

Volatile cues from pathogenic, mutualistic and saprotrophic fungi cause specific, fungus-dependent responses in poplar

Peiyuan Zhu¹ , Baris Weber¹ , Maaria Rosenkranz^{1,2} , Andrea Ghirardo¹  and Jörg-Peter Schnitzler^{1,3,*} 

¹Research Unit Environmental Simulation, Helmholtz Munich, Ingolstädter Landstraße 1, 85764 Neuherberg, Oberschleißheim, Bavaria, Germany
²Institute of Plant Sciences, Ecology and Conservation Biology, University of Regensburg, Universitätsstraße 31, 93053 Regensburg, Bavaria, Germany
³Ecosystem Physiology, Faculty of Environment and Natural Resources, University Freiburg, Georges-Köhler-Allee 053/054, 79110 Freiburg, Baden-Württemberg, Germany

*Corresponding author (joergpeter.schnitzler@helmholtz-munich.de)

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Plants are exposed to complex interactions with belowground organisms, yet how they differentiate between mutualistic and pathogenic fungi before physical contact remains largely unknown. We exposed the roots of young *Populus × canescens* (Aiton) Sm. plants to volatile organic compounds (VOCs) emitted by fungi with contrasting lifestyles: the pathogenic *Heterobasidion annosum* (Fr.) Bref., the saprotroph *Postia placenta* (Fr.) M.J. Larsen & Lombard and the ectomycorrhizal fungus *Laccaria bicolor* (Maire) P.D. Orton. Volatile organic compound analysis of the shared rhizosphere headspace and leaf emissions revealed that poplar plants could perceive and respond to fungal identity solely through airborne cues. The root-zone headspace contained fungus-specific sesquiterpene fingerprints that remained similar after 3 and 6 weeks of co-cultivation: Pathogen-derived VOCs induced constantly high sesquiterpene emissions from the root-zone, whereas mycorrhizal VOCs caused low but targeted emissions of specific sesquiterpenes. In contrast, saprotrophic VOCs caused a temporal shift in root-zone VOC pattern, with increased sesquiterpene emissions after 6 weeks. Fungal VOC exposure also altered leaf VOC emissions, enriching alkanes, esters and monoterpenes. Initially, leaf VOC emissions were fungal lifestyle-specific but they converged over time, indicating systemic signal integration of belowground signals. These findings demonstrate that trees can discriminate ‘friend-versus-foe’ through VOCs alone, extending pattern-recognition theory beyond contact-dependent cues. Multivariate analyses suggested organ-specific chemical strategies: roots function as chemosensors decoding fungal volatiles, while systemic adjustments shape aboveground VOC profiles. Understanding the plant response to fungal VOCs may offer potential for developing early pathogen diagnostics and further elucidate the volatile-mediated plant–fungus interactions.

Keywords belowground-aboveground signaling, chemical ecology, lifestyle-specific recognition, plant defense, *Populus*, volatile organic compounds (VOCs), sesquiterpenes

Introduction

Plant–fungus interactions are an essential component of the structure and processes of terrestrial ecosystems (Philippot et al. 2013). Despite their microscopic nature, fungi play a crucial role in characterizing plant physiology, nutrient cycling and robustness of the ecosystems (Lehmann and Kleber 2015, Heijden and Hartmann 2016, Bradford et al. 2016). The interactions between plants and fungi generally include three types: mutualistic, pathogenic and saprotrophic, each of which is able to regulate plant growth and development in a unique manner (Priyashantha et al. 2023). Whereas mycorrhizal fungi help plants stimulate acquisition of

nutrients and stress endurance (Augé 2001, Smith et al. 2010, van der Heijden et al. 2015), pathogens impair host performance by triggering immune responses or transmitting effectors that interfere with host physiology (Doehlemann et al. 2017, Shao et al. 2021), and saprophytes contribute to decomposing and reforming the soil structure (van der Wal et al. 2013, Baldrian 2017, Clocchiatti et al. 2020). Plant roots are colonized by diverse fungal communities that vary significantly across environmental contexts, including soil properties, geographic factors and biotic interactions (Jiang et al. 2020, Lin et al. 2024). Controlled experiments demonstrate that specific environmental factors, such as neighboring biotic elements and abiotic conditions,

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directly influence fungal assemblages associated with plant roots (Mony et al. 2021, Mészárosóvá et al. 2024). This established neighboring effect raises a deeper mechanistic question regarding the ability of plants to differentiate between their fungal neighbors, particularly before direct contact is made. Volatile organic compounds (VOCs), a class of small airborne metabolites, represent a possible mechanism for pre-contact recognition in fungal–plant interactions. Volatile organic compounds can diffuse through both soil airspaces and aqueous phases, with apolar VOCs being transmitted more rapidly in liquid environments due to their lack of hydration spheres (Weisskopf et al. 2021), thereby carrying chemically rich information over distances (Peñuelas et al. 2014). These compounds are important messengers in cross-kingdom communication among plants, fungi and other microorganisms to convey information such as identity, physiological state or potential dangers (Pichersky and Gershenzon 2002, Escobar-Bravo et al. 2023). Fungal VOC profiles are highly diverse and lifestyle-specific (Guo et al. 2021), and different effects have been observed: some species emit volatiles that promote plant growth or defense priming (Sharifi and Ryu 2021), but others also release suppressive or inhibitory signals (Bitas et al. 2013). Among these compounds, sesquiterpenes (SQTs), a prominent subclass of terpenoids, are particularly abundant in fungal emissions and are frequently detected in plant responses. These C₁₅ compounds have been shown to mediate lifestyle-specific interactions, such as promoting root growth in mutualistic ectomycorrhizal associations via apolar SQTs produced by fungi like *Laccaria bicolor* (Maire) P.D. Orton (Ditengou et al. 2015) or acting as antimicrobial agents in defenses against pathogens (Sánchez-Fernández et al. 2016).

While VOC-mediated pre-contact recognition offers clear ecological advantages, plants still retain a robust contact-dependent recognition system. Pattern recognition receptors (PRRs), such as LysM-type receptors, detect fungal-associated molecular patterns like chitin and activate immune responses through mitogen-activated protein kinase (MAPK) signaling cascades (Spoel and Dong 2012, Couto and Zipfel 2016, Liu et al. 2023). Recent studies show these receptors also fine-tune responses to modulate between defense and symbiosis, depending on context and dosage (Tan et al. 2025). Volatile organic compound mediated signaling may offer a complementary early warning layer to this classical microbe-associated molecular pattern (MAMP)-based recognition (Boller and Felix 2009). Additionally, under fungal challenge, plants often upregulate and emit sesquiterpenes that can function both as antimicrobial agents and intra-plant signals (Taniguchi et al. 2014, Frank et al. 2021, Laupheimer et al. 2023). However, the chemical overlap between fungal-derived and plant-derived sesquiterpenes creates a fundamental challenge for plants to distinguish fungal signals from endogenous volatile emissions when molecular structures are highly similar.

To investigate the capacity of plants to resolve such ambiguity and assign ecological value to fungal VOCs, we selected grey poplar, *Populus × canescens* (Aiton) Sm., as a plant model. This species is known for forming ectomycorrhizal symbioses with *L. bicolor*, a well-characterized mutualist that influences root development and suppresses jasmonate signaling via the MiSSP7 effector (Felten et al. 2009, Daguerre et al. 2020). As representatives of other lifestyles, we included the saprotroph *Postia placenta* (Fr.) M.J. Larsen & Lombard, involved in lignocellulose decay (Martínez et al. 2009), and the necrotrophic pathogen *Heterobasidion*

annosum (Fr.) Bref., known for aggressive root and stem infection (Hu et al. 2020, Tudoran et al. 2024). These fungi form ecologically distinct relationships with poplar and are known to produce lifestyle-informative VOCs (Müller et al. 2013, Guo et al. 2021).

Despite accumulating evidence of VOC-mediated plant–fungal communication, such as fungal volatiles reprogramming root architecture in ectomycorrhizal systems (Ditengou et al. 2015) and inducing short-term growth responses (Jiang et al. 2021), several questions remain unanswered. First, it is not yet known whether plants can reliably respond differentially to fungi with fundamentally different lifestyles (pathogenic, mutualistic, saprotrophic) using belowground volatile cues alone under standardized experimental conditions, particularly when distinguishing complex bouquets rather than individual compounds (Weisskopf et al. 2021). Second, it remains unclear whether these lifestyle-specific signals trigger distinct and predictable VOC responses in aboveground plant parts and how they are spatially coordinated between roots and leaves (organ-specific versus unified strategies). Third, it remains unclear how VOC-triggered responses unfold temporally, whether they are transient or sustained, and whether they evolve from initial perception to long-term systemic adjustments (Briusson et al. 2023).

To address these questions, we employed a non-contact ‘pot-in-pot’ system that excluded direct physical interaction between plant roots and fungi and focused exclusively on volatile signaling. *P. × canescens* roots were exposed to VOCs from each fungus, and both root-zone and leaf emissions were tracked over time using GC–MS analysis. Multivariate statistical analysis was employed to assess treatment discrimination and identify key discriminatory VOCs.

We tested three hypotheses: (I) poplar responses to fungal VOCs are dependent on fungal lifestyle, resulting in distinct volatile compositions in belowground and aboveground compartments; (II) these chemical interactions will exhibit temporal evolution from initial discrimination to adaptive integration over time, consistent with organ-specific and time-dependent effects observed in fungal volatile studies (Moisan et al. 2020, Singh et al. 2021, Olimi et al. 2025); and (III) sesquiterpenes dominate the discriminatory volatile classes in these interactions. By demonstrating that trees interpret airborne fungal signals before hyphal contact, this work widens the concept of pattern recognition beyond physical contact, offers a basis for non-invasive VOC-based diagnostics for early pathogen detection, and contributes to our understanding of plant–fungal chemical communication with implications for forest health monitoring.

Materials and methods

Plant and microbial materials and growth conditions

This experiment used wild-type *Populus × canescens* (INRA clone 717–1B4). For propagation, plantlets from the established sterile stock were sectioned into segments under a laminar flow hood. These segments were then cultured in glass jars containing half-strength Murashige & Skoog (MS) medium, which had been adjusted to a pH of 5.8, and contained 30 g L^{−1} sucrose and 7 g L^{−1} agar (Duchefa Biochemie, Haarlem, The Netherlands). The glass

jars containing the plants were placed in a growth chamber at 20/16 °C (day/night), with 10-h photoperiod (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD), for 2 months. Following the in vitro stage, the rooted plantlets were carefully transferred to pots containing an autoclaved substrate mixture (soil:vermiculite:perlite at a ratio of 3:1:1, v:v:v) and then all the plants were moved to a sun simulator (Kaling et al. 2014) with the following conditions: day/night temperature of 22/16 °C, relative humidity of 60/80% and light conditions of 10/14 h, 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD for 3 months to acclimatize to the different chamber conditions. The plants in the tray were first half-covered with plastic covers to maintain high humidity; the covers were removed gradually within 2 weeks.

Three fungal strains were used in the experiments based on their ecological lifestyles: the pathogen *H. annosum* (stock collection Karin Pritsch, EUS, Helmholtz Munich); the mycorrhizal fungus *L. bicolor* (S238N), and the saprotrophic *Postia placenta* (stock collection K. Pritsch). The fungal cultures were grown in 35 mm $\times$ 10 mm culture dishes (Corning 430588, Corning Inc., NY, USA), which contained a modified Melin-Norkrans (MMN) medium (1% glucose, 0.25% NH_4 -tartrate, 0.025% $(\text{NH}_4)_2\text{SO}_4$, 0.05% KH_2PO_4 , 0.015% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.0025% NaCl, 0.01% FeCl_3 , 0.001% thiamine hydrochloride and 1% Gelrite at a pH of 5.2) (Guo et al. 2021).

Pot-in-pot exposure system and experimental setup

A custom 'pot-in-pot' (PiP) system (Figure 1a) was developed to expose poplar roots to fungal VOCs without direct contact. The lower 2 cm of the upper pot was drilled with 28 evenly distributed 1 mm diameter holes to allow gas exchange between the upper and lower pots. Each PiP system consists of an upper pot containing 180 g of substrate (soil:vermiculite:perlite mixture at a ratio of 3:1:1, by volume) and a lower pot for the medium and fungi. All components were wrapped in aluminum foil and autoclaved at 121 °C for 20 min, 2 days prior to transferring the plants from the glass jar to the PiP system.

The experiment followed a 2×4 factorial design. The first factor was the presence or absence of a grey poplar plant in the upper pot. The second factor consisted of three fungal treatments and one control in the lower pot: a fungus-free MMN medium control (blank), and treatments containing fungi: *H. annosum*, *L. bicolor* and *P. placenta*. Each treatment had six biological replicates ($n = 6$), making a total of 48 PiP systems. The replicates of each treatment group were placed together in a single tray. To minimize position effects, different trays with poplar were systematically rotated within the climate chamber clockwise every three days.

To ensure continuous and active VOC emissions, the fungal plates in the lower pots were replaced weekly or when contamination on the fungal plate was observed. The fresh plates were pre-cultivated for different periods of time (*P. placenta*: 4 days; *H. annosum*: 8 days; *L. bicolor*: 28 days) to allow the mycelium to fully colonize the medium surface prior to replacement.

Volatile organic compound collection

Volatile organic compounds were collected at two time points: 3 weeks and 6 weeks after the onset of exposure of roots to fungal VOC emissions. For the belowground collection of VOCs from the

root-zone headspace (which includes emissions from both plant roots and fungal cultures), a polydimethylsiloxane (PDMS) Twister (Gerstel GmbH, Mülheim an der Ruhr, Germany) was magnetically attached to the exterior bottom surface of the upper pot, directly above the fungal culture, and positioned in the headspace of the lower pot. The junction between upper and lower pots was sealed with Teflon tape to create a gas-tight seal, and volatiles were collected for 16 h. This sampling approach captured the combined chemical environment, including fungal emissions and any root-derived compounds, allowing characterization of the complete root-zone VOC profile that plants experience during fungal exposure. Fungal monoculture treatments served as controls to distinguish fungal-derived compounds from plant-specific responses.

Aboveground VOC collection was performed immediately before sampling by first attaching a PDMS Twister to the abaxial surface of a mature leaf and then enclosing the entire aerial part of the plant in a sealed 2 L PTFE (Teflon) bag. This created a gas-tight headspace that was physically separated from the root zone, ensuring that the measured volatiles originated solely from aboveground plant tissues. Volatiles were sampled for 2 h under ambient light conditions. After sampling, all Twisters were stored in sealed glass vials at 4 °C until analysis.

Volatile organic compound analyses

Volatile organic compounds were analyzed on a GERSTEL Thermal Desorption Unit (TDU) with a Cooled Injection System (CIS 4), coupled to an Agilent 7890A gas chromatograph (GC) and a 5975C mass selective detector (MSD). Prior to analysis, an internal standard (860 pmol of δ -2-carene in hexane) was spiked onto each Twister. Each Twister was then thermally desorbed in the TDU from 30 °C (0.1 min) to 270 °C (2 min) at a rate of 280 °C min^{-1} . Desorbed analytes were cryofocused in the CIS at -50 °C before being injected in splitless mode. For injection, the CIS was heated to 270 °C (2 min) at 12 °C s^{-1} . The transfer line was held at 250 °C.

Gas chromatographic separation was performed on a DB-5MS column (70 m $\times$ 0.25 mm i.d. — 0.25 μm film thickness; Agilent J&W) with helium as the carrier gas at a constant flow of 1.0 mL min^{-1} . The GC oven program was as follows: 40 °C, ramped at 10 °C min^{-1} to 130 °C (held 5 min), then at 80 °C min^{-1} to 175 °C, then at 2 °C min^{-1} to 200 °C, then at 4 °C min^{-1} to 220 °C, and finally at 100 °C min^{-1} to 300 °C (held 5 min), for a total run time of 37.86 min.

The MSD was operated in electron-impact (EI) mode at 70 eV, with source and quadrupole temperatures at 230 °C and 150 °C, respectively. Data were acquired in a combined SIM/scan mode after an 8.3 min solvent delay. Full scan mode monitored m/z 35–250. Two SIM groups were used to enhance sensitivity for monoterpenes (m/z 93, 121, 136; starting at 8.3 min) and sesquiterpenes (m/z 93, 161, 204; starting at 14.9 min). Isoprene could not be measured due to its extremely high volatility and insufficient retention on the PDMS Twister sorbent material.

Compounds were identified by comparing their mass spectra and retention indices with the NIST 2020 spectral library and authenticated standards. Quantification was performed using external calibration curves. Limits of detection (LOD) and quantification (LOQ) for the main VOCs were estimated from calibration data using $\text{LOD} = 3.3 \times \text{SD}/\text{slope}$ and $\text{LOQ} = 10 \times \text{SD}/\text{slope}$, where SD is the standard deviation of the response at the lowest non-zero calibration level ($n = 3$), and slope was obtained from linear

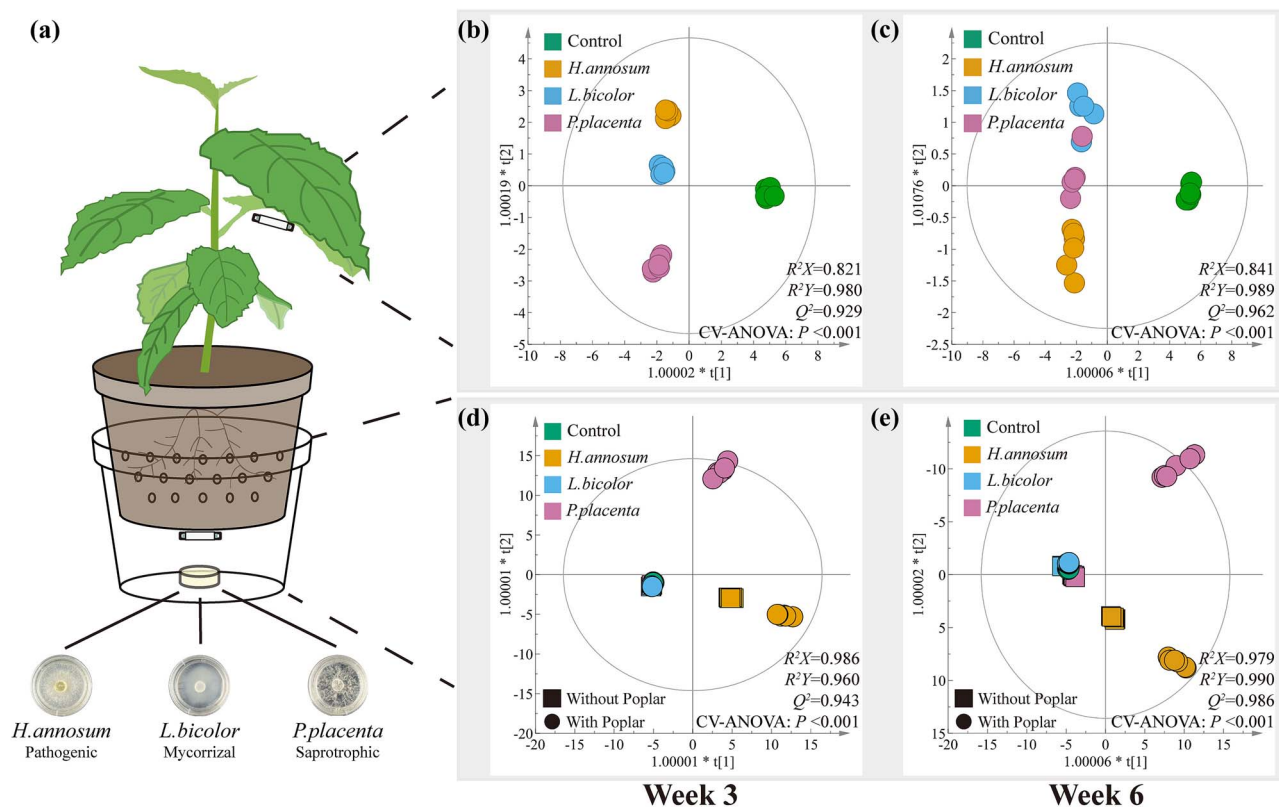


Figure 1 Experimental design and analysis of volatile organic compound (VOC) profiles in poplar exposed to volatiles from functionally diverse fungi. (a) Schematic showing poplar roots (upper pot) and fungal culture plates (lower pot) sharing headspace through perforated (upper) pot (28 × 1 mm holes) without physical contact. Fungal lifestyle: *H. annosum* = pathogenic; *L. bicolor* = mycorrhizal; *P. placenta* = saprotrophic. (b, c) OPLS-DA score plots of aboveground (leaf) VOC emissions at weeks 3 (b; $R^2X = 0.821$, $R^2Y = 0.980$, $Q^2 = 0.929$) and 6 (c; $R^2X = 0.841$, $R^2Y = 0.989$, $Q^2 = 0.962$) of co-cultivation. (d, e) OPLS-DA score plots of belowground VOC emissions at weeks 3 (d; $R^2X = 0.986$, $R^2Y = 0.960$, $Q^2 = 0.943$) and 6 (e; $R^2X = 0.979$, $R^2Y = 0.990$, $Q^2 = 0.986$); round symbols = with poplar, square symbols = without poplar. Each point represents one biological replicate ($n = 6$ per treatment). Model components were: aboveground VOCs—3 predictive components (PC) (week 3, panel b) and 3 PC (week 6, panel c); belowground VOCs—6 PC (weeks 3 and 6, panels d and e). Model parameters indicate good fit and predictive ability, with all models validated by permutation testing and cross-validation. All models were significant ($P < 0.05$, CV-ANOVA).

regression fitted to non-zero calibration points (Table S1 available as Supplementary Data at *Tree Physiology* Online). Emission rates were calculated based on the peak area of each compound relative to the peak area of the internal standard (δ -2-carene) and further normalized according to sampling duration and either aboveground leaf area or belowground Petri dish area (Ghirardo et al. 2011, 2020).

Statistical analysis

All statistical analyses were conducted on the time-resolved volatilomics data, which were processed as two separate matrices: the belowground dataset (56 compounds, Table S2 available as Supplementary Data at *Tree Physiology* Online) and the aboveground dataset (57 compounds, Table S3 available as Supplementary Data at *Tree Physiology* Online). Prior to analysis, all emission rate data were $\log_{10}$ -transformed to approximate a normal distribution, then mean-centered and Pareto-scaled. An exploratory principal component analysis (PCA) was initially performed on each dataset to inspect data variation and identify potential outliers. One sample falling outside the 95% Hotelling's T^2 confidence ellipse was excluded from further analysis.

For both the aboveground and belowground VOC datasets, separate Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) models were then performed using SIMCA-P (v. 13.0.3.0, Umetrics, Umeå, Sweden). In these supervised models, the respective matrix of VOC emission rates served as the independent variables (X), while the categorical variable indicating the fungal treatment (*H. annosum*, *L. bicolor*, *P. placenta* or control) was set as the dependent variable (Y). The performance and reliability of the OPLS-DA models were assessed using cumulative explained variance (R^2Y), predictive ability (Q^2Y), root mean square error of estimation (RMSEE) and root mean square error of cross-validation (RMSECV). Model significance was tested using a cross-validated analysis of variance (CV-ANOVA, $P < 0.05$) (Eriksson et al. 2008, 2013). OPLS-DA score plots were then used to illustrate the differentiation of poplar VOC profiles based on fungal exposure.

Further statistical analyses and data visualizations were conducted in R (v.4.3.3; R Core Team, 2023). For normally distributed data, differences between groups were tested using a one-way ANOVA followed by a Tukey's HSD post hoc test ($P < 0.05$). For univariate comparisons of non-normally distributed transformed data, a Kruskal-Wallis test was used, with Bonferroni-adjusted post hoc comparisons for emission-rate data. Hierarchical

clustering analysis was performed using complete linkage with Euclidean distances. The resulting patterns of VOC abundance across treatments were visualized as heatmaps using the 'pheatmap' package in R. Non-metric multidimensional scaling (NMDS), an ordination method, was used to visualize VOC community structure based on Bray–Curtis dissimilarity using the 'vegan' package in R. The homogeneity of multivariate dispersions was tested using the 'betadisper' function before conducting PERMANOVA for significance testing, with Holm adjustment applied to pairwise comparisons. β -diversity was calculated as the mean Bray–Curtis dissimilarity between samples within each treatment group compared with all other treatment groups (between-group beta diversity), averaged across all pairwise sample comparisons and expressed as mean $\pm$ SEM.

Results

Plant-mediated detection of fungal lifestyle through distinct fungal VOC signatures

Exposure of poplar to VOCs from fungi with contrasting lifestyles showed distinct below- and above ground volatile emission profiles (Figure 1). To quantify the separation, we performed OPLS-DA on VOC profiles from the root-zone headspace (between the air-permeable pot and the fungal culture; 56 compounds, Table S2 available as Supplementary Data at *Tree Physiology* Online) and above ground plant tissues (57 compounds, Table S3 available as Supplementary Data at *Tree Physiology* Online). This analysis revealed that fungal VOC exposure significantly altered root-zone volatile composition ($P < 0.001$, CV-ANOVA) relative to controls (roots alone or fungal monocultures without plant contact) (Figure 1). These VOC fingerprints clearly distinguished the pathogenic (*H. annosum*), mycorrhizal (*L. bicolor*) and saprotrophic (*P. placenta*) interactions from one another and from all control groups (Figure 1b–e).

In aboveground volatile profiles at week 3, the three fungal lifestyle treatments formed distinct clusters that were clearly separated from the controls along the first predictive component (PC1), while the different fungal lifestyles were further separated from one another along PC2 (Figure 1b). By week 6, differences among fungal treatments had decreased, although all co-cultivation treatments remained distinguishable from controls (Figure 1c). In contrast to the temporal changes observed aboveground, the VOC patterns in the headspace between the perforated pot (allowing air exchange) and the fungal culture remained temporally stable. At week 3, the OPLS-DA showed distinct separation among fungal lifestyles and controls (Figure 1d). Importantly, the presence of poplar roots shifted VOC profiles away from their respective monoculture baselines for the pathogenic and saprotrophic fungi, demonstrating that plant presence actively alters the volatile environment rather than simply reflecting inherent differences between fungal lifestyles. The saprotrophic *P. placenta* exhibited the greatest difference between its monoculture and co-culture states, indicating the strongest plant–fungus chemical interaction. At week 6, this plant-mediated separation became even more pronounced (Figure 1e). The distance between monoculture and co-culture conditions increased or persisted over time for all fungi, with

minimal change for *L. bicolor* (Figure 1d and e). *Postia placenta* co-cultures remained uniquely separated from all other treatments, with greater magnitude of shift compared with mycorrhizal or pathogenic interactions. High compound overlap between time points (*L. bicolor* 100%, *H. annosum* 93%, *P. placenta* 84%) confirmed temporal consistency in these interaction-specific, rather than simply fungal-specific, volatile profiles (Figure S1 available as Supplementary Data at *Tree Physiology* Online).

Together, the three fungal species representing different lifestyles and the responses from different root-zone and leaf responses showed different fingerprints of VOCs profile: the belowground VOC signatures remained temporally stable and treatment-specific, while the leaf VOC profiles gradually became more similar over time, but still maintained significant differences from the control group.

Belowground fungal lifestyle determines plant-modulated volatile signatures with distinct chemical class specialization

To identify the specific compounds driving these discriminant patterns, we performed a hierarchical cluster analysis of the individual volatile compounds. Across all treatments and time points, we detected 56 VOCs in the root-zone headspace, mainly sesquiterpenes (84%). Hierarchical clustering identified specific compound clusters that distinguished the three fungal lifestyles in co-cultivation with poplar (Figure 2). Compound-overlap analysis showed minimal sharing among lifestyles (typically < 5 shared compounds): in monoculture at week 3, *H. annosum* exhibited 15 unique compounds, whereas *L. bicolor* and *P. placenta* showed 2 and 0, respectively (Figure S2a available as Supplementary Data at *Tree Physiology* Online). Upon co-cultivation with poplar, all fungi emitted broader and more complex signatures (Figure S2b available as Supplementary Data at *Tree Physiology* Online); *H. annosum* maintained the highest uniqueness (16 compounds), and *P. placenta* increased from 0 to 14. These patterns persisted at week 6, with *H. annosum* reaching 14 unique compounds and *P. placenta* maintaining 13 (Figure S2c and d available as Supplementary Data at *Tree Physiology* Online).

Temporal comparisons indicated substantial consistency in VOC emissions: 30/56 compounds (54%) showed consistent responses across weeks 3 and 6, while 16 increased and 10 decreased in abundance. Pairwise Spearman correlation matrices revealed denser, positively correlated modules under poplar exposure, most pronounced for *H. annosum* and *P. placenta*, consistent with coordinated metabolic responses rather than random additions (Figures S3 and S4 available as Supplementary Data at *Tree Physiology* Online).

Treatment-specific changes were dominated by sesquiterpenoids (Figure 2a and b). In co-cultivation, additional sesquiterpenes were detected with *H. annosum* (e.g. isodene (ID 14), cedrene (ID 37); Figure 2a; Table S2 available as Supplementary Data at *Tree Physiology* Online), and *P. placenta* shifted from low monoculture VOC emissions to a more diverse sesquiterpenoid profile (e.g. silphinene (ID 13), β -caryophyllene (ID 36); Figure 2a; Table S2 available as Supplementary Data at *Tree Physiology* Online). In contrast, exposure to *L. bicolor* showed limited changes in overall composition under co-cultivation (Figure 2a and b).

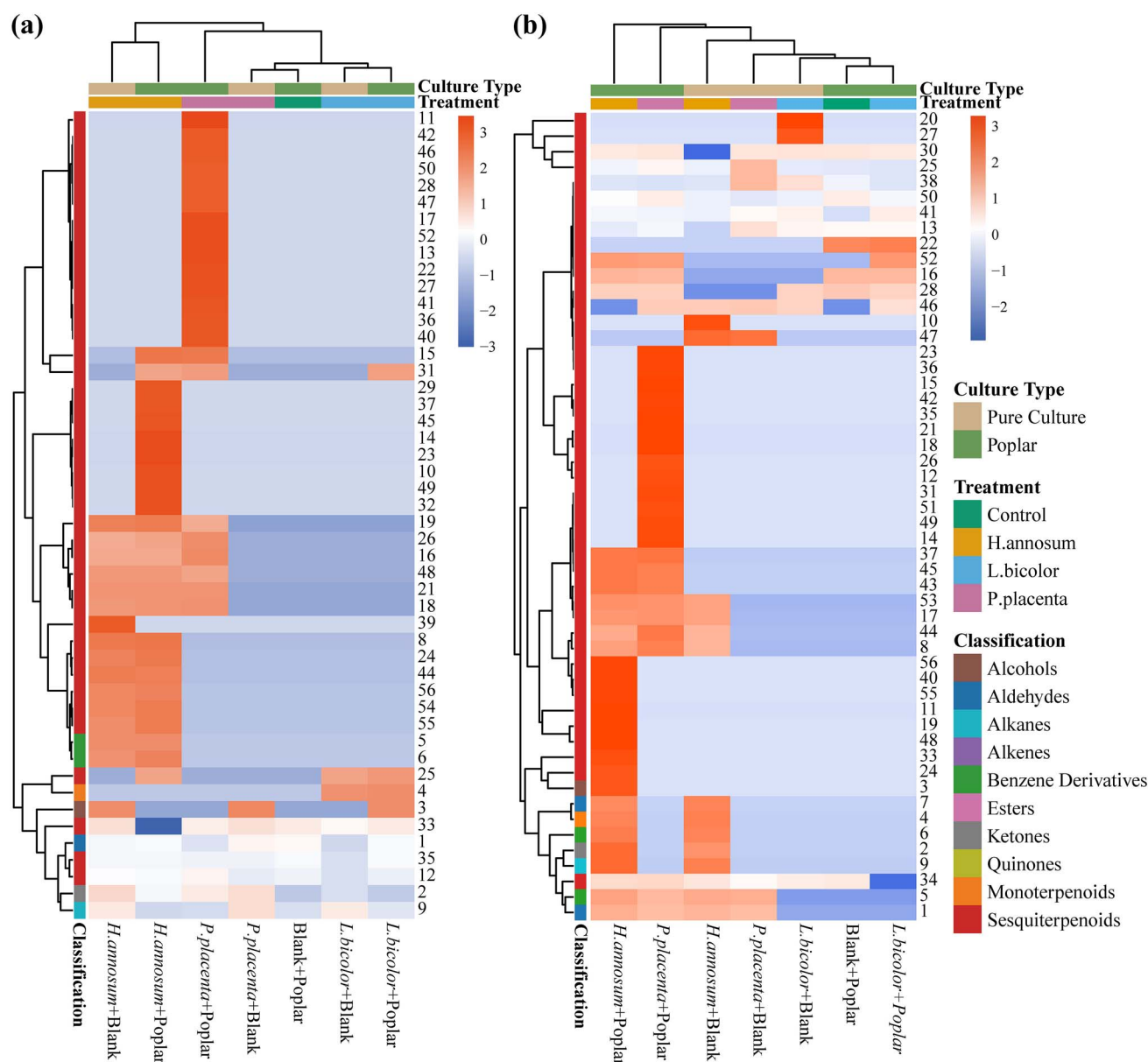


Figure 2 Hierarchical clustering reveals distinct belowground VOC profiles under contact-free fungal co-cultivation. (a, b) Bi-dimensional clustered heatmaps showing belowground Z-score normalized volatile compound abundances at weeks 3 (a) and 6 (b). Rows represent individual compounds (IDs in Table S2 available as Supplementary Data at Tree Physiology Online); columns show treatment groups including pure fungal cultures (*H. annosum*, *L. bicolor*, *P. placenta*) and co-cultures with poplar. Hierarchical clustering used Euclidean distance and complete linkage. Side annotation tracks indicate chemical classification, and top annotation tracks show culture type and treatment groups. Heatmap cell values represent Z-score-normalized volatile compound abundances. Data represent mean values from $n = 6$ biological replicates per treatment.

Control treatments (plants without fungal exposure and fungi without plants) exhibited low VOC emissions (Figure 2a and b).

Compartment-specific chemical strategies reveal temporal convergence in aboveground volatile responses

To test whether fungal volatiles detected from below ground trigger systemic aboveground responses, we analyzed 57 volatiles released from *P. × canescens* leaf tissue. Hierarchical cluster analysis of volatiles revealed distinct, fungal-specific leaf emissions (Figure 3), showing systemic chemical responses to different fungal VOCs exposed below ground. Although VOCs

detected in the belowground compartment were dominated by sesquiterpenes (84%), the aboveground emissions exhibited higher chemical diversity, with alkanes (35%) and monoterpenes (25%) being the most abundant compound classes (isoprene could not be measured due to methodological limitations). In contrast to the below ground compartment, where VOC profiles remained treatment-resolved at both week 3 and week 6 (Figure 4c and d), above ground VOC profiles showed increasing similarity across treatments over time, as evidenced by NMDS (Figure 5c and d).

At week 3 of exposure, the heatmap analysis revealed pronounced lifestyle-specific clustering patterns in aboveground volatile profiles (Figure 3a). The pathogenic *H. annosum* induced a distinctive systemic signature in the leaves,

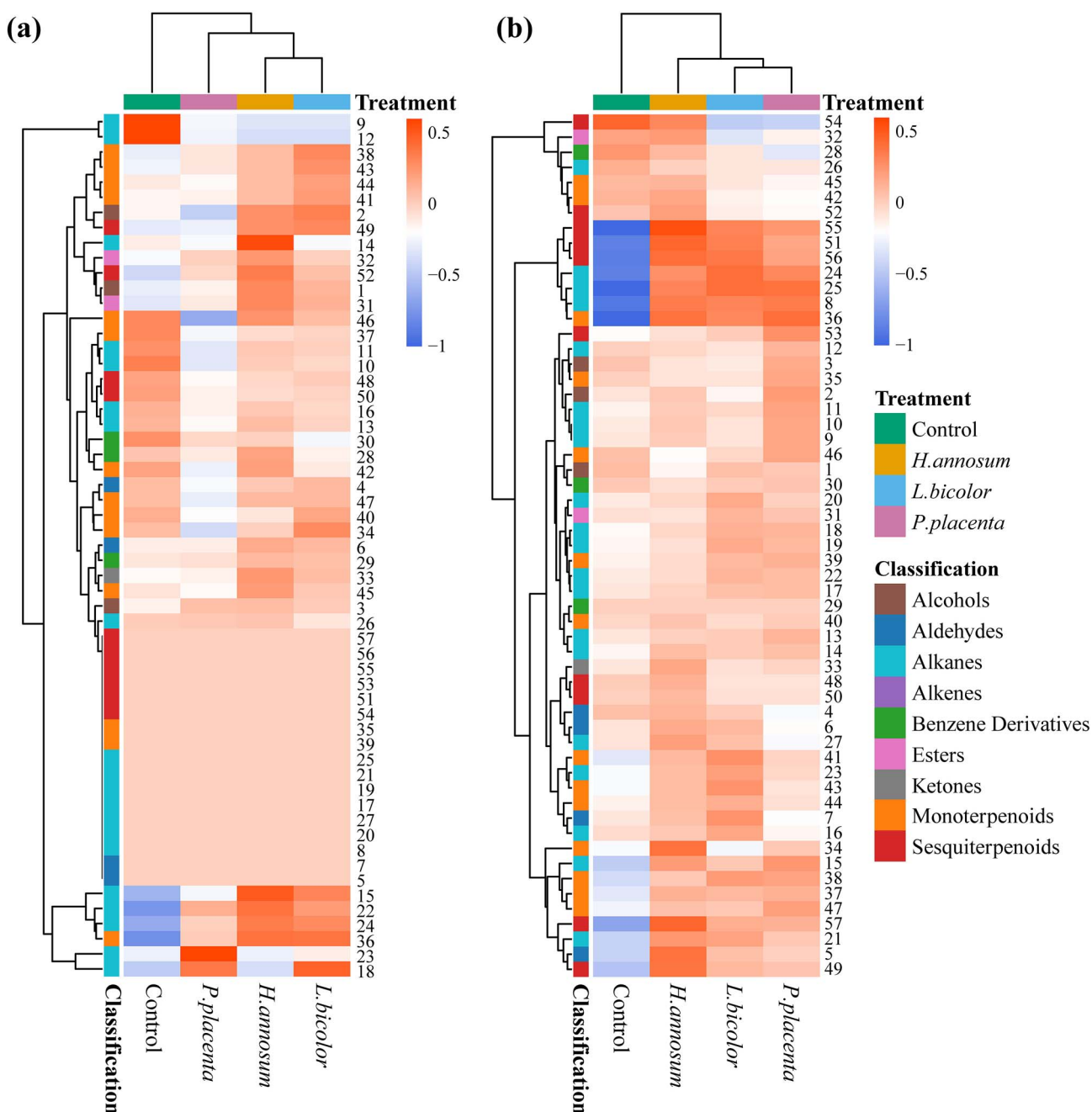


Figure 3 Hierarchical clustering of aboveground leaf VOC profiles in poplar exposed to fungal volatiles belowground. (a, b) Heatmaps showing Z-score normalized volatile compound abundances from poplar leaves at weeks 3 (a) and 6 (b) of co-cultivation. Rows represent individual compounds (IDs on right, see Table S3 available as Supplementary Data at *Tree Physiology* Online); columns show treatments where plants were exposed to volatiles from different fungal cultures (*H. annosum*, *L. bicolor*, *P. placenta*) or control medium via the non-contact pot-in-pot system. Hierarchical clustering was performed using Euclidean distance and complete linkage. Side annotation tracks indicate chemical classification of compounds, and top annotation tracks show fungal treatments. Heatmap cell values represent Z-score-normalized volatile compound abundances. Data represent mean values from $n = 6$ biological replicates per treatment.

characterized by broad upregulation across chemical classes, with 3-methylundecane (compound 14) detected at elevated levels only in the *H. annosum* treatment, and particularly strong increases in esters such as (Z)-3-hexen-1-yl acetate (compound 31), which increased to 6.83 compared with 4.20 nmol m⁻² s⁻¹ in controls (Figure 3a; Table S3 available as Supplementary Data at *Tree Physiology* Online). The ectomycorrhizal *L. bicolor*

induced a selective leaf response, with elevated monoterpenoids but little changes in alkanes and aldehydes. In contrast, the exposure of saprotrophic *P. placenta* volatiles did not lead to a strong aboveground leaf response, as seen from the minimal deviation from control levels across most chemical classes, with many compounds decreased in abundance compared with the control. Specifically, tridecane (compound 23) showed a clear

increase at week 3 under *P. placenta* exposure and was higher than in the other treatments (Figure S2 and Table S3 available as Supplementary Data at *Tree Physiology* Online).

By week 6, fungal VOC-discrimination decreased, as evidenced by reduced dendrogram distances in the hierarchical clustering analysis (Figure 3b). The treatment-specific branches from week 3 shifted toward more overlapping patterns, with reduced separation between groups. This convergence appeared particularly in the monoterpenoid and sesquiterpenoid classes, where initial distinct signatures transitioned to more consistent levels across treatments. However, some sesquiterpenes (compounds 49, 57), a monoterpene (compound 34) and an aldehyde (compound 5) showed higher abundance in the *H. annosum* treatment relative to others. For the overlapping compound bouquets, different lifestyle fungi still led to distinct patterns.

Despite this temporal convergence, all fungal treatments were clearly distinguishable from control conditions at both time points, but their differences increased at week 6, as seen in the dendrogram distances for sesquiterpenes and alkanes, indicating a remarkable systemic impact of belowground volatile exposure on leaf metabolism. This temporal dynamic in aboveground responses contrasted markedly with the persistent lifestyle-specific discrimination observed in belowground volatile profiles throughout the experimental period.

Emission rate quantification reveals compartmentalized VOC emissions with opposing temporal dynamics

Quantitative emission profiling revealed spatial specialization and temporal divergence in plant–fungal chemical interactions (Figures 4 and 5). Root-zone headspace emissions spanned a range from 0.5 to 32.1 pmol m^{−2} s^{−1} and exhibited fungal lifestyle-dependent signatures. At week 3, co-cultivation with *H. annosum* resulted in the highest belowground emissions (31.6 pmol m^{−2} s^{−1}), with sesquiterpenoids accounting for 98% (Figure 4a). *Laccaria bicolor* showed low emissions in both monoculture (1.71 pmol m^{−2} s^{−1}) and co-cultivation with poplar (2.16 pmol m^{−2} s^{−1}). By week 6, *P. placenta* showed higher emissions in co-cultivation with poplar (9.80 pmol m^{−2} s^{−1}) than in monoculture (6.31 pmol m^{−2} s^{−1}) (Figure 4b).

The NMDS of belowground VOC profiles showed lifestyle-specific structuring across time points (Figure 4c and d; model fit (stress) = 0.035 and 0.071, respectively). Treatment groups formed distinct, non-overlapping clusters, with stable patterns from week 3 to week 6 as clusters maintained relative positions and separation distances. Multivariate analysis showed significant treatment separation (PERMANOVA: week 3: $F_{6,34} = 725.86$, $R^2 = 0.992$, $P = 0.001$; week 6: $F_{6,35} = 409.58$, $R^2 = 0.986$, $P = 0.001$). β -Diversity (calculated as Bray–Curtis dissimilarity between samples within each treatment group) analysis showed declining within-group variability over time, with fungal treatments converging to low Bray–Curtis dissimilarities by week 6, while controls retained higher heterogeneity (Table S4 available as Supplementary Data at *Tree Physiology* Online).

Leaf volatile emissions ranged from 4.6–7.5 nmol·m^{−2} s^{−1} and were dominated by esters (46–68%) and monoterpenoids (Figure 5a and b). Among the esters, (Z)-3-hexen-1-yl acetate emissions were higher in the *H. annosum* treatment (6.83 nmol·m^{−2} s^{−1})

compared with controls (4.20 nmol·m^{−2} s^{−1}) and other fungal treatments in week 3 (Figure 5a). However, methyl salicylate (MeSA) emission in the control (2.29 nmol·m^{−2} s^{−1}) was higher than in the *H. annosum* treatment (1.68 nmol·m^{−2} s^{−1}). By week 6, these emissions converged across treatments, with (Z)-3-hexen-1-yl acetate levels close to 1 nmol·m^{−2} s^{−1} in all groups, while MeSA increased overall, with elevations particularly evident in pathogenic treatment (6.13 nmol·m^{−2} s^{−1}) relative to ectomycorrhizal and saprotrophic groups.

No significant differences in total leaf emission rates were observed among treatments at either time point. The NMDS ordination showed temporal convergence (Figure 5c and d; stress = 0.134 and 0.062, respectively). At week 3, treatments formed partially overlapping but distinguishable clusters (Figure 5c). By week 6, fungal treatments showed increased overlap with each other, while control plants remained distinguishable from fungal-exposed plants along NMDS1 (Figure 5d). Treatment effects were significant across time points (PERMANOVA: week 3: $F_{3,20} = 3.51$, $R^2 = 0.345$, $P = 0.002$; week 6: $F_{3,18} = 6.00$, $R^2 = 0.500$, $P = 0.001$). Fungal treatments remained distinguishable from controls (root: $P < 0.001$; leaf: $P < 0.01$) without physical contact.

Discussion

Our study demonstrates that poplars can distinguish among a pathogenic, mutualistic and saprotrophic fungi solely through sensing fungal VOCs, triggering a systemic response that alters aboveground leaf VOC emissions. This finding suggests a key mechanism by which plants navigate the complex chemical dialog of the rhizosphere, where interactions are shaped not only by soluble root exudates that mediate mutualisms (Yu et al. 2022), but also by airborne signals that enable pre-contact recognition between fungi and plants (Ding et al. 2020, Feng et al. 2025). This finding aligns with the broader view that microbial volatiles function as inter-kingdom infochemicals (Schulz-Bohm et al. 2017, Weisskopf et al. 2021).

We addressed the question of whether plant responses to fungal VOCs are generalized or specific. Systemic leaf volatile emissions reflected lifestyle-specific, rather than generalized responses to fungal presence. The results showed strategy-resolved patterns rather than generalized stress responses. The OPLS-DA and hierarchical clustering of leaf volatiles revealed distinct chemical signatures for each fungal lifestyle (Figure 3). Pathogenic *H. annosum* induced a broad upregulation across multiple compound classes, ectomycorrhizal *L. bicolor* triggered selective monoterpenoid responses, and saprotrophic *P. placenta* did not cause remarkable changes compared with controls. These differentiated systemic patterns indicate non-additive outcomes and suggest specific plant recognition mechanisms at the plant–fungus interface, phenomena that are increasingly recognized in complex plant–microbe interactions where plants can produce synergistic benefits (Hestrin et al. 2019, Rawat et al. 2025) through deep molecular regulation (Gao et al. 2023).

Regarding temporal stability, belowground VOC profiles maintained discrimination over 6 weeks, with consistent separation in NMDS ordinations (stress 0.035–0.071; Figure 4c and d) and declining Bray–Curtis dissimilarity (PERMANOVA $P = 0.001$; Table S4 available as Supplementary Data at *Tree Physiology* Online). This persistence aligns with the belowground aspect of

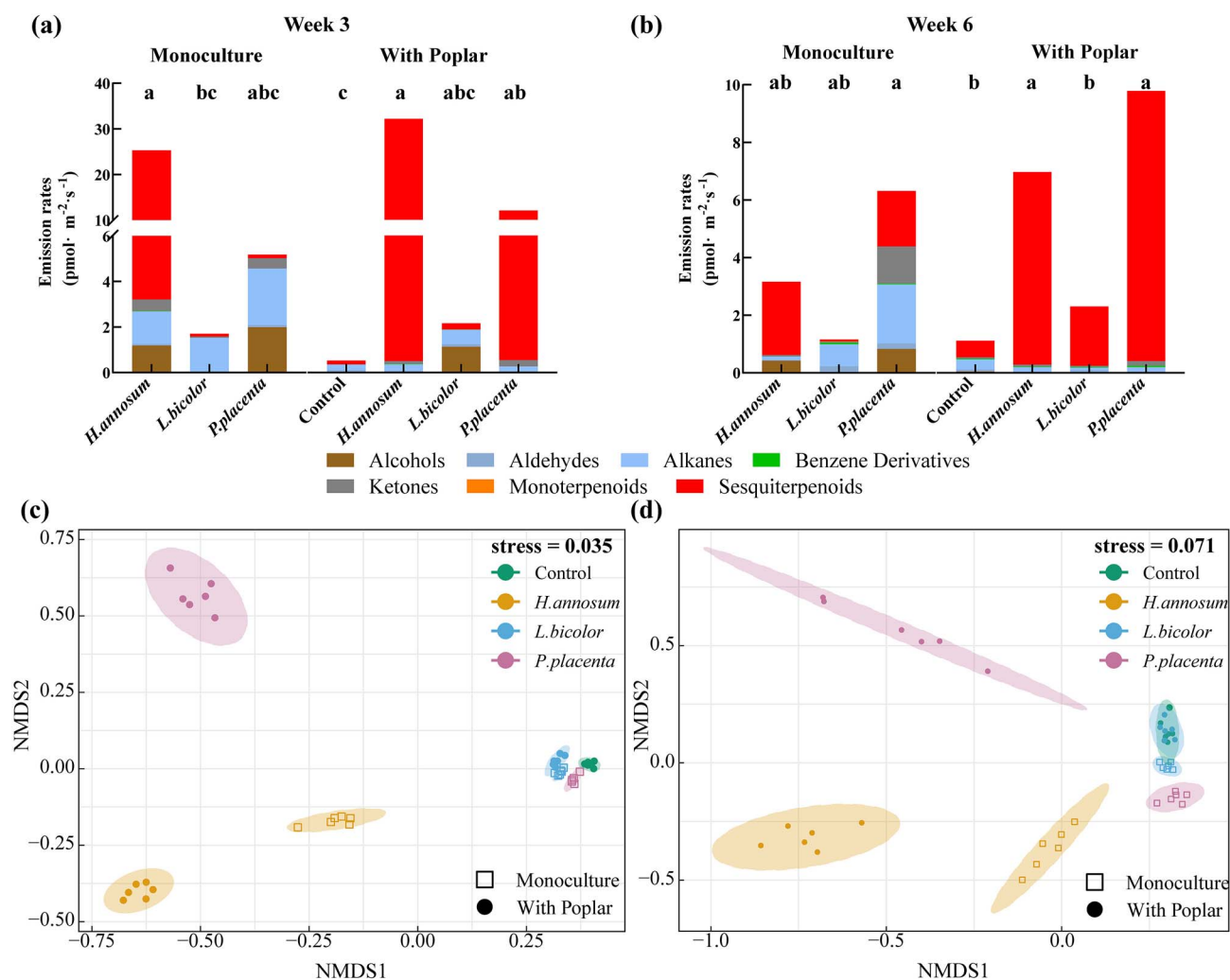


Figure 4 Effects of contact-free fungal co-cultivation on belowground VOC emission profiles and NMDS of VOC profiles over time. (a, b) Stacked bar plots depicting total VOC emission rates ($\text{pmol m}^{-2} \text{s}^{-1}$) at week 3 (a) and week 6 (b), categorized by chemical functional groups. Left panels show emissions from fungal monocultures; right panels illustrate VOC emissions from the root-zone headspace (including emissions from both poplar roots and fungal cultures) in a contact-free pot-in-pot system where poplar plants were exposed to fungal VOCs. Co-cultivations include *H. annosum*, *L. bicolor* and *P. placenta*, with monocultural controls. Different lowercase letters above denote significant differences among treatments ($P < 0.05$, Kruskal–Wallis test followed by Dunn’s post hoc test with Bonferroni correction). (c, d) Non-metric multidimensional scaling (NMDS) ordination plots showing β -diversity of belowground VOC profiles at week 3 (c) and week 6 (d), based on Bray–Curtis dissimilarity indices. Points represent individual replicates, colored and shaped by treatment group as indicated in the legends. Ellipses (dashed for monocultures, solid for poplar exposures) enclose group clusters. Stress values reflect ordination fit (week 3: 0.035; week 6: 0.071). Overall treatment effects were assessed by PERMANOVA. Week 3: $F_{6,34} = 12.96$, $R^2 = 0.696$, $P = 0.001$; Week 6: $F_{6,35} = 5.02$, $R^2 = 0.463$, $P = 0.001$. Data are from $n = 6$ biological replicates per treatment.

our hypothesis I, contrasting transient fluctuations observed in other systems, such as rapid protein phosphorylation changes in response to green leaf volatiles (Tanarsuwongkul et al. 2024) or short-term microbial gene expression shifts induced by fungal VOCs (Chang et al. 2024), and emphasizing sustained ecological strategies in our non-contact setup. On spatial coordination versus compartmentalization, responses were clearly divided: from root-zone sesquiterpene-dominated profiles ($0.5\text{--}32.1 \text{ pmol m}^{-2} \text{s}^{-1}$) were detected, while leaves shifted to alkane/ester/monoterpene blends ($4.6\text{--}7.5 \text{ nmol m}^{-2} \text{s}^{-1}$; Figures 4a, b and 5a, b). This compartmentalization supports a model of localized sensing in roots and systemic integration in leaves, which aligns with the introduction’s emphasis on whole-plant coordination. This finding also corresponds with the aboveground temporal convergence predicted in hypothesis II, as evidenced by NMDS

clustering (stress 0.134–0.062; Figure 5c and d). Notably, the dominance of sesquiterpenes in roots (comprising 84% of compounds) partially meets hypothesis III, though leaf diversity suggests broader chemical classes contribute to systemic responses.

Our study revealed that exposure to the fungal pathogen *H. annosum* induced high belowground sesquiterpene emissions ($31.6 \text{ pmol m}^{-2} \text{s}^{-1}$ at week 3, persisting through week 6; Figure 4a and b), including additional compounds such as isodene and cedrene (Figure 2a and b). This response may be mediated by rapid intracellular calcium (Ca^{2+}) transients, a known early signaling event in plant–pathogen interactions that activates downstream defense pathways (Zhang et al. 2014); however, direct quantification of these events was not performed. The sustained high sesquiterpene emissions are consistent with a

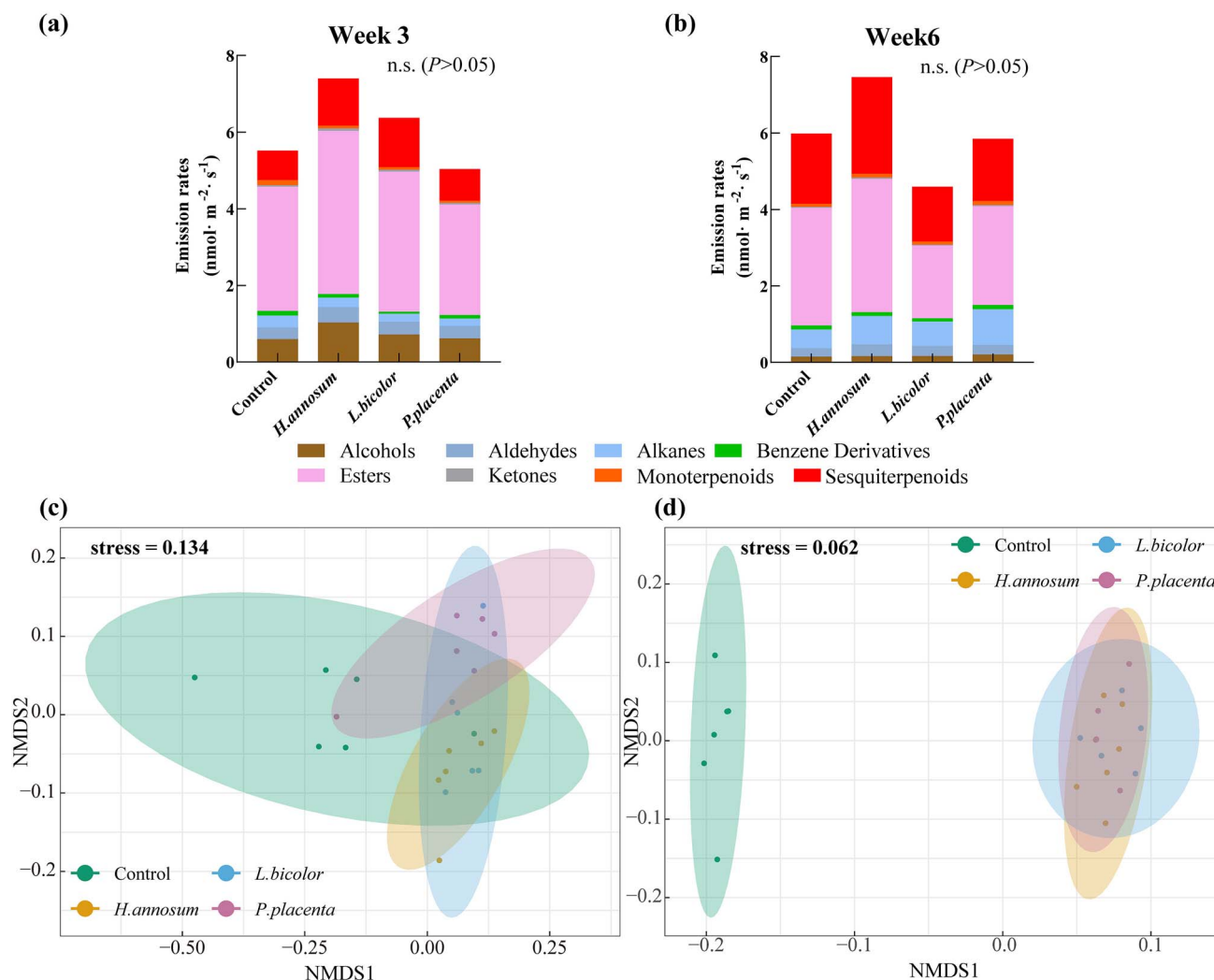


Figure 5 Effects of fungal VOC exposure on aboveground leaf VOC emission profiles and NMDS of VOC profiles over time. (a, b) Stacked bar plots depicting VOC emission rates at week 3 (a) and week 6 (b) of co-cultivation, categorized by chemical functional groups. Treatments include poplar plants without fungal exposure (control) and those exposed to fungal VOCs from *H. annosum*, *L. bicolor* or *P. placenta* in a contact-free pot-in-pot system. No significant differences in emission rates were observed among treatments at either week 3 or week 6 (n.s., $P > 0.05$, Kruskal–Wallis test followed by Dunn’s post hoc test with Bonferroni correction). (c, d) Non-metric multidimensional scaling (NMDS) ordination plots showing β -diversity of aboveground VOC profiles at week 3 (c) and week 6 (d), based on Bray–Curtis dissimilarity indices. Points represent individual replicates, colored and shaped by treatment group as indicated in the legends. Ellipses enclose group clusters. Stress values reflect ordination fit (week 3: 0.134; week 6: 0.062). Overall treatment effects were assessed by PERMANOVA. Week 3: $F_{3,20} = 3.51$, $R^2 = 0.345$, $P = 0.002$; Week 6: $F_{3,18} = 6.00$, $R^2 = 0.500$, $P = 0.001$. Data are means from $n = 6$ biological replicates per treatment.

barrier program that can be initiated in advance (Aratani et al. 2023). From a cost–benefit perspective, such chronic elevation represents an investment in preemptive barriers, reflecting a fundamental growth–defense tradeoff shaped by evolutionary optimization (Monson et al. 2022). This hypothesis is further supported by findings that species tend to maximize only one of several possible defense strategies (Sancho-Knapik et al. 2023). From an ecological perspective, this strategy of deploying high levels of constitutive antifungal terpenes has been shown to be a proven mechanism for preventing necrotrophic invasion, thereby contributing to forest pathogen resistance (Wang et al. 2024).

In contrast, the ectomycorrhizal fungus *L. bicolor* elicited consistently low emissions ($1.71 \text{ pmol m}^{-2} \text{ s}^{-1}$; Figure 4a and b) across all time points and showed minimal compositional changes upon co-cultivation. This restrained VOC profile does

not suggest a lack of communication but rather reflects a highly efficient ‘less is more’ signaling strategy. This is supported by studies showing that even low-abundance sesquiterpenes from *Laccaria* are potent enough to reprogram host root architecture (Ditengou et al. 2015). This targeted approach, possibly modulated by effectors like MiSSP7 (Daguerre et al. 2020), may optimize mutualistic establishment by avoiding the activation of broad-spectrum host defenses while maintaining effective chemical communication.

The saprotrophic *P. placenta* exhibited dramatic plasticity in response to plant presence, with VOC emissions increasing from near-zero monoculture levels to $11\text{--}12 \text{ pmol m}^{-2} \text{ s}^{-1}$ when co-cultivated with poplar, maintaining elevated emissions ($\sim 9.80 \text{ pmol m}^{-2} \text{ s}^{-1}$) through week 6 (Figure 4a and b). This response was accompanied by compositional shifts toward

sesquiterpenes such as silphinene (Figure 2a and b). Rather than indicating pathogenic behavior, this dynamic response likely reflects the opportunistic resource-sensing capacity typical of saprotrophic fungi, where metabolic investment scales with substrate availability (Marañón-Jiménez et al. 2021). The temporal increase suggests adaptive metabolic flexibility rather than aggressive host exploitation, consistent with the decomposer lifestyle that relies on detecting and efficiently utilizing available organic matter (Metz et al. 2025).

Exposure to fungal VOCs belowground induced systemic changes in leaf emissions aboveground. Initially, plants discriminated between fungal lifestyles, for instance ester upregulation under *H. annosum* (e.g. (Z)-3-hexen-1-yl acetate) (Figure 3a and b). Over time, these initially distinct VOC profiles showed convergence patterns (PERMANOVA $P = 0.001$ – 0.002 ; Figure 5c and d), reflecting a shift from acute green leaf volatile (GLV)-type responses toward more persistent salicylate-linked signaling, as indicated by increased methyl salicylate (MeSA) levels at week 6. This temporal progression from immediate GLV responses to sustained MeSA signaling is consistent with plant defense signaling patterns, where early volatile responses transition to more persistent systemic signals over prolonged exposure (Frank et al. 2021, Gong et al. 2023, Laupheimer et al. 2023).

The present findings reveal volatile-mediated surveillance as a complementary early layer of plant–microbe interactions, extending beyond contact-dependent PRR-MAMP recognition (Medina-Puche et al. 2020). Perception may involve membrane-associated candidates and KAI2-related pathways proposed for volatile/karrikin sensing in other contexts (Bythell-Douglas et al. 2017); we did not assay receptors here, so genetic tests in *Populus* will be required in the future (Stirling et al. 2024). The shared terpenoids may complicate cue origin, yet enable pattern-level decoding of mixture features (Zeng et al. 2022). In fact, this ambiguity may be ecologically beneficial, enabling shared chemical channels for compatibility signaling and threat recognition (Kanchiswamy et al. 2015, Sharma et al. 2017). Ecologically, such a system can contribute to microbiome assembly by allowing root VOCs to filter communities and facilitate feedbacks like neighbor facilitation (Gfeller et al. 2019, Mészárosová et al. 2024), though further studies are needed to validate these adaptive benefits.

Several limitations should be acknowledged. First, the simplified ‘pot-in-pot’ system cannot replicate the spatial heterogeneity, real-soil gradients and multi-species dynamics that influence VOC diffusion in natural environments (Parlin et al. 2022). Second, our 6-week study window captures only early interaction dynamics but may overlook long-term acclimation processes (Beckman et al. 2023). Third, the origin of individual compounds remains ambiguous without source tracing; future studies should employ stable isotopes to assign VOCs to plant or fungal sources (Ghirardo et al. 2011, Honeker et al. 2023). Fourth, the bidirectionality of VOC signaling needs validation via reciprocal assays and genetic approaches (e.g. terpene synthase (TPS) knockouts). Additional methodological concerns include culture plate replacement or batch effects, VOC adsorption to experimental material, and differences in diffusion coefficients between sealed PIP systems and natural soil pore structures. To address these limitations future studies should incorporate mesocosms with diverse fungi, proteomics analysis to identify plant receptors, and field studies to evaluate the generality of these findings beyond *Populus*.

In conclusion, our study identified three key findings of plant volatile-mediated recognition. (i) Plants detect and discriminate among fungal lifestyles based solely on airborne VOC cues, demonstrating specific rather than generalized responses that likely confer evolutionary advantage by enabling early threat detection in complex rhizosphere environments. (ii) Chemical signaling exhibited organ-specific patterns, with belowground responses maintaining discriminatory specificity while aboveground responses showed convergent patterns, indicating a balance between selective recognition and metabolic efficiency that extends beyond binary classification systems. (iii) Functional compartmentalization was observed, with sesquiterpenes dominating the discriminatory volatile classes, where root systems served as primary sensory organs and leaf tissues as integrative response centers. These results establish volatile-mediated surveillance as a complementary early-stage recognition mechanism in trees, widening the concept of pattern recognition beyond physical contact and offering potential for VOC-based diagnostics in forest health monitoring.

Supplementary Data

Supplementary data for this article are available at *Tree Physiology* Online.

Author contributions

P.Z., M.R. and J.-P.S. designed research; P.Z. performed research with B.W. (GC-MS); P.Z. analyzed data; P.Z., M.R., A.G. and J.-P.S. interpreted data; and P.Z. wrote the paper with input from all authors.

Conflict of interest

None declared.

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Data availability

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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