

1 **Root volatiles in plant-plant interactions I: Characterization of root sesquiterpene emissions from**

2 ***Centaurea stoebe* and their effects on other plants**

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12 Running title: Plant-plant interactions mediated by *Centaurea stoebe* root volatiles

13 **Abstract**

14 Volatile organic compounds (VOCs) emitted by plant leaves can influence the physiology of neighboring plants.  
15 In contrast to interactions above ground, little is known about the role of VOCs in belowground plant-plant  
16 interactions. Here, we characterize constitutive root volatile emissions of the spotted knapweed (*Centaurea*  
17 *stoebe*) and explore the impact of these volatiles on the germination and growth of different sympatric plant  
18 species. We show that *C. stoebe* roots emit high amounts of sesquiterpenes, with estimated release rates of (*E*)-  
19  $\beta$ -caryophyllene above  $3 \mu\text{g g}^{-1} \text{dw h}^{-1}$ . Sesquiterpene emissions show little variation between different *C. stoebe*  
20 populations, but vary substantially between different *Centaurea* species. Through root transcriptome sequencing,  
21 we identify six root-expressed sesquiterpene synthases (TPSs). Two root-specific TPSs, CsTPS4 and CsTPS5,  
22 are sufficient to produce the full blend of emitted root sesquiterpenes. Volatile exposure experiments demonstrate  
23 that *C. stoebe* root volatiles have neutral to positive effects on the germination and growth of different sympatric  
24 neighbors. Thus, constitutive root sesquiterpenes produced by two *C. stoebe* TPSs are associated with facilitation  
25 of sympatric neighboring plants. The release of root VOCs may thus influence *C. stoebe* abundance and plant  
26 community structure in nature.

27 **Keywords**

28 Root volatiles, sesquiterpene synthase, associational effects, neighborhood effects, Plant-plant interactions

## 29 **Introduction**

30 Plants influence their environment to maximize their fitness. One strategy by which plants can manipulate their  
31 environment is to produce and release chemicals such as volatile organic compounds (VOCs) (Pichersky & Gang  
32 2000). VOCs can for instance protect plants against biotic and abiotic stress (Gouinguéné & Turlings 2002;  
33 Loreto & Schnitzler 2010; Pichersky & Gershenzon 2002; Peñuelas et al. 2014). VOCs can also influence defense  
34 and growth of neighboring plants (Karban, Yang & Edwards 2014; Kegge et al. 2015; Ninkovic 2003; Pierik et  
35 al. 2003). Although the benefits of VOC-mediated plant-plant interactions for the emitter are subject to debate  
36 (Heil 2014; Morrell & Kessler 2017), VOC-mediated plant-plant interactions are increasingly recognized to  
37 influence plant ecology in natural and agricultural systems (Ninkovic, Markovic & Dahlin 2016). While most  
38 work on plant VOCs has focused on the phyllosphere, an increasing number of studies demonstrate that plant  
39 VOCs also have important roles in the rhizosphere. Root VOCs can for instance influence the behavior of  
40 herbivorous insects (Robert et al. 2012) and nematodes (Rasmann et al. 2005) and affect soil bacterial and fungal  
41 communities (Kleinheinz et al. 1999; Wenke, Kai & Piechulla 2010). In petri dish experiments, root VOCs have  
42 also been shown to negatively affect seed germination and seedling growth (Ens et al. 2009; Jassbi,  
43 Zamanizadehnajari & Baldwin 2010). Whether root VOCs mediate plant-plant interactions under more realistic  
44 conditions remains to be determined (Delory et al. 2016).

45 With more than 30,000 different structures, terpenoids are the most diverse class of secondary metabolites in the  
46 plant kingdom (Hartmann 2007) and are an integral part of plant VOC blends (Gershenzon & Dudareva 2007).  
47 Most volatile terpenoids are hemiterpenes ( $C_5$ ), monoterpenes ( $C_{10}$ ) and sesquiterpenes ( $C_{15}$ ) (Nagegowda 2010).  
48 Volatile terpenes have various ecological effects and function in plant-plant, plant-insect and plant-microbe  
49 interactions (Cheng et al. 2007). Terpenoids are derived from two common  $C_5$  precursor molecules, isopentenyl  
50 diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). In higher plants, IPP and DMAPP  
51 are formed through two different pathways, the mevalonic acid (MVA) and the methylerythritol phosphate  
52 (MEP) pathway. IPP and DMAPP are then further converted into geranyl diphosphate (GPP) and farnesyl  
53 diphosphate (FPP) as precursors for mono- and sesquiterpenes, respectively. The reaction for the final conversion  
54 to mono- and sesquiterpenes is catalyzed by terpene synthases (TPSs), which require a divalent cation to mediate  
55 the terpene formation (Cheng et al. 2007; Nagegowda 2010). As key enzymes for the production of terpenes,

56 TPSs have been characterized in plants (Degenhardt, Köllner & Gershenzon 2009; Jia et al. 2018), insects (Beran  
57 et al. 2016), fungi (Quin, Flynn & Schmidt-Dannert 2014), bacteria (Yamada et al. 2015), and amoebae (Chen et  
58 al. 2016). In plants it is known that TPS expression can be regulated in a tissue specific manner. Furthermore,  
59 TPSs often catalyze the formation of multiple products, which contributes to the substantial structural diversity  
60 of terpenoids (Tholl 2006).

61 In this study we characterize root VOCs emitted by the spotted knapweed (*Centaurea stoebe*). The tetraploid  
62 cytotype of *C. stoebe* is invasive in northern America (Treier et al. 2009), whereas the diploid cytotype is  
63 classified as threatened (vulnerable) in Switzerland according to the International Union for Conservation of  
64 Nature (IUCN). A previous study found that *C. stoebe* root chemicals affect the physiology of *Taraxacum*  
65 *officinale* agg. roots and their suitability for root feeding *Melolontha melolontha* larvae (Huang et al. 2018). As  
66 no direct root contact was needed to trigger these effects, we hypothesized that *C. stoebe* may affect neighboring  
67 plants through the release of root VOCs. In this study, we analyze the volatile blend of *C. stoebe* roots and identify  
68 sesquiterpenes as dominant root VOCs. Through root transcriptome sequencing and heterologous expression, we  
69 identify TPSs that are associated with this phenotype. Furthermore, we assess the impact of *C. stoebe* roots on  
70 the germination and growth of different sympatric plant species. The results of this study also provide a  
71 mechanistic basis to determine the impact of *C. stoebe* root sesquiterpenes on *T. officinale* and its interaction  
72 with *M. melolontha* larvae (companion paper Huang et al., under review). This work thus sheds light on the  
73 genetic basis and ecological consequences of VOC-mediated plant-plant interactions below ground.

## 74 **Methods and Materials**

### 75 ***Study system***

76 *Centaurea stoebe* L. (diploid) plants were grown from seeds purchased from UFA-SAMEN (Winterthur,  
77 Switzerland), unless specified otherwise. Seeds of *Anthemis tinctoria* L., *Centaurea scabiosa* L., *Centaurea jacea*  
78 L., *Cichorium intybus* L., *Daucus carota* L., *Dianthus carthusianorum* L., *Echium vulgare* L., *Festuca valesiaca*  
79 Gaudin, *Ranunculus bulbosus* L., *Taraxacum officinale* agg were obtained from the same vendor. *Medicago*  
80 *sativa* L. was obtained from Sativa Rheinau AG (Rheinau, Switzerland) and *Cardaria draba* (L.) Desv., was  
81 obtained from Templiner Kräutergarten (Templin, Germany). *Centaurea valesiaca* (DC.) Jord. seeds were

82 collected from a natural population in Raron (VS, Switzerland) and provided by Adrian Möhl (Info Flora) and  
83 Markus Fischer (University of Bern). Two *C. stoebe* populations Hu-11 (tetraploid, Hungary) and Ro-11  
84 (tetraploid, Romania), as well as *Koeleria macrantha* (Ledeb.) Schult. (MT, USA) were provided by Yan Sun  
85 and Heinz Müller-Schärer (University of Fribourg). Detailed information on these *C. stoebe* populations can be  
86 found in Mráz et al.(2012). Plant growth conditions are described in the corresponding experimental sections  
87 below.

### 88 ***Characterization of C. stoebe root volatiles***

89 To determine root volatile release by *C. stoebe*, plants were grown individually in sand under controlled  
90 conditions in a growth chamber (day length: 16 h; temperature: 20-22 °C; humidity: 65%) for seven weeks. The  
91 root system of each plant was then washed, separated from the shoot with a scalpel and dried with a paper towel  
92 (n = 8). Subsequently the roots were weighted and the cut at the root-shoot junction was sealed with Teflon tape  
93 before analysis to avoid contamination of the headspace with wound-released VOCs. The roots were then  
94 carefully inserted into 20 mL screw top glass vials (Gerstel, Sursee, Switzerland) and closed with airtight screw  
95 caps (septum Silicone/PTFE; Gerstel, Sursee, Switzerland). The vials were incubated for 1 min at 20 °C. Volatiles  
96 were then collected by exposing a SPME fiber (coated with 100 µm polydimethylsiloxane; Supelco, Bellefonte,  
97 PA, USA) to the headspace for 1.8 s. Volatiles were thermally desorbed (220 °C for 1 min) in the inlet of an  
98 Agilent 7820A series GC coupled to an Agilent 5977E MSD (source 230 °C, quadrupole 150 °C, ionization  
99 potential 70 eV, scan range 30–550; Palo Alto, CA, USA). After each run, the SPME fiber was baked out for 2  
100 min at 220 °C. VOCs were separated on a capillary GC-MS column (HP5-MS, 30m, 250µm ID, 2.5µm film;  
101 Agilent Technologies, Palo Alto, CA, USA) with He as carrier gas at a flow rate of 1 mL/min. Initial column  
102 temperature was set to 60 °C for 1 min followed by three temperature gradients: (i) 7 °C/min to 150 °C, (ii) 3  
103 °C/min to 165 °C and (iii) 30 °C/min to 250 °C and hold at this temperature for 3 min. VOCs were tentatively  
104 identified by comparing mass spectra to library entries of the National Institute of Standards and Technology  
105 (NIST 14). (*E*)-β-caryophyllene was identified by comparing mass spectrum and retention time to a synthetic  
106 standard (≥ 98.5 %, Sigma-Aldrich, Buchs SG, Switzerland). The first eluting petasitene was cross-validated by  
107 comparing mass spectra and retention times with a petasitene peak detected in a *Petasites hybridus* (L.) P. Gaertn.

108 & al. root extract (Saritas, von Reuss & König 2002). The other petasitene-like sesquiterpenes were tentatively  
109 identified by comparing mass spectra to petasitene from *P. hybridus*.

### 110 ***Quantification of terpene emissions***

111 To quantify the emission of (*E*)- $\beta$ -caryophyllene from *C. stoebe* roots, we first constructed volatile dispensers  
112 with known (*E*)- $\beta$ -caryophyllene release rates. The dispensers were constructed by adding 5  $\mu$ L pure (*E*)- $\beta$ -  
113 caryophyllene (> 98.5%, GC, Sigma-Aldrich, Buchs SG, Switzerland) into a 0.1 mL micro-insert (15 mm top;  
114 VWR, Dietikon, Switzerland). Teflon tape was wrapped around a 1  $\mu$ L capillary (Drummond, Millan SA, Plan-  
115 Les-Ouates, Switzerland), which was then plugged into the insert and sealed with more Teflon tape. The  
116 dispenser was stored for one day at room temperature before use to establish constant release rates. The (*E*)- $\beta$ -  
117 caryophyllene emission rate of the dispenser was quantified as previously described (D'Alessandro & Turlings  
118 2005). In short: the dispenser was placed into a glass bottle attached to a flow through system, whereby the  
119 outflow was coupled to a Super-Q trap to collect the volatile compounds. After 4 hours of volatile collection, the  
120 analytes were eluted from the trap with dichloromethane spiked with nonyl acetate as internal standard. The  
121 eluate was analyzed by gas chromatography-mass spectrometry (GC-MS) and compared to an (*E*)- $\beta$ -  
122 caryophyllene dilution series which was directly injected into the GC-MS, thus allowing to compute the (*E*)- $\beta$ -  
123 caryophyllene release rate of the dispensers. For the GC-MS analysis, 1  $\mu$ L of sample was injected into the inlet  
124 of the GC-MS system followed by separation and analysis as described above. To ensure an accurate (*E*)- $\beta$ -  
125 caryophyllene quantification, a single calibrated dispenser was incubated in SPME vials for different incubation  
126 periods (1, 5, 7.5, 10, 12.5, 20 min). The linear relationship between (*E*)- $\beta$ -caryophyllene release and MS signal  
127 ( $R^2 = 0.98$ ) was used to calculate *C. stoebe* root (*E*)- $\beta$ -caryophyllene emission. To calculate the release per g dry  
128 weight (DW), we dried the roots after analysis (80°C for 48h) and weighed them using a microbalance (n = 8).

### 129 ***Hexane tissue extraction and analysis***

130 To analyze the composition and abundance of VOCs in *C. stoebe* root and leaf extracts, plants were grown in  
131 'Tonsubstrat' (Klasmann-Deilmann, Geeste, Germany) in a greenhouse (light: 14h; temperature: day 21-23 °C  
132 night 19-21 °C; humidity: 50-60 °C) for ten weeks. Tissue samples were obtained by washing the roots and  
133 leaves, drying them with paper towel and wrapping root and leaf tissue separately into aluminum foil, flash-

134 freezing them in liquid nitrogen and storing them at -80 °C. All samples were ground with mortar and pestle under  
135 liquid nitrogen, and approximately 100 mg of frozen tissue powder per sample were put into a 1 mL glass vial.  
136 One mL of hexane with nonyl acetate as internal standard (10 ng\* $\mu$ l) was immediately added to the samples (n  
137 = 10 for each tissue). The samples were shaken at 200 rpm for 1 h at room temperature, followed by a  
138 centrifugation step of 20 min at 5,300 rpm. 600  $\mu$ L of supernatant per sample were pipetted into new tubes and  
139 stored at -20 °C. Characterization of VOCs in the extracts was carried out on an Agilent 6890 series GC coupled  
140 to an Agilent 5973 mass selective detector (source 230 °C, quadrupole 150 °C, ionization potential 70 eV; Palo  
141 Alto, CA, USA) and a flame ionization detector operating at 300 °C. He (MS) and H<sub>2</sub> (FID) were used as carrier  
142 gases. The VOC separation took place on a DB-5MS capillary column (Agilent, Santa Clara, CA, USA, 30 m x  
143 0.25 mm x 0.25  $\mu$ m). After injection of 1  $\mu$ L of tissue extract, the following temperature program was run: initial  
144 temperature of 45 °C was hold for 2 min followed by two temperature ramps, (i) 6 °C/min to 180 °C and (ii) 100  
145 °C/min to 300 °C and hold for 2 min. For volatile quantification, the peak areas of the GC-FID chromatograms  
146 were integrated. The area of each compound was taken relative to the area of the internal standard and corrected  
147 for the weight of the extracted tissue. For compound identification, root and leaf samples were also run on the  
148 GC-MS. In parallel an n-alkane standard solution was run with the same method, which enabled to calculate the  
149 linear retention indices (RI) following the procedure published by van den Dool & Kratz (1963). Tentative  
150 identification was carried out by comparing mass spectra and RI of a given peak to known compounds in plant  
151 extracts of *Aloysia sellowii* (Briq.) Moldenke and *Phoebe porosa* (Nees & Mart.) Mez., which were kindly  
152 provided by Prof. W.A. König, University of Hamburg. For compounds not found in these plant extracts, mass  
153 spectra and RI were matched to the library entries of the National Institute of Standards and Technology (NIST  
154 14). Corresponding retention indices (RI) can be found in the supplementary materials (Tab. S1). Daucadiene  
155 was tentatively identified by comparison to the mass spectra in the NIST library. Although the mass spectra  
156 showed high similarity, the RI was not as described for the best match to the NIST library (trans-dauca-4(11),8-  
157 diene), suggesting that the detected compound might be another daucadiene diastereoisomer.

### 158 ***Terpene emission of C. stoebe populations and related species***

159 To study if root sesquiterpene production differs between *C. stoebe* ecotypes and between congeneric plant  
160 species, plants of three *C. stoebe* populations, as well as four different species of the genus *Centaurea* were grown

161 in sand under controlled conditions (day length: 16 h; temperature: 20-22 °C; humidity: 65%) for five weeks.  
162 Two tetraploid populations (Hu-11, Ro-11) and one diploid population (UFA) were compared (n = 5-7). As  
163 congeneric species, *C. jacea*, *C. scabiosa* and *C. valesiaca*, which grow in distinct habitats were used (Landolt  
164 et al. 2010) (n = 4-8). Roots were prepared as described above for VOC characterization. The glass vials containing  
165 the roots were immediately stored on a cooling block at 2 °C of an autosampler system (MPS; Gerstel, Sursee,  
166 Switzerland) connected to the GC-MS system. Immediately prior to analysis, the samples were transferred to an  
167 incubator set to 30 °C, in which VOCs were subsequently collected by exposition of an SPME fiber to the  
168 headspace for 1.8 s. Next, the compounds were analyzed on the GC-MS system as mentioned above for VOC  
169 characterization.

#### 170 ***Transcriptome sequencing and analysis***

171 To explore the molecular basis of *C. stoebe* sesquiterpene production, we performed root transcriptome  
172 sequencing. *C. stoebe* root tissue was harvested, washed, dried, wrapped in aluminum foil and flash frozen in  
173 liquid nitrogen and ground to a fine powder. Total RNA was isolated from root powder following the  
174 manufacturer's protocol of the InviTrap® Spin Plant RNA Mini Kit (Stratagene molecular, Berlin, Germany). A  
175 TruSeq RNA-compatible library was prepared and PolyA enrichment was performed before sequencing the  
176 transcriptome on an IlluminaHiSeq 2500 with 10 Mio reads (250 base pair, paired end). Reads were quality  
177 trimmed using Sickle with Phred quality score of >20 and a minimum read length of 60. *De novo* transcriptome  
178 assembly was performed with the pooled reads using Trinity (version 2.2.0) running at default settings. Raw  
179 reads were deposited in the NCBI Sequence Read Archive (SRA) under the BioProject accession (to be inserted  
180 at a later date). To identify putative terpene synthase genes, the root transcriptome was screened using a  
181 TBLASTN search with the (*E*)- $\beta$ -caryophyllene synthase MrTPS1 from *Matricaria recutita* (Irmisch et al. 2012)  
182 as query.

#### 183 ***Sequence analysis and tree reconstruction***

184 Multiple sequence alignment of the identified TPS genes from *C. stoebe* and characterized TPS genes from *M.*  
185 *recutita* was computed using the MUSCLE codon algorithm implemented in MEGA6 (Tamura et al. 2013).  
186 Based on the alignment, a tree was reconstructed with MEGA6 using a maximum likelihood algorithm (GTR



187 model). Codon positions included were 1st+2nd+3rd+noncoding. All positions with <80% site coverage were  
188 eliminated. A bootstrap resampling analysis with 1000 replicates was performed to evaluate the topology of the  
189 generated tree.

#### 190 ***Cloning and heterologous expression of CsTPS genes***

191 To evaluate the TPS activity of the putative CsTPS genes, cDNA was produced. Then, focal genes were cloned  
192 into an expression vector and heterologously expressed in *Escherichia coli*. Subsequently, proteins were isolated  
193 and used for enzyme activity assays. To obtain plant material for RNA extraction, *C. stoebe* plants were grown  
194 in sand under controlled conditions (day length: 16 h; temperature: 20-22 °C; humidity: 65%) for eight weeks.  
195 Roots were gently washed, dried with a paper towel, cut 2 mm below root initiation, wrapped in aluminum foil  
196 and immediately flash frozen in liquid nitrogen. Afterwards, roots were ground with mortar and pestle under  
197 constant cooling with liquid nitrogen and stored at -80 °C before further processing. RNA extraction was carried  
198 out according to the manufacturer's protocol with an innuPrep Plant RNA Kit (Analytik Jena, Jena, Germany).  
199 For cDNA synthesis, 2 µg of RNA was treated with DNase (Thermo scientifics, CA, USA). First-strand DNA  
200 was synthesized with oligo dT<sub>12-18</sub> primers and Super Script™ III reverse transcriptase (Invitrogen, Carlsbad,  
201 CA, USA). The open reading frames (ORF) of the putative *C. stoebe* terpene synthases were amplified with the  
202 primer pairs listed in the supplementary (Tab. S2) and cloned into a pASK-IBA37plus plasmid (IBA-  
203 Lifesciences, Göttingen, Germany) by restriction digest and ligation. NEB 10-beta competent *E. coli* cells (New  
204 England Biolabs, Ipswich, MA, USA) were then transformed with these vectors. In order to obtain the cloned  
205 *CsTPS* sequences and to check the transformation events, the inserted fragments were sequenced by Sanger  
206 sequencing.

207 For heterologous expression, NEB 10-beta cells containing the *CsTPS* constructs were grown at 37°C to an OD<sub>600</sub>  
208 of 0.8. Subsequently protein expression was induced by adding anhydrotetracycline (IBA-Lifesciences,  
209 Göttingen, Germany) to a final concentration of 200 ng\*mL<sup>-1</sup>. Expression took place for 18 h at 18 °C. Cells  
210 were harvested by centrifugation and resuspended in assay buffer (10 mM Tris HCl, 1mM DTT and 10 %  
211 (vol/vol) glycerol (pH 7.5)). To disrupt the cells, they were treated 4 x 20 s at 60 % power with a sonicator  
212 (Bandelin Sonoplus HD 2070, Berlin, Germany). Samples were then centrifuged at 4 °C for 1 h at 14,000 g to

213 separate the soluble proteins from cell debris. A further purification was made by passing the proteins through  
214 an illustra NAP-5 column (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK).

215 Enzyme activity assays were performed to test the terpene production of the different CsTPS. Activity assays  
216 were carried out by adding 50  $\mu$ L of assay buffer and 50  $\mu$ L of purified crude bacterial protein extract with 10  
217 mM MgCl<sub>2</sub> and 10  $\mu$ M (*E,E*)-FPP into a threaded 1 mL glass vial with a cap containing a Teflon septum. The  
218 reaction mix was incubated for 1 h at 30 °C. During the incubation period, VOCs were sampled with a SPME  
219 fiber. For volatile analysis, the collected volatiles were desorbed directly in the inlet (240 °C) of the GC-MS  
220 system. An Agilent 6890 series GC coupled to an Agilent 5973 MSD (source 230 °C, quadrupole 150 °C,  
221 ionization potential 70 eV; Palo Alto, CA, USA) was used for analysis. He was used as carrier gas at a rate of 1  
222 mL\*min<sup>-1</sup>. The volatile separation took place on a DB-5MS capillary column (Agilent, Santa Clara, CA, USA,  
223 30 m x 0.25 mm x 0.25  $\mu$ m). The initial oven temperature of 80 °C was hold for 2 min, followed by a ramp of 7  
224 °C/min to 180 °C and a second ramp of 100 °C/min to 300 °C where the temperature was held for 1 min.

#### 225 ***qRT-PCR analysis of CsTPS genes***

226 To determine the expression levels of individual CsTPS genes, RNA was extracted, converted into cDNA and  
227 further used for qRT-PCR. Total RNA was isolated from the same root and leaf tissue samples as for hexane  
228 extraction. This was made following the InviTrap<sup>®</sup> Spin Plant RNA Mini Kit (Stratec molecular, Berlin,  
229 Germany). Next, 1  $\mu$ g of the RNA was DNase I treated followed by first-strand cDNA synthesis using RevertAid  
230 H Minus Reverse Transcriptase with oligo (dT)<sub>18</sub> primers (Thermo scientific, CA, USA). cDNA was diluted 1:10  
231 before used for qRT-PCR. To find an appropriate reference gene, *actin1* and *EF1 $\alpha$*  sequences of *Arabidopsis*  
232 *thaliana* were taken as query for a screen in the *C. stoebe* Trinity assembly with the software Blast2GO 4.1 (Götz  
233 et al. 2008) running at default settings. Two primer combinations were designed for each homologous reference  
234 gene. *EF1 $\alpha$*  was found to be the most robust reference gene. Next, for each of the CsTPS genes, a qPCR primer  
235 pair was designed. All primers are listed in supplementary (Tab. S2). Primer specificity was tested by means of  
236 melting curve analysis and gel electrophoresis. qRT-PCR was carried out on a LightCycler<sup>®</sup> 96 Instrument  
237 (Roche, Basel, Switzerland) using the KAPA 480 SYBR FAST qPCR Master Mix (Kapa Biosystems, Boston,  
238 USA). Primer efficiency was determined using a linear standard curve approach. For very low expressed genes,

239 this was repeated with samples spiked with plasmids containing the genes of interest. Biological replicates were  
240 all run in technical triplicates. Three samples had to be excluded from the analysis due to poor RNA quality or  
241 very low expression of the reference gene, resulting in a total of 7 biological replicates for *CsTPS4* as well as  
242 *CsTPS5* and 5 biological replicates for *CsTPS1*. Relative transcript abundance was analyzed as fold change ( $2^{-\Delta\Delta Ct}$ ). As *CsTPS1* showed dissimilar melting peaks for root and shoot PCR amplicons, the fragments were  
243 subsequently sequenced by Sanger sequencing.

### 245 ***Impact of C. stoebe* root VOCs on neighboring plants**

246 To evaluate the influence of *C. stoebe* root volatiles on the germination and growth of neighboring plants, we  
247 used an experimental setup that excluded direct root contact or the transfer of exudates, but allowed *C. stoebe*  
248 root VOCs to diffuse to the neighboring plants. The system consisted of mesh cages (12 × 9 × 10 cm, length ×  
249 width × height) made of Geotex fleece (Windhager, Austria), which were placed in pairs into rectangular plastic  
250 pots (Fig. 4A). A covered airgap between the cages allowed for the diffusion of VOCs between the rhizospheres  
251 of plants growing in the soil-filled mesh cages. Water was supplied carefully to soil in the mesh cages avoid  
252 leaching and exchange of root exudates across the airgap. The Geotex fleece of the mesh cages was sufficient to  
253 stop roots from growing out of the mesh cages, thus eliminating direct root contact between the plants. Diffusion  
254 of *C. stoebe* VOCs into the airgap was confirmed by SMPE (companion paper Huang et al., under review). Plants  
255 for this experiment were grown in a greenhouse (light: 14h; temperature: day 16-24 °C, night 16-22 °C, mean  
256 temperature over growth period 20 °C; humidity: 30-60 °C) in potting soil consisting of five parts ‘Landerde’  
257 (RICOTER, Aarberg, Switzerland), four parts ‘Floratorf’ (Floragard, Oldenburg, Germany) and one part sand  
258 („Capito“ 1-4 mm, LANDI Schweiz AG, Dotzigen, Switzerland). The “sender” mesh cages in the plastic pots  
259 where either left plant free or planted with three week old *C. stoebe* seedlings. After 25 days, different plant  
260 species were planted into the “receiver” mesh cages (10 seeds per cage, n = 12 for each species). As receiver  
261 species, 11 commonly co-occurring species of *C. stoebe* were selected: *Anthemis tinctoria*, *Cardaria draba*,  
262 *Centaurea stoebe*, *Cichorium intybus*, *Dianthus carthusianorum*, *Echium vulgare*, *Festuca valesiaca*, *Koeleria*  
263 *macrantha*, *Medicago sativa*, *Ranunculus bulbosus*, *Daucus carota*, and *Taraxacum officinale* agg. The pots were  
264 watered every one to three days. Pots were turned 180° and randomized fortnightly. Potential bias through above  
265 ground effects of *C. stoebe* was ruled out by arranging the pots on the table so that each receiver had a *C. stoebe*

266 as neighbor either only above ground in a separate pot (control) or aboveground and belowground in the same  
267 pot (treatment). The total number of germinated seeds was recorded after 4 weeks. The first germinated seedling  
268 was retained, all the others were removed. After nine weeks of growth, the plants were harvested. Roots and  
269 leaves were washed, separated and dried at 80 °C until constant weight to determine dry mass.

## 270 **Data Analysis**

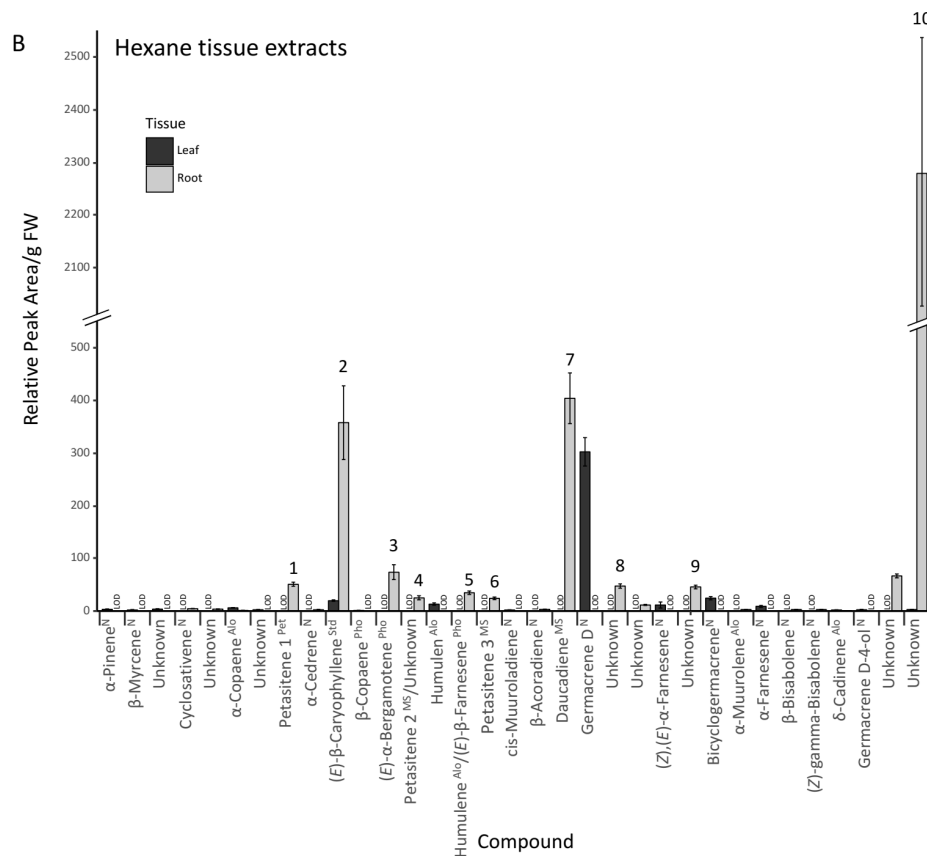
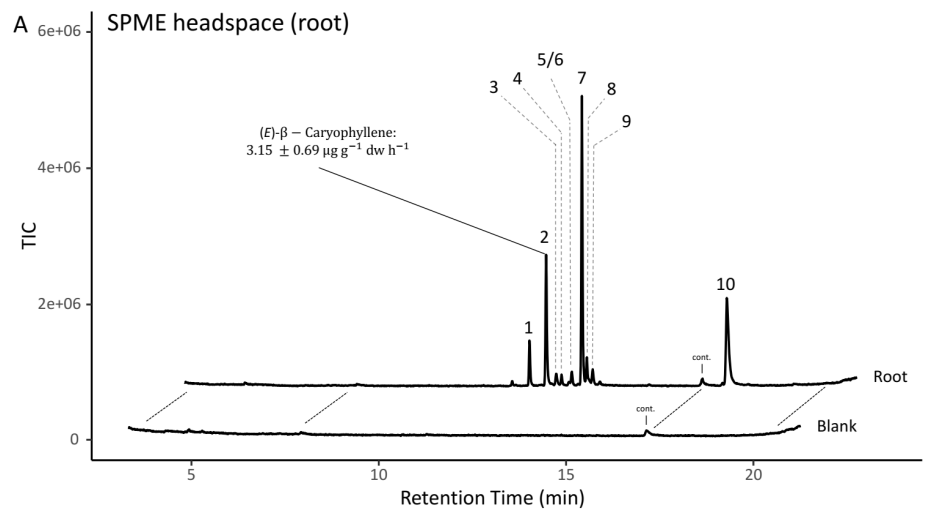
271 Statistical assumptions such as normal distribution and homoscedasticity of error variance were checked and  
272 square root or  $\log_e$  transformed if the assumptions were not met. Differences in relative peak area per g FW  
273 between root and leaf tissue in hexane extracts were tested with a Wilcoxon signed rank test. To test for  
274 differences in sesquiterpene abundance among *C. stoebe* populations and *Centaurea* species for a given  
275 compound, Analysis of Variance (ANOVA) of a fitted linear model was performed and if significant followed  
276 by LS means pairwise comparisons with  $p$  value adjustment. Differences in expression levels between root and  
277 leaf tissue were tested by Wilcoxon signed rank tests. A possible effect of the emitter on the germination was  
278 analyzed by fitting a generalized linear model with a quasibinomial distribution to the data and performing an  
279 ANOVA ( $n = 12$  per species and treatment). Dry biomass of roots and leaves were investigated by fitting a linear  
280 model and conducting an ANOVA ( $n = 12$  per species and treatment, 9 out of 244 plants died and were therefore  
281 excluded from the analysis). For each species, the effect of the emitter plant on biomass production was tested  
282 by means of a Student's  $t$ -test followed by  $p$  value correction for multiple comparison (Benjamini & Hochberg  
283 1995). Statistical analysis and data visualization was conducted with R 3.4.3 (R Core Team 2017), with  
284 'lsmeans', 'car' 'plyer' and 'ggplot2' packages (Lenth 2016; Wickham 2009, 2011; Fox & Weisberg 2011).

## 285 **Results**

### 286 **Characterization of *C. stoebe* VOCs**

287 Analysis of the volatile blend emitted by intact *C. stoebe* roots revealed an abundant sesquiterpene fraction (Fig.  
288 1A) with (*E*)- $\beta$ -caryophyllene and daucadiene (most likely a diastereoisomere of trans-dauca-4(11),8-diene) as  
289 the predominant compounds. The sesquiterpenes (*E*)- $\alpha$ -bergamotene, humulene, (*E*)- $\beta$ -farnesene, three putative  
290 petasitene isomers (petasitene 1-3), and an unknown sesquiterpene were emitted as well. (*E*)- $\beta$ -caryophyllene  
291 emission was quantified at  $3.15 \pm 0.69 \mu\text{g g}^{-1} \text{dw h}^{-1}$  (mean  $\pm$  SE). Hexane root tissue extracts contained

292 comparable sesquiterpene profiles, with (*E*)- $\beta$ -caryophyllene and daucadiene as major compounds (Fig. 1B).  
293 Additionally, low quantities of other sesquiterpenes such as cyclosativene,  $\beta$ -acoradiene,  $\alpha$ -farnesene, and  $\beta$ -  
294 bisabolene were found in these extracts, which were not detected in the volatile blend of intact roots. Besides  
295 sesquiterpenes, there were other compounds eluting from the column, mostly at later time points. The most  
296 abundant of these compounds showed a terpenoid-like structure and was tentatively identified as a sesquiterpene  
297 lactone ( $m/z = 232$ ). The other late eluting analytes were neither known nor present in the volatile blend of intact  
298 roots and therefore not analyzed further. Sesquiterpenes were much more abundant in root hexane extracts than  
299 leaf extracts (Fig. 1B). Only four compounds were detected in both leaves and roots, namely  $\alpha$ -copaene, (*E*)- $\beta$ -  
300 caryophyllene,  $\delta$ -cadinene and the putative sesquiterpene lactone. (*E*)- $\beta$ -caryophyllene and the putative  
301 sesquiterpene lactone were present in much higher concentrations in the roots than the leaves (Wilcoxon signed  
302 rank test:  $n = 10$ ,  $p = 0.002$ ), while  $\alpha$ -copaene,  $\delta$ -cadinene were more abundant in the leaves (Wilcoxon signed  
303 rank test:  $n = 10$ ,  $p = 0.002$ ). In contrast to root tissue, we also detected three monoterpenes in *C. stoebe* leaves:  
304  $\alpha$ -pinene,  $\beta$ -myrcene and an unknown monoterpene. Compared to sesquiterpenes, monoterpene signals were low  
305 in abundance.

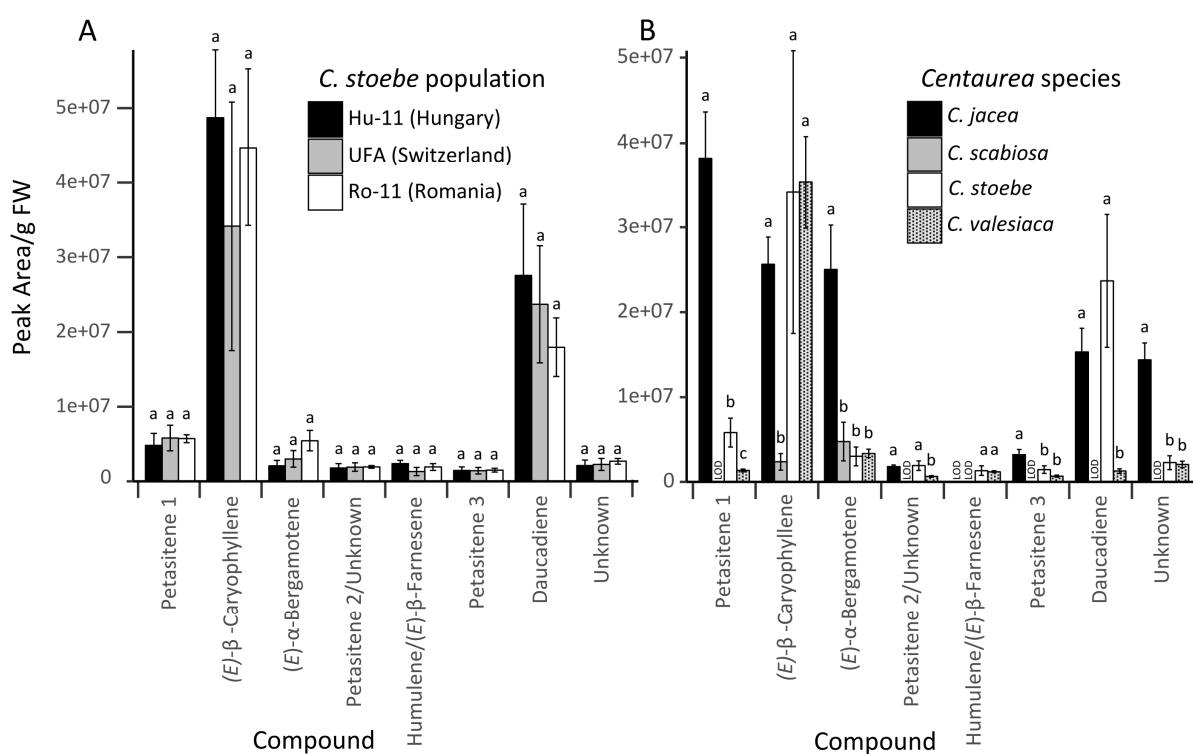


306

307 **Fig. 1. *Centaurea stoebe* roots release high amounts of sesquiterpenes.** (A) Representative SPME-GC-MS  
 308 chromatogram of VOCs emitted by intact *C. stoebe* roots. (*E*)-β-caryophyllene emission rate is displayed as mean ±  
 309 SE (n = 8; dw, dry weight). (B) Relative peak area per g fresh weight (FW) of compounds found in hexane tissue  
 310 extracts shown as mean ± SE (n = 10). TIC, total ion current; 1, petasitene 1; 2, (*E*)-β-caryophyllene; 3, (*E*)-α-  
 311 bergamotene; 4, petasitene 2; 5, humulene and (*E*)-β-farnesene; 6, petasitene 3; 7, daucadiene; 8, unknown  
 312 sesquiterpene; 9, unknown non terpenoid; 10, unknown sesquiterpene lactone-like compound; cont, contamination;  
 313 LOD, below limit of detection; Identification: N, NIST library, comparison of mass spectra and retention index (RI);  
 314 MS, inspection of mass spectra (RI other than literature); Std, comparison of mass spectra an RI with pure standard  
 315 compound; and comparison of mass spectra an RI with known compounds of Alo, *Aloysia sellowii* ; Pet, *Petasites*  
 316 *hybridus*; Pho, *Phoebe porosa*.

317 **Emission Pattern of *C. stoebe* Populations and other *Centaurea* Species**

318 Sesquiterpenes released by intact roots of three different *C. stoebe* populations did not differ significantly in  
 319 quality and quantity (Fig. 2A), suggesting that this trait is conserved within *C. stoebe*. By contrast, congeneric  
 320 *Centaurea* species emitted distinct terpene bouquets compared to *C. stoebe* (Fig. 2B). The volatile blend of the  
 321 closely related *C. valesiaca* was most similar to *C. stoebe*, with petasitene 1, petasitene 2 and daucadiene being  
 322 emitted in lower quantities by *C. valesiaca* than by *C. stoebe*. *C. jacea* emitted sesquiterpenes similar to *C. stoebe*  
 323 but in different quantities: the release of petasitene 1, petasitene 3, (*E*)- $\alpha$ -bergamotene and of an unknown  
 324 compound was significantly increased in *C. jacea* compared to *C. stoebe*. Finally, we detected (*E*)- $\beta$ -  
 325 caryophyllene and (*E*)- $\alpha$ -bergamotene, but not any of the other sesquiterpenes in the headspace of *C. scabiosa*  
 326 roots. Thus, sesquiterpene release from the roots seems to be conserved in *C. stoebe* ecotypes, but varies  
 327 qualitatively and quantitatively between different *Centaurea* species.



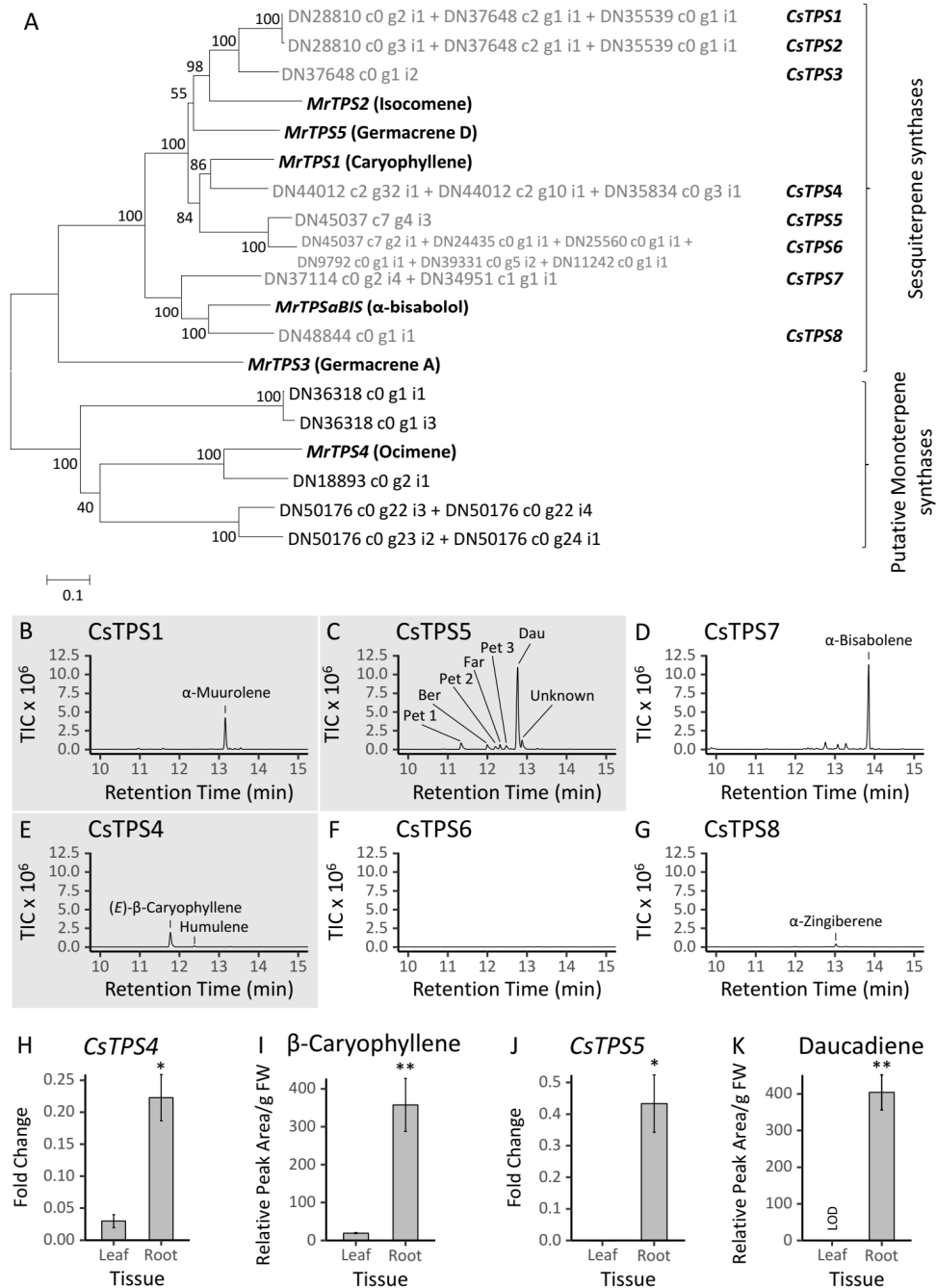
328  
 329 **Fig. 2. Root sesquiterpene release is conserved within *C. stoebe*, but varies between different *Centaurea* species.**  
 330 (A) Peak area per g fresh weight (FW) of *C. stoebe* populations shown as mean  $\pm$  SE (n = 5; except for Hu-11, n = 7).  
 331 Letters show significant differences among populations within one compound (Analysis of Variance (ANOVA)  
 332 followed by pairwise comparison of LS means,  $p_{adj} < 0.05$ ). (B) Peak area per g fresh weight (FW) of *Centaurea*  
 333 species shown as mean  $\pm$  SE (*C. jacea* and *C. scabiosa*, n = 8; *C. stoebe*, n = 5; *C. valesiaca*, n = 4). Letters show  
 334 significant differences among species within one compound (Analysis of Variance (ANOVA) followed by pairwise  
 335 comparison of LS means,  $p_{adj} < 0.05$ ). LOD, below limit of detection.

### 336 *Terpene Synthases of C. stoebe*

337 To understand the genetic basis of sesquiterpene formation in *C. stoebe* roots, known sequences of *M. recutita*  
338 terpene synthases (TPS) were used to find homologous genes in the *C. stoebe* root transcriptome. This led to the  
339 identification of eight potential sesquiterpene synthases (CsTPSs, Fig. 3A). Apart from CsTPS2 and CsTPS3, for  
340 which ORF amplification and transformation into *E. coli* was unsuccessful, all TPSs were successfully cloned  
341 and expressed in *E. coli*. CsTPS protein activity assays showed that CsTPS1, CsTPS4, CsTPS5, CsTPS7, and  
342 CsTPS8 exhibit sesquiterpene synthase activity. No activity was found for CsTPS6 (Fig. 3B-G). CsTPS1  
343 catalyzed the formation of  $\alpha$ -muurolene, and CsTPS4 produced (*E*)- $\beta$ -caryophyllene and humulene. CsTPS5  
344 produced daucadiene as main compound and (*E*)- $\alpha$ -bergamotene, (*E*)- $\beta$ -farnesene, three petasitenes,  $\beta$ -  
345 acoradiene,  $\beta$ -bisabolene, (*Z*)- $\gamma$ -bisabolene, as well as an unknown sesquiterpene as byproducts. All the  
346 compounds produced by CsTPS1, CsTPS4 and CsTPS5 were found in hexane root extracts of *C. stoebe*.  
347 Furthermore, the compounds produced by CsTPS4 and CsTPS5 cover all highly emitted volatiles from intact  
348 roots. Comparison of retention indices and mass spectra revealed that CsTPS7 produced (*E*)- $\alpha$ -bisabolene (RI  
349 1545) and CsTPS8 produced  $\alpha$ -zingiberene (RI 1497) as main compounds. The two compounds were not detected  
350 in tissue extracts or the headspace of intact roots.

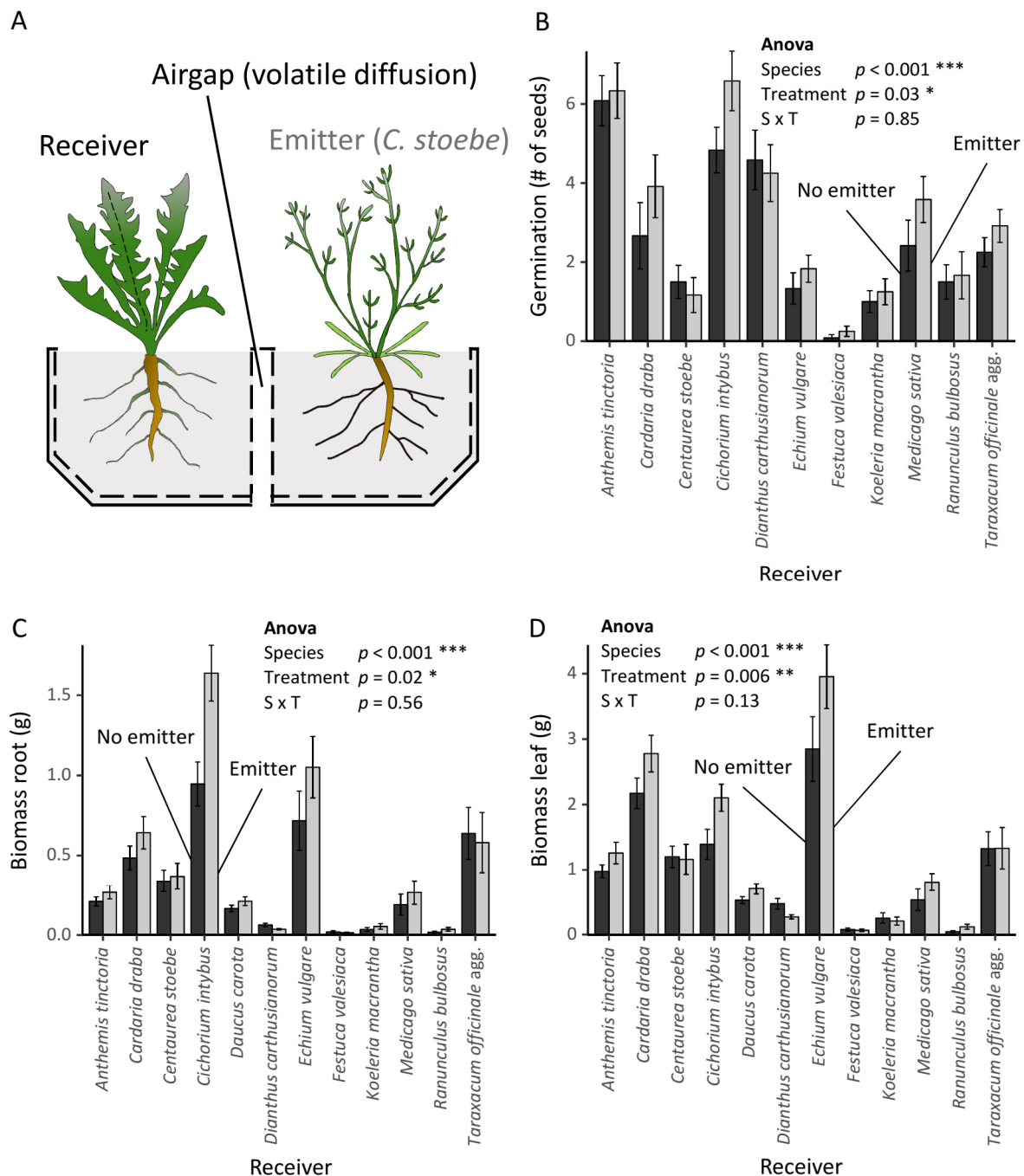
351 The predominant sesquiterpenes (*E*)- $\beta$ -caryophyllene and daucadiene are produced in high amounts in the roots,  
352 but not in the leaves (Fig. 3I/K). The same pattern was found for the expression of *CsTPS4* and *CsTPS5*, the two  
353 TPSs putatively responsible for the production of these VOCs (Fig. 3H/J). The mRNA levels in root compared  
354 to leaf tissue revealed a 7.5-fold increase in *CsTPS4* (Wilcoxon signed rank test:  $n = 7, p = 0.016$ ) and a >5,000-  
355 fold increase for *CsTPS5* (Wilcoxon signed rank test:  $n = 7, p = 0.016$ ). Low expression of *CsTPS1* was detected  
356 in the leaves and roots. Melting point analysis indicated that different fragments were amplified in the different  
357 tissues. Fragment sequencing revealed that the root fragment corresponds to *CsTPS1*, whereas the leaf fragment  
358 only showed 89% sequence similarity to *CsTPS1*. No other sequence in the *C. stoebe* root transcriptome besides  
359 *CsTPS1* was found to match the leaf fragment, suggesting that it may stem from a TPS gene that is specifically  
360 expressed in the leaves.





361

362 **Fig. 3. Two terpene synthases account for major *Centaurea stoebe* root sesquiterpenes.** (A) To find potential *C.*  
 363 *stoebe* terpene synthases (*CsTPS*s), sequences of *Matricaria recutita* terpene synthases (*MrTPS*) were taken to screen  
 364 for homologous genes in the *C. stoebe* root transcriptome. The phylogenetic tree shows contigs of potential *CsTPS*s as  
 365 end nodes and their related *MrTPS* genes. (B-G) SPME-GC-MS analysis of *CsTPS* protein activity assays with (*E,E*-  
 366 FPP as substrate. Compounds of highlighted chromatograms (B, C, E) were also found in *C. stoebe* hexane root  
 367 extracts. mRNA abundance for *CsTPS4* (H) and *CsTPS5* (J) and relative peak area per g fresh weight (FW) of their  
 368 main products (*E*)- $\beta$ -caryophyllene (I) and daucadiene (K) in hexane root extracts. Shown are mean  $\pm$  SE (qRT-PCR,  
 369  $n = 7$ ; Tissue extracts,  $n = 10$ ). Differences in means were tested by Wilcoxon signed rank tests, levels of significance:  
 370  $p < 0.01$  \*\*,  $p < 0.05$  \*. TIC, total ion current; Pet, petasitene; Ber, (*E*)- $\alpha$ -bergamotene; Far, (*E*)- $\beta$ -farnesene; Dau,  
 371 daucadiene. LOD, below limit of detection



372  
 373 **Fig. 4. *Centaurea stoebe* root volatiles increase germination and growth of sympatric neighbors.** (A) Experimental  
 374 setup to evaluate the influence of *C. stoebe* (“emitter”) root volatiles on receiver plant species. As control, the emitter  
 375 compartment was filled with soil, but no plant was grown in it (“no emitter”). (B) Number of receiver seeds that  
 376 germinated up to four weeks after they were sown. Analysis of Variance (ANOVA) output of generalized linear model  
 377 is shown (distribution, quasibinomial;  $n = 11$  per species and treatment). Dry biomass of receiver roots (C) and leaves  
 378 (D) after nine weeks of growth. ANOVA output of linear model is shown (levels of significance:  $p < 0.001$  \*\*\*,  $p <$   
 379  $0.01$  \*\*,  $p < 0.05$  \*;  $n = 12$  per species and treatment)

### 380 ***Effect of C. stoebe* Root Volatiles on Neighboring Plants**

381 To test whether *C. stoebe* root VOCs influence the germination and performance of neighboring plants, we  
382 exposed seeds and germinating plants of different sympatric species to *C. stoebe* rhizosphere VOCs for several  
383 weeks. An overall positive effect of *C. stoebe* root VOCs on the germination of the different sympatric plant  
384 species was observed ( $p = 0.03$ , Fig. 4B). Furthermore, nine weeks after sowing, root biomass ( $p = 0.02$ , Fig. 4C)  
385 and leaf biomass ( $p = 0.006$ , Fig. 4D) were significantly increased in the presence of *C. stoebe* root VOCs.  
386 Individual comparisons revealed no significant effects for single species, even though visual inspection indicated  
387 that the magnitude of the effects varied from neutral to positive for the different species.

### 388 **Discussion**

389 Plants are known to produce a variety of VOCs that play important roles in biotic interactions (Peñuelas et al.  
390 2014; Pichersky et al. 2002). Physiological changes in plants exposed to VOCs from neighboring plants for  
391 instance are well documented above ground (Arimura, Shiojiri & Karban 2010; Heil & Karban 2010; Karban et  
392 al. 2014). In contrast, there is a gap of knowledge regarding VOC-mediated plant-plant interactions below ground  
393 (Delory et al. 2016). In this study, we characterized the volatiles emitted by *C. stoebe* and identified two terpene  
394 synthases which are sufficient to produce the full sesquiterpene blend emitted by intact roots. Furthermore, we  
395 show that *C. stoebe* root VOCs enhance germination and biomass production of sympatric neighbors. Here, we  
396 discuss these findings from physiological and ecological points of view and reflect on the potential role of root  
397 VOCs in determining the rarity of *C. stoebe* in its native environment.

398 Plants can release terpenoids constitutively or in response to environmental stress (Keeling & Bohlmann 2006).  
399 Our headspace analyses show that *C. stoebe* releases sesquiterpenes specifically and constitutively from its roots.  
400 The emission rate of the sesquiterpene (*E*)- $\beta$ -caryophyllene was measured at  $3.15 \pm 0.69 \mu\text{g g}^{-1} \text{dw h}^{-1}$  (mean  $\pm$   
401 SE), leading to a situation where 2 seconds of exposure to a few mg of *C. stoebe* roots already saturated our  
402 analytical equipment. For comparison, (*E*)- $\beta$ -caryophyllene release from herbivore-attacked maize roots is likely  
403 in the lower ng range per plant (Hiltbold et al. 2011). Only few studies so far provide absolute quantification of  
404 root VOC emission rates, and we are not aware of any report showing below ground sesquiterpene release rates  
405 at the levels reported here. Monoterpenes have been shown to be released in substantial quantities by roots. *Pinus*

406 *pinea* roots for instance release monoterpenes at rates up to  $26 \pm 5 \mu\text{g g}^{-1} \text{dw h}^{-1}$  (mean  $\pm$  SE) (Lin, Owen &  
407 Peñuelas 2007). Thus, *C. stoebe* constitutively releases relatively high amounts of sesquiterpenes from its roots.  
408 Terpenoids are produced by terpene synthases (TPSs) (Bohlmann, Meyer-Gauen & Croteau 1998). We identified  
409 two CsTPSs whose products correspond to the root-emitted sesquiterpenes in *C. stoebe*. (*E*)- $\beta$ -caryophyllene  
410 occurs in many plant species and it has been reported several times to be produced by the same terpene synthase  
411 as humulene (Cai et al. 2002; Irmisch et al. 2012; Köllner et al. 2008; Yang et al. 2013). In *C. stoebe*, we also  
412 found these two compounds to be produced by the same TPS (CsTPS4). Examining the expression level of  
413 *CsTPS4* in roots and leaves of *C. stoebe* showed the same pattern as the distribution of the compound: low  
414 quantities of RNA and (*E*)- $\beta$ -caryophyllene in leaves and significantly higher quantities of both in roots. The  
415 second TPS involved in producing the volatile bouquet is CsTPS5 with daucadiene as main product. Enzyme  
416 activity assays of this enzyme led to the production of several sesquiterpenes, all of which were also present in  
417 *C. stoebe* roots. The sesquiterpenes produced by CsTPS5 were not found in the leaves, and *CsTPS5* was not  
418 expressed in this tissue. Regulation of sesquiterpene synthesis through transcriptional control of *TPSs* is well  
419 established (Tholl 2006) and likely also accounts for the differences in leaf and root sesquiterpene profiles in *C.*  
420 *stoebe*. Taken together, we show that two highly expressed, root-specific TPSs can account for the full root  
421 sesquiterpene blend of *C. stoebe*.

422 *In vitro* studies found negative effects of root VOCs on seed germination (Ens et al. 2009; Jassbi et al. 2010).  
423 Using a soil-based system that allows for the passive diffusion of VOCs between sender and receiver plants, we  
424 demonstrate that *C. stoebe* volatiles have no negative effects on the germination and growth of 11 sympatric plant  
425 species. Root VOC exposure even resulted in an overall increase in the germination and growth of other plants.  
426 A degradation product of (*E*)- $\beta$ -caryophyllene has been shown to exhibit a broad antifungal activity (Hubbell,  
427 Wiemer & Adejare 1983) and other root VOCs are also known to influence microbial communities, which again  
428 can alter plant performance (Wenke et al. 2010; Inderjit & Weiner 2001; Kleinheinz et al. 1999). Thus, the  
429 positive effect of *C. stoebe* root VOCs on the receiver plants could either be a direct effect mediated through the  
430 impact of the VOCs on the physiology of the seeds and growing plants, or an indirect effect mediated through  
431 soil microbial communities (Hu et al. 2018b). Of note, *C. stoebe* VOCs do not only modulate plant performance,  
432 but can also change root physiology and herbivore resistance, as shown in the companion paper to this study

433 (companion paper Huang et al., under review). Thus, the effects of *C. stoebe* VOCs on neighboring plants are  
434 likely multifaceted and may change the interactions of neighboring plants with other organisms. How root VOCs  
435 interact with bioactive soluble exudates, which can also be important for plant and herbivore performance (Hu et  
436 al. 2018a), remains to be studied.

437 The release of VOCs can benefit the emitter by intoxicating and repelling herbivores, attracting natural enemies  
438 and priming defenses in systemic tissues (De Moraes, Mescher & Tumlinson 2001; Frost, Mescher, Carlson, De  
439 Moraes 2008; Erb et al. 2015; Schuman, Barthel & Baldwin 2012; Ye et al. 2018). To what extent the release of  
440 VOCs is beneficial for the emitter in the context of plant-plant interactions, however, is less clear. Here, we show  
441 that the release of sesquiterpenes from the roots may have negative consequences for *C. stoebe* plants, as it  
442 increases the germination and growth of a variety of sympatric competitors. Strikingly, and in contrast to what  
443 has been observed in other plant systems (Degen et al. 2004; Schuman et al. 2009), sesquiterpene release seems  
444 to be conserved within different *C. stoebe* ecotypes. The benefit of this potentially conserved phenotype for *C.*  
445 *stoebe* is currently unclear. Germination and growth of *C. stoebe* itself does not seem to be improved through  
446 VOC exposure, for instance. However, it is possible that the high release rates protect the plant from herbivores  
447 and pathogens in addition to the known resistance factors in this species (Landau, Müller-Schärer & Ward 1994).  
448 Furthermore, as shown in the companion paper (companion paper Huang et al., under review), the VOCs may  
449 trigger susceptibility to herbivores in neighboring species. Knocking down *CsTPS4* and *CsTPS5* could help to  
450 understand the potential benefits of root sesquiterpene production in the future.

451 According to the IUCN red list, *C. stoebe* is classified as threatened in Switzerland while it is invasive in the  
452 United States. Substantial work has been conducted to evaluate whether *C. stoebe* may suppress competitors in  
453 the invasive range through allelopathic effects (Duke et al. 2009; Ridenour & Callaway 2001). It has for instance  
454 been demonstrated that *C. stoebe* suffers substantially from competition by its neighbors in its native range, but  
455 not in the invasive range (Callaway et al. 2011). It will be interesting to study VOC emissions of invasive ecotypes  
456 and effects on competitors in the invasive range in the future. In the native range, the increased growth of  
457 neighboring species triggered by *C. stoebe* root VOCs may contribute to its rarity.

458 In conclusion, this work demonstrates that two TPSs are sufficient to explain the high constitutive sesquiterpene  
459 emissions of *C. stoebe*, and that the release of these VOCs, as dominant constituents of the full root VOC blend,  
460 do not negatively affect neighboring plants, but increase their growth and germination. Thus, below ground plant-  
461 plant interactions mediated by plant volatiles may affect competition and coexistence in natural plant  
462 communities.

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### 468 **Authors Contributions**

469 V.G, T.G.K. and M.E. designed the experiments. M.H. and T.G.K. sequenced, assembled and analyzed the *C.*  
470 *stoebe* root transcriptome. V.G. and C.F. performed experiments. V.G., T.G.K. and M.E. analyzed data. V.G. and  
471 M.E. wrote the first draft of this manuscript.

### 472 **Data accessibility**

473 Raw data associated with this study can be downloaded from Dryad [to be inserted at a later stage] and the NCBI  
474 Sequence Read Archive (SRA) [to be inserted at a later stage].

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- 627

628 **Supplementary materials**

**Table S1:** Compounds found in *Centaurea stoebe* hexane tissue extracts listed as means  $\pm$  SE of relative peak area per g fresh weight.

Compound	Class	Root	Leaf	RI	Identification	CsTPS
$\alpha$ -Pinene	M	0 $\pm$ 0	3.5 $\pm$ 0.58	933	NIST	
$\beta$ -Myrcene	M	0 $\pm$ 0	2.27 $\pm$ 0.47	991	NIST	
Unknown	M	0 $\pm$ 0	3.79 $\pm$ 0.6	1049		
Cyclosativene	S	4.36 $\pm$ 0.41	0 $\pm$ 0	1371	NIST	
Unknown	S	3.84 $\pm$ 0.36	0 $\pm$ 0	1374		
$\alpha$ -Copaene	S	1.31 $\pm$ 0.11	6.02 $\pm$ 0.46	1380	Aloysia	
Unknown	S	0 $\pm$ 0	2.64 $\pm$ 0.2	1394		
Petasitene 1	S	50.71 $\pm$ 3.79	0 $\pm$ 0	1399	Petasites	CsTPS5
$\alpha$ -Cedrene	S	2.56 $\pm$ 0.65	0 $\pm$ 0	1418	NIST	
( <i>E</i> )- $\beta$ -Caryophyllene	S	358.02 $\pm$ 69.75	19.72 $\pm$ 1.51	1424	Pure standard	CsTPS4
$\beta$ -Copaene	S	0 $\pm$ 0	1.24 $\pm$ 0.12	1434	Aloysia	
( <i>E</i> )- $\alpha$ -Bergamotene	S	73.45 $\pm$ 14.08	0 $\pm$ 0	1439	Phoebe	CsTPS5
Petasitene 2/ Unknown	S	25.04 $\pm$ 3.75	0 $\pm$ 0	1449	MS	CsTPS5
Humulen	S	0 $\pm$ 0	13.23 $\pm$ 2.2	1459	Aloysia	CsTPS4
Humulene/( <i>E</i> )- $\beta$ -Farnesene	S	34.68 $\pm$ 3.1	0 $\pm$ 0	1459	Aloysia/Phoebe	CsTPS4/5
Petasitene 3	S	24.08 $\pm$ 2.39	0 $\pm$ 0	1464	MS	CsTPS5
cis-Muuroadiene	S	0 $\pm$ 0	2.11 $\pm$ 0.21	1468	NIST	
$\beta$ -Acoradiene	S	3.33 $\pm$ 0.24	0 $\pm$ 0	1472	NIST	CsTPS5
Daucadiene	S	404.11 $\pm$ 47.9	0 $\pm$ 0	1479	NIST	CsTPS5
Germacrene D	S	0 $\pm$ 0	302.89 $\pm$ 26.83	1486	NIST	
Unknown	S	47.12 $\pm$ 4.11	0 $\pm$ 0	1486		CsTPS5
Unknown		11.53 $\pm$ 1.04	0 $\pm$ 0	1489		
( <i>Z</i> ),( <i>E</i> )- $\alpha$ -Farnesene	S	0 $\pm$ 0	11.44 $\pm$ 5.51	1496	NIST	
Unknown		45.51 $\pm$ 3.45	0 $\pm$ 0	1492		
Bicyclogermacrene	S	0 $\pm$ 0	25.67 $\pm$ 2.98	1502	NIST	
$\alpha$ -Muurolene	S	2.92 $\pm$ 0.13	0 $\pm$ 0	1504	Aloysia	CsTPS1
$\alpha$ -Farnesene	S	0 $\pm$ 0	8.87 $\pm$ 1.86	1510	NIST	
$\beta$ -Bisabolene	S	2.82 $\pm$ 0.33	0 $\pm$ 0	1512	NIST	CsTPS5
( <i>Z</i> )- $\gamma$ -Bisabolene	S	2.75 $\pm$ 0.45	0 $\pm$ 0	1519	NIST	CsTPS5
$\delta$ -Cadinene	S	0.86 $\pm$ 0.08	2.12 $\pm$ 0.23	1528	Aloysia	
Germacrene D-4-ol		0 $\pm$ 0	2.39 $\pm$ 0.9	1582	NIST	
Unknown		66.45 $\pm$ 3.69	0 $\pm$ 0	1665		
Unknown		2281.68 $\pm$ 254.96	2.93 $\pm$ 0.47	1671		

Compound class is indicated if monoterpene (M) or sesquiterpene (S). Retention indices (RI) were calculated according to van den Dool and Kratz (1963). The compound identification was made by comparison of mass spectra only (MS), by comparison to MS and RI found in the NIST library (NIST) or by comparison of MS and RI to known compounds in plant extracts. Furthermore, the putative enzymes producing these compounds are listed (CsTPS)

**Table S2:** Primers used for cloning of *CsTPS* genes and for qRT-PCR. The reference gene primer pair used for the analysis is highlighted in grey.

Label	Sequence (5'...3')	Primer type	Comment
CsTPS1-IBA-F	ATGGTAGGTCTCAGCGCATGTCTTTTAAACAAGAAGATGTTATC	clonig	
CsTPS1-IBA-R	ATGGTAGGTCTCATATCAATTCATAGCATCAATGAGGAGAGAC	clonig	reverse primer for CsTPS1 & CsTPS2
CsTPS2-IBA-F	ATGGTAGGTCTCAGCGCATGTCTTTGAACGAGAAGATGTTAT	clonig	
CsTPS3-IBA-F	ATGGTACGTCTCAGCGCATGCCTCTTACACAAGAAGATGTTA	clonig	
CsTPS3-IBA-R	ATGGTACGTCTCATATCAATTAATAGCATTAAATGAAAAGAGATTTTA	clonig	
CsTPS4-IBA-F	ATGGTAGGTCTCAGCGCATGTCTCTTAAACAAGAAGAAGTTATT	clonig	
CsTPS4-IBA-R	ATGGTAGGTCTCATATCACAACAACTAATATCATGAACGAGCAAAG	clonig	
CsTPS5-IBA-F	ATGGTACGTCTCAGCGCATGCTAATATCAAGTAAATACATACATC	clonig	
CsTPS5-IBA-R	ATGGTACGTCTCATATCATATATCCATAGGATGAATGAGCAAAG	clonig	
CsTPS6-IBA-F	ATGGTACGTCTCAGCGCATGAATCTGATCGGTCTCAGATCG	clonig	
CsTPS6-IBA-R	ATGGTACGTCTCATATCATATATGCATAGGATGAATGAACAACG	clonig	
CsTPS7-IBA-F	ATGGTAGGTCTCAGCGCATGTCTTACAAAGTCTCAGTTGTTT	clonig	
CsTPS7-IBA-R	ATGGTAGGTCTCATATCACACGTTTATGGGATTCACGAGAAG	clonig	
CsTPS8-IBA-F	ATGGTAGGTCTCAGCGCATGTCAACTTTTCTGGTTTCTACTAA	clonig	
CsTPS8-IBA-R	ATGGTAGGTCTCATATCAAACGGGGCCGGGTGAACAAG	clonig	
CsACT-F-1	TGGCTTTGGGATTCAGTGCC	qRT-PCR	
CsACT-R-1	GGATGACATGGAAAAGATTTGGCA	qRT-PCR	
CsACT-F-2	TGAGTCATCTTCTCTGTGGC	qRT-PCR	
CsACT-R-2	CACACTTTCTACAACGAGCTCC	qRT-PCR	
CsEF1 $\alpha$ -F-1	GGCATCGATGACTGTGCAGT	qRT-PCR	
CsEF1 $\alpha$ -R-1	CATGGGTGCTCGACAAACTTA	qRT-PCR	
CsEF1 $\alpha$ -F-2	AGACATCTGGAGTGGGAGA	qRT-PCR	
CsEF1 $\alpha$ -R-2	TGACTGGTACAAGGGTCCAAC	qRT-PCR	
CsTPS1-F	AGTTTGGTTGGCATGGGTGA	qRT-PCR	
CsTPS1-R	GCCCGCCATATCATCCATGA	qRT-PCR	
CsTPS4-F	TGCATTCGTGAGTAGCGGTT	qRT-PCR	
CsTPS4-R	GCCTTTCGAGAGCCCATTTG	qRT-PCR	
CsTPS5-F	GGATGCGTGTGACCTCCTTT	qRT-PCR	
CsTPS5-R	GTGCCATGTTGACCACACAC	qRT-PCR	
CsTPS6-F	ATCGGTCTCAGATCGTCCCT	qRT-PCR	
CsTPS6-R	CGCTGATCCACTTCCCTACC	qRT-PCR	
CsTPS7-F	CAAATGGGCAAAGAGGGGC	qRT-PCR	
CsTPS7-R	CGACCAAATCGTCTTCGTGC	qRT-PCR	
CsTPS8-F	ATGGCCCAGGATCCTTGTTG	qRT-PCR	
CsTPS8-R	TGTAACGCACTGCCTTAGC	qRT-PCR	