

1 **Tissue-specific volatile-mediated defense regulation in maize leaves and roots**

2 Cong van Doan^{1,2}, Tobias Züst¹, Corina Maurer¹, Xi Zhang^{1,3}, Ricardo A.R. Machado¹, Pierre Mateo¹,
3 Meng Ye¹, Bernardus C.J. Schimmel¹, Gaétan Glauser⁴, Christelle A.M. Robert^{1,2*}

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5 ¹ Institute of Plant Sciences, University of Bern, Bern, Switzerland

6 ² Oeschger Centre for Climate Change Research (OCCR), University of Bern, Bern, Switzerland

7 ³ Key Laboratory of Plant Stress Biology, State Key Laboratory of Cotton Biology, School of Life
8 Sciences, Henan University, Kaifeng 475004, China.

9 ⁴ Neuchâtel Platform of Analytical Chemistry, Université de Neuchâtel, Neuchâtel, Switzerland

10

11 * For correspondence:

12 Christelle Robert

13 Tel: +41 31 631 31 55

14 Email: christelle.robert@ips.unibe.ch

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21 **SUMMARY**

- 22 • Plant leaves that are exposed to herbivore induced plant volatiles (HIPVs) respond by increasing
23 their defenses. Whether this phenomenon also occurs in the roots is unknown.
- 24 • Using maize (*Zea mays*), whose leaves respond strongly to leaf HIPVs, we measured the impact
25 of root HIPVs, emanating from plants infested by the banded cucumber beetle (*Diabrotica*
26 *balteata*), on constitutive and herbivore-induced levels of root soluble sugars, starch, total
27 soluble proteins, free amino acids, volatile and non-volatile secondary metabolites, defense gene
28 expression, growth and root herbivore resistance of neighboring plants.
- 29 • HIPV exposure did not alter constitutive or induced levels of any of the measured root traits.
30 Furthermore, HIPV exposure did not reduce the performance and survival of banded cucumber
31 beetle larvae on maize or teosinte. Cross-exposure experiments revealed that maize roots, in
32 contrast to maize leaves, neither emit nor respond strongly to defense-regulating HIPVs.
- 33 • Together, these results demonstrate that volatile-mediated defense regulation is restricted to the
34 leaves of maize and teosinte, a finding which is in line with the lower diffusibility of volatiles
35 in the soil and the availability of other, potentially more efficient information conduits below
36 ground.

37

38 **Keywords:** belowground plant-herbivore interactions, maize, plant-plant interactions, priming,
39 volatiles.

40 INTRODUCTION

41 Upon herbivory, plants emit volatile organic compounds that can repel herbivores and attract their
42 natural enemies (Baldwin, 2010; Turlings & Erb, 2018). These herbivore-induced plant volatiles
43 (HIPVs) can also be perceived by unattacked plant tissues and neighboring plants, resulting in the direct
44 activation and/or priming of defense and resistance (Farmer, 2001; Baldwin *et al.*, 2006; Frost *et al.*,
45 2008; Heil & Ton, 2008; Heil, 2014; Erb, 2018; Turlings & Erb, 2018; Bouwmeester *et al.*, 2019).
46 Numerous HIPVs have been found to regulate defenses, including green leaf volatiles such as (Z)-3-
47 hexenal, (Z)-3-hexen-1-ol, and (Z)-3-hexenyl acetate (HAC), aromatic compounds such as indole, and
48 terpenoids such as ocimene (Farmer, 2001; Engelberth *et al.*, 2004; Erb *et al.*, 2015; Riedlmeier *et al.*,
49 2017; Ameye *et al.*, 2018). HIPVs can regulate redox signalling genes (González-Bosch, 2018), early
50 defense signalling genes and proteins such as MAP kinases (Ton *et al.*, 2007; Erb *et al.*, 2015; Hu *et al.*,
51 2019; Ye *et al.*, 2019), the biosynthesis of stress hormones such as jasmonates (Ton *et al.*, 2007; Heil &
52 Ton, 2008; Hirao *et al.*, 2012) and the expression of direct and indirect defenses (Zeringue, 1987;
53 Zeringue, 1992; Bate & Rothstein, 1998; Arimura *et al.*, 2000; Arimura *et al.*, 2001; Engelberth *et al.*,
54 2004; Farag *et al.*, 2005; Kessler *et al.*, 2006; Kost & Heil, 2006; Ton *et al.*, 2006; Karban, 2011; Kim
55 *et al.*, 2011; Erb *et al.*, 2015; Martinez-Medina *et al.*, 2016; Freundlich & Frost, 2018; Tugizimana *et*
56 *al.*, 2018).

57 Although defense regulation by HIPVs has been documented extensively in plant leaves, much less is
58 known about this phenomenon in the roots (Delory *et al.*, 2016). To the best of our knowledge, no study
59 so far investigated the impact of root HIPVs on defense and resistance of neighboring plants. Roots emit
60 specific volatile blends when attacked by herbivores (Rasmann *et al.*, 2005; Ali *et al.*, 2010; Delory *et*
61 *al.*, 2016). These volatiles can diffuse through the soil and alter the behaviour of herbivores and natural
62 enemies (Hiltpold & Turlings, 2008; Xavier *et al.*, 2017; Gfeller *et al.*, 2019). Recent work also found
63 that constitutively released root volatiles can affect growth and defense expression in neighboring plants
64 (Huang *et al.*, 2018; Gfeller *et al.*, 2019). Thus, it is conceivable that roots may also respond to root
65 HIPVs in anticipation of root herbivore attack.

66 To test this hypothesis, we investigated HIPV-mediated root interactions in maize, one of the three most
67 important crops worldwide (Shiferaw *et al.*, 2011). Maize plants are regularly attacked by root
68 herbivores such as rootworms, which can cause substantial damage and yield losses (Tinsley *et al.*,
69 2016). Maize leaves are highly responsive to leaf HIPVs such as indole and (Z)-3-hexenyl acetate
70 (Engelberth *et al.*, 2004; Erb *et al.*, 2015; Hu *et al.*, 2019). Upon herbivore attack, maize roots emit
71 distinct blends of HIPVs that contain terpenes such (E)- β -caryophyllene, humulene and copaene
72 (Rasmann *et al.*, 2005; Robert *et al.*, 2012b; Robert *et al.*, 2012a), but no detectable amounts of indole
73 or GLVs. (E)- β -caryophyllene can diffuse up to 20 cm.h⁻¹ in the soil matrix (Xavier *et al.*, 2017). To test
74 if maize roots can use root HIPVs to prepare their defense system for incoming herbivore attack, we
75 first assessed the impact of root HIPVs on maize primary metabolism and defense markers in the absence

76 of herbivory. Second, we assessed the impact of root HIPVs on root-herbivory induced changes in
77 primary metabolism and defense markers. Third, we tested the effect HIPVs on plant growth and
78 resistance. Fourth, we conducted cross-exposure experiments to assess the impact of leaf HIPVs on root
79 resistance and *vice versa*. These experiments found no evidence for HIPV-mediated induction of root
80 defenses, and suggest that roots do not respond to HIPVs by increasing their resistance to herbivores.

81 **MATERIALS AND METHODS**

82 *Plants and insects*

83 Maize seeds (*Zea mays* L., var. “Delprim”) were provided by Delley Semences et Plantes (DSP, Delley,
84 CHE). Maize seeds were sown in plastic pots (diameter, 4cm; height, 11.2 cm; Patz GmbH
85 Medizintechnik, Dorsten-Wulfen; DE) as described in (Erb *et al.*, 2011). The seedlings were fertilized
86 twice a week after germination with MioPlant Vegetal and Herbal Fertilizer (Migros, CHE). Twelve-
87 day old plants with three fully developed leaves were used for the experiments. Eggs of the banded
88 cucumber beetle *Diabrotica balteata* (Coleoptera: Chrysomelidae) were kindly provided by Oliver
89 Kindler (Syngenta, Stein, CHE). Hatching larvae were reared on freshly germinated maize seedlings
90 (var. Akku, DSP, CHE). Second-instar larvae were used in the experiments. The larval instars were
91 determined according to the head capsule size as previously described (George & Hintz, 1966). Plant
92 infestations were performed by placing six larvae in two 4-5 cm deep holes in the sand. Eggs of the
93 Egyptian cotton leafworm *Spodoptera littoralis* were provided by the University of Neuchâtel and reared
94 on artificial diet until use.

95 *Characterization of root HIPV production by emitter plants*

96 To determine the HIPV profile emitted by root-infested plants over time, maize plants were placed into
97 L-shaped glass pots (diameter: 5 cm; depth: 11 cm; Verre & Quartz Technique SA, Neuchâtel, CHE).
98 Moist white sand (Migros, CHE) was added to fill the pots. The L-pots were wrapped in aluminium foil
99 to keep the root system in the dark and prevent degradation of volatile compounds. Two days later, half
100 the plants were infested with six second-instar *D. balteata* larvae. Control and infested maize roots were
101 collected after one, two, three, four or eight days (n=5-7 per treatment and per day). The roots were
102 ground in liquid nitrogen using a mortar and a pestle. An aliquot of 100 mg was used to measure root
103 volatile production by solid phase micro extraction gas chromatography coupled to mass spectrometry
104 (SPME-GC-MS, Agilent 7820A GC coupled to an Agilent 5977E MS, Agilent Technologies, Santa
105 Clara, CA, USA). Briefly, a 100 µm polydimethylsiloxane SPME fibre (Supelco, Bellefonte, PA, USA)
106 was inserted through the septum of the root containing glass vial (20 mL Precision Thread Headspace-
107 Vial and UltraClean 18 mm Screw caps, Gerstel GmbH & Co., Mülheim an der Ruhr, DE) and exposed
108 to the vial headspace for 40 min at 50°C. The fibre was inserted into the GC injection port (220°C) and
109 desorbed. Chromatography was performed using an apolar column (DB1-MS, 30 m, 0.25 mm internal
110 diameter, 0.25 µm film thickness; J & W Scientific, Folsom, CA, USA). Helium was used as carrier gas

111 at a constant pressure of 50.6 kPa. The column temperature was maintained at 60 °C for 1 min and then
112 increased to 250 °C at 5 °C min⁻¹ followed by a final stage of 4 min at 250 °C. Volatile identification
113 was obtained by comparing their mass spectra with those of the NIST05 Mass Spectra Library.

114 ***Root herbivore migration timing***

115 To determine the most realistic experimental timing for the response phase of neighboring plants, we
116 evaluated the time window during which *D. balteata* root herbivores are most likely to migrate from an
117 infested to a neighboring plant. Maize plants were potted into 100 mL pots with two 5 mm diameter
118 openings at the bottom. Each pot was placed in a plastic cup (12 x 25 x 10 cm WxLxH, OBI Group
119 Holding SE & Co.KGaA, Schaffhausen, CHE) filled with a 3 cm high layer of tap water. All plants
120 (n=6) were infested with six second-instar *D. balteata* larvae. The larvae moving away from the plant
121 through the openings or from the top of the pot were therefore trapped in water and collected daily.

122 ***Exposure to belowground HIPVs***

123 To test whether plant exposure to belowground HIPVs induces a response in neighboring plants,
124 belowground two-arm olfactometers were used as previously described (Robert *et al.*, 2012a). Briefly,
125 maize plants were placed into L-shaped glass pots (diameter: 5 cm; depth: 11 cm). Moist white sand
126 (Migros, CHE) was added to fill the pots. The L-pots were wrapped in aluminium foil to keep the root
127 system in the dark and prevent degradation of volatile compounds. Two days later, pots containing plants
128 of similar sizes were connected in pairs using two Teflon connectors and one glass connector (length, 8
129 cm; diameter, 2.2 cm, VQT, Neuchâtel, CHE). The Teflon connectors contained a fine metal screen
130 (2300 mesh; Small Parts Inc., Miami Lakes, FL, USA) to restrain the larvae from moving to the second
131 plant. The glass connectors remained empty to only allow volatile compounds to diffuse through the
132 system. Each pair included one emitter plant and one receiver plant. Emitter plants were either infested
133 with six second-instar *D. balteata* larvae or remained uninfested. Receiver plants were exposed to
134 emitter plants for four days prior to any treatment. After this four days exposure period, receiver plants
135 were either infested with six root herbivore larvae or left uninfested depending on the experiments. All
136 pairs remained connected until collection of the samples.

137 ***Root responses to root HIPVs***

138 To evaluate how exposure to HIPVs affects the metabolism of maize plants in absence and presence of
139 herbivores, two independent experiments were conducted. In the first experiment, primary metabolism
140 and defenses of receiver plants were characterized after four days exposure to HIPVs in absence of
141 herbivory (n=9 per treatment). In the second experiment, receiver plants were infested with six second-
142 instar *D. balteata* larvae, and primary metabolism and defenses were measured 1, 3, 6, 9 and 12 hr after
143 the onset of herbivory (n=3-7). In all experiments, maize roots were collected, gently washed with tap
144 water, flash frozen in liquid nitrogen and ground to a fine powder for further analyses. Plant primary

145 metabolism was assessed by measuring sucrose, glucose, fructose and starch using enzymatic assays
146 (Velterop & Vos, 2001; Smith & Zeeman, 2006; Machado *et al.*, 2013), soluble proteins using
147 colorimetric assays (Bradford, 1976; Jongasma *et al.*, 1994), free amino acids using HPLC-MS (Li *et al.*,
148 2018), and the expression of the carbohydrate transporters *Zm-stp1*, *Zm-zifl2* by q-RT-PCR (Robert *et al.*
149 *et al.*, 2012b) (Supporting Information Table S1). Plant secondary metabolism was characterized by
150 performing untargeted metabolomic analyses by UHPLC-qTOF-MS (Hu *et al.*, 2018), measuring
151 concentrations of benzoxazinoids by UHPLC-qTOF-MS (Hu *et al.*, 2018), and volatile emissions by
152 GC-MS as described above. Plant defense expression was characterized by measuring stress hormones
153 by UHPLC-MS/MS (Glauser *et al.*, 2014) and defense marker genes, including genes involved in
154 volatile production (*Zm-tps23*, *Zm-igl*); hormonal signalling (*Zm-saur2*, *Zm-nced*, *Zm-orp7*, *Zm-lox5*
155 *Zm-acs6*) and direct defenses (*Zm-cysII*, *Zm-cyst*, *Zm-serpin*, *Zm-mpi*, *Zm-bx1*, *Zm-pal*, *Zm-pr1*) by q-
156 RT-PCR (Robert *et al.* 2012b). For a more detailed description of these genes, refer to (Robert *et al.*
157 2012b) and Supplementary Information Table S1.

158 ***Plant and herbivore performance following root exposure to root HIPVs***

159 To determine whether exposure to root HIPVs impacts the performance of root herbivores, belowground
160 two-arm olfactometers were used as described above. After four days exposure to control or infested
161 emitter plants, all receiver plants were infested with six pre-weighed root herbivore larvae (n=18 per
162 treatment). Four days later, all larvae feeding on receiver plants were recovered and weighed. Maize
163 roots from the plants were collected for damage evaluation (Oleson *et al.*, 2005) and weighed.

164 ***Cross-exposure experiment***

165 To assess whether priming is tissue-specific, cross exposure experiments were conducted by exposing
166 roots or leaves to volatiles emitted by either control or infested roots or leaves of emitter plants (n=4-5
167 per treatment). All plants were potted in L-pots as described above. Emitter plants were either infested
168 with six second-instar *D. balteata* (root herbivory), three fourth-instar *S. littoralis* larvae (leaf herbivory)
169 or left uninfested. All plants were covered with plastic bags (Bratbeutel Tangan N°34, Genossenschaft
170 Migros Aare, Urtenen-Schönbühl, CHE). Emitter and receiver plants were paired using the glass
171 connectors described above. The glass connectors were used to connect roots to roots, roots to leaves,
172 leaves to roots or leaves to leaves. To connect a leaf compartment, a 3 cm opening was made in the
173 plastic bag to insert the connector. The bag was then sealed around the glass connector with a rubber
174 band and tape. The headspace of emitter plants was connected to a multiple air-delivery system via
175 PTFE tubing. Purified air was pushed in the system at a flow rate of 0.3 L.min⁻¹. After 17 hr exposure
176 to emitter plants (from 5 pm to 10 am the next day), all systems were disconnected and bags removed.
177 Three pre-weighed *S. littoralis* or six pre-weighed second-instar *D. balteata* larvae were added to
178 receiver plants and new plastic bags were added to all plants. After 2 days, all larvae were collected and
179 weighed.

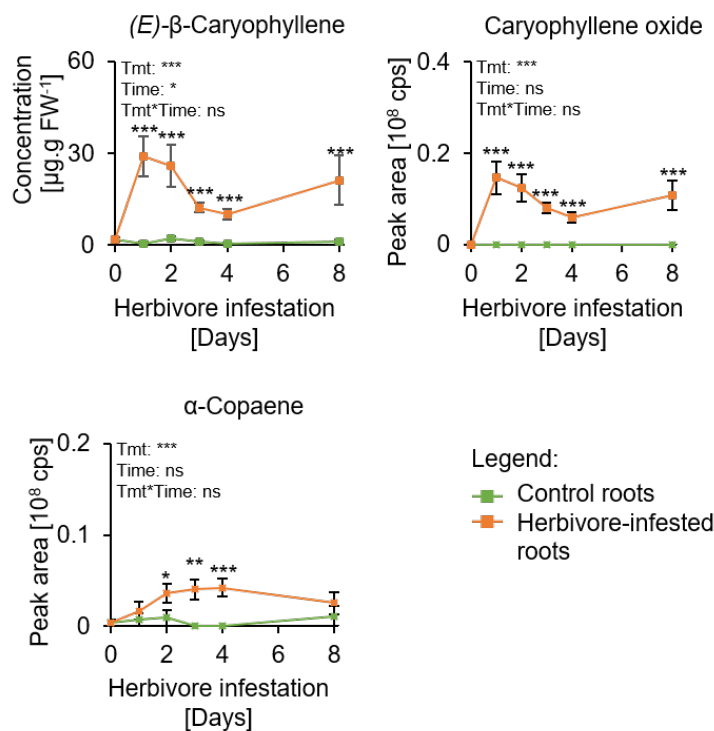
180 *Statistical Analyses*

181 Statistical analyses were conducted using R (version 3.5.3, <https://www.r-project.org>) and Sigma Plot
182 (version 13, Systat Software, San Jose, CA). All data were first tested for normality and
183 heteroscedasticity of error variance using Shapiro-Wilk and Brown-Forsythe tests. Data fitting
184 normality and variance equality assumptions were analyzed using Analysis of Variance (ANOVA). Data
185 that did not fit normality and equality of variance were analyzed using Mann-Whitney Rank Sum tests
186 (U tests) and ANOVAs on ranks. Metabolomic and volatile data were analyzed using principal
187 component analyses (PCA) followed by PPLS-DA and permutation tests.

188 **RESULTS**

189 *Root herbivory induces a distinct bouquet of root volatiles*

190 To characterize belowground HIPVs, we measured root volatile production from the plants over 8 days
191 infestation. Root-herbivore infested plants produced distinct bouquets of volatile compounds over the
192 entire exposure period, including high amounts of (*E*)- β -caryophyllene, caryophyllene oxide and
193 copaene (Fig. 1).



194

195 **Figure 1. Root herbivory induces terpene volatiles from maize root.** (*E*)- β -caryophyllene, caryophyllene oxide, and α -
196 copaene emissions by control (green) and infested maize roots (orange) after 0-8 days (Mean \pm se, Two way ANOVA, n=5-7).
197 (*E*)- β -Caryophyllene was identified and quantified using a standard curve of the pure compound. Caryophyllene oxide and α -
198 copaene were identified by using the NIST library (Match >85%). Tmt: Treatment. cps: Counts per second. Stars indicate
199 significant differences (*: $p \leq 0.05$).

200

201 ***Root herbivores migrate away from infested plants 1-4 days after the start of infestation***

202 To assess the probability of a neighboring plant to be attacked, we measured larval migration from the
203 plants over time. Root herbivore larvae migrated away from the first day on: After one day, 23.3% of
204 the larvae were recovered outside the pots, and after four days, more than 60% had migrated away from
205 the plant (Supplementary Information Fig. S1). Thus, response plants were exposed to root HIPVs for
206 four days in subsequent experiments.

207 ***Root HIPVs do not directly induce defenses in neighboring root systems***

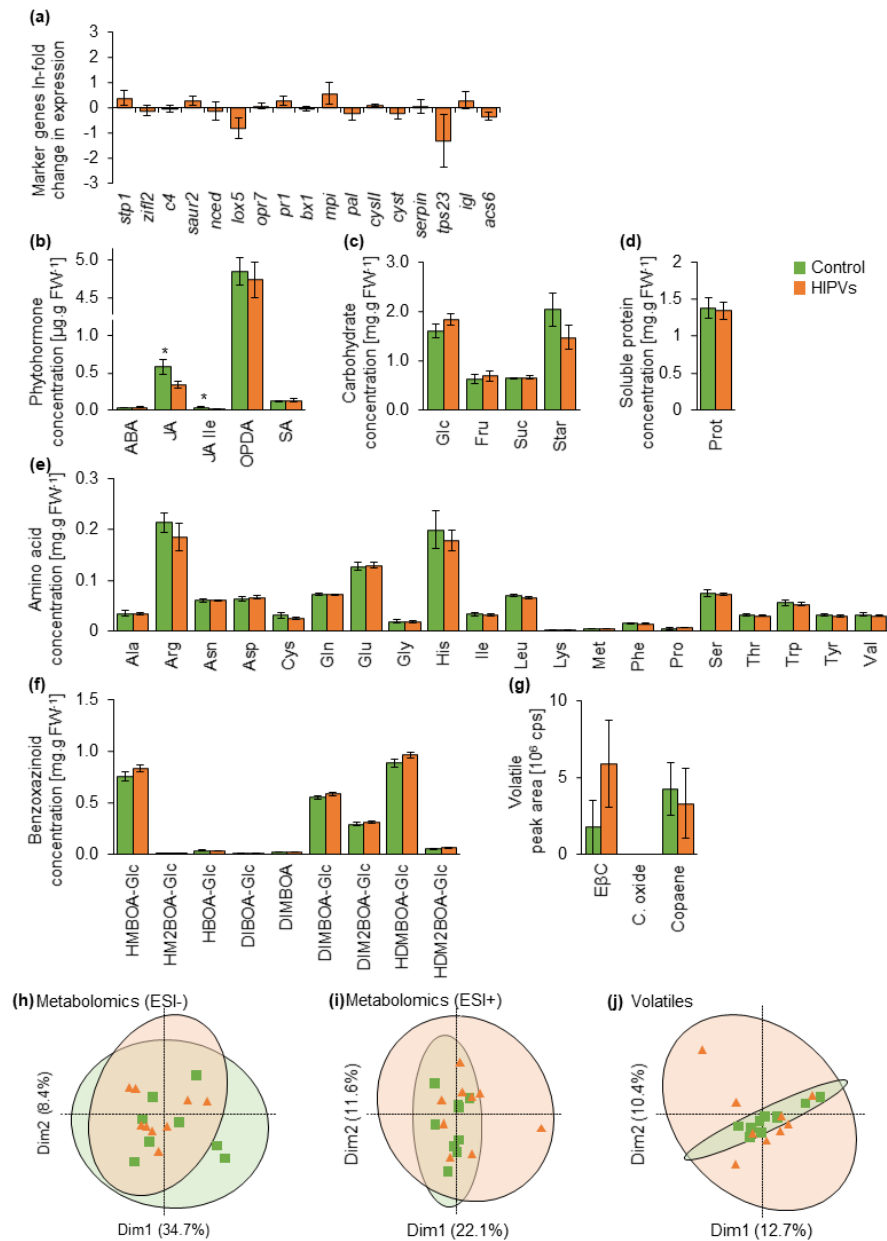
208 To evaluate whether belowground exposure to root HIPVs induces physiological changes in neighboring
209 plants, we characterized the primary metabolism and defenses of maize roots exposed to control or root-
210 herbivore infested volatiles over four days. The expression of marker genes involved in plant primary
211 and secondary metabolism was not significantly altered by HIPV exposure (Fig. 2a). Phytohormone
212 production was similar between control and HIPV-exposed roots, except for jasmonic acid (JA) and its
213 isoleucine conjugate (JA-Ile), for which levels were slightly lower in HIPV-exposed roots than control
214 roots (Fig. 2b). Individual and total soluble sugars, starch, protein, and amino acid concentrations were
215 not affected by exposure to root HIPVs (Figs. 2c-e). Also, no significant effects on benzoxazinoids, the
216 most abundant root secondary metabolites, were observed (Fig. 2f). Untargeted metabolomics (511 and
217 1763 features were detected in negative and positive modes, respectively) did not reveal differential
218 clustering of chemicals (Figs. 2h-i). Finally, root volatile production remained unchanged between
219 control and HIPV-exposed plants (Figs. 2g and j).

220 ***Root HIPVs do not change root defense induction in neighboring root systems***

221 To investigate whether belowground HIPV-exposure alters responses to herbivory in the roots of
222 neighboring plants, we characterized root responses to infestation by *D. balteata*. Marker genes involved
223 in plant response to root herbivory (Robert *et al.*, 2012b) responded similarly in control and HIPV-
224 exposed maize plants, with the exception of *acs6* (Fig. 3a). The production of abscisic acid (ABA), oxo-
225 phytodienoic acid (OPDA) and JA and JA-Ile increased upon root herbivory but was not influenced by
226 HIPV exposure (Fig. 3b). Carbohydrate concentrations were similar in control than in HIPV-exposed
227 plants although HIPV-exposed plants overall had lower fructose concentrations than control plants (Fig.
228 3c). Soluble proteins, and amino acids responded to herbivory independently of HIPV exposure (Figs.
229 3d-e). Untargeted metabolomics (443 and 1906 features detected in negative and positive modes,
230 respectively) and benzoxazinoid profiling did not reveal differential clustering or differences in
231 concentrations (Figs. 3f, h-i). Volatiles were induced similarly by herbivory, independently of previous
232 exposure to HIPVs (Figs. 3g and j).

233

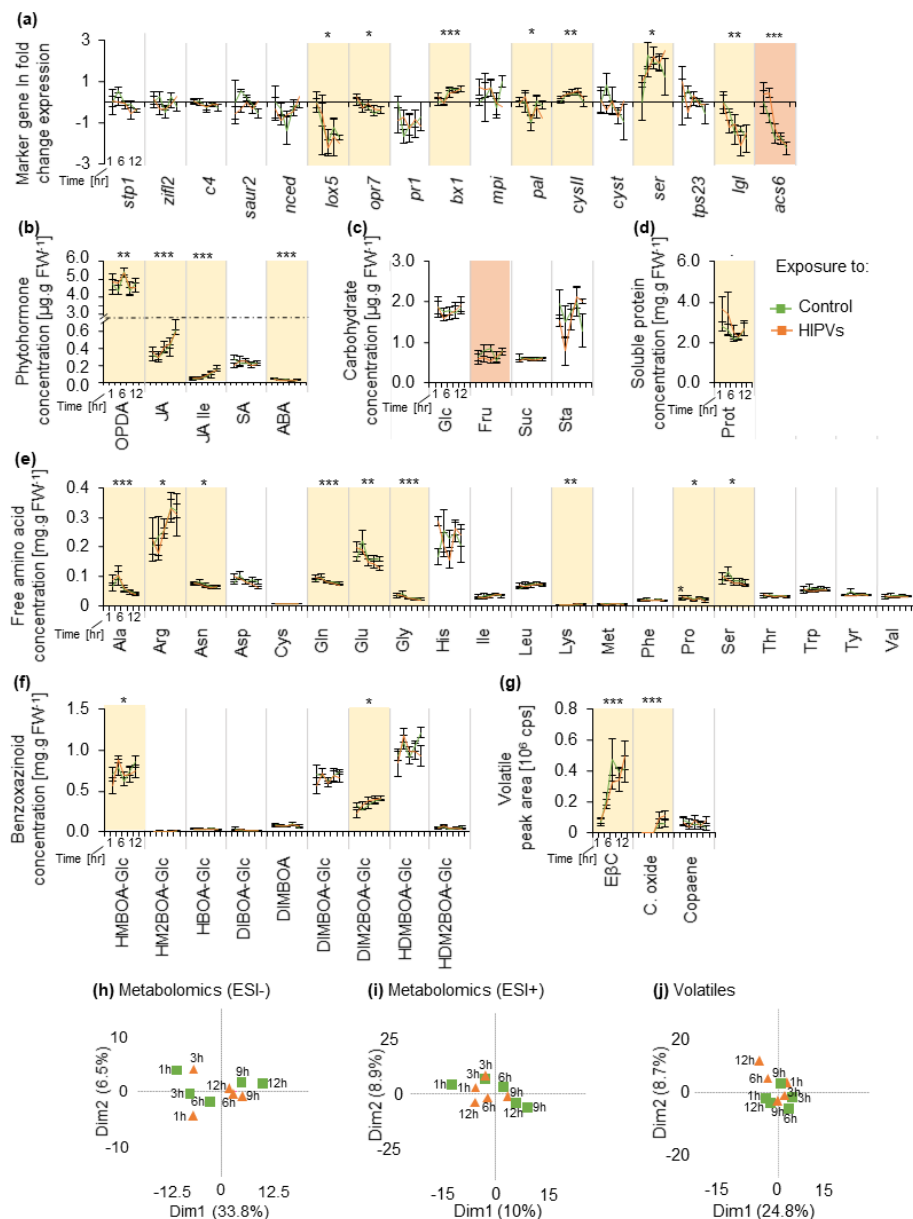
Figure 2. Responses of maize roots to herbivore-induced plant volatiles from neighboring roots



234

235 **Figure 2. Belowground herbivore-induced plant volatiles (HIPVs) do not affect plant metabolism in absence of**
 236 **herbivory.** (a) Ln fold changes in gene expression (Mean \pm se, Student's t-tests and Mann-Whitney U tests, n = 9) in maize
 237 roots exposed for four days to plants infested with six *Diabrotica balteata* larvae (HIPVs) relative to maize roots exposed to
 238 control plants (control, green) or to plants infested with six *D. balteata* larvae (HIPVs, orange). (b-f) Concentrations (Mean
 239 \pm se, Student's t-tests and Mann-Whitney U tests, n = 9) of (c) glucose, fructose, sucrose, and starch, (d) proteins, (e) amino
 240 acids, and (f) benzoxazinoids in roots of maize plants exposed for four days to control plants (control, green) or to plants
 241 infested with six *D. balteata* larvae (HIPVs, orange). (h-i) Principal Component Analysis of all features detected (PLS DA, n
 242 = 9) in roots of maize plants exposed for four days to control plants (control, green) or to plants infested with six *D. balteata*
 243 larvae (HIPVs, orange) using untargeted metabolomic analysis in (h) negative (511 features) and (i) positive modes (1763
 244 features). (j) Principal Component Analysis of volatile emissions (PLS DA, n = 9) and (g) terpene volatile emissions by roots
 245 of maize plants exposed for four days to control plants (control, green) or to plants infested with six *D. balteata* larvae (HIPVs,
 246 orange). E β C: (*E*)- β -caryophyllene. C. oxide: Caryophyllene oxide. Stars indicate significant differences (*: $p \leq 0.05$).
 247

Figure 3.



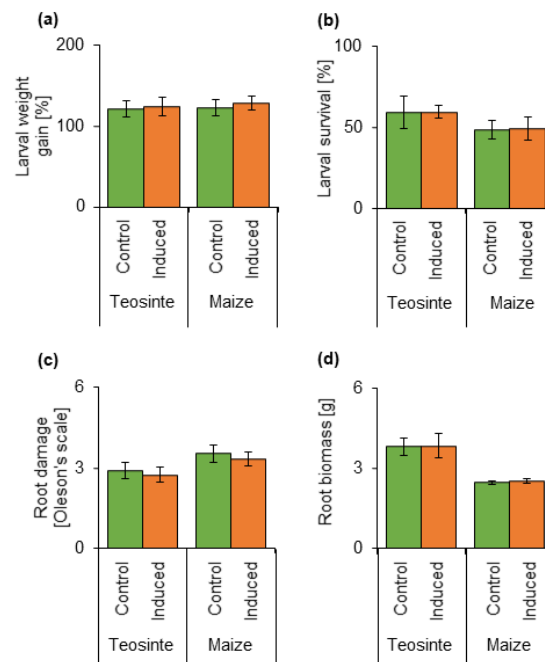
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249 **Figure 3. Exposure to an infested neighboring plant does not change the plant response to *D. balteata*'s attack.** (a) Ln
 250 fold changes in gene expression (Mean \pm se, Two way ANOVA, n=3-7) in maize roots exposed for four days to plants infested
 251 with six *Diabrotica balteata* larvae relative to maize roots exposed to control plants prior attack by *D. balteata* for 1-12 hours.
 252 (b) Phytohormone production (Mean \pm se, Two way ANOVA, n=3-7) maize roots exposed for four days to control plants
 253 (control, green) or to plants infested with six *D. balteata* larvae (HIPVs, orange) prior attack by *D. balteata* for 1-12 hours. (c-
 254 f) Concentrations (Mean \pm se, Two way ANOVA, n = 3-7) of (c) glucose, fructose, sucrose, and starch, (d) proteins, (e) amino
 255 acids, and (f) benzoxazinoids in maize roots exposed for four days to control plants (control, green) or to plants infested with
 256 six *D. balteata* larvae (HIPVs, orange) prior attack by *D. balteata* for 1-12 hours. (h-i) Principal Component Analysis of all
 257 features detected (PLS DA, n = 3-7) in maize roots exposed for four days to control plants (control, green) or to plants infested
 258 with six *D. balteata* larvae (HIPVs, orange) prior attack by *D. balteata* for 1-12 hours, using untargeted metabolomic analysis
 259 in (h) negative (443 features) and (i) positive modes (1906 features). (j) Principal Component Analysis of volatile emissions
 260 (PLS DA, n = 3-7) and (g) terpene volatiles emissions by maize roots exposed for four days to control plants (control, green)
 261 or to plants infested with six *D. balteata* larvae (HIPVs, orange) prior attack by *D. balteata* for 1-12 hours. Only averages per
 262 treatment are presented in principal component analyses. E β C: (*E*)- β -caryophyllene. C. oxide: Caryophyllene oxide. Yellow
 263 shading and stars indicate significant differences over time (*: $p \leq 0.05$, **: $p \leq 0.01$; ***: $p \leq 0.001$). Orange shading indicate
 264 significant differences between exposure treatments ($p \leq 0.05$). No interaction between time and exposure was found to be
 265 significant.

266 *Belowground HIPVs do not increase plant resistance to root herbivory in maize and teosinte*

267 To investigate whether exposure to root HIPVs increases plant resistance in maize or its wild ancestor
268 teosinte, we measured herbivore performance and root damage on control and HIPV-exposed root
269 systems. Exposure to HIPVs emitted by one or three neighboring plants did not alter the herbivore
270 performance, survival, root damage and root fresh mass in both maize and teosinte (Figs. 4, S2).

Figure 4.



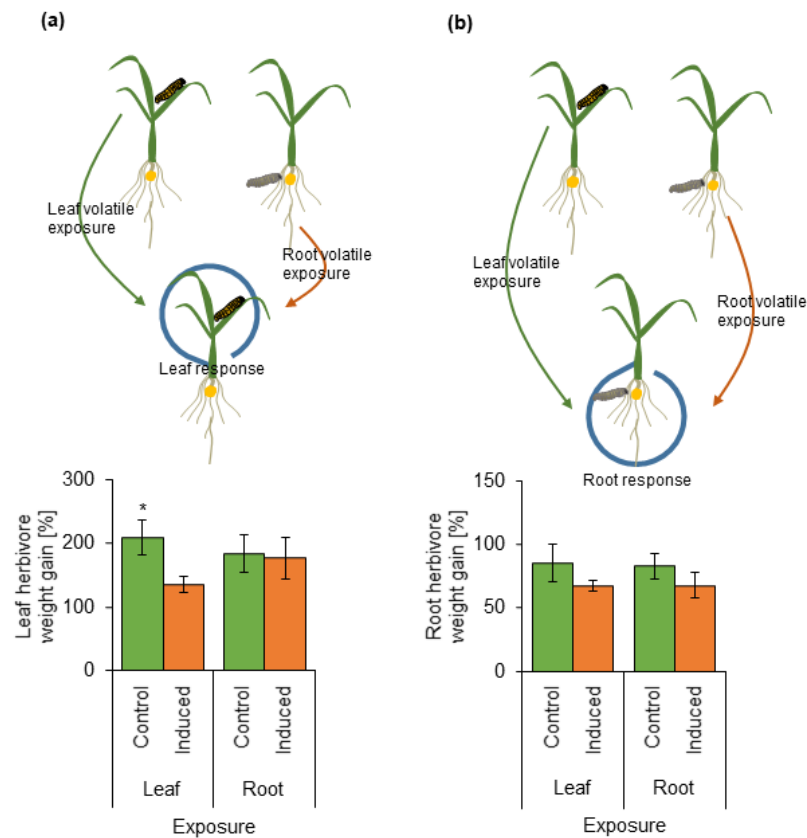
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272 **Figure 4. Exposure to an infested neighboring plant does not alter plant defense to herbivory.** (a) Relative larval weight
273 gain (Mean \pm se, Student's t-tests) of the root herbivore *Diabrotica balteata* feeding for four days on maize (n=17-18) or
274 teosinte (n=8-9) previously exposed for four days to control plants (control, green) or to plants infested with six *D. balteata*
275 larvae (HIPVs, orange). (b) Proportions (Mean \pm se, Student's t-tests) of *D. balteata* recovered after 4 days infested on maize
276 (n=18) and teosinte (n=9) previously exposed for four days to control plants (control, green) or to plants infested with six *D.*
277 *balteata* larvae (HIPVs, orange). (c) *D. balteata* damage scaling (Mean \pm se, Student's t-tests) after four days infestation of
278 maize (n=18) and teosinte (n=9) plants previously exposed for four days to control plants (control, green) or to plants infested
279 with six *D. balteata* larvae (HIPVs, orange). (d) Root fresh mass after four days infestation by the root herbivore *D. balteata*
280 (Mean \pm se, Student's t-tests) of maize (n=18) and teosinte (n=9) previously exposed for four days to control plants (control,
281 green) or to plants infested with six *D. balteata* larvae (HIPVs, orange).

282 *Roots are impaired in the emission and perception of resistance-inducing HIPVs*

283 To assess whether roots can perceive and respond to defense-inducing HIPVs, we conducted a cross-
284 experiment where leaf or root tissues were exposed to HIPVs of either leaves or roots prior infestation.
285 Leaf exposure to leaf HIPVs, but not to root HIPVs, lead to a decreased performance of *S. littoralis*
286 caterpillars (Fig. 5a). Root exposure to either leaf or root HIPVs did not affect the root herbivore
287 performance (Fig. 5b). Thus, root HIPVs do not trigger resistance in roots or leaves, and roots, in contrast
288 to leaves, do not respond to leaf HIPVs through an increase in resistance. This result suggests that roots
289 are impaired in both emission and perception of resistance-inducing HIPVs.

Figure 5.



290

291 **Figure 5. Only leaf exposure to leaf HIPVs leads to a decreased performance of *Spodoptera littoralis* caterpillars.** (a)
292 Relative larval weight gain (Mean \pm se, Two way ANOVA, n=4-5) of the leaf herbivore *S. littoralis* feeding for two days on
293 leaves previously exposed for one night to control plants (control, green) or to plants infested with six *D. balteata* larvae
294 (HIPVs, orange). (b) Relative larval weight gain (Mean \pm se, Two way ANOVA, n=4-5) of the root herbivore *D. balteata*
295 feeding for two days on roots previously exposed for one night to control plants (control, green) or to plants infested with six
296 *D. balteata* larvae (HIPVs, orange). Stars indicate significant differences within leaf herbivore performance (*: $p \leq 0.05$).

297 DISCUSSION

298 The current work shows that HIPV-mediated defense priming occurs in maize leaves, but not roots. The
299 lack of root HIPV response contrasts with the well characterized responses in maize leaves (Engelberth
300 *et al.*, 2004; Baldwin *et al.*, 2006; Heil & Silva Bueno, 2007; Rodriguez-Saona *et al.*, 2009; 2013;
301 Skoczek *et al.*, 2017) and is discussed in detail below.

302 Leaves of many different species are known to respond to HIPVs by increasing their defense investment,
303 and, sometimes also reduce their growth. A recent study furthermore found that volatiles that are
304 constitutively emitted by *Centaurea stoebe* lead to changes in root carbohydrate and protein levels in
305 *Taraxacum officinale* (Gfeller *et al.*, 2019; Huang *et al.*, 2019). However, *C. stoebe* is an unusually
306 strong constitutive emitter of root terpenes, and whether plants respond to herbivory-induced changes
307 in volatile as a form of “eavesdropping” remains unknown. Our study demonstrates that HIPV-exposed
308 maize roots do not display any of the defense responses displayed by maize leaves and leaves of other

309 plant species (Farmer, 2001; Baldwin *et al.*, 2006; Frost *et al.*, 2008; Heil & Ton, 2008; Heil, 2014; Erb,
310 2018; Turlings & Erb, 2018; Bouwmeester *et al.*, 2019). Despite prolonged exposure of maize roots to
311 distinct blends of root HIPVs, we did not observe direct induction or priming of stress hormones,
312 primary and secondary metabolites in these roots. On the contrary, we observed that root HIPVs slightly
313 suppressed constitutive JA-Ile levels. This suppression however was gone 1 hr after herbivore attack.
314 Defense marker genes were also not differentially expressed, with the exception of the ethylene
315 biosynthesis gene *acs6*, which was less suppressed upon herbivore attack in HIPV exposed roots.
316 However, these differences were not associated with measurable changes in metabolite accumulation,
317 resistance or plant growth, despite the well-established roles of jasmonates and ethylene in root growth
318 (Staswick *et al.*, 1992; Schaller, 2012; Huang *et al.*, 2017; Dubois *et al.*, 2018) and defense (McConn *et*
319 *al.*, 1997; Bonaventure *et al.*, 2011; Erb *et al.*, 2012). This absence of phenotypic consequences could
320 be because the changes in JA-Ile and ethylene biosynthesis were too small and/or transient. Root
321 resistance and plant growth were not affected in teosinte either, suggesting that the absence of HIPV
322 responsiveness in maize roots is not due to plant domestication. From these results, we conclude that
323 maize roots, in contrast to leaves, do not strongly respond to root HIPVs.

324 What are the physiological mechanisms that could be responsible for the tissue-specific absence of
325 responsiveness of maize roots to root HIPVs? Our experiments suggest two mutually non-exclusive
326 mechanisms: Absence of defense-inducing HIPVs and lack of HIPV responsiveness. Regarding the first
327 mechanism, our experiments show that maize roots do not release any HIPVs that have been shown to
328 mediate priming in maize leaves: GLVs and indole (Farmer, 2001; Engelberth *et al.*, 2004; Erb *et al.*,
329 2015; Riedlmeier *et al.*, 2017; Ameye *et al.*, 2018). Instead, their HIPV profile is dominated by
330 sesquiterpenes (Robert *et al.*, 2012a). Sesquiterpenes have been associated with priming in tomato,
331 beans (Arimura *et al.*, 2000; Arimura *et al.*, 2001; Zhang *et al.*, 2019), but not in maize (Ruther &
332 Fürstenau, 2005). This suggests that maize roots do not produce HIPV blends capable of triggering
333 defense responses in neighbors. Why maize roots do not release GLVs and indole remains to be
334 elucidated. GLVs are produced via the hydroperoxide lyase (HPL) branch of the oxylipin pathway
335 (Kenji, 2006). The first step of GLV biosynthesis is to deacylate galactolipids to release the omega-3
336 and omega-6 fatty acids, α -linolenic acid and linoleic acid (Matsui *et al.*, 2000; Kombrink, 2012). The
337 hydroperoxidation of α -linolenic and of linoleic acid results in the production of Z-3-hexenal and n-
338 hexenal respectively (Moataz *et al.*, 2017). Yet, maize roots contains only trace amounts of linolenic
339 acid in favour of high concentrations of linoleic acid (Bernklau & Bjostad, 2008). This limitation in
340 linolenic acid contents in the roots may explain the absence of Z-3-hexenal, as well as its alcohol and
341 acetyl GLV downstream products (Z-3 and E-2 hexenol, Z-3 and E-2 hexenyl acetate). The lack of indole
342 release is likely due to a different mechanism, as indole-3-glycerol-phosphate, the precursor of indole
343 (Frey *et al.*, 2009), is abundant in maize roots. However, the indole-3-glycerol phosphate lyase, which
344 is responsible for volatile indole production (Frey *et al.*, 2000) seems to be suppressed upon *D. balteata*
345 attack in the roots, which may explain the absence of volatile indole in the headspace of attacked roots.

346 Regarding the second mechanism, our experiments show that maize roots do not seem capable of
347 increasing their resistance in response to bioactive HIPV blends which are capable of inducing resistance
348 in the leaves. This suggests that maize roots can either not perceive or not translate HIPVs into resistance
349 responses. A better understanding of HIPV perception and early signalling will help to test these
350 hypotheses in the future.

351 From an adaptive point of view, the question arises why maize plants did evolve the capacity to perceive
352 HIPVs in their leaves, but not their roots. A possible explanation may be that the transfer of HIPVs
353 between plants in the rhizosphere is unreliable. First, volatile dispersal, conversion or degradation in the
354 soil strongly depends on matrix properties (Hayward *et al.*, 2001; Owen *et al.*, 2007; Perry *et al.*, 2007;
355 Hiltbold & Turlings, 2008; Seo *et al.*, 2009; Ramirez *et al.*, 2010; Peñuelas *et al.*, 2014; Xavier *et al.*,
356 2017). Volatile compounds, such as indole, linalool, α -pinene, and limonene, can be degraded upon
357 release and used as source of carbon for soil dwelling micro-organisms (Misra *et al.*, 1996; Arora *et al.*,
358 2015; Arora *et al.*, 2015; Ma *et al.*, 2018; Owen *et al.*, 2007; Arora *et al.*, 2015; Ma *et al.*, 2018). Second,
359 root HIPVs may be less reliable signals, as soil microorganisms produce a wide variety of volatile
360 compounds. Terpenes such as copaene, (*E*)- β -caryophyllene and caryophyllene oxide are also produced
361 by soil micro-organisms (Insam & Seewald, 2010; Wenke *et al.*, 2010; Schenkel *et al.*, 2015; Delory *et*
362 *al.*, 2016). Thus, we propose that the unreliable transfer and the low specificity of root HIPVs may have
363 impeded the evolution of HIPV perception in maize roots. Instead, alternative strategies to eavesdrop
364 on neighbors may have emerged, including mycorrhizal networks (Perry, 1995; Selosse *et al.*, 2006; van
365 der Heijden & Horton, 2009; Jung *et al.*, 2012; Song *et al.*, 2013; Shahzad *et al.*, 2015; Song *et al.*,
366 2019).

367 In summary, our work shows that plant-plant interactions mediated by herbivore-induced plant volatiles
368 are tissue specific and restricted to the leaves in wild and cultivated maize, and that this tissue-specificity
369 is likely driven by a lack of bioactive cues and a lack of perception capacity of roots. We suggest that
370 the low reliability and specificity of volatiles as danger cues in the rhizosphere together with the
371 availability of other information transfer networks may have impeded the evolution of eavesdropping
372 mechanisms in plant roots.

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377 **AUTHOR CONTRIBUTIONS**

378 CAMR designed the project. CAMR supervised the project. CvD, TZ, CM, XZ, RARM, RM, MY,
379 BCJS, and GG performed the experiments. CvD, CAMR, TZ, RARM and GG analyzed the data. CvD
380 and CAMR wrote the first draft. All authors reviewed and approved the manuscript.

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619 **Supplementary Information**

620 **Figure S1. The root herbivore *Diabrotica balteata* migrate away from infested plants.** Proportion
621 of larvae escaping from the maize plant after infestation (Mean \pm se, One sample t-test, n=6). Stars
622 indicate significant differences (*: $p \leq 0.05$, **: $p \leq 0.01$; ***: $p \leq 0.001$).

623 **Figure S2. Exposure to HIPVs from through infested neighbors does not alter plant defense to**
624 **herbivory. (a)** Relative larval weight gain (Mean \pm se, Student's t-tests) of the root herbivore
625 *Diabrotica balteata* feeding for four days on maize (n=9) previously exposed for four days to
626 control plants (control, green) or to plants infested with six *D. balteata* larvae (HIPVs, orange).
627 **(b)** Proportions (Mean \pm se, Student's t-tests, n=9) of *D. balteata* recovered after 4 days infested
628 on maize previously exposed for four days to control plants (control, green) or to plants infested
629 with six *D. balteata* larvae (HIPVs, orange). **(c)** Root fresh mass after four days exposure to
630 control (green) or to plants infested with six *D. balteata* larvae (HIPVs, orange) and then
631 infested for four days by the root herbivore *D. balteata* (Mean \pm se, Student's t-tests, n=9).

632 **Table S1. Primer list for q-RT-PCR used to assess the plant response in this study** (Peng *et al.*, 2005; Ton
633 *et al.*, 2007; Gao *et al.*, 2008; Erb *et al.*, 2009; Robert *et al.*, 2012b; Remy *et al.*, 2014; Hajiahmadi *et al.*, 2017);
634 *NCBI Gene: 100193700**).

