Article

Ectomycorrhizal Influence on the Dynamics of Sesquiterpene Release by *Tricholoma vaccinum*

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Abstract: *Tricholoma vaccinum* is an ectomycorrhizal basidiomycete with high host specificity. The slow-growing fungus is able to produce twenty sesquiterpenes, including α-barbatene, sativene, isocaryophyllene, α-cuprenene, β-cedrene, β-copaene, 4-epi-α-acoradiene, and chamigrene in axenic culture. For the three major compounds, Δ6-protoilludene, β-barbatene, and an unidentified oxygenated sesquiterpene (*m/z* 218.18), changed production during co-cultivation with the ectomycorrhizal partner tree, *Picea abies*, could be shown with distinct dynamics. During the mycorrhizal growth of *T. vaccinum*–*P. abies*, Δ6-protoilludene and the oxygenated sesquiterpene appeared at similar times, which warranted further studies of potential biosynthesis genes. In silico analyses identified a putative protoilludene synthesis gene, *pie1*, as being up-regulated in the mycorrhizal stage, in addition to the previously identified, co-regulated geosmin synthase, *ges1*. We therefore hypothesize that the sesquiterpene synthase *pie1* has an important role during mycorrhization, through Δ6-protoilludene and/or its accompanied oxygenated sesquiterpene production.

Keywords: *Tricholoma vaccinum*; ectomycorrhiza; volatilome; sesquiterpene synthases

1. Introduction

Basidiomycete, mushroom-forming fungi are well-known producers of sesquiterpenoids [1,2]. This class of natural products includes well-known plant compounds, e.g., zingiberene, from the spice and traditional medicinal plant, ginger (*Zingiber officinale*), or the sesquiterpene lactones such as the anti-malaria compound artemisinin [1]. After backbone assembly, sesquiterpenoids undergo modifications to form, e.g., the fresh soil odor compound geosmin or many essential oils [1]. Aside from plants, volatiles including sesquiterpenes are prevalent in fungi, where the mushroom odor compounds 3-octanone and 1-octen-3-ol are found in addition to merosesquiterpenoids, the latter being formed from precursors with mixed origin in biosynthesis through both the mevalonate and non-mevalonate pathways [3]. A wealth of compounds was isolated and identified, e.g., from plant endophytic fungi [4,5]. Sesquiterpenes from fungi play a significant role in their headspace chemistry [6], and just like other volatile organic compounds (VOCs), they are regarded as infochemicals that activate inter- and intraspecies communication [2,7,8]. Their potential functions include interactions with other organisms, from immunomodulatory/pathogenicity-related functions, through interspecific interactions, to mutual symbiosis [9,10]. Production might be triggered by environmental, as well as developmental cues [11–13]. Hence, the function of sesquiterpene volatiles in mutually symbiotic interactions was addressed to unravel the role of these compounds as natural communication molecules in these co-evolved systems.
**Tricholoma vaccinum** is a late-stage ectomycorrhizal fungus, characterized by slow growth and high host specificity towards *Picea abies*, while during saprophytic growth it degrades plant litter [14,15]. It is genetically amenable, and can be grown in culture under axenic conditions with its natural host [14]. While fast-growing ectomycorrhizal fungi have been used widely to study symbiosis, late-stage mycorrhizal fungi have rarely been used [15,16]. However, since they are replacing fast-growing generalist mycorrhizal partners in later successional stages, the communication system, including volatiles, may be more specific with these [16]. Hence, *T. vaccinum* was used in this study as a model for late-stage ectomycorrhizal fungi. The production of multiple compounds, including volatiles and phytohormones, has been shown [16,17].

The synthesis of isoprenoids can proceed via the methyl erythritol 4-phosphate (MEP/DOXP) pathway, starting from farnesol-pyrophosphate [18,19], or, as known for fungi, using the mevalonate pathway [5]. Isotope labeling has shown the synthesis of geosmin to occur via the mevalonate pathway for the basidiomycete *T. vaccinum* [16]. A sesquiterpene cyclase then generates a cationic intermediate that undergoes a series of transformations, until a stable product is formed that consists of several products with different skeletal scaffolds after rearrangement [5,13]. In the case of meloebiosynthesis, \( \Delta^6 \)-protoilludene is the first stable product [20]. The ecological role of fungal sesquiterpenes may include acting as pheromones, allomones, or kairomones [21]. In ectomycorrhizae, thu-jopsene, a sesquiterpene from *Laccaria bicolor*, was found to enhance lateral root elongation and root hair prolongation in *Populus*, and to promote superoxide anion radical formation in the meristematic zone of the root tip [21,22]. Dehydroaromadendrene, \( \beta \)-cubebene, and longicyclene have been reported to promote the symbiosis between *Tuber borchii* and *Tilia americana* without contact, at the same time eliciting hyphal elongation [23].

Coding for a terpene synthase, the production of geosmin by the ectomycorrhizal *T. vaccinum* has been found to occur, depending on the activity of ges1. The knockdown of this gene reduced the amount of geosmin in the headspace of the fungus [24]. In this previous study, we focused on the major products, while in this study we attempted to learn more about the full spectrum of volatiles involved in the mycorrhization process.

The sampling of below-ground headspace, excluding the contamination by above-ground, tree-needle-derived compounds, requires static sampling with microfibers for solid-phase microextraction (SPME) [23,25,26]. We adapted the technique for mycorrhizal interactions to compare axenic versus ectomycorrhizal VOC representation in the headspace. We then identified a comprehensive set of potential biosynthetic sesquiterpene synthase genes by analyzing differential expression in mycorrhiza. With this approach, we identified \( \gamma \)-protoilludene (2) and its putative protoilludene synthase, pie1, as one of the major biosynthetic pathways during *T. vaccinum* ectomycorrhizal interaction with its host, *P. abies*.

### 2. Materials and Methods

#### 2.1. Cultivation of *T. vaccinum* and Germination of Seeds of *P. abies*

*T. vaccinum* GK6514 (SF004731, Jena Microbial Resource Collection, Jena, Germany) was cultivated on an agar medium (Modified Melin Norkrans MMNb) [27] at 23 °C for 4 weeks. *Picea abies* (Karst.) seeds (Landesforst Mecklenburg-Vorpommern, Germany) were immersed in tap water over night, surface-sterilized using 30% H\(_2\)O\(_2\) for 60 min, washed, and germinated [28].

#### 2.2. Sampling of Below-Ground Volatiles

A solid MMNa medium (MMNb with 0.5 g/L (NH\(_4\))\(_2\)HPO\(_4\), 2 g/L D-glucose, and without malt extract) [27] was used for axenic *T. vaccinum* cultures and *P. abies*—*T. vaccinum* co-cultivation in a climate chamber with a day/night cycle of 12 h at 23/17 °C at 80% humidity. For separating below-ground from above-ground headspaces, the two chambers were separated by a Teflon cork through which the stem could be passed. The stem was then sealed with agar to prevent the passage of volatiles (Figure 1). The fungus was then inoculated into the lower chamber to allow contact with the root. The volatiles
were sampled via inlet Teflon caps using SPME extraction (Supelco, Bellafonte, PA, USA) coated with three different stationary phases (polydimethylsiloxane 100 µm, polydimethylsiloxane/divinylbenzene 65 µm, and divinylbenzene/carboxene/polydimethylsiloxane 50/30 µm). The fibers were conditioned according to the manufacturer instructions. To prepare the fibers for sampling, they were cleaned by inserting them into the inlet of the gas chromatograph (GC) for 5 min at 250 °C.

Figure 1. Experimental set up for the sampling of root volatile organic compounds. The green tree parts are visible and separated from the below-surface root fungus system, where sampling was made possible through the inlet Teflon cap visible at the right.

2.3. GC–MS Analysis of Volatiles

The headspace VOCs were sampled regularly for 48 h over a period of 6 weeks using SPME fibers. Individual fibers were used for re-sampling one biological replicate, as SPME fibers may differ in their loading capacity. The SPME fibers were then applied to desorption in the GC-inlet, and the compounds analyzed by gas chromatography–mass spectrometry (GC–MS; Trace 1310 GC and ISQ LT MS detector, Thermo Fisher Scientific GmbH, Dreieich, Germany), equipped with a ZB5 column (30 m × 0.25 mm × 0.25 µm) with a 10 m Guardian End (Phenomenex, Aschaffenburg, Germany). Measurements were executed in electron impact (EI) mode with 70 eV (m/z 33 to 450) at 1.5 mL/min helium. The GC injector (split ratio 1:20), transfer line, and ion source were set at 230 °C, 280 °C, and 250 °C, respectively. The volatiles were eluted under programmed conditions from 40 °C (hold for 2 min), followed by a 10 °C/min increase to 230 °C (cleaning step 50 °C/min increase to 300 °C) [29].

2.4. Identification of Sesquiterpenes of T. vaccinum

The mixed n-alkanes C8 through C20 in n-hexane (Sigma-Aldrich, Saint Louis, MI, USA) were measured under the same conditions as the samples. Retention indices (RI) were calculated, and all compounds were preliminary identified by RI. Wherever possible, the identity was confirmed by direct comparison to authentic references. In addition, their mass spectra (MS) and RIs were compared to mass spectral libraries (NIST/EPA/NIH; Wiley, Hoboken, NJ, USA), Massfinder MS 4 and 4 with the Adams collection RI data, in
combination with mass spectral library (NIST/EPA/NIH) 53 MS search. RIs deviating more than ±2 from the authentic references and ±5 from the database were regarded as mismatches, and not considered [7,29]. Spectra of the fungal volatile α-barbatene (6) were obtained using an authentic standard (kindly provided by Prof. Stefan von Reuss, Neuchatel, Switzerland), while Δ6-protoilludene (2) was identified using headspace volatiles from Ophiostoma picea (provided by Dineshkumar Kandasamy, MPI Chemical Ecology, Jena, Germany).

2.5. Homology Searches and Phylogenetic Tree Reconstruction

Sequences of fungal sesquiterpene synthases of Antrodia cinnamomea, Armillaria gallica, Boreostereum vibrans, Coprinopsis cinerea, Coniophora puteana, Fomitopsis pinicola, Lignosus rhinoceros, Omphalotus olearius, and Stereum hirsutum [30] were employed for phylogenetic tree reconstruction. Sesquiterpene synthase genes of Coprinopsis cinerea (Cop1 through Cop6) were used in a Blast search (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 1 March 2022) against the genome of T. vaccinum (available at genome.jgi.doe.gov/portal, accessed on 1 March 2022). Sequence alignments were computed using ClustalW [31], and alignment and phylogenetic reconstructions were performed as implemented on GenomeNet (https://www.genome.jp/tools/ete (accessed on 1 March 2022)) [32]. The maximum likelihood tree was inferred using PhyML (v20160115) [33]. Branch supports were computed out of 100 bootstrapped trees. The phylogenetic tree dataset was thereafter designed using iTOL (www.itol.embl.de, accessed on 23 January 2022).

2.6. Expression Analyses by qRT-PCR for Putative Sesquiterpene Synthase Genes

The influence of co-cultivation on putative sesquiterpene synthase gene expression was analyzed in 3-week-old co-cultures compared to axenic T. vaccinum by qPCR. Total RNA was extracted (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany) [34], cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), and changes in gene expression for nine candidate genes (g334, g1826, g1880, g2958, g5920/ges1, g4529, g7856, g6053 and g10283) were monitored. For reference, cis1, act1, and tef1 were used with three technical replicates (for primers used, Table 1). Quantification was carried out using qPCR (Cepheid Thermocycler, Sunnyvale, CA, USA) with Maxima SYBR Green 2X Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). Primer efficiencies were calculated using a dilution series, and expression ratios were normalized as described [35]. Statistical significance was checked using a Student’s t-test.

Table 1. Oligonucleotide primers used in this study.

| Gene   | Forward Primer Sequence | Reverse Primer Sequence |
|--------|-------------------------|-------------------------|
| g334   | CCCAGTGCATCCATGTGTGA    | CTGCGCTGCAATCACACACT    |
| g1826  | GCAACCCCATGCCGATATAAA   | ATCAGGAAGTGCCGATAAG     |
| g1880  | TCACCAACATGTCGACTTT    | CCTGTGCGATCAAGCATAC    |
| g2958  | CGACAAACCCGATACTCAAAAAT | GGTCTGTAAGAGACGATGCC    |
| g4529  | AGGAGGTGTGTGATCTT     | GGTAGCGCGACAGTAAGATAG  |
| g5920  | GCCACCCAGCGAAGATTTAT  | CAGACGCGAGAGGCAGATG    |
| g6053  | GTGTGCGATCTCGGATATT   | GCGTCGACGTCGTTGATT     |
| g7586  | TCGACTGCTGGCCCAAATAAT | GTGTCGTAAGACGATGCCAT    |
| g10283 | GATGGAATGGGACTGCGTT   | GACGTAACCAAGCAGGTAT    |
| act1   | ACAACATGTGCTCCGGTACTTCT | TTCGCTACAGGAGGCAACCAT    |
| cis1   | CAAATCTCTGCGAGGAGCTTG | ACCCTTCTGTGAAAGGAGGACT |
| tef1   | GGCAACTTTATGTTGCTGTGAACAA | GACCTTCTGTGAAAGGAGGCTT |

3. Results

3.1. Sesquiterpenes Produced by Axenically Grown T. vaccinum

Upon cultivation, T. vaccinum produced more than 20 detectable VOCs (Figure 2). From those, Δ6-protoilludene (2; the numbers refer to each compound identified in Figure 2),
sativene (3), isocaryophyllene (4), β-cedrene (5), α-barbatene (6), α-copaene (7), thujopsene (8), β-barbatene (10), 4-epi-α-acoradiene (13), chamigrene (15), and α-cupranene (16) could be identified (Supplementary Table S1; for mass spectrometry data see Supplementary Figure S1). Among the unidentified sesquiterpenes, one of the major compounds was tentatively assigned as a sesquiterpene ketone. The three major components of the volatilome of T. vaccinum were the VOCs Δ6-protoilludene (2) eluting at 13.57 ± 0.03 min, β-barbatene (10; 14.47 ± 0.03 min), and the unidentified sesquiterpene ketone (17; 16.67 ± 0.03 min), with β-barbatene appearing as the dominating peak. For the major compounds, Δ6-protoilludene (2), β-barbatene (10), and the putative sesquiterpene ketone (17), a role in signaling during mycorrhization was checked.

3.2. The Volatilome of T. vaccinum Is Modified by Mycorrhization

The volatiles produced were monitored over six weeks during growth under mycorrhizal conditions. With this approach, the peak areas of the three major components could be correlated with their production rates during the co-cultivation of T. vaccinum with its host, P. abies. The minor sesquiterpenes were below detection limits at different time points. However, the major compounds were tractable throughout, showing distinct dynamics for Δ6-protoilludene (2), α-6 and β-barbatene (10), and the putative sesquiterpene ketone (17). A decrease in intensity was observed progressively over incubation times for β-barbatene (10). Both Δ6-protoilludene (2) and the major unidentified sesquiterpene ketone (17) appeared at increasing amounts in co-culture with the host, P. abies, where they appeared at increasing amounts in co-culture with the host, P. abies, where they...
3.2. The Volatilome of T. vaccinum Is Modified by Mycorrhization

The volatiles produced were monitored over six weeks during growth under the cultivation conditions used here to identify a higher diversity of sesquiterpenes, the previously observed geosmin was not produced. With this, we could predict the up-regulated gene g2958, now referred to as VOCs, we tested the differential expression of these nine genes in mycorrhiza by qRT-PCR with a four-fold increase under co-cultivation conditions. Most strikingly, under the cultivation conditions used here to identify a higher diversity of sesquiterpenes, the previously observed geosmin was not produced.

Figure 3. Dynamics of the production of major sesquiterpenes. Quantification in axenic (blue) and ectomycorrhizal (orange) conditions for (2) \( \Delta^6 \)-protoilludene (A), (10) \( \beta \)-barbatene (B), and the (17) sesquiterpene ketone (C) are given; \( n = 5 \).

3.3. Multiple Genes for Sesquiterpene Synthases Are Present in the T. vaccinum Genome

A predictive framework based on the known C. cinerea sesquiterpene synthases Cop1 through Cop6 was employed to identify nine genes encoding putative sesquiterpene synthases from the T. vaccinum genome at 30 to 70% similarity, including the already identified geosmin synthase, ges1 [16]. Since we were interested in mycorrhiza-specific VOCs, we tested the differential expression of these nine genes in mycorrhiza by qRT-PCR (Figure 4). While most genes showed similar expression under both conditions, one (gene identifier g5920; ges1, see [24]) appeared with six-fold higher amounts, and a second gene (g2958) with a four-fold increase under co-cultivation conditions. Most strikingly, under the cultivation conditions used here to identify a higher diversity of sesquiterpenes, the previously observed geosmin was not produced.

Phylogenetic tree reconstruction using basidiomycete sesquiterpene synthase genes [5,30] placed the geosmin synthase ges1 into a cluster of functional clade I with enzymes known to catalyze the 1,10 cyclization of \( E,E \)-farnesyl pyrophosphate (Figure 5). Another gene, g7586, was associated with clade II enzymes catalyzing the 1,10 cyclization of 3R-nerolidyl pyrophosphate. Gene g2958 (as well as g6053) is also statistically significantly up-regulated in mycorrhiza. These gene products were placed in the tree to cluster at the base of clade III proteins that 1,11-cyclize \( E,E \)-farnesyl pyrophosphate. In addition to these two basal lineages, products of g334, g4529, and g10283 were found together with other characterized \( \Delta^6 \)-protoilludene synthases. The members of clade IV, coding for known barbatene synthases, and catalyzing the 1,6 cyclization of 3R/S-nerolidyl pyrophosphate, included g1880 and g1826. With this, we could predict the up-regulated g2958, now referred to as pie1, into a group of genes known to encode \( \Delta^6 \)-protoilludene synthases.
Figure 4. Expression of sesquiterpene synthases with log2-fold changes measured by qRT-PCR in *T. vaccinum*. Axenic (blue) and mycorrhiza with *P. abies* (orange) were measured. The gene identifiers for the nine genes, coding for putative sesquiterpene synthases, are given; *n* = 3. Statistical significance is indicated with a star.

Figure 5. Phylogenetic clustering of putative basidiomycete sesquiterpene synthases. The protein sequences identified from the *T. vaccinum* genome are labeled in red; conceptually translated protein sequences of other basidiomycetes are from *Antrodia cinnamomea*, *Armillaria gallica*, *Boreostereum vibrans*, *Coprinopsis cinerea*, *Coniophora puteana*, *Fomitopsis pinicola*, *Lignosus rhinoceros*, *Omphalotus olearius*, *Stereum hirsutum*, and *Schizophyllum commune*; gene identifiers are given in brackets if no protein names are available.
4. Discussion

In order to identify the sesquiterpenes produced by the ectomycorrhizal fungus *T. vaccinum*, a sampling device was used that allowed for both axenic culture or mycorrhiza volatile extraction without interference from above-ground tree VOCs. The ectomycorrhizal basidiomycete *T. vaccinum* was observed without and with its specific partner, Norway spruce (*P. abies*). Basidiomycete sesquiterpenes Δ^6^-protoilludene (2), α- (6) or β-barbatene (10), have been observed previously, while the oxygenated sesquiterpene, putatively identified here as a ketone (17), has not yet been reported [36]. Early in the axenic culture of *T. vaccinum*, β-barbatene (10) was seen as the most abundant compound, in concordance with sesquiterpenes appearing during mycelial growth in other ectomycorrhizal basidiomycetes [37]. The decline in barbatene concentrations observed after new growth had been established might hint at a reaction to mechanical damage incurred during mycelial plug cutting and transfer, as it has been shown for the typical mushroom odor compounds 1-octen-3-ol and 3-octanone, which increase by up to 10-fold after mushroom cutting [38]. In *Fomitopsis*, oct-1-ene and octan-3-one, as well as β-barbatene (10), responded to the chopping of one-week old fruiting bodies, but also under natural conditions at the sporulation stage [39]. Since *T. vaccinum* does not form fruiting bodies under co-cultivation conditions, a response to mechanical stress seems the likely reason for its increased production after the transfer of mycelial plugs.

While VOCs have so far mostly been recorded from mushrooms, and hence during sporulation [13,20,21,40], *T. vaccinum* produced Δ^6^-protoilludene during mycelial, vegetative growth. Protoilludene was long thought to be a biomarker for basidiomycetes, until it was identified from other fungi as well, and it has also been associated with bark beetle attack [41–43]. In our study, Δ^6^-protoilludene (2) production was highly induced under mycorrhizal conditions, a dynamic that is shared with a potential oxidation product, a sesquiterpene ketone (17).

In contrast to β-barbatene (10), the production of Δ^6^-protoilludene (2), after an initial increase, steadily declines over time in mycorrhiza, as is the case for the putative sesquiterpene ketone (17), suggesting that both compounds may be produced from a sesquiterpene by subsequent oxidation involving a cytochrome P450 enzyme. The putative ketone compound has a mass of m/z 218.18, indicative of the presence of a scaffold containing a single oxygen atom. Most oxygenations in sesquiterpenes are catalyzed by specific cytochrome P450 monooxygenases, often encoded adjacent to the respective sesquiterpene synthase gene [44]. We hypothesize that the oxygenated sesquiterpene may be a derivative of protoilludene. Both Δ^6^-protoilludene (2) and the oxygenated sesquiterpene (17) formed in increased amounts in *T. vaccinum* under co-cultivation with its spruce host, which may suggest that these are host communication molecules.

Most basidiomycetes possess inherent abilities to produce sesquiterpenes with varied and unpredictable scaffolds [1,7,45]. This is linked to promiscuity in sesquiterpene synthases, and associated with linked genes coding for modifying enzymes. Two sesquiterpene synthases of *Stereum hirsutum* (gene identifiers 64702, 73029), both belonging to clade III, produced germacrene A-derived products via 1,10 cyclization, which is found with members of clade I, while Δ^6^-protoilludene (2) is usually seen to originate from 1,11 cyclization by members of clade III. Thus, some sesquiterpenes can be produced through multiple mechanistic pathways. Additionally, members of clades I and II sesquiterpene synthases are well known for their non-specific product formation [7]. The sequence ambiguity that characterizes most sesquiterpenes of fungi challenges the in silico functional characterization.

We identified the genes for several sesquiterpene synthases of 30–70% similarity to the *C. cinerea* enzymes Cop1–6. While most genes were expressed under axenic and co-cultivation conditions alike, ges1 (g5920), as well as a second gene (g2958), showed a six- and four-fold upregulation, respectively, in mycorrhiza. While ges1 was clustering with Cop1, Cop2, and Cop3 in clade I, known to be involved in germacrene A production, the newly identified, up-regulated putative sesquiterpene synthase clusters at the base of clade III. This prospective protoilludene synthase, pie1 (g2958), shares a good clustering score.
with the well-characterized protoilludene synthase from Armillaria gallica, using a sequence similarity network construction [46,47].

5. Conclusions

This study identified sesquiterpenes and their biosynthetic enzymes from the ectomycorrhizal fungus T. vaccinum. The applied sampling allowed for the identification of minor compounds of the volatilome that had not been detected previously. The sesquiterpenes Δ⁶-protoilludene (2) and an oxygenated (potentially derivative) sesquiterpene ketone (17) specifically increased in amount in mycorrhiza, as was the transcript for the prospective synthase pie1. With this work, we identified putative signaling compounds and an accordingly regulated gene. This will pave the way to unravel host communication via VOCs in the ectomycorrhizal root system, both during establishment and in mycorrhizal morphotypes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jof8060555/s1: Table S1: Characterization of 20 volatiles produced by T. vaccinum in axenic culture; Figure S1: Mass spectra with relative abundance for the sesquiterpenes; Figure S2: Dynamics of the production of major sesquiterpenes.

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References
1. Vickers, C.E.; Sabri, S. Isoprene. In Biotechnology of Isoprenoids. Advances in Biochemical Engineering/Biotechnology; Springer: Berlin/Heidelberg, Germany, 2015; Volume 148, pp. 289–317, ISSN 0724-6145.
2. Wirth, S.; Krause, K.; Kunert, M.; Broska, S.; Paetz, C.; Boland, W.; Kothe, E. Function of sesquiterpenes from Schizophyllum commune in interspecific interactions. PLoS ONE 2021, 16, e0245623. [CrossRef] [PubMed]
3. De Pinho, P.G.; Ribeiro, B.; Gonçalves, R.F.; Baptista, P.; Valentião, P.; Seabra, R.M.; Andrade, P.B. Correlation between the pattern volatiles and the overall aroma of wild edible mushrooms. J. Agric. Food Chem. 2008, 56, 1704–1712. [CrossRef] [PubMed]
4. Amirzakariya, B.Z.; Shakeri, A. Bioactive terpenoids derived from plant endophytic fungi: An updated review (2011–2020). Phytochemistry 2022, 197, 113130. [CrossRef] [PubMed]
5. Gressler, M.; Löhr, N.A.; Schäfer, T.; Lawrinowitz, S.; Seibold, P.S.; Hoffmeister, D. Mind the mushroom: Natural product biosynthetic genes and enzymes of Basidiomycota. Nat. Prod. Rep. 2021, 38, 702–722. [CrossRef]
6. Riipinen, I.; Yli-Juuti, T.; Pierce, J.R.; Petäjä, T.; Worsnop, D.R.; Kulmala, M.; Donahue, N. Aerosols—the contribution of organics to atmospheric nanoparticle growth. Nat. Geosci. 2012, 5, 453–458. [CrossRef]
7. Ichinose, H.; Kitaoka, H. Insight into metabolic diversity of the brown-rot basidiomycete Postia placenta responsible for sesquiterpene biosynthesis: Semi-comprehensive screening of cytochrome P450 monoxygenase involved in protoilludene metabolism. Microbi. Biotechnol. 2018, 11, 952–965. [CrossRef]
8. Hung, R.; Lee, S.; Bennett, J.W. Fungal volatile organic compounds and their role in ecosystems. Appl. Microbiol. Biotechnol. 2015, 99, 3395–3405. [CrossRef]
9. Pham, H.T.; Doan, T.P.; Kim, H.W.; Kim, T.W.; Park, S.Y.; Kim, H.; Lee, M.; Kim, K.H.; Oh, W.K.; Lim, Y.W.; et al. Cyclohumulanoid sesquiterpenes induced by the noncompetitive coculture of Phellinus orientoasiaticus and Xyloclen flaviporus. J. Nat. Pred. 2022, 85, 511–518. [CrossRef]
10. Kreuzenbeck, N.B.; Seibel, E.; Schwitalla, J.W.; Fricke, J.; Conlon, B.H.; Schmidt, S.; Hammerbacher, A.; Köllner, T.G.; Poulsen, M.; Hoffmeister, D.; et al. Comparative genomic and metabolomic analysis of Termitomyces species provides insights into the terpenoid of the fungus cultivar and the characteristic odor of the fungus garden of Macrotermes natalensis termites. mSystems 2022, 7, e012142. [CrossRef]

11. Wirth, S.; Kunert, M.; Ahrens, L.M.; Krause, K.; Broska, S.; Paetz, C.; Kniemeyer, O.; Jung, E.M.; Boland, W.; Kothe, E. The regulator of G-protein signalling Thn1, links pheromone response to volatile production in Schizophyllum Commun. Environ. Microbiol. 2018, 20, 3684–3699. [CrossRef]

12. Freihorst, D.; Brunsch, M.; Wirth, S.; Krause, K.; Kniemeyer, O.; Linde, J.; Kubert, M.; Boland, W.; Kothe, E. Smelling the difference: Transcriptome, proteome and volatile changes after mating. Fungal Genet. Biol. 2018, 112, 2–11. [CrossRef] [PubMed]

13. Kramer, R.; Abraham, W.R. Volatile sesquiterpenes from fungi: What are they good for? Phytochem. Rev. 2012, 11, 15–37. [CrossRef]

14. Henke, C.; Jung, E.-M.; Kothe, E. Hortic. net formation of Tricholoma vaccinum–spruce ectomycorrhiza in hydroponic cultures. Environ. Sci. Pollut. Res. Int. 2015, 22, 19394–19399. [CrossRef] [PubMed]

15. Sammer, D.; Krause, K.; Gube, M.; Wagner, K.; Kothe, E. Hydrophobins in the life cycle of the ectomycorrhizal basidiomycete Tricholoma vaccinum. PLoS ONE 2016, 11, e0167773. [CrossRef]

16. Abdulsalam, O.; Wagner, K.; Wirth, S.; Kunert, M.; David, A.; Kallenbach, M.; Boland, W.; Kothe, E. Krause, K. Phytohormones and volatile organic compounds, like geosmin, in the ectomycorrhizas of Tricholoma vaccinum and Norway spruce (Picea abies). Mycorrhiza 2021, 31, 173–188. [CrossRef]

17. Wagner, K.; Linde, J.; Krause, K.; Gube, M.; Koestler, T.; Sammer, D.; Kniemeyer, O.; Kothe, E. Tricholoma vaccinum host communication during ectomycorrhiza formation. FEMS Microbiol. Ecol. 2015, 91, fiv120. [CrossRef]

18. Banerjee, A.; Sharkey, T.D. Methylerythritol 4-phosphate (MEP) pathway metabolic regulation. J. Biol. Chem. 2003, 278, 20677–20685. [CrossRef] [PubMed]

19. Lin, H.C.; Hewage, R.T.; Lu, Y.C.; Chooi, Y.H. Biosynthesis of bioactive natural products from Basidiomycota. Org. Biomol. Chem. 2019, 17, 1027–1036. [CrossRef]

20. Engels, B.; Heinig, U.; Grothe, T.M.; Stadler, M.; Jennewein, S. Cloning and characterization of an Armillaria gallica cDNA encoding protoiludene synthase, which catalyzes the first committed step in the synthesis of antimicrobial melleolides. J. Biol. Chem. 2011, 286, 6871–6878. [CrossRef]

21. Mineri, D.; Mazzini, V.; Fani, F. Volatile organic compounds: From figurants to leading actors in fungal symbiosis. FEMS Microbiol. Ecol. 2021, 97, 67. [CrossRef]

22. Ditengou, F.A.; Mäurer, A.; Rosenkranz, M.; Felten, J.; Lasok, H.; van Doorn, M.; Legue, V.; Palme, W.K.; Schnitzler, J.-P.; Polle, A. The volatile signalling by sesquiterpenes from ectomycorrhizae fungi reprogrammes root architecture. Nat. Comm. 2015, 6, 6279. [CrossRef] [PubMed]

23. Menotta, M.; Gioacchini, A.M.; Amicucci, A.; Buffalini, M.; Sisti, D.; Stocchi, V. Headspace solid-phase microextraction with gas chromatography and mass spectrometry in the investigation of volatile organic compounds in an ectomycorrhizae synthesis system. Rap. Commun. Mass Spect. 2004, 18, 206–210. [CrossRef] [PubMed]

24. Abdulsalam, O.; Ueberschaar, N.; Krause, K.; Kothe, E. Geosmin synthase ges1 knock-down by siRNA in the dikaryotic fungus Tricholoma vaccinum. J. Basic Microbiol. 2022, 62, 109–115. [CrossRef] [PubMed]

25. Shashenkov, E.E.; Martinez, J.R. Sampling volatile compounds from natural products with headspace/solid-phase micro-extraction. J. Biochem. Biophys. Methods. 2007, 70, 235–242. [CrossRef]

26. Gioacchini, A.M.; Menotta, M.; Bertini, L.; Rossi, I.; Zeppa, S.; Zambonelli, A.; Piccoli, G.; Stocchi, V. Solid-phase microextraction gas chromatography/mass spectrometry: A new method for species identification of truffles. Rapid Commun. Mass Spectrom. 2005, 19, 2365–2370. [CrossRef]

27. Kottke, I.; Guttenberger, M.; Hampp, R.; Oberwinkler, F. An in vitro method for establishing mycorrhizae on coniferous tree seedling. Trees 1987, 9, 191–194. [CrossRef]

28. Krause, K.; Kothe, E. Use of RNA fingerprinting to identify fungal genes specifically expressed during ectomycorrhizal interaction. J. Basic Microbiol. 2006, 46, 387–399. [CrossRef]

29. Meents, A.K.; Chen, S.P.; Reichelt, M.; Lu, H.; Bartram, S.; Yeh, K.W. Volatile DMNT systemically induces jasmonate-independent direct anti-herbivore defense in leaves of sweet potato (Ipomoea batatas) plants. Sci. Rep. 2019, 9, 1–14. [CrossRef]

30. Mischko, W.; Hirte, M.; Fuchs, M.; Mehlen, B.; Brück, T.B. Identification of sesquiterpene synthases from the Basidiomycota Coniophora puteana for the efficient and highly selective β-copaene and cubebol production in E. coli. Microb. Cell Fact. 2018, 17, 164. [CrossRef]

31. Thompson, J.D.; Gibson, T.J.; Higgins, D.G. Multiple sequence alignment using ClustalW and ClustalX. Curr. Protoc. Bioinf. 2003, 1, 2.3.1–2.3.22. [CrossRef]

32. Huerta-Cepas, J.; Serra, F.; Bork, P. ETE 3: Reconstruction, analysis, and visualization of phylogenomic data. Mol. Biol. Evol. 2016, 33, 1635–1638. [CrossRef] [PubMed]

33. Guindon, S.; Dufayard, J.F.; Lefort, V.; Anisimova, M.; Hordijk, W.; Gascuel, O. New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. Syst. Biol. 2010, 59, 307–321. [CrossRef] [PubMed]

34. Erdmann, S.; Freihorst, D.; Raudaskoski, M.; Schmidt-Heck, W.; Jung, E.-M.; Senftleben, D.; Kothe, E. Transcriptome and functional analysis of mating in the basidiomycete Schizophyllum commune. Eukaryot. Cell 2012, 11, 571–589. [CrossRef] [PubMed]
35. Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucl. Acids Res.* **2001**, *29*, e45. [CrossRef] [PubMed]

36. Rösecke, J.; Pietsch, M.; König, W.A. Volatile constituents of wood-rotting basidiomycetes. *Phytochemistry* **2000**, *54*, 747–750. [CrossRef]

37. Lee, S.Y.; Ryu, S.H.; Choi, I.G.; Kim, M. Biosynthesis of eudesmane-type sesquiterpenoids by the wood-rotting fungus, *Polyporus brumalis*, on specific medium, including inorganic magnesium source. *J. Korean Wood Sci. Technol.* **2016**, *44*, 253–263. [CrossRef]

38. Combet, E.; Henderson, J.; Eastwood, D.C.; Burton, K.S. Influence of sporophore development, damage, storage, and tissue specificity on the enzymic formation of volatiles in mushrooms (*Agaricus bisporus*). *J. Agric. Food Chem.* **2009**, *57*, 3709–3717. [CrossRef]

39. Fält, J.; Jonsell, M.; Nordlander, G.; Borg-Karlson, A.K. Volatiles of bracket fungi *Fomitopsis pinicola* and *Fomes fomentarius* and their functions as insect attractants. *J. Chem. Ecol.* **1999**, *25*, 1181–1195. [CrossRef]

40. Kirschner, R.; Oberwinkler, F. A new *Ophiostoma* species associated with bark beetles infesting Norway spruce. *Can. J. Bot.* **1999**, *77*, 247–252. [CrossRef]

41. Azeem, M.; Rajarao, G.K.; Terenius, O.; Nordlander, G.; Nordenhem, H.; Nagahama, K.; Norin, E.; Borg-Karlson, A.K. A fungal metabolite masks the host plant odor for the pine weevil (*Hylobius abietis*). *Fung. Ecol.* **2015**, *13*, 103–111. [CrossRef]

42. De Sena Filho, J.G.; Quin, M.B.; Schmidt-Dannert, C. Genome of *Diaporthe* sp. provides insights into the potential inter-phylum transfer of a fungal sesquiterpenoid biosynthetic pathway. *Fungal Biol.* **2016**, *120*, 1050–1063. [CrossRef] [PubMed]

43. Chow, J.Y.; Tian, B.X.; Ramamoorthy, G.; Hillerich, B.S.; Seidel, R.D.; Almo, S.C.; Jacobson, M.P.; Poulter, C.D. Computational-guided discovery and characterization of a sesquiterpene synthase from *Streptomyces clavuligerus*. *Proc. Natl. Acad. Sci. USA* **2015**, *18*, 5661–5666. [CrossRef]