Received: 22 October 2018 | Revised: 18 January 2019 | Accepted: 19 January 2019
DOI: 10.1111/pce.13532

ORIGINAL ARTICLE

Root volatiles in plant–plant interactions I: High root sesquiterpene release is associated with increased germination and growth of plant neighbours

Valentin Gfeller[1] | Meret Huber[2] | Christiane Förster[2] | Wei Huang[1,3] | Tobias G. Köllner[2] | Matthias Erb[1]

[1] Institute of Plant Sciences, University of Bern, 3013 Bern, Switzerland
[2] Department of Biochemistry, Max Planck Institute for Chemical Ecology, 07745 Jena, Germany
[3] Key Laboratory of Aquatic Plant and Watershed Ecology, Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan 430074Hubei, China

Correspondence
Matthias Erb, Institute of Plant Sciences, University of Bern, Altenbergrain 21, 3013 Bern, Switzerland.
Email: matthias.erb@ips.unibe.ch

Present Address
Meret Huber, Institute of Plant Biology and Biotechnology, University of Münster, Schlossplatz 7-8, 48143 Münster, Germany.

Funding information
European Commission FP7 People: Marie-Curie Actions, Grant/Award Number: 704334

Abstract

Volatile organic compounds (VOCs) emitted by plant leaves can influence the physiology of neighbouring plants. In contrast to leaf VOCs, little is known about the role of root VOCs in plant–plant interactions. Here, we characterize constitutive root VOC emissions of the spotted knapweed (*Centaurea stoebe*) and explore the impact of these VOCs on the germination and growth of different sympatric plant species. We show that *C. stoebe* roots emit high amounts of sesquiterpenes, with estimated release rates of (*E*)-β-caryophyllene above 3 μg g^{-1} dw hr^{-1}. Sesquiterpene emissions show little variation between different *C. stoebe* populations but vary substantially between different *Centaurea* species. Through root transcriptome sequencing, we identify six root-expressed sesquiterpene synthases (TPSs). Two root-specific TPSs, CsTPS4 and CsTPS5, are sufficient to produce the full blend of emitted root sesquiterpenes. VOC-exposure experiments demonstrate that *C. stoebe* root VOCs have neutral to positive effects on the germination and growth of different sympatric neighbours. Thus, constitutive root sesquiterpenes produced by two *C. stoebe* TPSs are associated with facilitation of sympatric neighbouring plants. The release of root VOCs may thus influence plant community structure in nature.

KEYWORDS
associational effects, neighbourhood effects, sesquiterpene synthase

1 | INTRODUCTION

Plants influence their environment to maximize their fitness. One strategy by which plants can manipulate their environment is to produce and release chemicals such as volatile organic compounds (VOCs; Pichersky & Gang, 2000). VOCs can, for instance, protect plants against biotic and abiotic stress (Gouinguené & Turlings, 2002; Loreto & Schnitzler, 2010; Peñuelas et al., 2014; Pichersky & Gershenzon, 2002). VOCs can also influence defence and growth of neighbouring plants (Karban, Yang, & Edwards, 2014; Kegge et al., 2015; Ninkovic, 2003; Pierik, Visser, de Kroon, & Voesenek, 2003). Although the benefits of VOC-mediated plant–plant interactions for the emitter are subject to debate (Heil, 2014; Morrell & Kessler, 2017), VOC-mediated plant–plant interactions are increasingly

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
© 2019 The Authors Plant, Cell & Environment Published by John Wiley & Sons Ltd

recognized to influence plant ecology in natural and agricultural systems (Ninkovic, Markovic, & Dahlin, 2016). Although most work on plant VOCs has focused on the phyllosphere, an increasing number of studies demonstrate that plant VOCs also have important roles in the rhizosphere. Root VOCs can, for instance, influence the behaviour of herbivorous insects (Robert et al., 2012) and nematodes (Rasmann et al., 2005) and affect soil bacterial and fungal communities (Kleinheinz, Bagley, St. John, Rughani, & McGinnis, 1999; Wenke, Kai, & Piechulla, 2010). In Petri dish experiments, root VOCs have also been shown to negatively affect seed germination and seedling growth (Ens, Bremner, French, & Korth, 2009; Jassbi, Zamanizadehnajari, & Baldwin, 2010). Whether root VOCs mediate plant–plant interactions under more realistic conditions remains to be determined (Delory, Delaplace, Fauconnier, & du Jardin, 2016).

With more than 30,000 different structures, terpenoids are the most diverse class of secondary metabolites in the plant kingdom (Hartmann, 2007) and are an integral part of plant VOC blends (Gershenzon & Dudareva, 2007). Most volatile terpenoids are hemiterpenes (C_5), monoterpenes (C_{10}), and sesquiterpenes (C_{15}; Nagegowda, 2010). Volatile terpenes have various ecological effects and function in plant–plant, plant–insect, and plant–microbe interactions (Cheng et al., 2007). Terpenoids are derived from two common C_5 precursor molecules, isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). In higher plants, IPP and DMAPP are formed through two different pathways, the mevalonic acid and the methylerythritol phosphate pathway. IPP and DMAPP are then further converted into geranyl diphosphate and farnesyl diphosphate (FPP) as precursors for monoterpenes and sesquiterpenes, respectively. The reaction for the final conversion to monoterpenes and sesquiterpenes is catalysed by terpene synthases (TPSs), which require a divalent cation to mediate the terpene formation (Cheng et al., 2007; Nagegowda, 2010). As key enzymes for the production of terpenes, TPSs have been characterized in plants (Degenhardt, Köllner, & Gershenzon, 2009; Jia, Köllner, Gershenzon, & Chen, 2018), insects (Beran et al., 2016), fungi (Quin, Flynn, & Schmidt-Dannert, 2014), bacteria (Yamada et al., 2015), and amoebae (Chen et al., 2016). In plants, it is known that TPS expression can be regulated in a tissue specific manner. Furthermore, TPSs often catalyse the formation of multiple products, which contributes to the substantial structural diversity of terpenoids (Tholl, 2006).

In this study, we characterize root VOCs emitted by the spotted knapweed (*Centaurea stoebe*). The tetraploid cytotype of *C. stoebe* is invasive in northern America (Treier et al., 2009), whereas the diploid cytotype is classified as threatened (vulnerable) in Switzerland according to the International Union for Conservation of Nature. A previous study found that *C. stoebe* root chemicals affect the physiology of *Taraxacum officinale* agg. roots and their suitability for root feeding *Melolontha melolontha* larvae (Huang, Zwimpfer, Hervé, Bont, & Erb, 2018). As no direct root contact was needed to trigger these effects, we hypothesized that *C. stoebe* may affect neighbouring plants through the release of root VOCs. In this study, we analyse the volatile blend of *C. stoebe* roots and identify sesquiterpenes as dominant root VOCs. Through root transcriptome sequencing and heterologous expression, we identify TPSs that are associated with this phenotype. Furthermore, we assess the impact of *C. stoebe* roots on the germination and growth of different sympatric plant species. This work thus sheds light on the genetic basis and ecological consequences of VOC-mediated plant-plant interactions below ground. The results of this study also provide a mechanistic basis to determine the impact of *C. stoebe* root sesquiterpenes on *T. officinale* and its interaction with *M. melolontha* larvae (Huang et al., 2019).

2 | METHODS AND MATERIALS

2.1 | Study system

C. stoebe L. (diploid) plants were grown from seeds purchased from UFA-SAMEN (Winterthur, Switzerland), unless specified otherwise. Seeds of *Anthemis tinctoria* L., *Centaurea scabiosa* L., *Centaurea jacea* L., *Cichorium intybus* L., *Daucus carota* L., *Dianthus carthusianorum* L., *Echium vulgare* L., *Festuca valesiaca* Gaudin, *Ranunculus bulbosus* L., and *T. officinale* agg were obtained from the same vendor. *Medicago sativa* L. was obtained from Sativa Rheinau AG (Rheinau, Switzerland) and *Cardaria draba* (L.) Desv. was obtained from Templiner Kräutergarten (Templin, Germany). *Centaurea valesiaca* (DC.) Jord. seeds were collected from a natural population in Raron (VS, Switzerland) and provided by Adrian Möhl (Info Flora) and Markus Fischer (University of Bern). Two *C. stoebe* populations Hu-11 (tetraploid, Hungary) and Ro-11 (tetraploid, Romania), as well as *Koeleria macrantha* (Ledeb.) Schult. (MT, USA), were provided by Yan Sun and Heinz Müller-Schärer (University of Fribourg). Detailed information on these *C. stoebe* populations can be found in Mráz et al. (2012). Plant growth conditions are described in the corresponding experimental sections below.

2.2 | Characterization of *C. stoebe* root volatiles

To determine root volatile release by *C. stoebe*, plants were grown individually in sand under controlled conditions in a growth chamber (day length: 16 hr; temperature: 20–22°C; humidity: 65%) for 7 weeks. Every 1 to 3 days, the plants were watered, and once a week, a nutrient solution (0.1% [*w/v*]; Plantaaktiv Typ K, Hauert, Grossaffoltern, Switzerland) was supplied. The root system of each plant was washed, separated from the shoot with a scalpel and dried with a paper towel (*n* = 8). Subsequently, the roots were weighted, and the cut at the root-shoot junction was sealed with Teflon tape before analysis to avoid contamination of the headspace with wound-released VOCs. The roots where then carefully inserted into 20-ml screw top glass vials (Gerstel, Sursee, Switzerland) and closed with airtight screw caps (septum Silicone/PTFE; Gerstel, Sursee, Switzerland). The vials were incubated for 1 min at 20°C. Volatiles were then collected by exposing an Solid Phase Microextraction (SPME) fibre (coated with 100-μm polydimethylsiloxane; Supelco, Bellefonte, PA, USA) to the headspace for 1.8 s. Volatiles were thermally desorbed (220°C for 1 min) in the inlet of an Agilent 7820A series gas chromatography (GC) coupled to an Agilent 5977E MSD (source 230°C, quadrupole 150°C, ionization potential 70 eV, scan range 30–550; Palo Alto, CA, USA). After each run, the SPME fibre was baked out for 2 min at 220°C. VOCs were

separated on a capillary gas chromatography–mass spectrometry (GC-MS) column (HP5-MS, 30 m, 250-μm ID, 2.5-μm film; Agilent Technologies, Palo Alto, CA, USA) with He as carrier gas at a flow rate of 1 ml min^{-1}. Initial column temperature was set to 60°C for 1 min followed by three temperature gradients: (a) 7°C min^{-1} to 150°C, (b) 3°C min^{-1} to 165°C, and (c) 30°C min^{-1} to 250°C and hold at this temperature for 3 min. VOCs were tentatively identified by comparing mass spectra to library entries of the National Institute of Standards and Technology (NIST 14). (*E*)-β-caryophyllene was identified by comparing mass spectrum and retention time to a synthetic standard (≥98.5%, Sigma-Aldrich, Buchs SG, Switzerland). The first eluting petasitene was cross-validated by comparing mass spectra and retention times with a petasitene peak detected in a *Petasites hybridus* (L.) G. Gaertn. & al. root extract (Saritas, von Reuss, & König, 2002). The other petasitene-like sesquiterpenes were tentatively identified by comparing mass spectra to petasitene from *P. hybridus*.

2.3 | Quantification of terpene emissions

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

2.4 | Hexane tissue extraction and analysis

To analyse the composition and abundance of VOCs in *C. stoebe* root and leaf extracts, plants were grown in soil (7: 20 mixture of Klasmann Tonsubstrat and Klasmann Kultursubstrat TS1; Klasmann-Deilmann, Geeste, Germany) in a greenhouse (light: 14 hr; temperature: day 21–23°C night 19–21°C; humidity: 50–60°C) for 10 weeks. Plants were watered as needed to keep the soil moist. No fertilizer was added. Tissue samples were obtained by washing the roots and leaves, drying them with paper towel and wrapping root and leaf tissue separately into aluminum foil, flash freezing them in liquid nitrogen, and storing them at −80°C. All samples were ground with mortar and pestle under liquid nitrogen, and approximately 100 mg of frozen tissue powder per sample was put into a 1-ml glass vial; 1 ml of hexane with nonyl acetate as internal standard (10 ng * μl^{-1}) was immediately added to the samples (*n* = 10 for each tissue). The samples were shaken at 200 rpm for 1 hr at room temperature, followed by a centrifugation step of 20 min at 5,300 rpm; 600 μl of supernatant per sample was pipetted into new tubes and stored at −20°C. Characterization of VOCs in the extracts was carried out on an Agilent 6890 series GC coupled to an Agilent 5973 mass selective detector (source 230°C, quadrupole 150°C, ionization potential 70 eV; Palo Alto, CA, USA) and a flame ionization detector operating at 300°C. He (MS) and H_2 Flame Ionization Detector (FID) were used as carrier gases. The VOC separation took place on a DB-5MS capillary column (Agilent, Santa Clara, CA, USA, 30 m × 0.25 mm × 0.25 μm). After injection of 1 μl of tissue extract, the following temperature programme was run: Initial temperature of 45°C was hold for 2 min followed by two temperature ramps, (a) 6°C min^{-1} to 180°C and (b) 100°C min^{-1} to 300°C and hold for 2 min. For volatile quantification, the peak areas of the GC-FID chromatograms were integrated. The area of each compound was taken relative to the area of the internal standard and corrected for the weight of the extracted tissue. For compound identification, root and leaf samples were also run on the GC-MS. In parallel, an n-alkane standard solution was run with the same method, which enabled to calculate the linear retention indices (RI) following the procedure published by Van den Dool and Kratz (1963). Tentative identification was carried out by comparing mass spectra and RI of a given peak to known compounds in plant extracts of *Aloysia sellowii* (Briq.) Moldenke and *Phoebe porosa* (Nees & Mart.) Mez., which were kindly provided by Prof. W. A. König, University of Hamburg. For compounds not found in these plant extracts, mass spectra and RI were matched to the library entries of the National Institute of Standards and Technology (NIST 14). Corresponding RI can be found in Table S1. Daucadiene was tentatively identified by comparison to the mass spectra in the NIST library. Although the mass spectra showed high similarity, the RI was not as described for the best match to the NIST library (trans-dauca-4(11),8-diene), suggesting that the detected compound might be another daucadiene diastereoisomer.

2.5 | Terpene emission of *C. stoebe* populations and related species

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

Centaurea, were grown in sand under controlled conditions (day length: 16 hr; temperature: 20–22°C; humidity: 65%) for 5 weeks. Every 1 to 3 days, the plants were watered, and once a week, a nutrient solution (0.1% (*w*/*v*); Plantaaktiv Typ K, Hauert, Grossaffoltern, Switzerland) was supplied. Two tetraploid populations (Hu-11 and Ro-11) and one diploid population (UFA) were compared (n = 5–7). As congeneric species, *C. jacea*, *C. scabiosa*, and *C. valesiaca*, which grow in distinct habitats, were used (Landolt et al., 2010; n = 4–8). Roots were prepared as described above for VOC characterization. Fresh biomass of roots and leaves were also determined. The glass vials containing the roots were immediately stored on a cooling block at 2°C of an autosampler system (Multi Purpose Sampler (MPS); Gerstel, Sursee, Switzerland) connected to the GC-MS system. Immediately prior to analysis, the samples were transferred to an incubator set to 30°C for 15 s, in which VOCs were subsequently collected by exposition of an SPME fibre to the headspace for 1.8 s. Next, the compounds were analysed on the GC-MS system as mentioned above for VOC characterization.

2.6 | Transcriptome sequencing and analysis

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

2.7 | Sequence analysis and tree reconstruction

Multiple sequence alignment of the identified *TPS* genes from *C. stoebe* and characterized *TPS* genes from *M. recutita* was computed using the MUSCLE codon algorithm implemented in MEGA6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Based on the alignment, a tree was reconstructed with MEGA6 using a maximum likelihood algorithm (General Time Reversible (GTR) model). Codon positions included were first + second + third + noncoding. All positions with <80% site coverage were eliminated. A bootstrap resampling analysis with 1,000 replicates was performed to evaluate the topology of the generated tree.

2.8 | Cloning and heterologous expression of CsTPS genes

To evaluate the TPS activity of the putative CsTPS genes, cDNA was produced. Then, focal genes were cloned into an expression vector and heterologously expressed in *Escherichia coli*. Subsequently, proteins were isolated and used for enzyme activity assays. To obtain plant material for RNA extraction, *C. stoebe* plants were grown in sand under controlled conditions (day length: 16 hr; temperature: 20–22°C; humidity: 65%) for 8 weeks. Every 1 to 3 days, the plants were watered, and once a week, a nutrient solution (0.1% [*w*/*v*]; Plantaaktiv Typ K, Hauert, Grossaffoltern, Switzerland) was supplied. Roots were gently washed, dried with a paper towel, cut 2 mm below root initiation, wrapped in aluminium foil, and immediately flash frozen in liquid nitrogen. Afterwards, roots were ground with mortar and pestle under constant cooling with liquid nitrogen and stored at −80°C before further processing. RNA extraction was carried out according to the manufacturer's protocol with an innuPrep Plant RNA Kit (Analytik Jena, Jena, Germany). For cDNA synthesis, 2 μg of RNA was treated with DNAse (Thermo scientifics, CA, USA). First-strand DNA was synthesized with oligo dT_{12-18} primers and Super Script™ III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The open reading frames of the putative *C. stoebe* TPSs were amplified with the primer pairs listed in Table S2 and cloned into a pASK-IBA37plus plasmid (IBA-Lifesciences, Göttingen, Germany) by restriction digest and ligation. NEB 10-beta competent *E. coli* cells (New England Biolabs, Ipswich, MA, USA) were then transformed with these vectors. In order to obtain the cloned *CsTPS* sequences and to check the transformation events, the inserted fragments were sequenced by Sanger sequencing.

For heterologous expression, NEB 10-beta cells containing the *CsTPS* constructs were grown at 37°C to an OD_{600} of 0.8. Subsequently, protein expression was induced by adding anhydrotetracycline (IBA-Lifesciences, Göttingen, Germany) to a final concentration of 200 ng * ml^{-1}. Expression took place for 18 hr at 18°C. Cells were harvested by centrifugation and resuspended in assay buffer (10-mM Tris HCl, 1-mM DTT, and 10% [vol/vol] glycerol [pH 7.5]). To disrupt the cells, they were treated 4 × 20 s at 60% power with a sonicator (Bandelin Sonoplus HD 2070, Berlin, Germany). Samples were then centrifuged at 4°C for 1 hr at 14,000 *g* to separate the soluble proteins from cell debris. A further purification was made by passing the proteins through an illustra NAP-5 column (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK).

Enzyme activity assays were performed to test the terpene production of the different CsTPS. Activity assays were carried out by adding 50 μl of assay buffer and 50 μl of purified crude bacterial protein extract with 10-mM $MgCl_2$ and 10-μM (*E*,*E*)-FPP into a threaded 1-ml glass vial with a cap containing a Teflon septum. The reaction mix was incubated for 1 hr at 30°C. During the incubation period, VOCs were sampled with an SPME fibre. For volatile analysis, the collected volatiles were desorbed directly in the inlet (240°C) of the GC-MS system. An Agilent 6890 series GC coupled to an Agilent 5973 MSD (source 230°C, quadrupole 150°C, ionization potential 70 eV; Palo Alto, CA,

USA) was used for analysis. He was used as carrier gas at a rate of 1 ml * min^{-1}. The volatile separation took place on a DB-5MS capillary column (Agilent, Santa Clara, CA, USA, 30 m × 0.25 mm × 0.25 μm). The initial oven temperature of 80°C was hold for 2 min, followed by a ramp of 7°C min^{-1} to 180°C and a second ramp of 100°C min^{-1} to 300°C where the temperature was held for 1 min.

2.9 | qRT-PCR analysis of CsTPS genes

To determine the expression levels of individual *CsTPS* genes, RNA was extracted, converted into cDNA, and further used for qRT-PCR. Total RNA was isolated from the same root and leaf tissue samples as for hexane extraction. This was made following the InviTrap® Spin Plant RNA Mini Kit (Stratec molecular, Berlin, Germany). Next, 1 μg of the RNA was DNase I treated followed by first-strand cDNA synthesis using RevertAid H Minus Reverse Transcriptase with oligo $(dT)_{18}$ primers (Thermo scientific, CA, USA). cDNA was diluted 1:10 before used for qRT-PCR. To find an appropriate reference gene, *actin1* and *EF1α* sequences of *Arabidopsis thaliana* were taken as query for a screen in the *C. stoebe* Trinity assembly with the software Blast2GO 4.1 (Götz et al., 2008) running at default settings. Two primer combinations were designed for each homologous reference gene. *EF1α* was found to be the most robust reference gene. Next, for each of the *CsTPS* genes, a qPCR primer pair was designed. All primers are listed in Table S2. Primer specificity was tested by means of melting curve analysis and gel electrophoresis. qRT-PCR was carried out on a LightCycler® 96 Instrument (Roche, Basel, Switzerland) using the KAPA 480 SYBR FAST qPCR Master Mix (Kapa Biosystems, Boston, USA). Primer efficiency was determined using a linear standard curve approach. For very low expressed genes, this was repeated with samples spiked with plasmids containing the genes of interest. Biological replicates were all run in technical triplicates. Three samples had to be excluded from the analysis due to poor RNA quality or very low expression of the reference gene, resulting in a total of seven biological replicates for *CsTPS4* as well as *CsTPS5* and five biological replicates for *CsTPS1*. Relative transcript abundance was analysed as fold change ($2^{-\Delta Ct}$). As *CsTPS1* showed dissimilar melting peaks for root and shoot PCR amplicons, the fragments were subsequently sequenced by Sanger sequencing.

2.10 | Impact of *C. stoebe* root VOCs on neighbouring plants

To evaluate the influence of *C. stoebe* root volatiles on the germination and growth of neighbouring plants, we used an experimental set-up that excluded direct root contact or the transfer of exudates, but allowed *C. stoebe* root VOCs to diffuse to the neighbouring plants. The system consisted of mesh cages (12 × 9 × 10 cm, length × width × height) made of Geotex fleece (Windhager, Austria), which were placed in pairs into rectangular plastic pots (Figure 4a). A covered airgap between the cages allowed for the diffusion of VOCs between the rhizospheres of plants growing in the soil-filled mesh cages. Water was supplied carefully to soil in the mesh cages to avoid leaching and exchange of root exudates across the airgap. The Geotex fleece of the mesh cages was sufficient to stop roots from growing out of the mesh cages, thus eliminating direct root contact between the plants. Diffusion of *C. stoebe* VOCs into the airgap was confirmed by SPME (Huang et al., 2019). Plants for this experiment were grown in a greenhouse (light: 14 hr; temperature: day 16–24°C, night 16–22°C, mean temperature over growth period 20°C; humidity: 30–60°C) in potting soil consisting of five parts "Landerde" (RICOTER, Aarberg, Switzerland), four parts "Floratorf" (Floragard, Oldenburg, Germany), and one part sand ("Capito" 1–4 mm, LANDI Schweiz AG, Dotzigen, Switzerland). The "sender" mesh cages in the plastic pots where either left plant free or planted with 3-week-old *C. stoebe* seedlings. After 25 days, different plant species were planted into the "receiver" mesh cages (10 seeds per cage, n = 12 for each species). As receiver species, 11 commonly co-occurring species of *C. stoebe* were selected: *A. tinctoria*, *C. draba*, *C. stoebe*, *C. intybus*, *D. carthusianorum*, *E. vulgare*, *F. valesiaca*, *K. macrantha*, *M. sativa*, *R. bulbosus*, *D. carota*, and *T. officinale* agg. Every 1 to 3 days, the plants were watered, and once a week, a nutrient solution (0.1% [*w/v*]; Plantaaktiv Typ K, Hauert, Grossaffoltern, Switzerland) was supplied. Pots were turned 180° and randomized fortnightly. Potential bias through above ground effects of *C. stoebe* was ruled out by arranging the pots on the table so that each receiver had a *C. stoebe* as neighbour either only above ground in a separate pot (control) or above-ground and belowground in the same pot (treatment). The total number of germinated seeds was recorded after 4 weeks. The first germinated seedling was retained; all the others were removed. After 9 weeks of growth, the plants were harvested. Roots and leaves were washed, separated, and dried at 80°C until constant weight to determine dry mass.

2.11 | Data analysis

Statistical assumptions such as normal distribution and homoscedasticity of error variance were checked and square root or $\log_e$ transformed if the assumptions were not met. Differences in relative peak area per g FW between root and leaf tissue in hexane extracts were tested with a Wilcoxon signed rank test. To test for differences in sesquiterpene abundance among *C. stoebe* populations and *Centaurea* species for a given compound, analysis of variance (ANOVA) of a fitted linear model was performed and if significant followed by Least-Square (LS) means pairwise comparisons with P value adjustment. Differences in expression levels between root and leaf tissue were tested by Wilcoxon signed rank tests. A possible effect of the emitter on the germination was analysed by fitting a generalized linear model with a quasibinomial distribution to the data and performing an ANOVA (n = 12 per species and treatment). Dry biomass of roots and leaves were investigated by fitting a generalized linear model (family: gamma, link: inverse) and conducting an ANOVA (n = 12 per species and treatment, nine out of 244 plants died and were therefore excluded from the analysis). For each species, the

effect of the emitter plant on biomass production was tested by means of a Student's *t* test followed by *P* value correction for multiple comparison (Benjamini & Hochberg, 1995). Statistical analysis and data visualization were conducted with R 3.4.3 (R Core Team, 2017), with "lsmeans," "car," "plyer," and "ggplot2" packages (Fox & Weisberg, 2011; Lenth, 2016; Wickham, 2009, 2011).

3 | RESULTS

3.1 | Characterization of *C. stoebe* VOCs

Analysis of the volatile blend emitted by *C. stoebe* roots revealed an abundant sesquiterpene fraction (Figure 1a) with (*E*)-β-caryophyllene and daucadiene (most likely a diastereoisomere of trans-dauca-4(11),8-diene) as the predominant compounds. The sesquiterpenes (*E*)-α-bergamotene, humulene, (*E*)-β-farnesene, three putative petasitene isomers (petasitene 1–3), and an unidentified sesquiterpene (Unknown 5) were emitted as well. (*E*)-β-caryophyllene emission was quantified at 3.15 ± 0.69 μg g^{-1} dw hr^{-1} (mean ± *SE*). Hexane root tissue extracts contained comparable sesquiterpene profiles, with (*E*)-β-caryophyllene and daucadiene as major compounds (Figure 1b). Additionally, low quantities of other sesquiterpenes such as cyclosativene, β-acoradiene, and β-bisabolene were found in these extracts, which were not detected in the volatile blend of *C. stoebe* roots. Besides sesquiterpenes, there were other compounds eluting from the column, mostly at later time points. The most abundant of these compounds showed a terpenoid-like structure and was tentatively identified as a sesquiterpene lactone (Unknown 9, m/z = 232). The other late eluting analytes were neither known nor present in the volatile blend of *C. stoebe* roots and therefore not analysed further. Sesquiterpenes were much more abundant in root hexane extracts than leaf extracts (Figure 1b). Only four compounds were detected in both leaves and roots, namely, α-copaene, (*E*)-β-caryophyllene, δ-cadinene, and the putative sesquiterpene lactone (Unknown 9). (*E*)-β-caryophyllene and the putative sesquiterpene lactone (Unknown 9) were present in much higher concentrations in the roots than the leaves (Wilcoxon signed rank test: n = 10, P = 0.002), whereas α-copaene and δ-cadinene were more abundant in the leaves (Wilcoxon signed rank test: n = 10, P = 0.002). In contrast to root tissue, we also detected three monoterpenes in *C. stoebe* leaves: α-pinene, β-myrcene, and an unknown monoterpene (Unknown 1). Compared with sesquiterpenes, monoterpene signals were low in abundance.

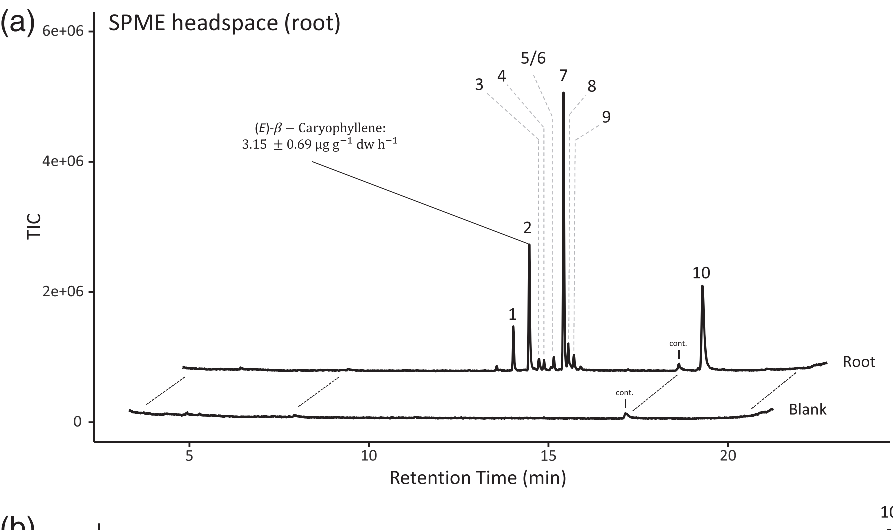
3.2 | Emission pattern of *C. stoebe* populations and other *Centaurea* species

Sesquiterpenes released by roots of three different *C. stoebe* populations did not differ significantly in quality and quantity (Figure 2a), suggesting that this trait is conserved within *C. stoebe*. By contrast, congeneric *Centaurea* species emitted distinct terpene bouquets compared with *C. stoebe* (Figure 2b). The volatile blend of the closely related *C. valesiaca* was most similar to *C. stoebe*, with petasitene 1, petasitene 2, and daucadiene being emitted in lower quantities by *C. valesiaca* than by *C. stoebe*. *C. jacea* emitted sesquiterpenes similar to *C. stoebe* but in different quantities: The release of petasitene 1, petasitene 3, (*E*)-α-bergamotene, and of an unknown compound was significantly increased in *C. jacea* compared with *C. stoebe*. Finally, we detected (*E*)-β-caryophyllene and (*E*)-α-bergamotene, but not any of the other sesquiterpenes in the headspace of *C. scabiosa* roots. No significant differences in biomass accumulation was found between the different species (Figure S1). Thus, sesquiterpene release from the roots seems to be conserved in *C. stoebe* ecotypes but varies qualitatively and quantitatively between different *Centaurea* species.

3.3 | TPSs of *C. stoebe*

To understand the genetic basis of sesquiterpene formation in *C. stoebe* roots, known sequences of *M. recutita* TPSs were used to find homologous genes in the *C. stoebe* root transcriptome. This led to the identification of eight potential sesquiterpene synthases (CsTPSs, Figure 3a). Apart from CsTPS2 and CsTPS3, for which open reading frame amplification and transformation into *E. coli* were unsuccessful, all TPSs were successfully cloned and expressed in *E. coli*. CsTPS protein activity assays showed that CsTPS1, CsTPS4, CsTPS5, CsTPS7, and CsTPS8 exhibit sesquiterpene synthase activity. No vactivity was found for CsTPS6 (Figure 3b–g). CsTPS1 catalysed the formation of α-muurolene, and CsTPS4 produced (*E*)-β-caryophyllene and humulene. CsTPS5 produced daucadiene as main compound and (*E*)-α-bergamotene, (*E*)-β-farnesene, three petasitenes, β-acoradiene, β-bisabolene, (*Z*)-γ-bisabolene, as well as an unknown sesquiterpene as byproducts. All the compounds produced by CsTPS1, CsTPS4, and CsTPS5 were found in hexane root extracts of *C. stoebe*. Furthermore, the compounds produced by CsTPS4 and CsTPS5 cover all highly emitted volatiles from *C. stoebe* roots. Comparison of RI and mass spectra revealed that CsTPS7 produced (*E*)-α-bisabolene (RI 1545) and CsTPS8 produced α-zingiberene (RI 1497) as main compounds. The two compounds were not detected in tissue extracts or the headspace of *C. stoebe* roots.

The predominant sesquiterpenes, (*E*)-β-caryophyllene and daucadiene, are produced in high amounts in the roots but only present in trace amounts, if at all, in the leaves (Figure 3i,k). The same pattern was found for the expression of *CsTPS4* and *CsTPS5*, the two TPSs putatively responsible for the production of these VOCs (Figure 3h,j). The mRNA levels in root compared with leave tissue revealed a 7.5-fold increase in *CsTPS4* (Wilcoxon signed rank test: n = 7, P = 0.016) and a >5,000-fold increase for *CsTPS5* (Wilcoxon signed rank test: n = 7, P = 0.016). Low expression of *CsTPS1* was detected in the leaves and roots. Melting point analysis indicated that different fragments were amplified in the different tissues. Fragment sequencing revealed that the root fragment corresponds to *CsTPS1*, whereas the leaf fragment only showed 89% sequence similarity to *CsTPS1*. No other sequence in the *C. stoebe* root transcriptome besides *CsTPS1* was found to match the leaf fragment, suggesting that it may stem from a *TPS* gene that is specifically expressed in the leaves.



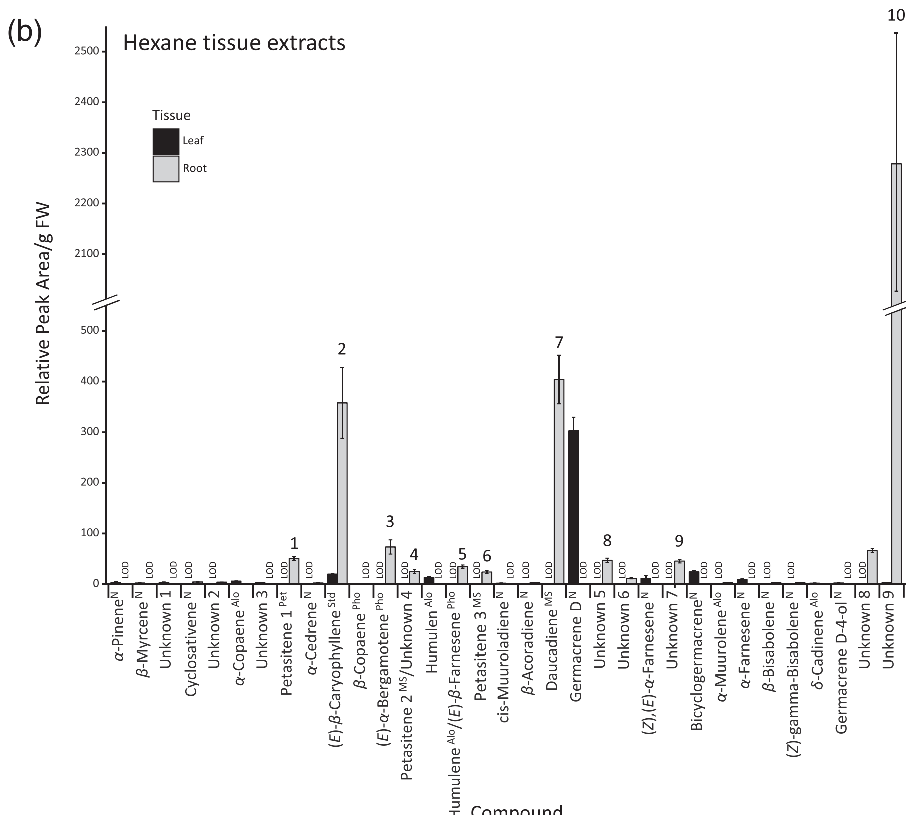


FIGURE 1 *Centaurea stoebe* roots release high amounts of sesquiterpenes. (a) Representative SPME-GC-MS chromatogram of volatile organic compounds emitted by *C. stoebe* roots. (*E*)-β-caryophyllene emission rate is displayed as mean ± *SE* (*n* = 8; dw, dry weight). (b) Relative peak area per g fresh weight (FW) of compounds found in hexane tissue extracts shown as mean ± *SE* (*n* = 10). TIC: total ion current; 1: petasitene 1; 2: (*E*)-β-caryophyllene; 3: (*E*)-α-bergamotene; 4: petasitene 2; 5: humulene and (*E*)-β-farnesene; 6: petasitene 3; 7: daucadiene; 8: Unknown 5 (sesquiterpene); 9: Unknown 7 (nonterpenoid); 10: Unknown 9 (sesquiterpene lactone-like compound); cont: contamination; LOD: below limit of detection; Identification: N: NIST library, comparison of mass spectra and retention index (RI); MS: inspection of mass spectra (RI other than literature); Std: comparison of mass spectra an RI with pure standard compound; and comparison of mass spectra an RI with known compounds of Alo: *Aloysia sellowii*; Pet: *Petasites hybridus*; Pho: *Phoebe porosa*; GC-MS: gas chromatography–mass spectrometry; SPME: solid phase micro extraction

3.4 | Effect of *C. stoebe* root volatiles on neighbouring plants

To test whether *C. stoebe* root VOCs influence the germination and performance of neighbouring plants, we exposed seeds and germinating plants of different sympatric species to *C. stoebe* rhizosphere VOCs for several weeks. Across all species, a positive effect of *C. stoebe* root VOCs on the germination of the different sympatric plant species was observed (*P* = 0.03, Figure 4b). Furthermore, 9 weeks after sowing, root biomass (*P* = 0.03, Figure 4c) and leaf

biomass (P = 0.04, Figure 4d) were significantly increased in the presence of *C. stoebe* root VOCs.

4 | DISCUSSION

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

Plants can release terpenoids constitutively or in response to environmental stress (Keeling & Bohlmann, 2006). Our headspace analyses show that *C. stoebe* releases sesquiterpenes specifically and constitutively from its roots. The emission rate of the sesquiterpene (*E*)-β-caryophyllene was measured at 3.15 ± 0.69 μg g^{-1} dw hr^{-1} (mean ± SE), leading to a situation where 2 s of exposure to a few mg of *C. stoebe* roots already saturated our analytical equipment. For comparison, (*E*)-β-caryophyllene release from herbivore-attacked maize roots is likely in the lower ng range per plant (Hiltpold, Erb, Robert, & Turlings, 2011). Only few studies so far provide absolute quantification of root VOC emission rates, and we are not aware of any report showing below ground sesquiterpene release rates at the levels reported here. Monoterpenes have been shown to be released in substantial quantities by roots. *Pinus pinea* roots, for instance, release monoterpenes at rates up to 26 ± 5 μg g^{-1} dw hr^{-1} (mean ± *SE*; Lin, Owen, & Peñuelas, 2007). Thus, *C. stoebe* constitutively releases relatively high amounts of sesquiterpenes from its roots.

Terpenoids are produced by TPSs (Bohlmann, Meyer-Gauen, & Croteau, 1998). We identified two CsTPSs whose products correspond to the root-emitted sesquiterpenes in *C. stoebe*. (*E*)-β-caryophyllene occurs in many plant species, and it has been reported several times to be produced by the same TPS as humulene (Cai et al., 2002; Irmisch et al., 2012; Köllner et al., 2008; Yang et al., 2013). In *C. stoebe*, we also found these two compounds to be produced by the same TPS (CsTPS4). Examining the expression level of *CsTPS4* in roots and leaves of *C. stoebe* showed the same pattern as the distribution of the compound: low quantities of RNA and (*E*)-β-caryophyllene in leaves and significantly higher quantities of both in roots. The second TPS involved in producing the volatile bouquet is CsTPS5 with daucadiene as main product. Enzyme activity assays of this enzyme led to the production of several sesquiterpenes, all of

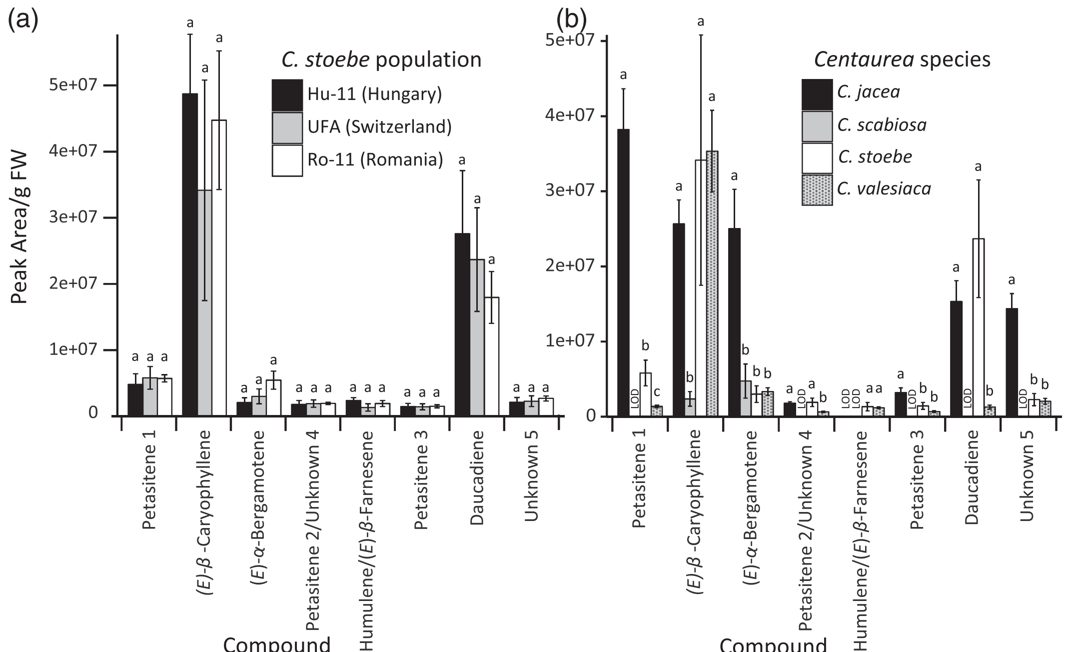


FIGURE 2 Root sesquiterpene release is conserved within *Centaurea stoebe* but varies between different *Centaurea* species. (a) Peak area per g fresh weight (FW) of *C. stoebe* populations shown as mean ± *SE* (n = 5; except for Hu-11, n = 7). Letters show significant differences among populations within one compound (analysis of variance followed by pairwise comparison of LS means, p_{adj} < 0.05). (b) Peak area per g FW of *Centaurea* species shown as mean ± *SE* (*Centaurea jacea* and *Centaurea scabiosa*, n = 8; *C. stoebe*, n = 5; *Centaurea valesiaca*, n = 4). Letters show significant differences among species within one compound (analysis of variance followed by pairwise comparison of LS means, p_{adj} < 0.05). LOD: below limit of detection

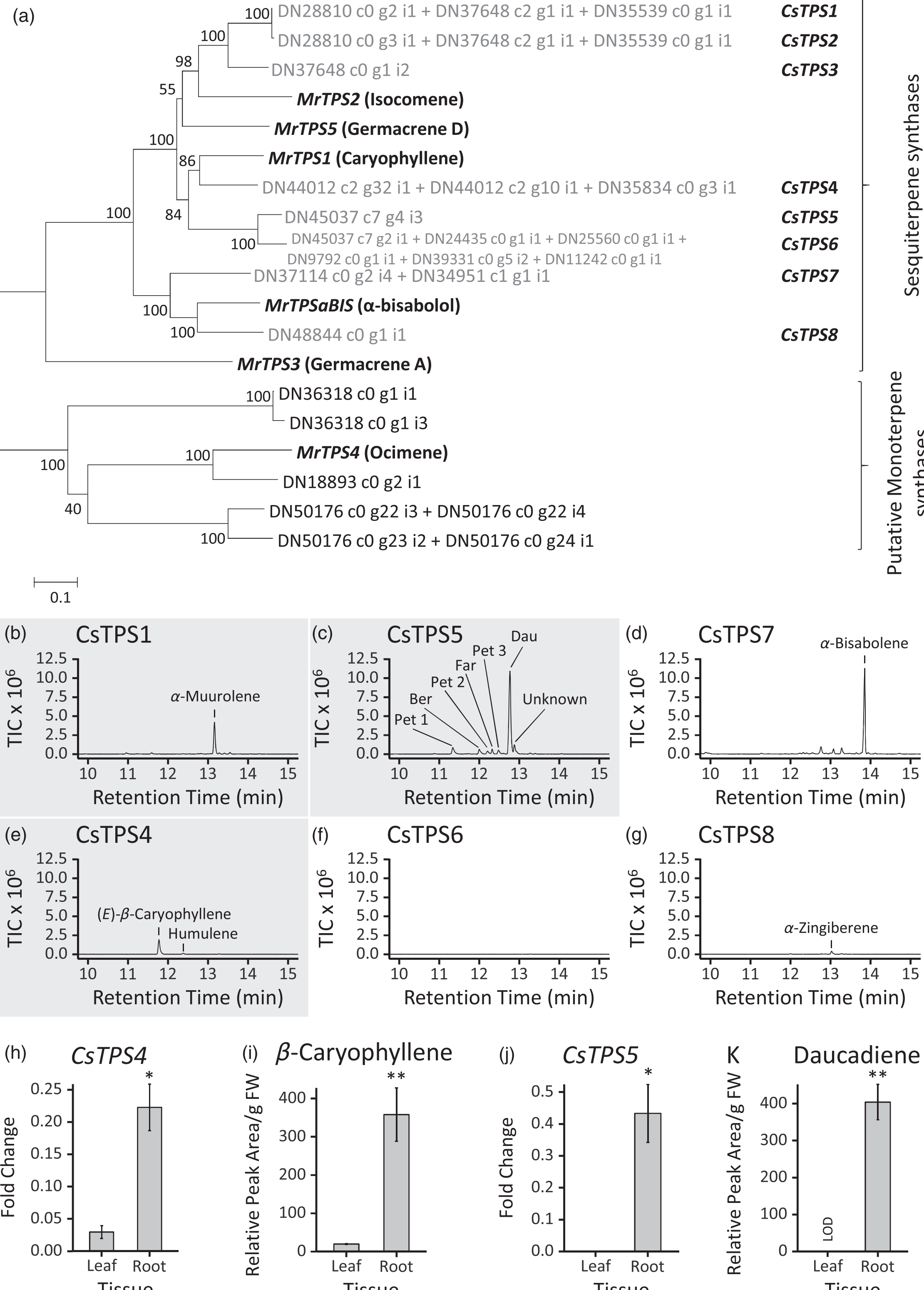


FIGURE 3 Two terpene synthases account for major *Centaurea stoebe* root sesquiterpenes. (a) To find potential *C. stoebe* terpene synthases (CsTPSs), sequences of *Matricaria recutita* terpene synthases (MrTPS) were taken to screen for homologous genes in the *C. stoebe* root transcriptome. The phylogenetic tree shows contigs of potential *CsTPS*s as end nodes and their related *MrTPS* genes. (b–g) SPME-GC-MS analysis of CsTPS protein activity assays with (*E,E*)-FPP as substrate. Compounds of highlighted chromatograms (b,c,e) were also found in *C. stoebe* hexane root extracts. mRNA abundance for *CsTPS4* (h) and *CsTPS5* (j) and relative peak area per g fresh weight (FW) of their main products (*E*)-β-caryophyllene (i) and daucadiene (k) in hexane root extracts. Shown are mean ± *SE* (qRT-PCR, n = 7; Tissue extracts, n = 10). Differences in means were tested by Wilcoxon signed rank tests, levels of significance: $^{**}P < 0.01$; $^{*}P < 0.05$. TIC: total ion current; Pet: petasitene; Be:, (*E*)-α-bergamotene; Far: (*E*)-β-farnesene; Dau: daucadiene; LOD: below limit of detection; GC-MS: gas chromatography–mass spectrometry; SPME: solid phase micro extraction

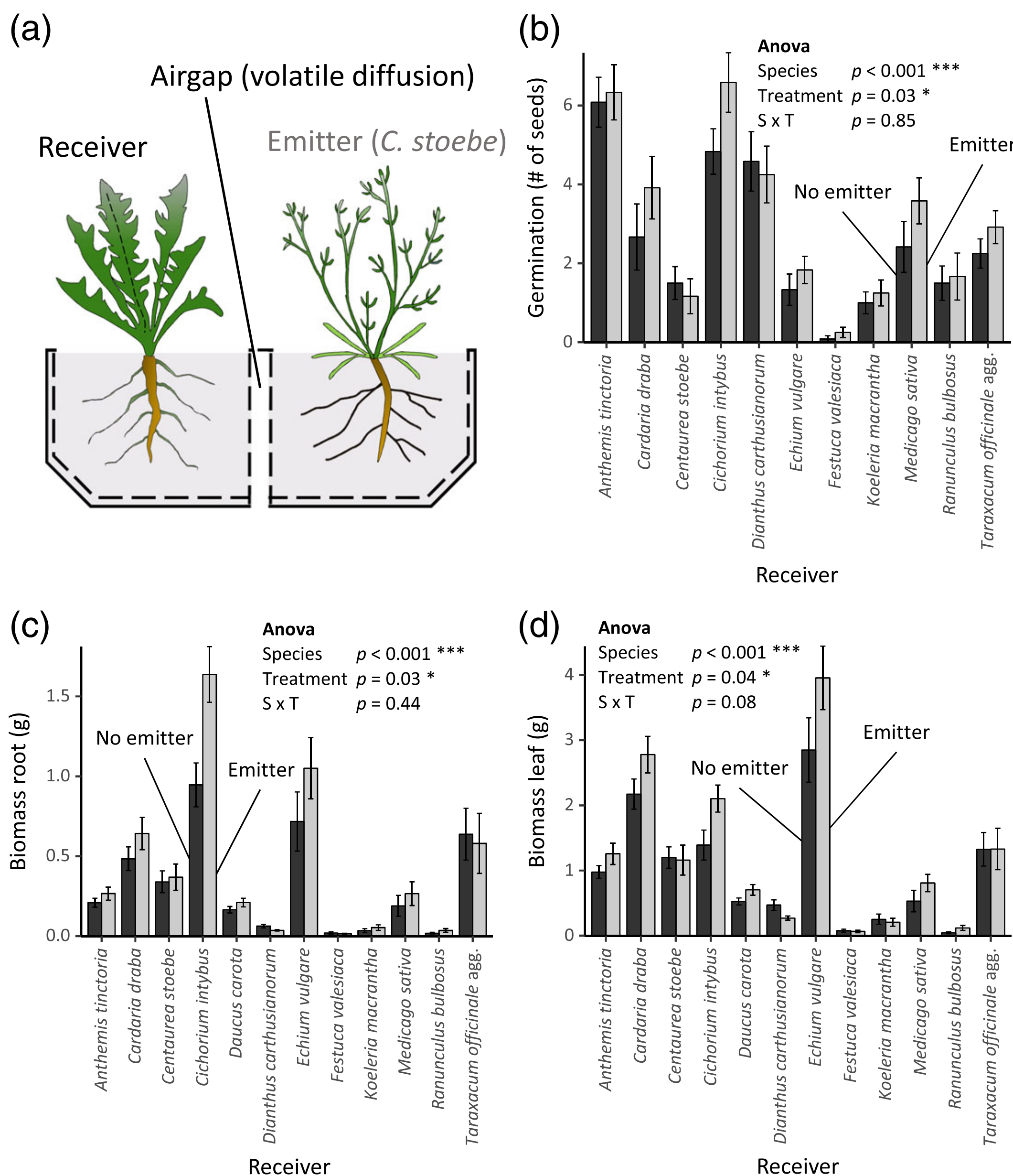


FIGURE 4 *Centaurea stoebe* root volatiles increase germination and growth of sympatric neighbours. (a) Experimental set-up to evaluate the influence of *C. stoebe* ("emitter") root volatiles on receiver plant species. As control, the emitter compartment was filled with soil, but no plant was grown in it ("no emitter"). (b) Number of receiver seeds that germinated up to 4 weeks after they were sown (mean ± *SE*). Analysis of variance output of generalized linear model is shown (distribution, quasibinomial; $n = 12$ per species and treatment). Dry biomass of receiver roots (c) and leaves (d) after 9 weeks of growth (mean ± *SE*). Analysis of variance output of generalized linear model is shown (GLM; family: gamma, link: inverse; $n = 12$ per species and treatment). Levels of significance: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ [Colour figure can be viewed at wileyonlinelibrary.com]

which were also present in *C. stoebe* roots. The sesquiterpenes produced by CsTPS5 were not found in the leaves, and *CsTPS5* was not expressed in this tissue. Regulation of sesquiterpene synthesis through transcriptional control of *TPSs* is well established (Tholl, 2006) and likely also accounts for the differences in leaf and root sesquiterpene profiles in *C. stoebe*. Taken together, we show that two predominantly root-expressed TPSs can account for the full root sesquiterpene blend of *C. stoebe*.

In vitro studies found negative effects of root VOCs on seed germination (Ens et al., 2009; Jassbi et al., 2010). Using a soil-based system that allows for the passive diffusion of VOCs between sender and receiver plants, we demonstrate that *C. stoebe* volatiles have no negative effects on the germination and growth of 11 sympatric plant species. Root VOC exposure even resulted in an overall increase in the germination and growth of other plants. A degradation product of (*E*)-β-caryophyllene has been shown to exhibit a broad antifungal activity (Hubbell, Wiemer, & Adejare, 1983), and other root VOCs are also known to influence microbial communities, which again can alter plant performance (Inderjit & Weiner, 2001; Kleinheinz et al., 1999; Wenke et al., 2010). Thus, the positive effect of *C. stoebe* root

VOCs on the receiver plants could either be a direct effect mediated through the impact of the VOCs on the physiology of the seeds and growing plants, or an indirect effect mediated through soil microbial communities (Hu, Robert, et al., 2018). Of note, *C. stoebe* VOCs do not only modulate plant performance but can also change root physiology and herbivore resistance, as shown in the companion paper to this study (Huang et al., 2019). Thus, the effects of *C. stoebe* VOCs on neighbouring plants are likely multifaceted and may change the interactions of neighbouring plants with other organisms. How root VOCs interact with bioactive soluble exudates, which can also be important for plant and herbivore performance (Hu, Mateo, et al., 2018), remains to be studied. Past studies proposed that *C. stoebe* soluble exudates may have allelopathic effects (Ridenour & Callaway, 2001), which may, in theory, counterbalance the positive effects of root VOCs by suppressing the growth of neighbouring plants. More experiments will be required to assess potential interactions between VOCs and soluble exudates in plant–plant interactions.

The release of VOCs can benefit the emitter by intoxicating and repelling herbivores, attracting natural enemies, and priming defenses in systemic tissues (De Moraes, Mescher, & Tumlinson, 2001; Erb et al., 2015; Frost, Mescher, Carlson, & Moraes, 2008; Schuman, Barthel, & Baldwin, 2012; Ye et al., 2018). To what extent the release of VOCs is beneficial for the emitter in the context of plant–plant interactions, however, is less clear. Here, we show that the release of sesquiterpenes from the roots may have negative consequences for *C. stoebe* plants, as it increases the germination and growth of a variety of sympatric competitors. Strikingly, and in contrast to what has been observed in other plant systems (Degen, Dillmann, Marion-Poll, & Turlings, 2004; Schuman, Heinzel, Gaquerel, Svatos, & Baldwin, 2009), sesquiterpene release seems to be conserved within different *C. stoebe* ecotypes. The benefit of this potentially conserved phenotype for *C. stoebe* is currently unclear. Germination and growth of *C. stoebe* itself does not seem to be improved through VOC exposure, for instance. However, it is possible that the high release rates protect the plant from herbivores and pathogens in addition to the known resistance factors in this species (Landau, Müller-Schärer, & Ward, 1994). Furthermore, as shown in the companion paper (Huang et al., 2019), the VOCs may trigger susceptibility to herbivores in neighbouring species. Knocking down *CsTPS4* and *CsTPS5* could help to understand the potential benefits of root sesquiterpene production in the future.

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

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

ACKNOWLEDGEMENTS

We thank Adrian Möhl (Info Flora) for advice on plant species growing in sympatry with *C. stoebe*. Additionally, we thank Yan Sun and Heinz Müller Schärer (University of Fribourg) as well as Adrian Möhl and Markus Fischer (University of Bern) for providing seeds. We thank Benjamin M. Delory for providing helpful comments on the preprint of this manuscript. This project was supported by the European Commission (MC-IEF no. 704334 to W. H.) and the University of Bern.

AUTHOR CONTRIBUTIONS

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

ORCID

Meret Huber https://orcid.org/0000-0002-8708-394X
Matthias Erb https://orcid.org/0000-0002-4446-9834

REFERENCES

Arimura, G.-i., Shiojiri, K., & Karban, R. (2010). Acquired immunity to herbivory and allelopathy caused by airborne plant emissions. *Phytochemistry*, *71*, 1642–1649. https://doi.org/10.1016/j.phytochem.2010.06.021

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society, Series B: Statistical Methodology*, *57*, 289–300.

Beran, F., Rahfeld, P., Luck, K., Nagel, R., Vogel, H., Wielsch, N., … Köllner, T. G. (2016). Novel family of terpene synthases evolved from trans-isoprenyl diphosphate synthases in a flea beetle. *Proceedings of the National Academy of Sciences of the United States of America*, *113*, 2922–2927. https://doi.org/10.1073/pnas.1523468113

Bohlmann, J., Meyer-Gauen, G., & Croteau, R. (1998). Plant terpenoid synthases: Molecular biology and phylogenetic analysis. *Proceedings of the National Academy of Sciences*, *95*, 4126–4133. https://doi.org/10.1073/pnas.95.8.4126

Cai, Y., Jia, J.-W., Crock, J., Lin, Z.-X., Chen, X.-Y., & Croteau, R. (2002). A cDNA clone for β-caryophyllene synthase from *Artemisia annua*. *Phytochemistry*, *61*, 523–529. https://doi.org/10.1016/S0031-9422(02)00265-0

Callaway, R. M., Waller, L. P., Diaconu, A., Pal, R., Collins, A. R., Mueller-Schaerer, H., & Maron, J. L. (2011). Escape from competition: Neighbors reduce *Centaurea stoebe* performance at home but not away. *Ecology*, *92*, 2208–2213. https://doi.org/10.1890/11-0518.1

Chen, X., Köllner, T. G., Jia, Q., Norris, A., Santhanam, B., Rabe, P., … Chen, F. (2016). Terpene synthase genes in eukaryotes beyond plants and fungi: Occurrence in social amoebae. *Proceedings of the National Academy of Sciences of the United States of America*, *113*, 12132–12137. https://doi.org/10.1073/pnas.1610379113

Cheng, A.-X., Lou, Y.-G., Mao, Y.-B., Lu, S., Wang, L.-J., & Chen, X.-Y. (2007). Plant terpenoids: Biosynthesis and ecological functions. *Journal of Integrative Plant Biology*, *49*, 179–186. https://doi.org/10.1111/j.1744-7909.2007.00395.x

D'Alessandro, M., & Turlings, T. C. J. (2005). In situ modification of herbivore-induced plant odors: A novel approach to study the attractiveness of volatile organic compounds to parasitic wasps. *Chemical Senses*, *30*, 739–753. https://doi.org/10.1093/chemse/bji066

De Moraes, C. M., Mescher, M. C., & Tumlinson, J. H. (2001). Caterpillar-induced nocturnal plant volatiles repel conspecific females. *Nature*, *410*, 577–580. https://doi.org/10.1038/35069058

Degen, T., Dillmann, C., Marion-Poll, F., & Turlings, T. C. J. (2004). High genetic variability of herbivore-induced volatile emission within a broad range of maize inbred lines. *Plant Physiology*, *135*, 1928–1938. https://doi.org/10.1104/pp.104.039891

Degenhardt, J., Köllner, T. G., & Gershenzon, J. (2009). Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. *Phytochemistry*, *70*, 1621–1637. https://doi.org/10.1016/j.phytochem.2009.07.030

Delory, B. M., Delaplace, P., Fauconnier, M.-L., & du Jardin, P. (2016). Root-emitted volatile organic compounds: Can they mediate belowground plant-plant interactions? *Plant and Soil*, *402*, 1–26. https://doi.org/10.1007/s11104-016-2823-3

Duke, S. O., Blair, A. C., Dayan, F. E., Johnson, R. D., Meepagala, K. M., Cook, D., & Bajsa, J. (2009). Is (−)-catechin a novel weapon of spotted knapweed (*Centaurea stoebe*)? *Journal of Chemical Ecology*, *35*, 141–153. https://doi.org/10.1007/s10886-008-9587-z

Ens, E. J., Bremner, J. B., French, K., & Korth, J. (2009). Identification of volatile compounds released by roots of an invasive plant, bitou bush (*Chrysanthemoides monilifera* spp. *rotundata*), and their inhibition of native seedling growth. *Biological Invasions*, *11*, 275–287. https://doi.org/10.1007/s10530-008-9232-3

Erb, M., Veyrat, N., Robert, C. A. M., Xu, H., Frey, M., Ton, J., & Turlings, T. C. J. (2015). Indole is an essential herbivore-induced volatile priming signal in maize. *Nature Communications*, *6*, 6273. https://doi.org/10.1038/ncomms7273

Fox, J., & Weisberg, S. (2011). *An R companion to applied regression* (Second ed.). Thousand Oaks CA: Sage.

Frost, C. J., Mescher, M. C., Carlson, J. E., & Moraes, C. M. (2008). Plant defense priming against herbivores: Getting ready for a different battle. *Plant Physiology*, *146*, 818–824. https://doi.org/10.1104/pp.107.113027

Gershenzon, J., & Dudareva, N. (2007). The function of terpene natural products in the natural world. *Nature Chemical Biology*, *3*, 408–414. https://doi.org/10.1038/nchembio.2007.5

Götz, S., García-Gómez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., … Conesa, A. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Research*, *36*, 3420–3435. https://doi.org/10.1093/nar/gkn176

Gouinguené, S. P., & Turlings, T. C. J. (2002). The effects of abiotic factors on induced volatile emissions in corn plants. *Plant Physiology*, *129*, 1296–1307. https://doi.org/10.1104/pp.001941

Hartmann, T. (2007). From waste products to ecochemicals: Fifty years research of plant secondary metabolism. *Phytochemistry*, *68*, 2831–2846. https://doi.org/10.1016/j.phytochem.2007.09.017

Heil, M. (2014). Herbivore-induced plant volatiles: Targets, perception and unanswered questions. *New Phytologist*, *204*, 297–306. https://doi.org/10.1111/nph.12977

Heil, M., & Karban, R. (2010). Explaining evolution of plant communication by airborne signals. *Trends in Ecology & Evolution*, *25*, 137–144. https://doi.org/10.1016/j.tree.2009.09.010

Hiltpold, I., Erb, M., Robert, C. A. M., & Turlings, T. C. J. (2011). Systemic root signalling in a belowground, volatile-mediated tritrophic interaction. *Plant, Cell & Environment*, *34*, 1267–1275. https://doi.org/10.1111/j.1365-3040.2011.02327.x

Hu, L., Mateo, P., Ye, M., Zhang, X., Berset, J. D., Handrick, V., … Erb, M. (2018). Plant iron acquisition strategy exploited by an insect herbivore. *Science*, *361*, 694–697. https://doi.org/10.1126/science.aat4082

Hu, L., Robert, C. A. M., Cadot, S., Zhang, X., Ye, M., Li, B., … Erb, M. (2018). Root exudate metabolites drive plant-soil feedbacks on growth and defense by shaping the rhizosphere microbiota. *Nature Communications*, *9*, 2738. https://doi.org/10.1038/s41467-018-05122-7

Huang, W., Gfeller, V., & Erb, M. (2019). Root volatiles in plant-plant interactions II: Root volatiles alter root chemistry and plant-herbivore interactions of neighboring plants. *Plant, Cell & Environment*. https://onlinelibrary.wiley.com/doi/abs/10.1111/pce.13534

Huang, W., Zwimpfer, E., Hervé, M. R., Bont, Z., & Erb, M. (2018). Neighbourhood effects determine plant-herbivore interactions belowground. *Journal of Ecology*, *106*, 347–356. https://doi.org/10.1111/1365-2745.12805

Hubbell, S. P., Wiemer, D. F., & Adejare, A. (1983). An antifungal terpenoid defends a neotropical tree (*Hymenaea*) against attack by fungus-growing ants (*Atta*). *Oecologia*, *60*, 321–327. https://doi.org/10.1007/BF00376846

Inderjit, & Weiner, J. (2001). Plant allelochemical interference or soil chemical ecology? *Perspectives in Plant Ecology, Evolution and Systematics*, *4*, 3–12.

Irmisch, S., Krause, S. T., Kunert, G., Gershenzon, J., Degenhardt, J., & Köllner, T. G. (2012). The organ-specific expression of terpene synthase genes contributes to the terpene hydrocarbon composition of chamomile essential oils. *BMC Plant Biology*, *12*, 84. https://doi.org/10.1186/1471-2229-12-84

Jassbi, A. R., Zamanizadehnajari, S., & Baldwin, I. T. (2010). Phytotoxic volatiles in the roots and shoots of *Artemisia tridentata* as detected by headspace solid-phase microextraction and gas chromatographic-mass spectrometry analysis. *Journal of Chemical Ecology*, *36*, 1398–1407. https://doi.org/10.1007/s10886-010-9885-0

Jia, Q., Köllner, T. G., Gershenzon, J., & Chen, F. (2018). MTPSLs: New terpene synthases in nonseed plants. *Trends in Plant Science*, *23*, 121–128. https://doi.org/10.1016/j.tplants.2017.09.014

Karban, R., Yang, L. H., & Edwards, K. F. (2014). Volatile communication between plants that affects herbivory: A meta-analysis. *Ecology Letters*, *17*, 44–52. https://doi.org/10.1111/ele.12205

Keeling, C. I., & Bohlmann, J. (2006). Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. *The New Phytologist*, *170*, 657–675. https://doi.org/10.1111/j.1469-8137.2006.01716.x

Kegge, W., Ninkovic, V., Glinwood, R., Welschen, R. A. M., Voesenek, L. A. C. J., & Pierik, R. (2015). Red:far-red light conditions affect the emission of volatile organic compounds from barley (*Hordeum vulgare*), leading to altered biomass allocation in neighbouring plants. *Annals of Botany*, *115*, 961–970. https://doi.org/10.1093/aob/mcv036

Kleinheinz, G. T., Bagley, S. T., St. John, W. P., Rughani, J. R., & McGinnis, G. D. (1999). Characterization of alpha-pinene-degrading microorganisms and application to a bench-scale biofiltration system for VOC

degradation. *Archives of Environmental Contamination and Toxicology*, *37*, 151–157. https://doi.org/10.1007/s002449900500

Köllner, T. G., Held, M., Lenk, C., Hiltpold, I., Turlings, T. C. J., Gershenzon, J., & Degenhardt, J. (2008). A maize (E)-β-caryophyllene synthase implicated in indirect defense responses against herbivores is not expressed in most American maize varieties. *The Plant Cell*, *20*, 482–494. https://doi.org/10.1105/tpc.107.051672

Landau, I., Müller-Schärer, H., & Ward, P. I. (1994). Influence of cnicin, a sesquiterpene lactone of *Centaurea maculosa* (Asteraceae), on specialist and generalist insect herbivores. *Journal of Chemical Ecology*, *20*, 929–942. https://doi.org/10.1007/BF02059588

Landolt, E., Bäumler, B., Erhardt, A., Hegg, O., Klötzli, F., Lämmler, W., … Wohlgemuth, T. (2010). Flora indicativa: Ecological inicator values and biological attributes of the flora of Switzerland and the Alps (2., völlig neu bearb. und erw. Aufl.). Bern: Haupt.

Lenth, R. V. (2016). Least-squares means: The R package lsmeans. *Journal of Statistical Software*, *69*, 1–33.

Lin, C., Owen, S., & Peñuelas, J. (2007). Volatile organic compounds in the roots and rhizosphere of *Pinus* spp. *Soil Biology and Biochemistry*, *39*, 951–960. https://doi.org/10.1016/j.soilbio.2006.11.007

Loreto, F., & Schnitzler, J.-P. (2010). Abiotic stresses and induced BVOCs. *Trends in Plant Science*, *15*, 154–166. https://doi.org/10.1016/j.tplants.2009.12.006

Morrell, K., & Kessler, A. (2017). Plant communication in a widespread goldenrod: Keeping herbivores on the move. *Functional Ecology*, *31*, 1049–1061. https://doi.org/10.1111/1365-2435.12793

Mráz, P., Garcia-Jacas, N., Gex-Fabry, E., Susanna, A., Barres, L., & Müller-Schärer, H. (2012). Allopolyploid origin of highly invasive *Centaurea stoebe* s.l. (Asteraceae). *Molecular Phylogenetics and Evolution*, *62*, 612–623. https://doi.org/10.1016/j.ympev.2011.11.006

Nagegowda, D. A. (2010). Plant volatile terpenoid metabolism: Biosynthetic genes, transcriptional regulation and subcellular compartmentation. *FEBS Letters*, *584*, 2965–2973. https://doi.org/10.1016/j.febslet.2010.05.045

Ninkovic, V. (2003). Volatile communication between barley plants affects biomass allocation. *Journal of Experimental Botany*, *54*, 1931–1939. https://doi.org/10.1093/jxb/erg192

Ninkovic, V., Markovic, D., & Dahlin, I. (2016). Decoding neighbour volatiles in preparation for future competition and implications for tritrophic interactions. *Perspectives in Plant Ecology, Evolution and Systematics*, *23*, 11–17. https://doi.org/10.1016/j.ppees.2016.09.005

Peñuelas, J., Asensio, D., Tholl, D., Wenke, K., Rosenkranz, M., Piechulla, B., & Schnitzler, J. P. (2014). Biogenic volatile emissions from the soil. *Plant, Cell & Environment*, *37*, 1866–1891. https://doi.org/10.1111/pce.12340

Pichersky, E., & Gang, D. R. (2000). Genetics and biochemistry of secondary metabolites in plants: An evolutionary perspective. *Trends in Plant Science*, *5*, 439–445. https://doi.org/10.1016/S1360-1385(00)01741-6

Pichersky, E., & Gershenzon, J. (2002). The formation and function of plant volatiles: Perfumes for pollinator attraction and defense. *Current Opinion in Plant Biology*, *5*, 237–243. https://doi.org/10.1016/S1369-5266(02)00251-0

Pierik, R., Visser, E. J. W., de Kroon, H., & Voesenek, L. A. C. J. (2003). Ethylene is required in tobacco to successfully compete with proximate neighbours. *Plant, Cell & Environment*, *26*, 1229–1234. https://doi.org/10.1046/j.1365-3040.2003.01045.x

Quin, M. B., Flynn, C. M., & Schmidt-Dannert, C. (2014). Traversing the fungal terpenome. *Natural Product Reports*, *31*, 1449–1473. https://doi.org/10.1039/C4NP00075G

R Core Team (2017). R: A language and environment for statistical computing. Vienna, Austria. Retrieved from https://www.R-project.org/

Rasmann, S., Köllner, T. G., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U., … Turlings, T. C. J. (2005). Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature*, *434*, 732–737. https://doi.org/10.1038/nature03451

Ridenour, W. M., & Callaway, R. M. (2001). The relative importance of allelopathy in interference: The effects of an invasive weed on a native bunchgrass. *Oecologia*, *126*, 444–450. https://doi.org/10.1007/s004420000533

Robert, C. A. M., Erb, M., Duployer, M., Zwahlen, C., Doyen, G. R., & Turlings, T. C. J. (2012). Herbivore-induced plant volatiles mediate host selection by a root herbivore. *The New Phytologist*, *194*, 1061–1069. https://doi.org/10.1111/j.1469-8137.2012.04127.x

Saritas, Y., von Reuss, S. H., & König, W. A. (2002). Sesquiterpene constituents in *Petasites hybridus*. *Phytochemistry*, *59*, 795–803. https://doi.org/10.1016/S0031-9422(01)00489-7

Schuman, M. C., Barthel, K., & Baldwin, I. T. (2012). Herbivory-induced volatiles function as defenses increasing fitness of the native plant *Nicotiana attenuata* in nature. *eLife*, *1*, 1–29.

Schuman, M. C., Heinzel, N., Gaquerel, E., Svatos, A., & Baldwin, I. T. (2009). Polymorphism in jasmonate signaling partially accounts for the variety of volatiles produced by *Nicotiana attenuata* plants in a native population. *The New Phytologist*, *183*, 1134–1148. https://doi.org/10.1111/j.1469-8137.2009.02894.x

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*, 2725–2729. https://doi.org/10.1093/molbev/mst197

Tholl, D. (2006). Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. *Current Opinion in Plant Biology*, *9*, 297–304. https://doi.org/10.1016/j.pbi.2006.03.014

Treier, U. A., Broennimann, O., Normand, S., Guisan, A., Schaffner, U., Steinger, T., & Müller-Schärer, H. (2009). Shift in cytotype frequency and niche space in the invasive plant *Centaurea maculosa*. *Ecology*, *90*, 1366–1377. https://doi.org/10.1890/08-0420.1

Van den Dool, H., & Kratz, P. D. (1963). A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *Journal of Chromatography*, *11*, 463–471. https://doi.org/10.1016/S0021-9673(01)80947-X

Wenke, K., Kai, M., & Piechulla, B. (2010). Belowground volatiles facilitate interactions between plant roots and soil organisms. *Planta*, *231*, 499–506. https://doi.org/10.1007/s00425-009-1076-2

Wickham, H. (2009). *ggplot2: Elegant graphics for data analysis*. New York: Springer-Verlag. https://doi.org/10.1007/978-0-387-98141-3

Wickham, H. (2011). The Split-Apply-Combine Strategy for Data Analysis. *Journal of Statistical Software*, *40*, 1–29.

Yamada, Y., Kuzuyama, T., Komatsu, M., Shin-Ya, K., Omura, S., Cane, D. E., & Ikeda, H. (2015). Terpene synthases are widely distributed in bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *112*, 857–862. https://doi.org/10.1073/pnas.1422108112

Yang, C.-Q., Wu, X.-M., Ruan, J.-X., Hu, W.-L., Mao, Y.-B., Chen, X.-Y., & Wang, L.-J. (2013). Isolation and characterization of terpene synthases in cotton (*Gossypium hirsutum*). *Phytochemistry*, *96*, 46–56. https://doi.org/10.1016/j.phytochem.2013.09.009

Ye, M., Veyrat, N., Xu, H., Hu, L., Turlings, T. C. J., & Erb, M. (2018). An herbivore-induced plant volatile reduces parasitoid attraction by changing the smell of caterpillars. *Science Advances*, *4*, eaar4767. https://doi.org/10.1126/sciadv.aar4767

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Fresh weight does not significantly differ among different *Centaurea* species and among *C. stoebe* populations used for analysis of root VOC emission. Fresh biomass of roots (A) and leaves (B) of *Centaurea* species are shown as mean ± SE (*C. jacea* and *C. scabiosa*, *n* = 8; *C. stoebe*, *n* = 5; *C. valesiaca*, *n* = 4). Fresh biomass of roots (C) and leaves (D) of *C. stoebe* populations are shown as mean ± SE (Hu-11, *n* = 7; UFA and Ro-11, *n* = 5). Analysis of Variance (ANOVA) output of linear model is shown.

Table S1: Compounds found in *Centaurea stoebe* hexane tissue extracts.

Table S2: Primers used for cloning of CsTPS genes and for qRT-PCR.

How to cite this article: Gfeller V, Huber M, Förster C, Huang W, Köllner TG, Erb M. Root volatiles in plant–plant interactions I: High root sesquiterpene release is associated with increased germination and growth of plant neighbours. *Plant Cell Environ*. 2019;42:1950–1963. https://doi.org/10.1111/pce.13532