gerstel) equipped with a sample tray holder and a needle heater for heating the vials. Gerstel Maestro software was used for autosampler control. Standards and dilutions were always handled with a Hamilton syringe with a volume of 1 µl. The molecules identified by GC-MS were grouped into the following classes: alkanes, aldehydes, alcohols, esters, sulfur, and furan compounds. In each class, a representative compound was selected as the basis of the calibration curves: *n*-tetradecane (S.-A.; 99 %), (*E*)-non-2-enal (SAFC; ≥ 93 %), (*E*)-non-2-en-1-ol (SAFC; ≥ 96 %), methyl benzoate (Fluka; ≥ 99.5 %), dimethyl sulfoxide (S.-A.; ≥ 99,9 %), and 2-pentylfuran (SAFC; 97 %). The calibration curves included at least four points and were performed in triplicate (Supplemental Table S1). For each measurement, the experiment was carried out according to the following process. A stock solution was diluted in methanol by using volumetric glassware. One microliter of each dilution then was placed in a vial (20 ml) with 1 µl of the internal standard (butylbenzene 0.86mgl^{-1} in methanol). After an equilibration period of 15 min at 30 °C, the fiber was exposed for 30 min (at 30 °C) before analysis as described above.

Wireworms Olfactory Orientation Bioassay

Wireworms were collected in November 2011 in Montardon (Pau, France), from the soil of grass edging a fallow plot and an untreated wheat field. Morphological criteria described in the keys of Cocquempot et al. (1999) and Pic et al. (2008) allowed *Agriotes sordidus* individuals to be identified. Each larva was kept individually in an 80 ml capped vial, with a mix of leaf mold and vermiculite (1/1 v/v, 16.5 % water) and a mix of meadow seeds (0.130–0.160 g, Prelac Bio, SCAR, Belgium). All vials were kept in the dark at 21.2±0.7 °C. Seven days before being tested, wireworms greater than 10 mm in length were individually isolated in vermiculite (16.5 % water). Wireworms were selected for testing from the isolated individuals according to their apparent activity: those visibly in the pre-molting or post-molting phases were excluded from the tests. In total, 60 larvae were submitted to the olfactometry bioassay.

Table 1 SPME analysis of VOCs emitted by excised 21-d-old barley roots

CAS number [(1)]	IUPAC Name	Identification [(2)]	Sample RI [(3)]	Reference RI [(4)]	A	B
					Relative area (%) ± SD; n=5	Estimation (ng/g RFW ± SD, n=5) [(5)]
75-18-3	Dimethyl sulfide	STD	N.D.	844[c]	25,8±6,3	38.8±9.1
66-25-1	Hexanal	STD	1075	1074[b]	2,2±0,4	9.9±1.0
106-70-7	Methyl hexanoate	STD	1179	1185[b]	1.1±0.9	0.13±0.07
6728-26-3	(*E*)-Hex-2-enal	STD	1206	1207[a]	0.59±0.32	1.7±0.7
3777-69-3	2-Pentylfuran	STD	1213	1229[a]	27.9±5.0	4.2±0.5
71-41-0	Pentan-1-ol	STD	1246	1244[d]	1.23±0.48	0.52±0.11
70424-13-4	2-(Pentenyl)furan[#]	MS	1287	-	1.01±0.22	0.46±0.03
1576-95-0	(*Z*)-Pent-2-en-1-ol	STD	1311	1313[d]	0.33±0.06	0.15±0.02
110-93-0	6-Methyl-hept-5-en-2-one	STD	1325	1319[e]	0.55±0.44	-
111-27-3	Hexan-1-ol	STD	1345	1351[f]	9.27±1.94	3.8±0.5
928-96-1	(*Z*)-Hex-3-en-1-ol	STD	1374	1351[a]	0.55±0.14	0.24±0.02
928-95-0	(*E*)-Hex-2-en-1-ol	STD	1389	1400[f]	2.26±0.75	0.9±0.2
3391-86-4	Oct-1-en-3-ol	STD	1438	1420[a]	0.22±0.04	0.09±0.02
104-76-7	2-Ethylhexan-1-ol	STD	1483	1504[g]	2.59±1.71	1.0±0.3
18829-56-6	(*E*)-Non-2-enal	STD	1524	1540[a]	1.71±0.93	7.6±2.0
67-68-5	Dimethyl sulfoxide	STD	1551	1553[h]	0.91±0.58	1.4±0.5
111-87-5	Octan-1-ol	STD	1552	1557[i]	0.51±0.25	0.22±0.04
557-48-2	(2*E*,6*Z*)-Nona-2,6-dienal	STD	1575	1597[j]	0.66±0.20	2.4±0.4
111-79-5	Methyl (*E*)-non-2-enoate	STD	1602	–	0.41±0.16	0.09±0.02
93-58-3	Methyl benzoate	STD	1614	1600[a]	3.75±2.12	0.92±0.27
143-08-8	Nonan-1-ol	STD	1656	1678[g]	0.58±0.08	0.26±0.03
10340-23-5	(*Z*)-Non-3-en-1-ol	STD	1680	1682[k]	4.44±2.98	1.8±0.5
31502-14-4	(*E*)-Non-2-en-1-ol	STD	1710	1722[l]	3.29±2.61	1.3±0.5
76649-25-7	Nona-3,6-dien-1-ol[#]	MS, RI	1733	1759[k]	0.79±0.46	0.33±0.07
7786-44-9	Nona-2,6-dien-1-ol[#]	MS, RI	1764	1776[l]	1.18±0.61	0.49±0.10
124-25-4	Tetradecanal	STD	1968	1940[m]	0.62±0.16	2.2±0.5
112-53-8	Dodecan-1-ol	STD	1971	1970[n]	0.30±0.07	0.14±0.02
104-61-0	Dihydro-5-pentyl-2(3*H*)-furanone	STD	2022	2024[j]	0.71±0.38	0.16±0.05
629-80-1	Hexadecanal[#]	MS, RI	2052	2020[o]	1.94±1.09	8.3±2.3

[(1)] CAS number of compounds listed in order of elution from a WAX factor 4 polar column. Source CAS: Scifinder® (Chemical Abstracts Service, Colombus, USA); [(2)] Identification methods: MS, comparison of mass spectra with those of Nist08 and Wiley 275 libraries; RI, comparison of retention indices with those reported in the literature (sources in section [(4)]); STD, comparison of retention time and mass spectra of available standards; [(3)] Retention indices on WAX factor 4 column, experimentally determined using a saturated n-alkane standard solution C7-C30; [(4)] Retention indices taken from [a] Jennings and Shibamoto 1980; [b] Sanchez-Ortiz et al. 2012; the others are taken from Pherobase : [c] Varming et al. 2004, [d] Umano et al. 2002, [e] Chung et al. 1993, [f] Ruther 2000, [g] Weingart et al. 2011, [h] Wei et al. 2001, [i] Valim et al. 2003, [j] Ferreira et al. 2001, [k] Hayata et al. 2003, [l] Weckerle et al. 2001, [m] Chisholm et al. 2003, [n] De Marques et al. 2000; [o] Kohara et al. 2006, [(5)] Estimation of the concentration was based on the response curves calculated for one representative molecule of the chemical family. This approach involved performing six calibration curves linear in the concentration ranges tested (correlation coefficients, always>0.99) # Compounds tentatively identified. Columns A and B represent respectively the relative area and concentration estimation of the VOCs

The bioassay set up (Fig. S1) consisted of a glass pipe (32 cm long, 3.6 cm internal diameter), with both extremities closed with GL45 caps (Duran, Belgium), which allowed the set-up to be filled and emptied with substrate. Two GL14 holes (Duran, Belgium) were present at a distance of 3 cm from both extremities to allow the introduction of stimuli from both sides. The entry point for the larvae was provided by a third lateral hole in the middle of the pipe, diametrically opposite to both lateral connections.

The set-up was filled with wet vermiculite, which was removed to a depth of 4 cm at the two ends of the equipment to leave room for the bait and control compartments (final vermiculite content: 64.4±0.9 g, 53.0±0.4%water). In each of the pipes, the bait consisted of the roots of 10 developed

barley seedlings gently removed from growth medium before they were introduced into the bait zone. The roots were positioned through one of the lateral tubular perforations at the distal end of the olfactometer. Plants were held in position with both aluminum foil and PTFE tape (EGEDA, Belgium). Thus, roots were the only plant material exposed to wireworms, as the rest of the plant was isolated out of the bioassay. Blank culture medium (240 mg – the average amount of medium still upon the roots after extraction from the medium) was introduced into the opposite side of the bait compartment. To prevent any contact between a wireworm and the roots, a gauze (3.6 cm diam., stainless steel; width: 0.042 mm; mesh: 0.036 mm; Haver, Belgium) was used to separate the substrate from the bait and control compartments. Tests were performed in batches of 10 olfactometers at a room temperature of 21.9±0.5 °C.

Bioassays were performed in the dark. Each wireworm was introduced individually 40 min after the bait was set in the system, and a red plastic sheet was placed on each bioassay during the test in order to suppress light biases. The position (left or right) of the baits on the laboratory bench was randomly assigned and noted, as was the position of the baits with respect to the side by which the olfactometers had been filled with substrate.

The position of the wireworms was recorded after 60 min. Any wireworm located within a distance of 3 cm from the entry point was considered as non-responding. We performed replicates until 50 responses were recorded, which took a total of 60 tested individuals. All material was cleaned with water and norvanol (VWR, Belgium) between each test. Observed frequencies relating to the choice of wireworm larvae in dual choice bioassays were compared to corresponding theoretical frequencies by applying a χ^2 *goodness-of-fit test*, using Minitab® release 14.2. Potential sources of bias (bait position on the laboratory bench and bait position with regard to the entry side of the substrate) were tested with *Fisher's exact test* for count data (one factor, four modalities) using R software, version 2.14.1 (2011-12-22; Development Core Team 2008). The orientation bioassay also was performed 'blank-to-blank', i.e. without baits and controls, in order to assess the nature of wireworm behavior in an odor-free testing environment. The results were treated with a χ^2 *goodness-of-fit test* with regard to wireworm position. The potential impact of the entry side of the substrate on the results was tested with a χ^2 test for independence, using Minitab® release 14.2.

Results

Identification of Volatile Organic Compounds in 21-d-Old Barley Roots We detected 29 volatile compounds in the headspace of isolated 21-d-old barley roots (Table 1, N=5 replicates). Confirmation of the occurrence of 24 VOCs was possible by comparison with the NIST 08 and Wiley 275 k databases, the library retention index, and standards. The retention index (RI) of dimethyl sulfide could not be precisely calculated as it was eluted in the very early phase of the chromatogram. Relative experimental RI deviations from the database's RI ranked from −1.4 to 1.7 %. Relative quantities were determined by determining the area of the compound peak relative to the total peaks area. In order to estimate the amounts of the 29 compounds listed in (Supplemental Table S1), response curves were calculated for major chemical families present in the list by using one representative compound of each family. This approach involved performing six linear calibration curves linear in the concentration ranges tested, as evidenced by the values of correlation coefficients, always>0.99 (Table 1).

In Figure S2 and the supplemental text, the development of the SPME-GC-MS method is described. An optimized protocol for SPME analysis of root volatiles was developed. An important point was the use of the DVB/CAR/PDMS fiber with a 50/30 μm coating, and a fixed equilibration and sampling time. Exactly 3 g root material were used, and a wax column proved to be suitable for separation of the volatiles. Figure S3 shows a total ion current chromatogram of a headspace sample of 21-d-old isolated barley roots.

Barley Root VOCs After Seven Days of Culture on Hoagland Gelified Medium Table 2 provides a list of the compounds that were emitted by 7-d-old barley roots cultivated on Hoagland gelified medium. Confirmation of the occurrence of 16 VOCs was possible by the injection of a standard, and 4 VOCs could be tentatively identified by their theoretical RI. Estimation of the amount of VOCs released was performed in the same way as described for the data shown in Table 1.

Barley Root VOCs After Seven Days of Culture in Sterile/Non-Sterile Conditions The potential contribution of microorganisms present in the environment of the roots in the degradation or emission of the VOCs was assayed by cultivating plants for 7 d in sterile vermiculite (ST) and in non-aseptic (NS) vermiculite fertilized with Hoagland solution. Thirty-three VOCs were identified from the roots of ST plants, 34 were detected as emitted from the roots of NS plants (Table 3, Fig. S4).

Statistical analysis of the relative area of VOCs emitted by ST and NS roots showed that six VOCs (pentan-3-one, pent-1-en-3-ol, 2-ethylhexan-1-ol, dodecan-1-ol, dihydro-5-pentyl-2(3*H*)-furanone, hexadecanal) were differentially detected (*two-tailed paired t-test*; $P<0.05$) in NS compared to ST conditions (Table 3), whereas (*E*)-pent-2-en-1-ol and methyl-dodecanoate were found only in the headspace of roots of NS plants.

Wireworms Olfactory Orientation Bioassay Seven-day-old barley roots grown in axenic conditions were used as volatile-emitting sources (10 plantlets/olfactometric test) for an orientation bioassay of the belowground pest insect *Agriotes sordidus*.

Table 2 SPME analysis of VOCs emitted by excised 7-d-old barley roots cultivated on Hoagland gelified medium

CAS number (1)	IUPAC name	Identification (2)	Sample RI (3)	Reference RI (4)	A	B
					Hoagland gelified medium Relative area (% ± SD, n=5)	Hoagland gelified medium Estimation (ng/g RFW ± SD, n=5) (5)
75-18-3	Dimethyl sulfide	STD	712	844[c]	5,03±1,03	13,55±4,51
3777-69-3	2-Pentylfuran	STD	1212	1229[a]	24,33±2,89	7,39±2,61
100-42-5	Ethenylbenzene#	MS, RI	1237	1273[m]	12,86±0,88	–
70424-13-4	2-(Pentenyl)furan#	MS	1284	–	0,98±0,30	1,22±0,16
1576-95-0	(*Z*)-Pent-2-en-1-ol	STD	1305	1313[d]	0,66±0,10	0,56±0,16
–	Oct-6-en-2-one#	MS	1313	–	0,98±0,25	–
111-27-3	Hexan-1-ol	STD	1339	1351[f]	4,81±0,25	3,68±1,27
928-95-0	(*E*)-Hex-2-en-1-ol	STD	1391	1400[f]	0,93±0,03	0,77±0,25
3391-86-4	Oct-1-en-3-ol	STD	1437	1420[a]	1,86±0,52	1,42±0,42
1569-60-4	6-Methylhept-5-en-2-ol#	MS	1451	–	1,68±0,43	1,28±0,36
104-76-7	2-Ethylhexan-1-ol	STD	1447	1504[g]	1,16±0,17	0,92±0,24
1731-84-6	Methyl nonanoate#	MS, RI	1479	1487[b]	1,62±0,37	0,66±0,16
18829-56-6	(*E*)-Non-2-enal	STD	1519	1540[a]	2,86±0,36	23,35±7,53
67-68-5	Dimethyl sulfoxide	STD	1543	1553[h]	0,45±0,15	–
111-87-5	Octan-1-ol	STD	1547	1557[i]	0,94±0,15	0,77±0,26
557-48-2	(2*E*,6*Z*)-Nona-2,6-dienal	STD	1550	1597[j]	1,05±0,21	7,55±5,32
111-79-5	Methyl (*E*)-non-2-enoate	STD	1592	–	4,50±0,92	1,95±0,47
93-58-3	Methyl benzoate	STD	1603	1600[a]	2,93±1,01	1,19±0,24
143-08-8	Nonan-1-ol	STD	1651	1678[g]	1,94±0,27	1,55±0,55
10340-23-5	(*Z*)-Non-3-en-1-ol	STD	1673	1682[k]	1,91±0,40	1,57±0,73
31502-14-4	(*E*)-Non-2-en-1-ol	STD	1704	1722[l]	11,81±3,84	9,68±5,86
7786-44-9	Nona-2,6-dien-1-ol#	MS, RI	1758	1776[l]	3,27±0,76	2,67±1,36
124-10-7	Methyl tetradecanoate	STD	1974	2034[e]	2,05±0,22	0,92±0,92
104-61-0	Dihydro-5-pentyl-2(3*H*)-furanone	STD	2010	2024[j]	0,90±0,33	0,32±0,05

(1) CAS number of compounds listed in order of elution from a WAX factor 4 polar column. Source CAS: Scifinder® (Chemical Abstracts Service, Colombus, USA); (2) Identification methods: MS, comparison of mass spectra with those of Nist08 and Wiley 275 libraries; RI, comparison of retention indices with those reported in the literature (sources in section (4)); STD, comparison of retention time and mass spectra of available standards; (3) Retention indices on WAX factor 4 column, experimentally determined using a saturated n-alkane standard solution C7-C30; (4) Retention indices taken from [a] Jennings and Shibamoto 1980; [b] Tressl et al. 1978; [c] Varming et al. 2004, [d] Umano et al. 2002, [e] Choi 2003, [f] Ruther 2000, [g] Weingart et al. 2011, [h] Wei et al. 2001, [i] Valim et al. 2003, [j] Ferreira et al. 2001, [k] Hayata et al. 2003, [l] Weckerle et al. 2001, [m] Sanz et al. 2001, (5) Estimation of the concentration was based on the response curves calculated for one representative molecule of the chemical family. This approach involved performing six calibration curves linear in the concentration ranges tested (correlation coefficients, always>0.99). tr: trace; N.D.: not determined; # Compounds tentatively identified. Columns A and B represent respectively the relative area and concentration estimation of the VOCs

We tested N=60 larvae; 10 larvae did not respond (16.5 %), 35 oriented towards barley roots, and 15 towards the control (χ^2=8; P=0.005).

The bait position with regard to both the substrate entry side and the left or right position of the roots on the laboratory bench did not significantly affect the response of the wireworms (*Fisher's test of exact count,* (P=0.861). The blank-to-blank experimentation confirmed the absence of biases in the experimental set-up. Fifteen wireworms orientated to the left, eleven to the right (χ^2=0.615; P=0.433), and nine did not respond (26 % of the tested individuals). The χ^2 test for independence showed that the entry side of the substrate had no impact on the results (χ^2=1.418; P=0.214).

Discussion

We developed an SPME method that enabled us to detect a wide range of VOCs released by barley roots. Furthermore, our method allowed the estimation of the amounts of VOCs emitted from roots. The physicochemical properties of a

Table 3 SPME analysis of VOCs emitted by excised 7-d-old barley roots cultivated in vermiculite

CAS number [(1)]	IUPAC name	Identification [(2)]	Sample RI [(3)]	Reference RI [(4)]	A		B	
					Hoagland fertilized vermiculite Sterile Relative area (%±SD, n=5)	Hoagland fertilized vermiculite Non Sterile Relative area (%±SD, n=5)	Hoagland fertilized vermiculite Sterile Estimation (ng/g RFW ±SD, n=5) [(5)]	Hoagland fertilized vermiculite Non Sterile Estimation (ng/g RFW ±SD, n=5) [(5)]
75-18-3	Dimethyl sulfide	STD	712	844[c]	14,34±7,99	14,20±7,79	20,34±13,82	24,83±11,89
96-22-0	Pentan-3-one	STD	965	971[b]	0,61±0,10	0,95±0,20	–	–
66-25-1	Hexanal	STD	1068	1074[m]	1,80±0,39	–	5,59±1,52	–
71-36-3	Butan-1-ol	STD	1140	–	0,31±0,10	0,39±0,13	0,18±0,03	0,24±0,03
616-25-1	Pent-1-en-3-ol[#]	MS	1153	–	0,77±0,09	0,60±0,13	0,37±0,11	0,35±0,08
6728-26-3	(*E*)-Hex-2-enal	STD	1199	1207[a]	0,57±0,24	0,42±0,16	–	–
3777-69-3	2-Pentylfuran	STD	1212	1229[a]	28,18±8,11	27,72±6,23	4,71±1,66	5,32±1,22
71-41-0	Pentan-1-ol	STD	1240	1244[d]	2,82±0,51	2,05±0,57	1,14±0,29	1,00±0,30
70424-13-4	(*Z*)-2-(Pentenyl)furan[#]	MS	1284	–	0,79±0,26	0,98±0,15	1,06±0,04	1,11±0,04
1576-96-1	(*E*)-Pent-2-en-1-ol	MS,RI	1297	1307[m]	–	0,54±0,08	–	0,32±0,05
1576-95-0	(*Z*)-Pent-2-en-1-ol	STD	1305	1313[d]	0,79±0,17	0,50±0,30	0,37±0,09	0,29±0,13
-	Oct-6-en-2-one[#]	MS	1313	–	0,69±0,30	0,71±0,28	–	–
111-27-3	Hexan-1-ol	STD	1339	1351[f]	5,23±0,46	4,79±0,38	2,08±0,57	2,27±0,68
928-95-0	(*E*)-Hex-2-en-1-ol	STD	1391	1400[f]	1,86±0,21	1,73±0,28	0,80±0,27	0,87±0,29
3391-86-4	Oct-1-en-3-ol	STD	1437	1420[a]	0,87±0,33	0,67±0,06	0,40±0,13	0,38±0,08
111-27-3	Heptan-1-ol	STD	1442	–	0,54±0,47	0,33±0,09	0,25±0,11	0,22±0,04
1569-60-4	6-Methylhept-5-en-2-ol[#]	STD	1451	–	1,46±1,27	0,60±0,35	0,57±0,40	0,32±0,12
104-76-7	2-Ethylhexan-1-ol	STD	1447	1504[g]	2,26±1,08	4,17±1,42	0,99±0,51	1,91±0,49
18829-56-6	(*E*)-Non-2-enal	STD	1519	1540[a]	3,03±0,44	2,64±0,91	11,78±4,65	12,67±7,95
67-68-5	Dimethyl sulfoxide	STD	1543	1553[h]	1,31±1,46	0,90±0,83	–	–
111-87-5	Octan-1-ol	STD	1547	1557[i]	2,68±1,96	1,02±0,33	1,18±0,97	0,53±0,15
557-48-2	(2*E*,6*Z*)-Nona-2,6-dienal	STD	1550	1597[j]	1,03±0,14	1,14±0,17	2,30±1,48	3,97±2,30
111-79-5	Methyl (E)-non-2-enoate	STD	1592	–	1,00±0,25	1,06±0,20	0,18±0,08	0,24±0,06
93-58-3	Methyl benzoate	STD	1603	1600[a]	1,97±0,34	1,90±0,52	0,40±0,11	0,48±0,17
143-08-8	Nonan-1-ol	STD	1651	1678[g]	1,88±0,37	1,53±0,12	0,81±0,30	0,76±0,15
10340-23-5	(*Z*)-Non-3-en-1-ol	STD	1673	1682[k]	3,29±0,30	3,93±0,68	1,33±0,34	1,92±0,79
31502-14-4	(*E*)-Non-2-en-1-ol	STD	1704	1722[l]	12,94±5,27	15,01±7,99	5,41±3,51	7,41±5,45
56805-23-3	Nona-3,6-dien-1-ol[#]	MS,RI	1741	1759[k]	1,10±1,12	0,63±0,12	0,49 ±0,46	0,37±0,15
7786-44-9	Nona-2,6-dien-1-ol[#]	MS, RI	1758	1776[l]	3,52±2,04	3,98±2,03	1,49±1,14	2,03±1,48
111-82-0	Methyl dodecanoate[#]	MS,RI	1794	1813[e]	–	0,34±0,13	–	0,03±0,03
60-12-8	2-Phenylethanol	STD	1893	1931[j]	0,37±0,20	0,21±0,08	0,21±0,07	0,17±0,04
112-53-8	Dodecan-1-ol	STD	1967	1970[n]	0,36±0,17	0,87±0,27	0,22±0,10	0,46±0,11

Table 3 (continued)

CAS number [(1)]	IUPAC name	Identification [(2)]	Sample RI [(3)]	Reference RI [(4)]	A		B	
					Hoagland fertilized vermiculite Sterile Relative area (%±SD, n=5)	Hoagland fertilized vermiculite Non Sterile Relative area (%±SD, n=5)	Hoagland fertilized vermiculite Sterile Estimation (ng/g RFW ±SD, n=5) [(5)]	Hoagland fertilized vermiculite Non Sterile Estimation (ng/g RFW ±SD, n=5) [(5)]
124-10-7	Methyl tetradecanoate	STD	1974	2034[e]	0,24±0,12	0,73±0,24	0,05±0,09	0,15±0,06
104-61-0	Dihydro-5-pentyl-2(3*H*)-furanone	STD	2010	2024[j]	0,35±0,12	0,55±0,11	0,02±0,01	0,10±0,04
629-80-1	Hexadecanal[#]	MS, RI	2023	2020[o]	0,39±0,19	1,03±0,34	–	–

[(1)] CAS number of compounds listed in order of elution from a WAX factor 4 polar column. Source CAS: Scifinder® (Chemical Abstracts Service, Colombus, USA); [(2)] Identification methods: MS, comparison of mass spectra with those of Nist08 and Wiley 275 libraries; RI, comparison of retention indices with those reported in the literature (sources in section [(4)]); STD, comparison of retention time and mass spectra of available standards; [(3)] Retention indices on WAX factor 4 column, experimentally determined using a saturated n-alkane standard solution C7-C30; [(4)] Retention indices taken from [a] Jennings and Shibamoto 1980; [b] Binder et al. 1990,[c] Varming et al. 2004, [d] Umano et al. 2002, [e] Choi 2003, [f] Ruther 2000, [g] Weingart et al. 2011, [h] Wei et al. 2001, [i] Valim et al. 2003, [j] Ferreira et al. 2001, [k] Hayata et al. 2003, [l] Weckerle et al. 2001, [m] Sanchez-Ortiz et al. 2012, [n] De Marques et al. 2000, [o] Kohara et al. 2006; [(5)] Estimation of the concentration was based on the response curves calculated for one representative molecule of the chemical family. This approach involved performing six calibration curves linear in the concentration ranges tested (correlation coefficients, always>0.99) tr: trace; N.D.: not determined; # Compounds tentatively identified. Columns A and B represent respectively the relative area and concentration estimation of the VOCs

VOC depend significantly on its chemical family. The sorption of a volatile compound on the SPME fiber depends on the functional groups, vapor pressure, and constitution of the headspace of the sample. This needs to be taken into account when considering the quantitative values presented in Tables 1, 2, and 3 (columns B). These quantitative values are just estimations based on calibration curves obtained from the analyses of compounds that are considered representative for the respective detected root volatile. Exact quantification of VOCs in a mixture of approximately thirty compounds is quite difficult as many VOCs have to be quantified simultaneously in the volatile blend.

The major volatiles emitted by barley aerial parts were described by Bukovinszky et al. (2005), Piesik et al. (2010, 2011a), and Wenda-Piesik et al. (2010), whereas VOCs emitted by barley roots have not been reported previously in the literature, nor has any description of their biological activity been published. In our research reported herein, a total of 29 compounds were identified from excised roots after 21 days of growth. Estimation of the concentration of barley VOCs on a fresh weight basis revealed a similar range of emission when compared to that reported for barley leaves under controlled conditions or for β-caryophyllene in maize roots (Hiltpold et al. 2011; Piesik et al. 2010). In our study, VOC profiling was performed on roots separated from the aerial parts of the plant. In order to validate the working conditions, roots were wounded manually and changes in the profile of VOCs were analyzed. This additional wounding of roots resulted in a dramatic increase in the amount of VOCs emitted with no major changes in the qualitative VOC profile (data not shown).

VOCs derived from polyunsaturated fatty acids constituted the largest number of VOCs of the barley root blend. These comprise hexanal, methyl hexanoate, *(E)*-hex-2-enal, 2-pentylfuran, pentan-1-ol, (*Z*)-2-(pentenyl)-furan, *(Z)*-pent-2-en-1-ol, hexan-1-ol, *(Z)*-hex-3-en-1-ol, *(E)*-hex-2-en-1-ol, oct-1-en-3-ol, 2-ethylhexan-1-ol, *(E)*-non-2-enal, octan-1-ol, (*2E,6Z*)-nona-2,6-dien-al, methyl *(E)*-non-2-enoate, nonan-1-ol, *(Z)*-non-3-en-1-ol, (*E*)-non-2-en-1-ol, nona-3,6-dien-1-ol, nona-2,6-dien-1-ol, and dihydro-5-pentyl-2(3H)-furanone (Min et al. 2003; Shiojiri et al. 2006). Most of these compounds have been largely described in leaves as direct or indirect defense molecules, produced in response to herbivory or wounding (Arimura et al. 2000). Similarly to our study, barley aerial volatiles emitted under unwounded conditions were mainly C18 fatty acid derived volatile compounds, such as (*E*)-2-hexenal and (*Z*)-hex-3-en-1-ol (Piesik et al. 2010; Wenda-Piesik et al. 2010). Fatty acid derived VOCs might have a basal level of emission under unattacked conditions. With respect to root VOCs, quite similar compounds (hexanal, (*E*)-2-hexenal, 2-pentylfuran, 2-ethylhexan-1-ol, octan-1-ol, (*E*)-non-2-enal) were identified in grapevine ground roots (Lawo et al. 2011). As these VOCs have been described in

the wound response, they are interesting candidates in the study of root - wireworms interactions. As 2-ethylhexan-1-ol has never been clearly demonstrated to be of plant origin, this compound might be regarded as a plastifying contaminant (Yi et al. 2009).

Apart from fatty acid derived VOCs, two sulfur-containing volatile molecules (dimethyl sulfide and dimethyl sulfoxide) were constituents of the volatile blend. These two compounds have not previously been described as emitted by barley. Dimethyl sulfide is released from wounded citrus and guava leaves (Rouseff et al. 2008). Sulfur compounds have been shown to be attractants of the fly Delia antiqua (Matsumoto 1970).

Surprisingly, we did not identify any terpenes, such as β-caryophyllene. Nevertheless, we detected exogenously applied monoterpenes and sesquiterpenes when using our method (data not shown). This means either that barley roots do not emit terpenes or emit them below detection limits. This result is in agreement with the measurement of VOCs emitted by barley leaves; no terpene was detected in the headspace of non-wounded barley leaves; however, terpenes were detected after wounding (Piesik et al. 2010; Wenda-Piesik et al. 2010).

The impact of microorganisms in the measured volatile blend of barley roots was low since 32 VOCs out 34 were the same between the NS and ST roots (Table 3). Similarly, 28 VOCs were present in similar amounts in the headspace of roots kept under the two conditions.

The orientation bioassay showed that wireworms exploit the emission of VOCs from 7-d-old barley roots and use it for location of host roots. The percentages of non-responding individuals in the blank-to-blank bioassay and in the root-baited bioassay tended to show a slightly increased activity of wireworms in the presence of a stimulus. As roots were still respiring, CO_2 obviously formed part of the blend. Its involvement in the attraction of Ctenicera destructor (Brown) and other wireworms has been demonstrated (Doane et al. 1975). However, CO_2 emission from roots is probably not a reliable cue for host root location by rhizophagous insects, since CO_2 is emitted from numerous sources in the soil and thus, lacks specificity. Nevertheless, it probably acts as a general signal or a search trigger. Moreover, chemically-mediated orientation due to volatile or non-volatile compounds of the rhizosphere often is proposed whenever root location by subterranean insects is investigated (Johnson and Gregory 2006; Reinecke et al. 2008;,Weissteiner et al. 2012; Wenke et al. 2010). Such cues could be considered within an integrated management perspective, which will never be the case for CO_2 which is present in all soils.

Future studies need to elucidate whether each of the barley root volatiles detected in our study serves as an attractant to a rhizophagous insect. The VOCs identified using the protocol described for 7-d-old sterile roots (Table 2) need to be tested alone and in combination in the olfactometers, in order to assess both their potential attractive properties and their possible synergistic interactions with each other and with CO_2. This can be achieved by using slow release systems such as alginate beads (Heuskin et al. 2011), with the advantage of suppressing carbon dioxide gradients.

The assessment of the role of volatile compounds in the chemical ecology of wireworms is promising, especially regarding the developed bioassay. Further experiments involving the natural enemies of wireworms, such as entomopathogenic nematodes, could lead to a higher trophic level and could also provide useful information. Such tri-trophic interactions have already been studied between the root pest *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), maize (*Zea mays*, L. Poaceae), and the entomopathogenic nematode *Heterorhabditis megidis* (Hiltpold et al. 2011).

Acknowledgments The authors thank Marc Camerman and Franck Michels for research assistance, Dr. Marie Fiers for providing standards. Gembloux Agro-Bio Tech (University of Liège) funded the present project (Rhizovol project).

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