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Barley (*Hordeum distichon* L.) roots synthesise volatile aldehydes with a strong age-dependent pattern and release (*E*)-non-2-enal and (*E*,*Z*)-nona-2,6-dienal after mechanical injury

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Abstract

In the context of chemical ecology, the analysis of the temporal production pattern of volatile organic compounds (VOCs) in root tissues and the emission rate measurement of root-emitted VOCs are of major importance for setting up experiments to study the implication of these compounds in biotic interactions. Such analyses, however, remain challenging because of the belowground location of plant root systems. In this context, this study describes the evolution of the root VOC production pattern of barley (*Hordeum distichon* L.) at five developmental stages from germination to the end of tillering and evaluates the emission of the identified VOCs in an artificial soil. VOCs produced by crushed root tissues and released by unexcavated root systems were analysed using dynamic sampling devices coupled to a gas chromatography-mass spectrometry methodology (synchronous SCAN/SIM). The results showed that, at each analysed developmental stage, crushed barley roots produced mainly four volatile aldehydes: hexanal; (*E*)-hex-2-enal; (*E*)-non-2-enal; and (*E*,*Z*)-nona-2,6-dienal. Higher total and individual VOC concentrations were measured in 3-day-old seminal roots compared with older phenological stages. For each developmental stage, the lipoxygenase (LOX) activity was greater for linoleic acid than α-linolenic acid and the greatest LOX activities using linoleic and α-linolenic acids as substrates were measured in 7- and 3-day-old roots, respectively. The analysis of VOCs released by barley roots into the soil showed that (*E*)-non-2-enal and (*E*,*Z*)-nona-2,6-dienal were the only VOCs emitted in quantifiable amounts by mechanically injured roots.

Keywords: (*E*)-hex-2-enal; (*E*)-non-2-enal; (*E*,*Z*)-nona-2,6-dienal; barley; hexanal; plant age; root VOC profiling.

1. Introduction

Volatile organic compounds (VOCs) are low molecular weight molecules with a high vapour pressure at ambient temperatures (Dudareva et al., 2013). Most VOCs emitted by plants can be classified into four chemical families, each related to specific biosynthetic pathways: terpenoids (mevalonic acid [MVA] and methylerythritol phosphate [MEP] pathways); fatty acid derivatives (lipoxygenase [LOX] pathway); benzenoid and phenylpropanoid compounds (shikimic acid pathway); and amino acid derivatives (Dudareva et al., 2013). These compounds can be emitted by various plant organs (flowers, fruits, leaves and roots) either locally or systemically in response to biotic and abiotic stresses and are known to play important roles in plant interactions with their surrounding environment (Dudareva et al., 2006; Gouinguené and Turlings, 2002; Hiltpold et al., 2013, 2011; Holopainen and Gershenzon, 2010; Loreto and Schnitzler, 2010). Many studies have provided clear evidence that VOC-mediated interactions also occur belowground between plant roots and soil organisms (for reviews, see Delory et al., 2016; Peñuelas et al., 2014; Turlings et al., 2012; Wenke et al., 2010). Root-emitted VOCs have been shown to attract insect herbivores (Guerin and Ryan, 1984; Palma et al., 2012; Robert et al., 2012; Sutherland and Hillier, 1974; Weissteiner et al., 2012) and plant parasitic nematodes (Ali et al., 2011; Farnier et al., 2012). Because insect-damaged roots release volatile cues attracting organisms of the third trophic level like entomopathogenic nematodes (Ali et al., 2011; Boff et al., 2002; Rasmann et al., 2005; van Tol et al., 2001) and insect predators (Ferry et al., 2007; Neveu et al., 2002), they are also implicated in indirect plant defences against pests.

Compared with aboveground plant organs, the quantitative analysis of VOCs emitted by root tissues remains challenging, mainly because of the belowground

location of plant root systems (Delory et al., 2016). Using static headspace sampling methods like solid phase microextraction (SPME), the volatile production capacity of belowground plant organs has been evaluated destructively on parts of isolated root systems (Fiers et al., 2013; Gfeller et al., 2013; Palma et al., 2012; Weissteiner et al., 2012) or on root samples that were flash-frozen and crushed in liquid nitrogen prior to volatile analysis (Erb et al., 2011; Hiltpold et al., 2011; Lawo et al., 2011; Laznik et al., 2011; Rasmann et al., 2005; Robert et al., 2012). Although easy to set up, such analyses do not allow the emission rate calculation of VOCs that are emitted in the soil by living and undamaged plant roots (Delory et al., 2016). Despite the significant volatile background existing due to the soil ecosystem surrounding the roots, both proton transfer reaction-mass spectrometry (PTR-MS) analyses (Crespo et al., 2012; Danner et al., 2015; van Dam et al., 2012) and gas chromatography-mass spectrometry (GC-MS) analyses performed after soil VOC collection on packed adsorbents (Ali et al., 2011, 2010; Hiltpold et al., 2011) have been successfully applied to the in situ analysis of root-emitted VOCs.

Previous experiments showed that fatty acid derivatives were the main VOCs emitted by isolated barley roots (Fiers et al., 2013; Gfeller et al., 2013). In higher plants, volatile fatty acid derivatives are produced mainly via the LOX pathway. Plant LOXs (EC 1.13.11.12) are classified as non-heme iron-containing enzymes that catalyse the stereospecific addition of molecular oxygen to either the 9^{th} (9-LOX) or 13^{th} (13-LOX) carbon atom of polyunsaturated fatty acids containing a (*Z*,*Z*)-penta-1,4-diene system (Dudareva et al., 2013; Gigot et al., 2010; Siedow, 1991). In plants, 9- and 13-LOXs use mainly linoleic acid and α-linolenic acid as substrates in order to produce 9- and 13-hydroperoxides, respectively (Maffei, 2010; Siedow, 1991). These LOX-derived products can subsequently be used by cytochrome P450 enzymes, such

as allene oxide synthases and hydroperoxide lyases (HPL), to produce jasmonates and C_6/C_9 volatile aldehydes, respectively (Dudareva et al., 2013; Grechkin and Hamberg, 2004; Maffei, 2010). HPL-derived aldehydes can be further converted to C_6/C_9 alcohols by alcohol dehydrogenases, and subsequently converted into their corresponding esters by alcohol acyltransferases (Dudareva et al., 2013; Gigot et al., 2010).

Because the LOX activity (Holtman et al., 1996) and the VOC production capacity of plant roots have been reported to change with plant ontogeny (Köllner et al., 2004; Palma et al., 2012; Tapia et al., 2007), characterising the temporal VOC production pattern of barley roots is needed for setting up experiments aimed at investigating the ecological roles of root-emitted VOCs, particularly in relation to plant defence. Currently, two main ecological theories offering conflicting predictions are used to explain patterns in costly defensive secondary metabolite production according to plant age: the optimal defence theory (ODT) and the growth-differentiation balance hypothesis (GDBH) (Barton and Koricheva, 2010; Quintero et al., 2013). Briefly, the ODT predicts that plants would invest more in the production of defensive secondary metabolites in young tissues and reproductive plant parts. In contrast, the GDBH predicts that plant organs at a given phenological stage will invest more in secondary metabolite production with increasing plant age (Barton and Koricheva, 2010; Boege and Marquis, 2005; Elger et al., 2009; Quintero et al., 2013; Radhika et al., 2008; Rostás and Eggert, 2008). Although the production of VOCs can be affected by plant age, such a temporal production pattern in barley roots has not yet been described and requires further investigations.

In this context, this paper reports on the evolution of the VOC production pattern of barley roots at five selected developmental stages from germination to the

123 end of tillering. In addition, we also estimated the emission rate of the main VOCs
124 released by barley roots into the soil environment using an in situ trapping device and
125 a GC-MS methodology allowing the identification and the quantification of VOCs
126 without extracting the roots from the soil. The significance of the results presented in
127 this work is further discussed in the context of plant ontogeny and belowground
128 chemical ecology.

129 **2. Methods**

130 **2.1.Chemicals**

131 Methanol (CAS 67-56-1, Grade gradient HiPerSolv CHROMANORM) was
132 bought from VWR BDH Prolabo® (Leuven, Belgium). Hexanal (98%, CAS 66-25-1),
133 3,5,5-trimethylhexanal (≥ 95%, CAS 5435-64-3), (*E*)-hex-2-enal (98%, CAS 6728-
134 26-3), (*E*)-non-2-enal (97%, CAS 18829-56-6), (*E*,*Z*)-nona-2,6-dienal (95%, CAS
135 557-48-2), 3-methyl-but-3-en-1-ol (≥ 97%, CAS 763-32-6), (*E*)-hept-2-enal (≥ 95%,
136 CAS 18829-55-5), hexan-1-ol (≥ 98%, CAS 111-27-3) and (*E*)-oct-2-enal (≥ 97%,
137 CAS 2548-87-0) were bought from Sigma-Aldrich (St. Louis, MO, USA). Pent-1-en-
138 3-ol (98%, CAS 616-25-1) and acetic acid (100%, CAS 64-19-7) were bought from
139 Avocado Research Chemicals Ltd and Merck Chemicals, respectively.

140 **2.2.Analysis of VOCs produced by barley roots at five developmental stages**

141 *2.2.1. Plant material*

142 Barley (*Hordeum distichon* L. 'Quench') caryopses with a mass of 40.6 mg ±
143 5% were selected for the experiments and allowed to germinate for 24 h on a double
144 layer of wet filter paper in the dark at a temperature of 21.9 ± 0.7°C. On the following
145 day, homogeneous 1-day-old seedlings were transplanted into polyvinyl chloride
146 (PVC) tubes that had been filled with a mixture (= substrate) of 2 mm-sieved sand
147 (80%, v/v) and 5 mm-sieved potting soil (20%, v/v). Plants to be analysed after 3 and

7 days were grown in 201 cm³ (h = 16 cm) and 295 cm³ (h = 15 cm) tubes containing 225 g and 275 g of substrate, respectively, and were watered every 2 days with 10 mL of tap water. Plants to be analysed at later developmental stages (17, 28 and 38 days) were grown in 1,909 cm³ tubes (h = 30 cm) filled with 2,140 g of substrate and watered every 2 days alternatively with 50 mL of tap water and 50 mL of standard Hoagland solution. Watering with Hoagland solution started 6 days after transplantation. Sowing depth and density were set at 3 cm and 1 seedling/tube. Plants were grown under controlled environmental conditions throughout the experiments (21.9 ± 0.7°C, 65.9 ± 1.7% RH, 16 h/8 h - L/D, PAR light intensity: 82.3 – 91.6 $\mu mol \cdot m^{-2} \cdot s^{-1}$, LED lighting). Plant age was expressed in growing degree days (GDD) according to Equation 1 where T_i is the daily average temperature (°C), T_t is the growing threshold temperature for barley (set at 5°C [Stewart and Dwyer 1987]) and n is the plant age expressed in days. Table 1 provides a summary of the main characteristics of the plants at the five selected developmental stages. The growth stages of barley were codified according to Zadoks et al. 1974. Each developmental stage was replicated four times.

$$GDD_n = \sum_{i=1}^{n} (T_i - T_t) \quad (1)$$

2.2.2. Sample preparation

When plants reached selected developmental stages (Table 1), the roots were extracted from the soil by carefully washing them with tap water, excising the shoots and rapidly freezing the roots in liquid nitrogen. The plant organs were then stored at -80°C. For each developmental stage, the roots were crushed in liquid nitrogen and 500 mg of root powder was placed in a 20 mL glass vial supplied with a silicone/PTFE septum (FilterService, Eupen, Belgium). Because of the low root

172 biomass at the young developmental stages, 10 and 2 barley root systems were pooled
173 for the 3- and 7-day-old barley seedlings, respectively. Before sealing, the atmosphere
174 in the vials was replaced by gaseous nitrogen. The sealed vials were then stored at -
175 80°C prior to VOC analysis and at -24°C on the day of the analysis. After the GC-MS
176 analyses, the samples were dried at 70°C until constant mass was reached. After
177 drying, the samples were allowed to cool at room temperature in a desiccator before
178 the dry weight was measured.

179 *2.2.3. Dynamic headspace sampling (DHS)-GC-MS*

180 Root VOC analyses were performed using a fully automated analytical
181 methodology comprising three main steps: VOC trapping using a DHS system
182 (Gerstel, Mülheim an der Ruhr, Germany), VOC separation using GC (7890A;
183 Agilent Technologies, Palo Alto, CA, USA) and VOC detection using a quadrupole-
184 type MS (5975C; Agilent Technologies, Palo Alto, CA, USA). Using a multipurpose
185 sampler (Gerstel, Mülheim an der Ruhr, Germany) and a 10 µL Hamilton gastight
186 syringe, 1 µL of a methanolic solution of 3,5,5-trimethylhexanal (300 ng/µL) was
187 added to the crushed roots as an internal standard (IS). The root samples were then
188 incubated at 25°C for 15 min under constant agitation (500 rpm). At the end of the
189 incubation process, VOCs were trapped in a cartridge containing 60 mg of Tenax TA
190 adsorbent (Gerstel, Mülheim an der Ruhr, Germany) for 10 min at 25°C with a helium
191 flow rate of 20 mL/min. Excess water was removed from the Tenax TA cartridges by
192 a dry purge performed for 3 min at 60°C with a helium flow rate of 20 mL/min. The
193 trapped VOCs were then thermally desorbed from Tenax TA traps using a thermal
194 desorption unit (TDU) (Gerstel, Mülheim an der Ruhr, Germany) running in splitless
195 mode for 10 min at 280°C. During thermal desorption, VOCs were cryofocused in a
196 CIS/PTV inlet (Gerstel, Mülheim an der Ruhr, Germany) cooled at -150°C with liquid

197 nitrogen. At the end of the desorption process, root VOCs were injected for 1.5 min in
198 solvent vent mode inside a polar GC column (VF-WAXms, 30 m length, 0.25 mm
199 i.d., 0.25 µm film thickness; Agilent Technologies, Palo Alto, CA, USA) by heating
200 the CIS/PTV inlet to 260°C for 5 min at a rate of 12°C/s. The vent flow was set at 50
201 mL/min. The GC oven had the following temperature program: 35°C for 2 min, ramp
202 5°C/min to 155°C, and ramp 20°C/min to 250°C held for 10 min. High purity helium
203 (99.999%, Air Liquide, Liège, Belgium) was used as carrier gas at a constant flow
204 rate of 1.5 mL/min. The MS was used in electron ionization mode (70 eV) with a gain
205 factor of 1 and operated synchronously in SCAN and SIM modes. In SCAN mode, the
206 MS scanned *m/z* ratios from 35 to 300 amu with a threshold value and a sampling rate
207 set at 500 and 2^1 (3,125 amu/s), respectively. Hexanal (*m/z* 56), (*E*)-hex-2-enal (*m/z*
208 69), (*E*)-non-2-enal (*m/z* 70) and (*E,Z*)-nona-2,6-dienal (*m/z* 70) were quantified in
209 SIM mode based on the internal standard (*m/z* 69 and 109) response. For each ion, the
210 dwell time was set at 100 ms. The source and the quadrupole temperature were set at
211 230°C and 150°C, respectively. Background VOCs were identified by analysing the
212 headspace of empty vials using the same methodology (blank measurements). GC-MS
213 data were analysed with the Agilent MSD ChemStation E.02.00.493 (Agilent
214 Technologies, Palo Alto, CA, USA).

215 *2.2.4. VOC identification and quantification*

216 VOCs produced by the barley roots were first identified by comparing recorded mass
217 spectra with those contained in the Wiley 275 mass spectral database. These
218 identifications were confirmed by comparing calculated non-isothermal retention
219 indices (RI) and MS data with those of authentic standards injected under the same
220 chromatographic conditions as described earlier. VOC identification was also
221 assessed by comparing calculated retention indices with those reported in the

literature (Ferreira et al., 2001; Jennings and Shibamoto, 1980). The RI were experimentally determined using a saturated *n*-alkanes (C7 - C30) standard solution (Sigma-Aldrich, St. Louis, MO, USA). Major VOCs in the root tissues were quantified using 3,5,5-trimethylhexanal as an internal standard (IS). This molecule was selected because (1) it is not produced by barley roots, and (2) it belongs to the same chemical family (aldehydes) and has a molecular weight close to that of the analytes that had to be quantified. VOC concentrations in the root tissues were calculated based on linear calibration curves linking the ratio of the analyte peak area to the IS peak area and the ratio of the injected mass of analyte to the injected mass of IS. GC-MS analyses were performed using the same parameters as described earlier, except that 1 µL of a methanolic solution containing each chemical standard at a defined concentration and a fixed amount of IS (90.6 ng) was directly injected into Tenax TA cartridges using a multipurpose sampler and a 10 µL Hamilton gastight syringe. VOCs were then thermally desorbed in a TDU running in solvent vent mode in order to avoid methanol injection into the GC column. Solvent venting was performed at 40°C for 3 min. For each VOC, a calibration curve was constructed with five equidistant points replicated four times [hexanal: 0 – 452 ng, $R^2 = 0.998$; (*E*)-hex-2-enal: 0 – 359.3 ng, $R^2 = 0.9906$; (*E*)-non-2-enal: 0 – 405.8 ng, $R^2 = 0.9894$; (*E*,*Z*)-nona-2,6-dienal: 0 – 267.8 ng, $R^2 = 0.9875$] (Fig. S1). Linear models were fitted with the *lm* function of R 3.1.2 (R Core Team, 2015).

2.3.LOX activity measurement

The evolution of the LOX activity in barley roots during plant ontogeny was studied using a spectrophotometric assay based on the absorbance at 234 nm of conjugated dienes (fatty acid hydroperoxides, HPOs) produced by the LOX activity from linoleic and α-linolenic acids (Surrey, 1964). Barley roots were crushed in liquid

nitrogen and 500 mg of root powder was homogenized for 1 h in 2.5 mL of 0.1 M sodium phosphate buffer (pH 7.5) at 7°C. After centrifugation at 21,000 × g for 30 min, the supernatant (= crude extract) was collected and stored on ice. For LOX activity measurement, the reaction mixture consisted of 100 µL of crude extract, 50 µL of a 10 mM linoleic or α-linolenic acid emulsion and 2,850 µL of oxygenated 0.1 M sodium phosphate buffer (pH 7.5). The absorbance at 234 nm was recorded every 1 s for 120 s with an UV-visible spectrophotometer (UV-1650PC, Shimadzu Corporation, Kyoto, Japan). Using a molar extinction coefficient of 25,000 $cm^{-1} \cdot M^{-1}$, one LOX activity unit corresponds to 1 µmol of HPOs formed per minute. The protein concentration in the crude extracts was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) based on the colorimetric method of Bradford (Bradford, 1976). The reaction mixture consisted of 780 µL of distilled water, 200 µL of the acidic dye reagent and 20 µL of crude extract. After 15 min, the absorbance of the reaction mixture at 595 nm was recorded. The protein concentration in the crude extracts was determined based on a calibration curve constructed using bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) concentrations in the reaction mixture ranging from 0 to 12.8 mg/L. For each developmental stage, four independent extracts were used for LOX activity and protein concentration measurements.

2.4.Analysis of VOCs emitted by barley roots in the soil

Because the volatile production pattern of crushed plant tissues does not accurately portray the volatile emission profile of intact plant organs (Rasmann et al., 2012), we designed an experimental protocol allowing the in situ collection and the quantitative analysis of VOCs emitted by barley roots without extracting the plant organs from the soil (Fig. 1). When studying root-emitted VOCs, this is of major

importance as some detected VOCs like C_6 and C_9 molecules produced via the LOX pathway have been reported to be rapidly emitted after tissue disruption (Matsui, 2006) and could therefore originate from the damages inflicted to the roots during the excavation process and would not have been emitted by undamaged roots (Delory et al., 2016; Jassbi et al., 2010). Briefly, caryopses were selected and allowed to germinate as described earlier. Then, 10 1-day-old homogeneous plantlets were transplanted into a glass container (internal volume: 500 mL) filled with 750 g of artificial soil and closed with a polypropylene cap perforated with one central 5 mm-hole used for plant watering and 10 1 cm-holes through which plants have grown for 15-16 days. The solid phase of the artificial soil consisted of 82.8% (m/m) of 2 mm-sieved clean sand previously heated at 200°C for 4 h and 17.2% (m/m) of 3 to 5 mm-glass beads. The glass beads were added to the soil in order to increase the soil's macroporosity. The humidity level of the sand fraction was adjusted to 10% (m/m) with a sterile standard Hoagland solution (1.6 g/L, pH 6.5; Hoagland's No. 2 Basal Salt Mixture, Sigma-Aldrich, St. Louis, MO, USA). The mass of each reactor was registered at the beginning of the experiment and was kept constant for the duration of the experiment by adding daily the required volume of standard Hoagland solution (0.8 g/L, pH 6.5).

The collection of VOCs emitted by the artificial soil alone and undamaged or mechanically damaged roots of 16 to 17-day-old seedlings was performed by pulling charcoal-filtered air out of the reactors at a rate of 80 mL/min through a glass tube containing 60 mg of Tenax TA using a Gilian GilAir® Plus air sampling pump (Sensidyne, LP, St. Petersburg, FL, USA). All connections were made via PTFE tubing and the reactors were sealed using a nonporous synthetic rubber paste (Terostat VII; Henkel AG & Co. KGaA, Düsseldorf, Germany) before the start of the VOC

297 collection period. In this work, we tested the ability of our analytical method to
298 measure the emission rates of VOCs released by roots that were mechanically
299 damaged prior to VOC collection using four nylon threads that were positioned along
300 the internal face of the reactors when they were filled with artificial soil. Undamaged
301 roots growing in an artificial soil and the artificial soil alone were used as controls.
302 After pumping for 4 h, Tenax TA cartridges were dried at 40°C for 3 min in the TDU.
303 Trapped VOCs were then thermally desorbed and analysed by GC-MS using the
304 parameters described earlier except that the MS operated with a threshold value of
305 150 and a gain factor of 1.5. In SIM mode, we defined 3 groups of *m/z* values in order
306 to detect and quantify target root-emitted VOCs (group 1 [hexanal]: *m/z* 44 and 56;
307 group 2 [(*E*)-hex-2-enal]: *m/z* 39 and 42; group 3 [(*E*)-non-2-enal and (*E*,*Z*)-nona-2,6-
308 dienal]: *m/z* 70). These *m/z* values were selected because of their high specificity for
309 the target root-emitted VOCs and their low specificity for the soil volatile
310 background. The analyses were replicated at least three times for each experimental
311 treatment. Soon after the volatile collection period, the roots were extracted from the
312 glass containers and were dried at 70°C until constant mass was reached. After
313 drying, the samples were allowed to cool at room temperature in a desiccator before
314 the dry weight was measured.

315 The quantification of each root-emitted VOC was performed using a linear
316 calibration curve linking the target SIM peak area to the mass of injected standard.
317 For each VOC, a calibration curve was constructed with five equidistant points
318 replicated three times [(*E*)-non-2-enal: 0 – 101.8 ng, $R^2 = 0.9887$; (*E*,*Z*)-nona-2,6-
319 dienal: 0 – 42.5 ng, $R^2 = 0.9833$] (Fig. S2). The linear models were fitted with the *lm*
320 function of R 3.1.2 (R Core Team, 2015).
321

2.5.Recovery of VOC standards injected into the soil

Because the concentration reached in the soil atmosphere by a VOC depends on its vapour pressure, its chemical stability, its emission rate by plant roots, and its interactions with the various components of the soil ecosystem (Delory et al., 2016), all VOCs will not be recovered with the same efficiency if a dynamic system is used to sample VOCs emitted by roots. In order to test the sensitivity and the selectivity of the method used in this study for the in situ analysis of root-emitted VOCs, we injected 1 µL of a synthetic mixture of VOCs (each compound had a final concentration of 300 ng/µL) into 4 glass containers filled with 750 g of artificial soil and closed with a polypropylene cap. A container that contained only artificial soil was used as a control to confirm that the injected VOCs were not present in the soil atmosphere. The composition of the solid phase of the soil was the same as previously described (see 2.4). The humidity level of the sand fraction was adjusted to 10% (m/m) with distilled water. Five VOCs were selected for this experiment: a sesquiterpene known to possess good diffusion properties in sand and soil ([*E*]-β-farnesene) (Hiltpold and Turlings, 2008), three fatty acid derivatives produced by crushed barley roots (hexanal, [*E*]-non-2-enal and [*E*,*Z*]-nona-2,6-dienal), and one VOC detected in the headspace of isolated barley roots (2-pentylfuran) (Gfeller et al., 2013). VOCs were injected 5 cm below the soil surface with a 1 µL Hamilton gastight syringe. Immediately after the injection, the glass containers were sealed using a nonporous synthetic rubber paste (Terostat VII; Henkel AG & Co. KGaA, Düsseldorf, Germany). VOCs were trapped using the same protocol as previously described (see 2.4). During the sampling of VOCs, the glass containers were placed inside a growth chamber (22.0 ± 0.1°C, 26.8 ± 0.5% RH). After pumping for 4 h, VOCs trapped on Tenax TA cartridges were thermally desorbed in a TDU, cryofocused in a CIS/PTV

inlet, separated by GC, and detected by MS. The MS operated in synchronous SCAN/SIM mode as described earlier (see 2.4). In order to detect the target VOCs, the following *m/z* ratios were followed by the MS operating in SIM mode: *m/z* 44 and 56 (hexanal), *m/z* 138 (2-pentylfuran), *m/z* 70 ([*E*]-non-2-enal and [*E,Z*]-nona-2,6-dienal) and *m/z* 204 ([*E*]-β-farnesene). The recovery rate (R) of each VOC was calculated according to Equation 2 where *A* is the SIM peak area obtained after the analysis of VOCs located in the soil atmosphere, and $\bar{A}_{300}$ is the mean SIM peak area (n = 3) obtained when a Tenax TA cartridge containing 300 ng of the compound is analysed using our GC-MS method (Eilers et al., 2015).

$$R = \frac{A}{\bar{A}_{300}} \times 100 \qquad (2)$$

2.6.Statistical analyses

Mean individual and total VOC concentrations, mean relative proportions of each major VOC identified in the chemical profiles, mean C_6/C_9 volatile aldehyde ratios (i.e., the ratio between the concentration of VOCs consisting of 6 carbon atoms [originating from a 13-LOX activity] and the concentration of VOCs consisting of 9 carbon atoms [originating from a 9-LOX activity]), and mean LOX activity measurements performed at five developmental stages were compared using one-way ANOVA followed by a Newman and Keuls test with plant age as a fixed factor. Similarly, the recovery rates of five VOCs were compared using one-way ANOVA followed by a Newman and Keuls test. All statistical analyses were performed using R 3.1.2/3.2.2 (R Core Team, 2015) with an alpha value of 5%.

3. Results

3.1. Identification of VOCs produced by barley roots at five phenological stages

For each developmental stage, GC-MS analyses showed that the barley roots produced mainly four volatile aldehydes: hexanal; (*E*)-hex-2-enal; (*E*)-non-2-enal; and (*E*,*Z*)-nona-2,6-dienal (Fig. 2, Table 2). In addition, pent-1-en-3-ol (CAS 616-25-1, RI_C = 1178, RI_{Std} = 1163), 3-methyl-but-3-en-1-ol (CAS 763-32-6, RI_C = 1259, RI_{Std} = 1251), (*E*)-hept-2-enal (CAS 18829-55-5, RI_C = 1326, RI_{Std} = 1321), hexan-1-ol (CAS 111-27-3, RI_C = 1357, RI_{Std} = 1355), (*E*)-oct-2-enal (CAS 2548-87-0, RI_C = 1426, RI_{Std} = 1426) and acetic acid (CAS 64-19-7, RI_C = 1457, RI_{Std} = 1478) were detected as minor compounds.

3.2. Quantification of VOCs produced by barley roots at five phenological stages

The total volatile aldehyde concentration was highest in 3-day-old barley seedlings and decreased markedly according to plant age (Fig. 3A). Compared with the youngest developmental stage, plant roots analysed at 470 and 640 GDD produced 36.9% and 85.3% fewer VOCs, respectively, which is highly significant statistically ($P < 0.001$). In addition, the ratio between concentrations of C_6 and C_9 volatile aldehydes in root tissues changed during plant development with a maximum value reached at 118 GDD (Fig. 3B). This result was linked to the predominance of both hexanal and (*E*)-hex-2-enal in the chemical profiles of 7-day-old barley roots (Fig. 4). The lowest values of this ratio were observed for roots analysed at 640 GDD. At this age, plant roots were characterised by a C_6/C_9 ratio that was less than one. This result can be explained by both an increase in the relative proportions of (*E*)-non-2-enal and (*E*,*Z*)-nona-2,6-dienal in the chemical profiles and a decrease in hexanal and (*E*)-hex-

2-enal synthesis in 38-day-old barley roots compared with other developmental stages (Fig. 4). In contrast, the mean C_6/C_9 volatile aldehyde ratios were always greater than one for plants younger than 640 GDD. Although the C_6/C_9 ratios measured at 51, 285 and 470 GDD did not differ statistically (Fig. 3B), the analysis illustrated in the Fig. 4 shows that the mean relative proportions of hexanal, (*E*)-hex-2-enal and (*E*,*Z*)-nona-2,6-dienal differed significantly in the chemical profiles analysed at these developmental stages. After 51, 118 and 470 GDD, hexanal was the major VOC found in barley roots and represented between 36.9 ± 2.9% and 41.2 ± 2.5% of the total VOC concentration. For other developmental stages, the hexanal proportions in the VOC profiles were significantly lower than that mentioned earlier ($P < 0.001$), but were statistically similar to each other. The mean relative proportions of (*E*)-hex-2-enal in the chemical profiles all differed statistically according to plant age ($P < 0.001$), ranging from 8.3 ± 1.7% for the oldest developmental stage to 35.3 ± 1.7% for plants analysed at 118 GDD. Like C_6 molecules, the mean relative proportions of (*E*)-non-2-enal and (*E*,*Z*)-nona-2,6-dienal differed at the five selected developmental stages in a very highly significant way ($P < 0.001$). After 640 GDD, (*E*)-non-2-enal was the major VOC found in barley roots and represented 50.1 ± 3.6% of the total VOC concentration. Roots analysed after 51, 285 and 470 GDD were characterised by intermediate (*E*)-non-2-enal levels ranging from 26.9 ± 2.4% to 30.2 ± 2.5% of the total volatile aldehyde concentration. With a mean value of 16.4 ± 1.7%, the lowest relative proportion of (*E*)-non-2-enal was found in barley roots harvested at 118 GDD. With regard to (*E*,*Z*)-nona-2,6-dienal, its contribution to the total VOC concentration ranged from 7.3 ± 0.6% to 17.9 ± 1.0% for plants analysed at 118 and 640 GDD, respectively. The mean relative proportions of (*E*,*Z*)-nona-2,6-dienal in the barley roots did not differ significantly at 285 and 470 GDD.

Like the total root VOC concentration, the hexanal (Fig. 3C), (*E*)-hex-2-enal (Fig. 3D), (*E*)-non-2-enal (Fig. 3E) and (*E,Z*)-nona-2,6-dienal (Fig. 3F) concentrations showed a strong age-dependent pattern. For each individual volatile, mean VOC concentrations measured from germination to the end of tillering differed in a very highly significant way statistically ($P < 0.001$). The mean hexanal concentration in the root tissues showed a strong decrease of 90.7% from the youngest to the oldest developmental stage. The highest hexanal concentration was found in 51 GDD-old barley seedlings with a mean concentration of 43.3 ± 2.3 µg/g dry wt, which was not significantly different from that measured at 118 GDD. Intermediate hexanal concentrations ranging from 25.7 ± 4.3 to 31.9 ± 5.2 µg/g dry wt occurred between 118 and 470 GDD (Fig. 3C). Mean (*E*)-hex-2-enal concentrations calculated between 51 and 470 GDD did not differ significantly from each other and ranged from 17.0 ± 3.0 to 27.6 ± 5.1 µg/g dry wt for roots analysed at 470 and 118 GDD, respectively. The lowest (*E*)-hex-2-enal concentration was observed in 640 GDD-old barley roots (Fig. 3D). With regard to the C_9 volatiles, they showed a very similar evolution pattern in relation to plant age (Fig. 3E and 3F). Barley roots were characterised by a low production of (*E*)-non-2-enal and (*E,Z*)-nona-2,6-dienal at 118 GDD. At this age, no C_9 molecule concentrations differed statistically from the lowest concentration measured at 640 GDD or from (*E*)-non-2-enal and (*E,Z*)-nona-2,6-dienal concentrations measured in 470 GDD-old barley roots. In addition, the (*E,Z*)-nona-2,6-dienal concentration observed at 285 GDD was similar to that measured in 118 GDD-old barley seedlings. Like C_6 volatiles, the highest C_9 volatile concentrations were measured at the youngest developmental stage and were 30.1 ± 4.8 and 15.7 ± 2.1 µg/g dry wt for (*E*)-non-2-enal and (*E,Z*)-nona-2,6-dienal, respectively. For (*E*)-non-2-enal, this value did not differ statistically from that observed at 285 GDD.

Similarly, the (*E*)-non-2-enal concentrations in 285 and 470 GDD-old barley roots did not differ significantly from each other ($P > 0.05$).

3.3.LOX activity measurement in barley roots

Because hexanal, (*E*)-hex-2-enal, (*E*)-non-2-enal and (*E,Z*)-nona-2,6-dienal are documented to originate from the enzymatic oxidation of linoleic and α-linolenic acids via the LOX pathway in plant tissues (Dudareva et al., 2013; Maffei, 2010), we measured the LOX activity in barley roots at the same developmental stages as those used for VOC analyses. The results showed that the specificity of LOX extracted from the roots of barley is greater for linoleic acid than for α-linolenic acid (Fig. 5). When linoleic acid was used as a substrate in the enzymatic assay, 125 GDD-old roots showed a significantly greater LOX activity (2.11 ± 0.26 units/mg protein) than that measured at the other developmental stages ($P = 0.012$). In contrast, the greatest LOX activity using α-linolenic acid as a substrate was measured in the roots of 53 GDD-old plants ($P < 0.001$) and reached a mean value of 0.97 ± 0.05 units/mg protein. Developmental stages older than 299 GDD did not show any statistical difference regarding LOX activity measurements.

3.4.Analysis of VOCs emitted by barley roots in the soil

The results showed that undamaged roots of barley plants growing in an artificial soil did not release additional VOCs compared with the odour profile obtained for the artificial soil alone (Fig. 6A and 6B). In contrast, 16 to 17-day-old barley roots released (*E*)-non-2-enal and (*E,Z*)-nona-2,6-dienal in the soil after mechanical injury (Fig. 6C). The identity of these molecules was confirmed by comparing SCAN mass spectral data and calculated RI with those of authentic standards. By external calibration, their emission rate in the soil was estimated to 13.8 ± 4.9 ng/g dry wt/h for (*E*)-non-2-enal and 4.7 ± 1.8 ng/g dry wt/h for (*E,Z*)-nona-2,6-

dienal (mean ± s.e., n = 6). Using SCAN MS data, traces amounts (not quantifiable) of hexanal were detected in some odour profiles associated with mechanically damaged roots.

3.5.Recovery of VOC standards injected into the soil

Because (1) both C_6 and C_9 volatile aldehydes were produced by crushed barley roots and (2) only C_9 volatile aldehydes were detected in quantifiable amounts when VOCs emitted by mechanically damaged roots were trapped in situ, we designed an experiment in order to test the sensitivity and the selectivity of our analytical method. To do so, we successively injected a synthetic VOC mixture into the soil, trapped VOCs using our dynamic sampling system, and calculated the recovery of each compound using a method similar to the one used by Eilers and co-workers (2015). We found that the five VOCs used in this experiment had significantly different recovery rates ($P < 0.001$) and can be divided into three groups (Fig. 7). The first group contained VOCs that were easily recovered from the soil. It contained the terpenoid (*E*)-β-farnesene (63.0 ± 4.9%) and the C_6 volatile hexanal (59.5 ± 3.6%). The two C_9 volatile aldehydes were contained in two different groups and were characterized by lower recovery rate values compared with the VOCs of the first group ([*E*]-non-2-enal: 32.2 ± 4.4%; [*E*,*Z*]-nona-2,6-dienal: 13.7 ± 5.9%). With regard to 2-pentylfuran (19.7 ± 4.9%), its recovery rate was not statistically different from that of (*E*)-non-2-enal and (*E*,*Z*)-nona-2,6-dienal.

4. Discussion

Crushed barley roots analysed at five developmental stages from germination to the end of tillering produced mainly four aldehydes as major VOCs: hexanal; (*E*)-hex-2-enal; (*E*)-non-2-enal; and (*E*,*Z*)-nona-2,6-dienal. Two previous studies mentioned the emission of these molecules by 7- to 21-day-old excised barley roots

493 using SPME (Fiers et al., 2013; Gfeller et al., 2013). There are major differences in
494 the number of identified VOCs when comparing the present results with those
495 obtained by SPME. In addition to the four volatile aldehydes discussed in this study,
496 Gfeller and co-workers (2013) identified 30 and 25 additional VOCs emitted in
497 significant amounts by isolated 7- and 21-day-old barley roots produced in
498 vermiculite, respectively. When testing the effect of barley root infection with one or
499 two pathogenic fungi (*Cochliobolus sativus* and *Fusarium culmorum*) on VOC
500 emission, only (*E*)-non-2-enal and (*E,Z*)-nona-2,6-dienal were detected in uninfected
501 9-day-old roots. (*E*)-hex-2-enal was reported to be emitted only by roots infected
502 simultaneously by *C. sativus* and *F. culmorum*. Hexanal was not reported to be
503 emitted by barley roots whatever the conditions tested (Fiers et al., 2013). Taken
504 together, these differences could be explained both by the preparation of biological
505 samples (crushed or excised roots) and by the analytical procedure used to sample
506 volatile compounds. The simpler VOC profile obtained under our experimental
507 conditions could result from the shorter sampling time limiting the sample
508 degradation process, as well as from the lower oxygen concentration in the headspace
509 of sealed vials, leading to lower oxidation of the biogenic VOCs in barley roots. In
510 addition, as the exposition of roots to light can rapidly induce the production of
511 reactive oxygen species (ROS) (Yokawa et al., 2011) and generates a stress to the
512 roots (Silva-Navas et al., 2015), one can hypothesise that the in vitro cultivation of
513 plants performed by Gfeller et al. (2013) and Fiers et al. (2013) and the SPME
514 collection of VOCs emitted by isolated and illuminated roots can lead to the
515 production of VOCs that would not have been emitted by unstressed roots. These
516 latter hypotheses are supported by the fact that we did not detect 2-pentylfuran in our
517 chemical profiles when crushed or potted roots were analysed, although it was one of

518 the major VOCs identified in the SPME analyses of sterile and non-sterile barley
519 roots (Fiers et al., 2013; Gfeller et al., 2013). Because 2-pentylfuran is a volatile
520 compound that can be produced via a non-enzymatic peroxidation of linoleic acid by
521 singlet oxygen (Min et al., 2003), it could possibly be an artefact produced during the
522 sampling of VOCs located in the headspace of isolated barley roots. With regard to
523 volatile fatty acid derivative emission, hexanal has been shown to be emitted in
524 significant amounts by isolated roots of the grass hybrid *Festuca pratensis* × *Lolium*
525 *perenne*, of which the aerial parts were colonized or not by the endophytic fungus
526 *Neotyphodium uncinatum* (Rostás et al., 2015). Volatile aldehydes have also been
527 detected in the roots of some dicotyledonous plant species. Hexanal has been
528 identified in the main root of *Agrimonia eupatoria* (Feng et al., 2013) and red clover
529 (*Trifolium pratense*) roots have been reported to produce hexanal and (*E*)-hex-2-enal
530 (Palma et al., 2012; Tapia et al., 2007). Hexanal, (*E*)-hex-2-enal and (*E*)-non-2-enal
531 have also been identified in crushed grapevine roots (*Vitis berlandieri* × *Vitis riparia*)
532 that were infested or not with phylloxera (*Daktulosphaira vitifoliae*) (Lawo et al.,
533 2011). In contrast, GC-MS and PTR-MS profiling of VOCs produced by *Arabidopsis*
534 *thaliana* hairy root cultures did not show any induction of C_6 aldehyde production
535 when the roots were submitted to mechanical wounding, *Pseudomonas syringae*
536 DC3000 infection or *Diuraphis noxia* infestation (Steeghs et al., 2004). These results
537 could possibly be explained by a mutation carried by the Columbia-0 ecotype of *A.*
538 *thaliana* affecting HPL activity and C_6 volatile synthesis (Erb et al., 2008). Similarly,
539 PTR-MS analyses did not reveal any C_6 volatile aldehyde production by *Brassica*
540 *nigra* roots infested by *Delia radicum* (Crespo et al., 2012).

541 In our study, VOC production by barley roots was characterised by a strong
542 age-dependent pattern. In comparison with plants analysed soon after germination or

543 at the seedling stage, barley roots analysed at the tillering stage synthesised fewer
544 VOCs. Such quantitative variations in VOC concentrations have been documented for
545 several plant species. In maize (*Zea mays*), the roots produced fewer sesquiterpenes
546 from mature plants than from seedlings (Köllner et al., 2004). In plant-plant
547 interaction studies, it has also been reported that young sagebrush (*Artemisia*
548 *tridentata*) plants are better emitters and respond more efficiently than older
549 individuals to volatile cues produced by conspecific damaged neighbours (Shiojiri and
550 Karban, 2006; Shiojiri et al., 2011). Higher VOC production in young developmental
551 stages has also been reported in lima bean (*Phaseolus lunatus*) (Radhika et al., 2008),
552 soybean (*Glycine max*) (Rostás and Eggert, 2008) and *Citrus* spp. (Azam et al., 2013),
553 as well as in the undomesticated species *Datura wrightii* (Hare and Sun, 2011; Hare,
554 2010). In contrast, some plant species such as sage (*Salvia officinalis*) and peppermint
555 (*Mentha* × *piperita*) showed a higher monoterpene content in leaves with increasing
556 leaf age (Croteau et al., 1981; Gershenzon et al., 2000). With regard to C_6 volatile
557 aldehydes, their production in soybean leaves fell significantly until full size was
558 reached; the total C_6 VOC production then markedly increased in older leaves and
559 reached similar levels to that produced by the youngest analysed leaves (Zhuang et
560 al., 1992). In a study of the general pattern of VOC production by plants as indirect
561 defences, a meta-analysis showed that VOC production declined significantly with
562 increasing plant age, thus supporting the ODT (Quintero et al., 2013). Although our
563 study did not investigate VOC production by barley roots after the tillering stage, our
564 results support the findings of the meta-analysis performed by Quintero and co-
565 workers (2013).

566 In addition to an overall decrease in VOC concentrations, our study suggested
567 that the composition of VOC blends produced by barley roots varied with plant age as

a result of quantitative changes in individual VOC concentrations. The composition of the VOC chemical profiles of various plant organs has also been reported to change across plant ontogeny in maize (Köllner et al., 2004), wheat (*Triticum aestivum*) (Batten et al., 1995), soybean (Boué et al., 2003; Zhu and Park, 2005; Zhuang et al., 1992), tomato (*Lycopersicon esculentum*) (Zhang et al., 2008), *Citrus* spp. (Azam et al., 2013), peppermint (Gershenzon et al., 2000), *D. wrightii* (Hare, 2010) and *Hymenaea courbaril* (Kuhn et al., 2004), as well as in the roots of variously aged red clover (Palma et al., 2012; Tapia et al., 2007). In this last example, hexanal and (*E*)-hex-2-enal were detected only in roots of the youngest developmental stages analysed (Palma et al., 2012).

The LOX extracted from barley roots was characterized by a higher specificity for linoleic acid. The LOX activity measurements were in line with the VOC production pattern produced by barley roots as the volatiles documented to derive from linoleic acid (hexanal and [*E*]-non-2-enal) represented between 57.4 and 73.5% of the VOC profiles. The LOX activity pattern seems to be developmentally regulated with 7- and 3-day-old barley roots possessing the greatest enzymatic activities for linoleic acid and α-linolenic acid, respectively. An influence of plant age on LOX activity has been previously demonstrated in developing barley roots younger than 9 days (Holtman et al., 1996). Using linoleic acid as a substrate, Holtman and co-workers (1996) showed that the LOX activity reached a maximum between 4 and 7 days after the start of germination. In our study, as the LOX activity measurements did not differ between plants older than 7 days, the temporal variation of the LOX activity is unlikely to be the only explanation for the decreasing VOC production pattern observed in older plants. Therefore, the influence of plant ontogeny on other

592 factors, such as substrate availability, substrate specificity and biosynthetic enzyme
593 expression (LOX and HPL) and activities (HPL) require further investigations.

594 In this study, we set up an experimental device and a GC-MS methodology
595 allowing both the identification (using SCAN MS data) and the quantification (using
596 SIM MS data) of VOCs emitted by cereal roots without extracting belowground plant
597 organs from the soil prior to VOC sampling. Using this technique, we were able to
598 show that undamaged barley roots did not release detectable amounts of VOCs, but
599 emitted (*E*)-non-2-enal and (*E*,*Z*)-nona-2,6-dienal after mechanical injury. Even if
600 hexanal and (*E*)-hex-2-enal represented 58.8% of the VOCs found in the headspace of
601 crushed 17-day-old roots, these molecules were not detected in quantifiable amounts
602 after mechanical injury. Because (1) only traces amounts of hexanal were detected
603 and (2) the recovery rates of (*E*)-non-2-enal and (*E*,*Z*)-nona-2,6-dienal in our system
604 were 46.0% and 77.0% lower than that of hexanal, it is likely that barley roots
605 released mainly C_9 volatile aldehydes after mechanical injury. Our findings contrast
606 with VOC analyses performed on aboveground barley tissues because they have been
607 reported to emit mainly C_6 aldehydes, alcohols and their corresponding esters when
608 the leaves were mechanically damaged or subjected to insect predation (*Oulema* spp.)
609 or fungal infection (*Fusarium* spp.) (Piesik et al., 2011, 2010). Although volatile
610 terpenes have been detected in the headspace of undamaged, mechanically damaged
611 and *Fusarium*-infected barley shoots (Kegge et al., 2015; Piesik et al., 2011), we were
612 not able to detect any terpene in the emission profiles of undamaged or mechanically
613 damaged barley roots, even if our in situ trapping system was able to recover more
614 than 60% of the (*E*)-β-farnesene injected into the soil as part of a synthetic mixture.

615 Given that LOX/HPL-derived volatiles (1) are rapidly formed after tissue
616 disruption (Matsui, 2006), (2) play important roles in plant chemical defences

(Dudareva et al., 2006; Matsui et al., 2006), (3) have low diffusion capacities in sand and soil (Hiltpold and Turlings, 2008), and (4) are emitted in the rhizosphere of mechanically damaged barley roots, it would be of great interest to study the involvement of the VOCs identified in this work in plant direct defences in terms of both the physiological and ecological implications. In addition, because VOCs released by barley roots into the soil can attract insect predators (Gfeller et al., 2013), this work paves the way for designing additional experiments aimed at investigating how the observed qualitative and quantitative variations in VOC production can benefit plants of different ages and influence the behaviour (attraction/repulsion) of phytophagous pests and insect predators.

Contributions

Conceived and designed the experiments: BMD, PD, PdJ, MLF; performed the experiments: BMD; analysed the data: BMD, PD, MLF; contributed reagents/materials/analysis tools: PD, PdJ, MLF; contributed to the writing of the manuscript: BMD, PD, PdJ, MLF.

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912

913 **Table 1.** Selected developmental stages for the analysis of VOCs produced by barley

914 roots.

Developmental stages		Age (days)	Age (GDD)	Description
Germination	07 – 10	3	51.1 ± 1.2	Coleoptile emerged from caryopsis; first leaf sometimes through the coleoptile
Seedling growth	11	7	117.8 ± 2.7	First leaf unfolded
	13	17	284.9 ± 2.1	Three leaves unfolded
Tillering	21 – 22	28	470.3 ± 2.4	Main shoot and one or two tillers
	22 – 24	38	639.8 ± 2.2	Main shoot and two to four tillers

915 Plant age data expressed in GDD are shown as mean ± s.d. Developmental stages are

916 codified according to Zadoks et al. 1974.

917

918 **Table 2.** VOCs produced by crushed barley roots.

CAS number	IUPAC name	RI_C	RI_{Std}	RI_{Ref}	Main *m/z* ratios in mass spectra
66-25-1	Hexanal	1092	1090	1084[a]	57 (70.5%), 56 (100%), 44 (98.5%), 43 (67.7%), 41 (95.4%)
6728-26-3	(*E*)-hex-2-enal	1230	1228	1207[a]	83 (72.8%), 69 (78.7%), 55 (88.1%), 42 (54.3%), 41 (100%), 39 (72.9%)
18829-56-6	(*E*)-non-2-enal	1529	1525	1540[a]	83 (78.7%), 70 (95.1%), 55 (97.0%), 43 (82.3%), 41 (100%)
557-48-2	(*E*,*Z*)-nona-2,6-dienal	1579	1574	1597[b]	70 (85.8%), 69 (78.4%), 67 (22.4%), 41 (100%), 39 (33.3%)

919 In addition to the comparison of mass spectral data and calculated retention indices

920 (RI_C) with those of authentic standards (RI_{Std}), VOC identification was assessed by

921 comparing calculated retention indices with those reported in the literature (RI_{Ref}). For

922 each identified molecule, the relative abundance of the main *m/z* ratios in mass spectra

923 corresponding to the chromatogram presented in Fig. 2A is shown in parentheses.

924 [a] RI_{Ref} was taken from (Jennings and Shibamoto, 1980)

925 [b] RI_{Ref} was taken from (Ferreira et al. 2001).

926 **Fig. 1.** Experimental device used for the sampling of VOCs emitted by 17-day-old
927 barley roots in an artificial soil.
928
929 **Fig. 2.** Typical chromatograms obtained for the GC-MS analysis of VOCs produced
930 by crushed 3-day-old barley roots. The mass spectrometer operated in synchronous
931 SCAN (A) and SIM (B) modes. IS, internal standard (3,5,5-trimethylhexanal); 1,
932 hexanal; 2, (*E*)-hex-2-enal; 3, (*E*)-non-2-enal; 4, (*E*,*Z*)-nona-2,6-dienal; a, pent-1-en-
933 3-ol; b, 3-methyl-but-3-en-1-ol; c, (*E*)-hept-2-enal; d, hexan-1-ol; e, (*E*)-oct-2-enal; f,
934 acetic acid.
935
936 **Fig. 3.** Evolution of the total volatile aldehyde concentration (A), C_6/C_9 volatile
937 aldehyde ratio (B) and the hexanal (C), (*E*)-hex-2-enal (D), (*E*)-non-2-enal (E) and
938 (*E*,*Z*)-nona-2,6-dienal (F) concentrations in barley roots according to plant age. Data
939 are shown as mean ± s.e. (n = 7 for plants analysed at 285 GDD, n = 8 for other
940 developmental stages). For each variable, a one-way ANOVA followed by a Newman
941 and Keuls test was performed using plant age as a fixed factor. Mean values sharing
942 the same letter did not statistically differ according to plant age ($P > 0.05$).
943
944 **Fig. 4.** Hexanal, (*E*)-hex-2-enal, (*E*)-non-2-enal and (*E*,*Z*)-nona-2,6-dienal relative
945 proportions in VOC profiles produced by barley roots according to plant age. Data are
946 shown as mean ± s.e. (n = 7 for plants analysed at 285 GDD, n = 8 for other
947 developmental stages). For each VOC, a one-way ANOVA followed by a Newman
948 and Keuls test was performed using plant age as a fixed factor. Mean values sharing
949 the same letter did not statistically differ according to plant age ($P > 0.05$).
950

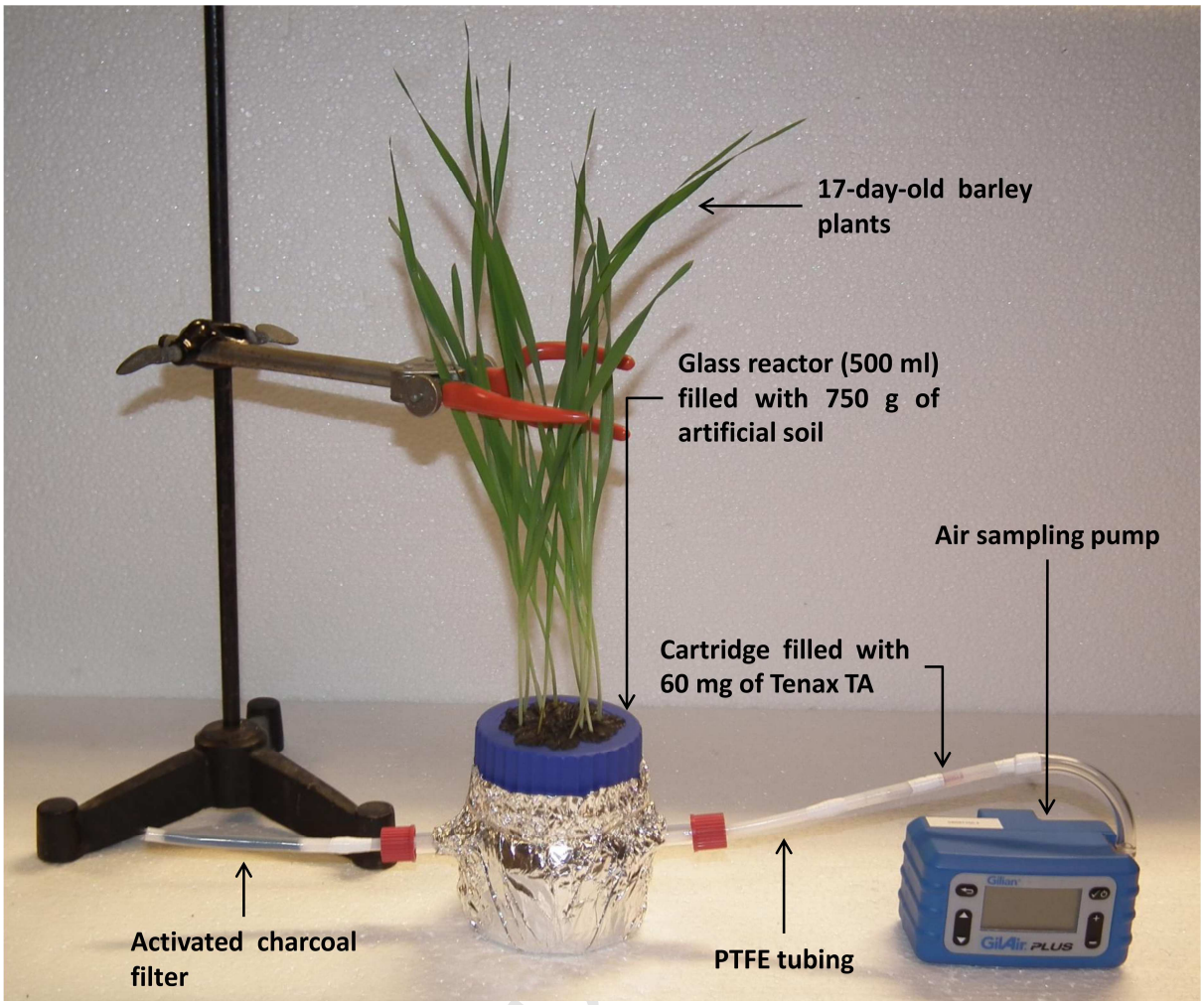
Fig. 5. Evolution of the LOX activity measured in barley roots according to plant age. Data are shown as mean ± s.e. (n = 4). For each fatty acid used as a LOX substrate, a one-way ANOVA followed by a Newman and Keuls test was performed using plant age as a fixed factor. Mean values sharing the same letter did not statistically differ according to plant age ($P > 0.05$).

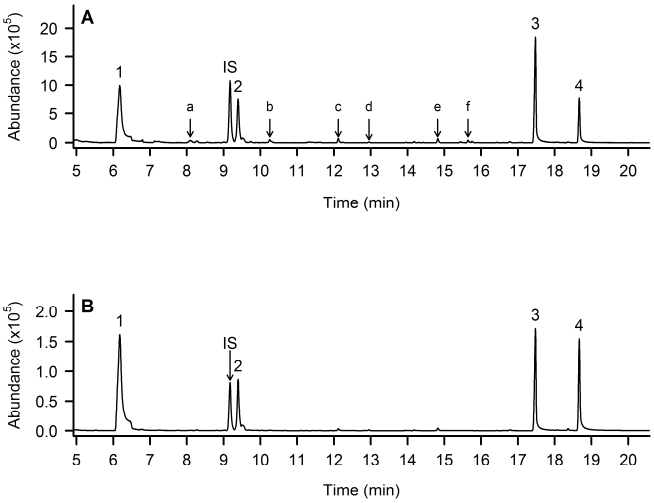
Fig. 6. Typical SIM chromatograms obtained for the GC-MS analysis of VOCs emitted by the artificial soil alone (A) and undamaged (B) or mechanically damaged (C) 17-day-old barley roots produced in an artificial soil. The mass spectrometer operated in synchronous SCAN and SIM modes. 3, (*E*)-non-2-enal; 4, (*E*,*Z*)-nona-2,6-dienal.

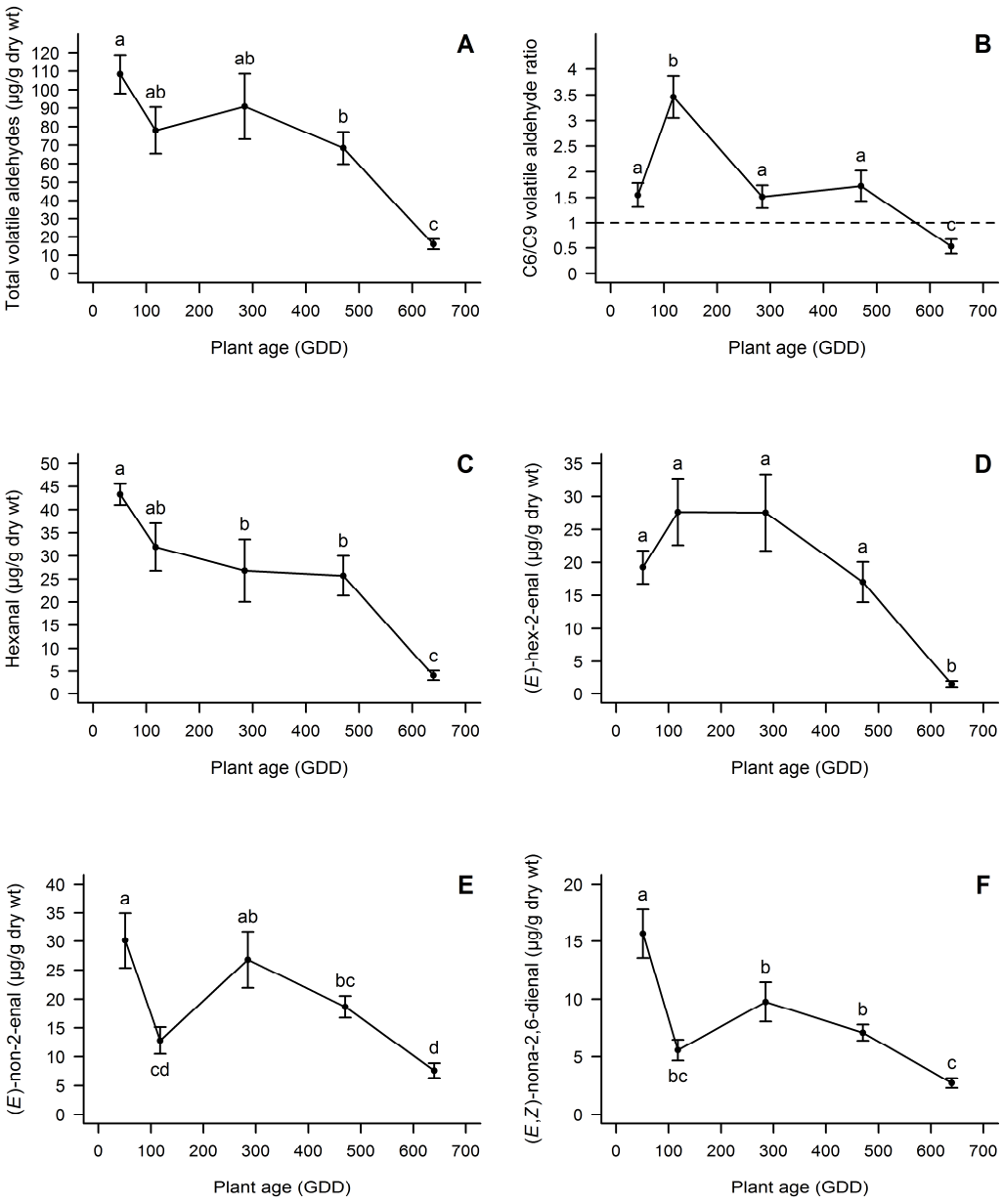
Fig. 7. Recovery rates of five VOC standards injected into the soil. Data are shown as mean ± s.e. (n = 4). Mean values were compared using one-way ANOVA followed by a Newman and Keuls test. Mean values sharing the same letter were not statistically different ($P > 0.05$).

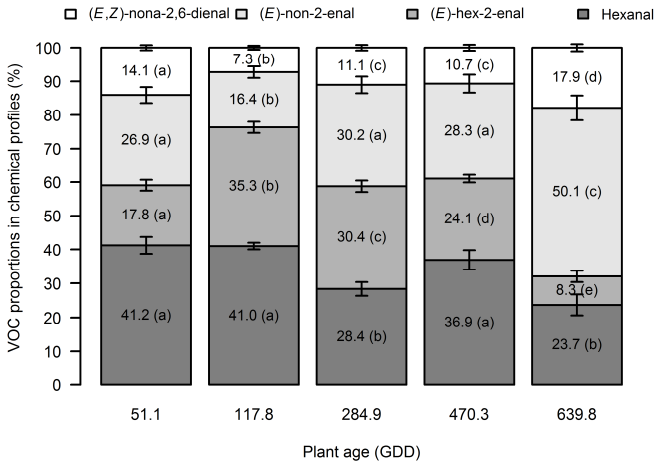
Fig. S1. Calibration curves used for the quantification of VOCs produced by crushed barley roots.

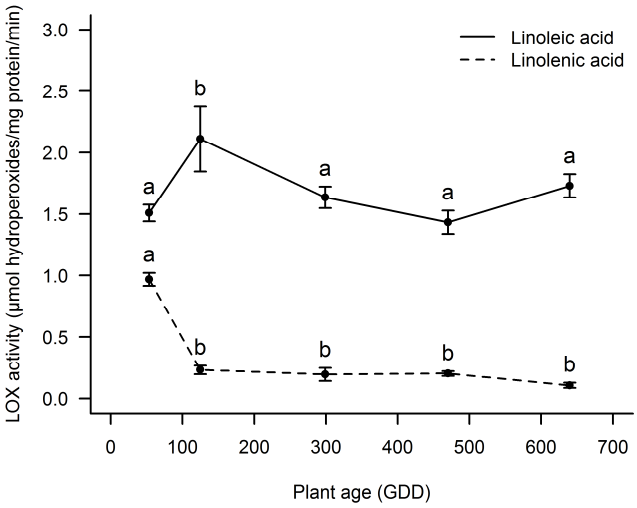
Fig. S2. Calibration curves used for the quantification of VOCs emitted in situ by mechanically damaged barley roots.

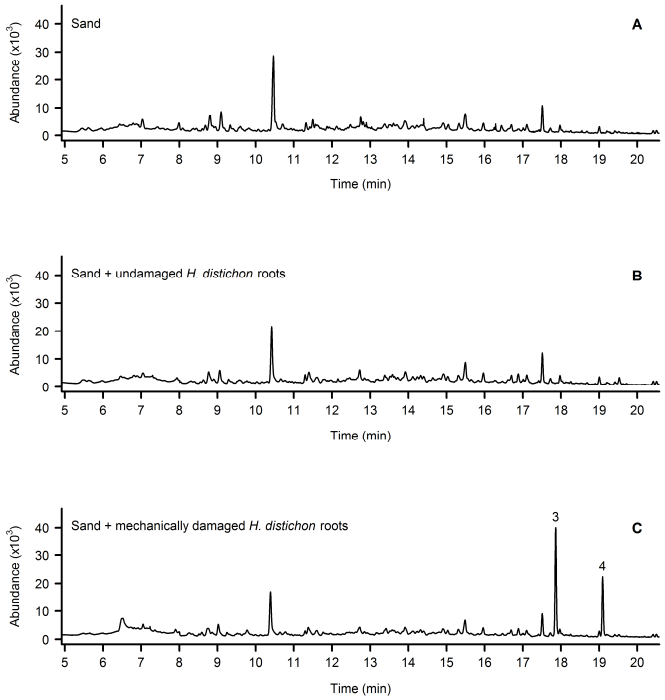

17-day-old barley plants
Glass reactor (500 ml) filled with 750 g of artificial soil
Air sampling pump
Cartridge filled with 60 mg of Tenax TA
Activated charcoal filter
PTFE tubing
Gilian
GilAir PLUS

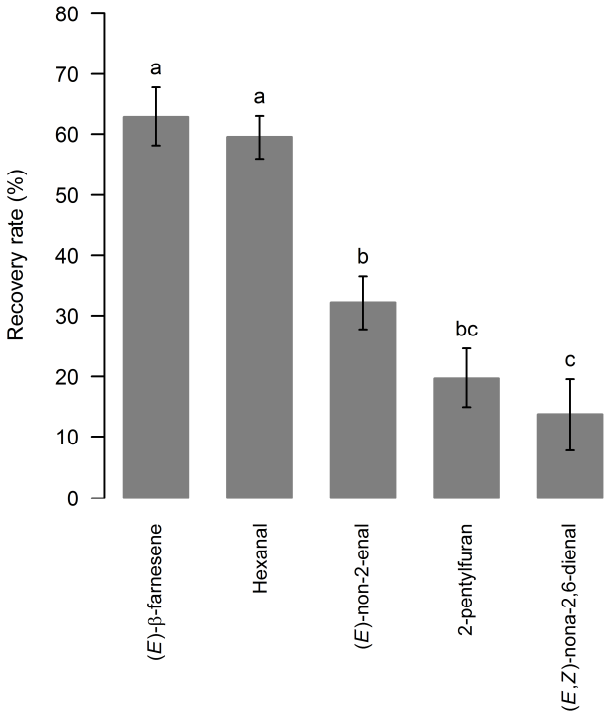



A
Total volatile aldehydes (µg/g dry wt)
Plant age (GDD)
B
C6/C9 volatile aldehyde ratio
Plant age (GDD)
C
Hexanal (µg/g dry wt)
Plant age (GDD)
D
(E)-hex-2-enal (µg/g dry wt)
Plant age (GDD)
E
(E)-non-2-enal (µg/g dry wt)
Plant age (GDD)
F
(E,Z)-nona-2,6-dienal (µg/g dry wt)
Plant age (GDD)









- VOCs produced by barley roots were analysed at 5 phenological stages.
- Hexanal, (*E*)-hex-2-enal, (*E*)-non-2-enal and (*E*,*Z*)-nona-2,6-dienal were the main identified VOCs.
- VOC production and LOX activity quantitatively varied with plant ontogeny.
- VOCs emitted by barley roots were trapped in situ and quantified by GC-MS.
- (*E*)-non-2-enal and (*E*,*Z*)-nona-2,6-dienal were emitted by mechanically damaged roots.

Barley (*Hordeum distichon* L.) roots synthesise volatile aldehydes with a strong age-dependent pattern and release (*E*)-non-2-enal and (*E*,*Z*)-nona-2,6-dienal after mechanical injury

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Contributions

Conceived and designed the experiments: BMD, PD, PdJ, MLF.

Performed the experiments: BMD.

Analysed the data: BMD, PD, MLF.

Contributed reagents/materials/analysis tools: PD, PdJ, MLF.

Contributed to the writing of the manuscript: BMD, PD, PdJ, MLF.