

Article

Volatile Organic Compound Profiling Supports the Detection of *Heterobasidion annosum* Colonization in Scots Pine Wood

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Abstract

Heterobasidion annosum is a destructive root- and butt-rot pathogen of coniferous forests, and its detection before the development of visible disease symptoms remains challenging. In this study, volatile organic compounds (VOCs) in the headspace of *H. annosum*-colonized Scots pine wood and fungal cultures were characterized by headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME/GC–MS) and compared with uncolonized pine wood and uninoculated culture medium. A total of 101 VOC signals were detected, and 98 received putative or tentative assignments. The colonized wood profile was the most diverse and comprised 64 signals, compared with 24 in uncolonized wood, 27 in the fungal culture, and 25 in the culture medium. Principal component analysis and hierarchical clustering of centered log-ratio-transformed replicate-level profiles revealed distinct compositional patterns among the four sample types. Global false-discovery-rate-adjusted q -values were below 0.05 for methyl 2-furoate, iso-longifolene, and 2-(2-furanyl)-1-propanol in the exploratory wood-profile contrast; the corresponding values for 2-methylfuran and β -barbatene were 0.0885 and 0.320. The study provides a preliminary multicomponent relative VOC profile for broader experimental and field validation.

Keywords: *Heterobasidion annosum*; volatile organic compounds (VOCs); HS-SPME/GC–MS; Scots pine wood; non-destructive diagnostics



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1. Introduction

Heterobasidion annosum (Fr.) Bref. is one of the most destructive fungal pathogens affecting coniferous trees in the temperate regions of the Northern Hemisphere [1]. As the causal agent of root and butt rot, it leads to considerable economic losses in forestry by reducing timber quality and causing premature stand mortality [2]. In Scandinavia, losses associated with *H. annosum* infections are estimated at hundreds of millions of euros annually [3].

The fungus primarily infects trees through freshly cut stump surfaces and spreads via root systems, making it particularly dangerous in pine and spruce stands [4]. Once established, the pathogen produces a characteristic white pocket rot that propagates from roots into butt logs and may advance toward the stem heartwood, severely compromising the mechanical stability of spruce or fir [5]. Transcriptomic analyses indicate an initial surge of lignocellulolytic activity followed by strong induction of lipid metabolism and antioxidant pathways, marking a shift from penetration to nutrient assimilation [6,7]. *Heterobasidion* spp. collectively infect more than 30 conifer hosts across the Northern Hemisphere and remove an estimated 800 million euros of stumpage value annually from European forestry [3,5,8,9]. Beyond timber degradation, the mortality of keystone species such as Scots pine and Norway spruce alters stand composition and facilitates secondary pests [10].

Early detection of *H. annosum* infection poses a significant challenge, as disease symptoms such as crown dieback, reduced growth and fruiting body emergence typically appear only in advanced stages of pathogenesis. Traditional diagnostic methods, based on morphological identification or biological testing, are time-consuming and often insufficiently sensitive. As a result, there is growing interest in analytical techniques that enable the identification of pathogens at earlier infection stages [11]. Detecting *Heterobasidion* spp. in forest stands is crucial for protecting forest ecosystems and forestry management [12,13]. Particularly important is the early identification of infected trees growing along roads crossing forest stands. Root and butt rot caused by *Heterobasidion* spp. may substantially reduce tree stability, increasing the probability of stem breakage or uprooting during storms and strong wind events [14–17]. Falling trees have repeatedly caused road blockages, damage to vehicles and infrastructure, and in some cases, serious injuries or fatalities [18,19]. Rapid and non-destructive methods capable of detecting infection before visible symptoms appear may have significant value not only for forest health monitoring but also for public safety and risk management.

Beyond direct economic losses, *Heterobasidion* root rot represents a major challenge for sustainable forest management [5,8]. Infected trees exhibit reduced growth, decreased timber quality, increased susceptibility to windthrow, and enhanced vulnerability to secondary pests and pathogens [20]. Because infections may persist for decades within root systems and spread underground between neighbouring trees, the early identification of infection centres is essential for effective sanitary management and disease containment [21]. The importance of the early detection of *Heterobasidion* infections extends beyond forest productivity and timber quality. Numerous incidents involving falling trees or uprooted root systems have resulted in vehicle damage, road blockages, injuries, and in some cases, fatalities. Therefore, the ability to identify infected trees before visible symptoms of decline appear may contribute not only to forest health management but also to public safety and risk reduction in forested landscapes. Consequently, there is a growing demand for rapid, non-destructive, and field-deployable diagnostic methods that could support forest health monitoring programmes and improve decision-making in managed forest ecosystems [22].

Routine diagnosis currently relies on visual assessment, fungal isolation, molecular identification, and increasingly on quantitative PCR- and LAMP-based assays. Visual inspection is inexpensive but usually detects only advanced stages of disease when crown decline or fruiting bodies become visible [23,24]. Culture-based methods provide reliable species identification but require destructive sampling and prolonged laboratory incubation [25]. Molecular techniques such as qPCR and LAMP offer high sensitivity and specificity; however, they require laboratory infrastructure, specialized reagents, and the extraction of biological material from roots or wood tissues. Consequently, these methods are not always suitable for rapid large-scale surveys in forest stands [26]. Volatile organic compound profiling may provide a complementary approach because it enables non-destructive

detection of metabolic changes associated with fungal colonization and may ultimately be adapted for portable field sensors or electronic nose technologies [27,28]. Such systems could support rapid screening of large forest areas and facilitate early disease detection before visible symptoms become apparent.

One promising approach is the analysis of volatile organic compounds (VOCs) produced by fungi. Many species, including *H. annosum*, emit characteristic scent profiles during growth and interaction with the host. Gas chromatography with mass spectrometry (GC-MS) allows for precise detection and identification of these compounds even at trace concentrations [29]. GC-MS is increasingly used in plant pathology diagnostics, offering non-invasive monitoring of pathogens in soil, wood or air [30]. Plant disease diagnostics based on GC-MS analyses enables the identification of distinctive volatile organic compound (VOC) profiles emitted by pathogenic fungi such as *Fusarium*, *Rhizoctonia* and *Phytophthora*. This facilitates early detection of infections in both forest and agricultural crops [31]. In the food industry, GC-MS is applied to detect mold associated metabolites in commodities like grains, fruits and dairy products, supporting assessments of freshness, spoilage and potential mycotoxin contamination [32,33]. Medical diagnostics enables detection of fungal infections, for example, pulmonary aspergillosis, by identifying specific metabolic markers in biological samples such as breath, serum or tissue extracts [34,35]. GC-MS can be also used to evaluate indoor air quality by detecting airborne fungal spores and secondary metabolites, particularly in humid environments where molds can pose health risks [36,37].

The aim of this study was to determine whether colonization of Scots pine wood by *Heterobasidion annosum* is associated with characteristic volatile organic compound (VOC) profiles, and to identify candidate compounds that may support the future development of non-destructive diagnostic methods. Particular attention was given to distinguishing VOCs associated with fungal colonization from those originating from pine wood and culture medium.

2. Materials and Methods

2.1. Sample Preparation

The isolates of *Heterobasidion annosum* used in this study originated from the fungal culture collection maintained at Adam Mickiewicz University in Poznań, Poland, and were kindly provided by Prof. Piotr Łakomy. A single *H. annosum* culture was maintained on 2% malt extract agar (MEA) at 22 ± 1 °C in darkness until actively growing mycelium was obtained. The experiment characterizes this culture under the stated laboratory conditions.

The wooden blocks were cut from the sapwood of hundred-year-old pines and had dimensions of $1 \times 2.5 \times 5$ cm. Scots pine (*Pinus sylvestris* L.) sapwood blocks were oven-dried to constant weight, autoclaved at 121 °C for 20 min, and cooled under sterile conditions [38,39].

The sterilized blocks assigned to colonization were placed directly on actively growing *H. annosum* cultures on MEA and incubated at 22 ± 1 °C in darkness for four weeks [3,8,39]. The uncolonized-wood reference comprised sterile pine wood. Four prepared sample types were analyzed: colonized pine wood, uncolonized pine wood, fungal culture on MEA, and uninoculated MEA. Three separately analyzed headspace samples were available per type ($n = 3$; 12 chromatograms in total), and the individual vessel/chromatogram was the analytical unit.

2.2. Analysis of Headspace Volatile Organic Compounds Using Solid-Phase Microextraction and Gas Chromatography–Mass Spectrometry (HS-SPME/GC–MS)

Colonized and uncolonized wood samples were placed separately in glass jars fitted with septum lids. Fungal-culture and uninoculated-MEA samples remained in Petri dishes sealed with parafilm. A 2 mm opening in the side wall of each Petri dish served as the access port for insertion of the SPME needle; the dish remained sealed except during needle insertion. All prepared headspace samples were conditioned for 24 h at 25 °C before extraction. Wood and culture-based samples were analyzed in their respective vessel formats.

In solid-phase microextraction (SPME), the sorption fibre with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) stationary phase was used. The needle of the SPME device (Supelco, Bellefonte, PA, USA) was inserted into the jar or Petri dish with the sample, and then the sorption fibre was introduced into the sample headspace for 30 min at 25 °C. The same fibre type, extraction temperature, and exposure duration were used throughout. Three separately analyzed samples were measured for each of the four sample types, giving 12 chromatograms.

Volatile organic compounds were desorbed from the SPME fibre in the GC injector for 10 min at 250 °C, in a splitless mode. The analyses of chemical composition were carried out using an Agilent 7890A gas chromatograph with an Agilent 5975C mass spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA). Chromatographic separation used an HP-5MS column (Agilent Technologies Inc., Santa Clara, CA, USA) with dimensions 30 m × 0.25 mm × 0.25 µm, and with 5%-phenyl-95%-methylpolysiloxane stationary phase. The oven was programmed from 35 to 250 °C at 5 °C/min. The helium flow rate through the column was 1 mL/min. The ionisation energy in the mass spectrometer was 70 eV. The temperatures of ion source and quadrupole were 230 °C and 150 °C, respectively. Full-scan data were acquired over m/z 29–600.

The total area of all peaks in the chromatogram was taken as 100%. The percentage content of each chemical compound in the total ion current (%TIC) registered by the mass spectrometer was calculated. These values represent relative VOC composition. VOC annotations were assigned by comparing EI mass spectra with the specified reference literature and mass-spectral libraries and by comparing experimental retention indices with literature data for a comparable stationary phase when available [40–43]. The annotations are reported as putative. A C5–C40 *n*-alkane mixture (1 µL) was analyzed under the same chromatographic conditions for retention-index calculation. The early-eluting feature assigned from its EI spectrum as *n*-butane preceded the lowest C5 standard and was classified as tentative. Experimental retention indices ($RI_{exp.}$) were calculated using the equation:

$$RI_{exp.} = 100[n + (t_x - t_n)/(t_{n+1} - t_n)],$$

where n is number of carbon atoms in the *n*-alkane eluted before the compound, t_x is the retention time of the compound, t_n is the retention time of the *n*-alkane eluted before the compound, and t_{n+1} is the retention time of the *n*-alkane eluted after the compound.

According to the analytical notes, the empty glass-jar blank yielded no peaks, whereas styrene occurred in the unused Petri-dish blank. Styrene and tetrachloroethylene were designated background-related signals and excluded from multivariate and univariate biological analyses; siloxane-derived signals were removed from the reported feature table.

2.3. Statistical Analysis

The statistical workflow used the replicate-level %TIC values for all 12 chromatograms. The complete workflow is implemented in the accompanying R script using stats, ggplot2, ggrepel, pheatmap, and readxl. Entries below detection were represented as 0%TIC for this

exploratory analysis. Styrene and tetrachloroethylene were retained in the descriptive table and excluded from multivariate and univariate biological inference, leaving 99 analyte features. All 12 supplied samples were included in the analysis.

The primary planned comparison was colonized versus uncolonized wood. For each analyte, a two-sided Welch two-sample t -test was applied to the three replicate-level %TIC values per group; Welch's unequal-variance formulation was used. Features with identical zero values in both groups were assigned $p = 1$. Mean differences are reported in percentage points with Welch 95% confidence intervals, test statistics, approximate degrees of freedom, raw p -values, and Benjamini–Hochberg-adjusted q -values. A single global correction was applied across all 99 tested features. As a sensitivity analysis, exact two-sided label-permutation p -values were computed from all 20 allocations of the six wood samples and adjusted across the same 99 features. The five compounds emphasized in the display panel form an exploratory subset, while statistical interpretation uses the single global correction across all features. Given $n = 3$ per group, the inferential results are treated as exploratory.

For descriptive multivariate visualization, each replicate profile was reclosed to a constant sum after removal of the two background-related features. Zero components were subjected to deterministic multiplicative replacement using 65% of the smallest positive component in that chromatogram, with proportional rescaling of its positive components, followed by centered log-ratio (CLR) transformation. Replicate-level PCA was performed with prcomp without unit-variance scaling. Hierarchical clustering used Euclidean distance in CLR space and Ward's minimum-variance method (ward.D2); the heatmap shows the 30 features with the largest replicate-level CLR variance, standardized feature-wise to z-scores. PCA and clustering provide descriptive summaries under the stated zero-replacement procedure.

3. Results

Four sample types were analyzed in this study: (i) *H. annosum*-colonized pine wood, (ii) uncolonized pine wood, (iii) *H. annosum* culture on MEA, and (iv) uninoculated MEA. Each type was represented by three separately analyzed samples, resulting in 12 chromatograms. Table 1 reports compound names, retention times, and relative abundances expressed as %TIC. Values detected in all three samples of a type are summarized as mean $\pm$ standard deviation; features detected in only one or two samples are shown as ranges. A dash denotes non-detection under the applied conditions. Detailed results of chemical analyses are provided in the Supplementary Materials.

Across the four prepared sample types, 101 VOC signals were detected; 98 received putative or tentative assignments and three remained unidentified. Colonized wood, uncolonized wood, fungal culture, and uninoculated MEA contained 64, 24, 27, and 25 detected features, respectively (Table 2). These counts summarize detected feature diversity under the applied conditions.

The colonized-wood composition contained a broader mixture of furan derivatives, esters, and terpenoids, whereas uncolonized wood had comparatively large relative contributions from 3-octanone and several aldehydes. The fungal-culture profile was dominated by 4-hydroxy-3-methyl-2-phenyl-2-cyclopenten-1-one, while aldehydes and carboxylic acids contributed strongly to the uninoculated-MEA profile. Styrene and tetrachloroethylene are reported descriptively in Table 1 and were classified as background-related signals for biological analysis.

Table 1. Volatile organic compounds detected in the analyzed samples. Relative abundances are expressed as percentages of total ion current (%TIC).

Group of Compounds	Retention Time (min)	Relative Abundance (%TIC): Mean $\pm$ Standard Deviation or Range			
		<i>H. annosum</i> -Colonized Pine Wood	Uncolonized Pine Wood	<i>H. annosum</i> Culture on MEA	Uninoculated MEA
Aliphatic hydrocarbons (alkanes and alkenes), including:	-	8.22 $\pm$ 0.23	12.97 $\pm$ 0.81	1.82 $\pm$ 0.53	9.97 $\pm$ 1.01
n-Butane (tentative)	1.781	1.35 $\pm$ 0.18	9.15 $\pm$ 0.86	1.82 $\pm$ 0.53	6.77 $\pm$ 0.40
1-Heptene	3.054	0.52 $\pm$ 0.14	-	-	-
1-Octene	4.691	5.59 $\pm$ 0.29	3.82 $\pm$ 0.10	-	-
n-Octane	4.872	0.75 $\pm$ 0.13	-	-	-
2,6-Dimethylundecane	16.490	-	-	-	0.75 $\pm$ 0.09
4,8-Dimethylundecane	17.889	-	-	-	1.08 $\pm$ 0.19
2,7,10-Trimethyldodecane	18.340	-	-	-	1.37 $\pm$ 0.45
Aromatic hydrocarbons, including:	-	-	-	3.39 $\pm$ 0.35	6.87 $\pm$ 0.76
Ethylbenzene	6.336	-	-	-	0.00–0.33
Styrene	7.101	-	-	3.39 $\pm$ 0.35	6.18 $\pm$ 0.68
Naphthalene	15.651	-	-	-	0.58 $\pm$ 0.19
Aliphatic alcohols, polyols, and ethers, including:	-	12.26 $\pm$ 1.34	14.36 $\pm$ 1.42	3.08 $\pm$ 0.62	16.78 $\pm$ 0.78
Ethanol	1.675	-	-	1.63 $\pm$ 0.48	8.90 $\pm$ 0.81
Isobutanol	2.432	0.00–0.21	-	-	-
1-Methoxy-2-propanol	2.912	-	-	-	5.50 $\pm$ 0.65
Isopentanol	3.682	0.38 $\pm$ 0.09	-	-	-
2-Methyl-1-butanol	3.745	0.00–0.30	-	-	-
1,2-Propanediol	3.871	0.34 $\pm$ 0.04	-	-	-
1-Pentanol	4.246	1.19 $\pm$ 0.28	1.95 $\pm$ 0.17	-	-
1-Hexanol	6.537	1.68 $\pm$ 0.19	0.87 $\pm$ 0.07	-	-
2,2-Dimethyl-1,3-dioxolane-4-methanol	8.476	0.46 $\pm$ 0.07	-	-	-
1-Butoxy-2-propanol	8.532	0.00–0.19	-	-	-
1-Heptanol	9.353	0.58 $\pm$ 0.11	-	-	-
1-Octen-3-ol	9.625	2.03 $\pm$ 0.27	8.54 $\pm$ 1.15	1.45 $\pm$ 0.24	-
3-Octanol	10.097	0.00–0.23	-	-	-
2-Ethyl-1-hexanol	11.086	1.64 $\pm$ 0.26	3.00 $\pm$ 0.39	-	2.39 $\pm$ 0.18
2,2,4-Trimethyl-1,3-pentanediol	14.693	1.22 $\pm$ 0.28	-	-	-
2-(2-Butoxyethoxy)ethanol	15.832	1.60 $\pm$ 0.40	-	-	-
2-Methyl-1-undecanol	16.497	0.82 $\pm$ 0.03	-	-	-
Aromatic alcohols, phenols, and ethers, including:	-	0.00–0.56	-	0.00–0.36	7.11 $\pm$ 0.59
p-Methylanisole	10.831	-	-	0.00–0.36	-
Benzeneethanol	13.613	0.00–0.21	-	-	-
2-Phenoxyethanol	16.691	0.00–0.18	-	-	-
1-Phenoxy-2-propanol	17.409	0.00–0.17	-	-	-
Ionol	24.381	-	-	-	7.11 $\pm$ 0.59

Table 1. Cont.

Group of Compounds	Retention Time (min)	Relative Abundance (%TIC): Mean $\pm$ Standard Deviation or Range			
		<i>H. annosum</i> -Colonized Pine Wood	Uncolonized Pine Wood	<i>H. annosum</i> Culture on MEA	Uninoculated MEA
Aliphatic aldehydes, including:	-	0.61 $\pm$ 0.07	10.60 $\pm$ 1.00	-	21.49 $\pm$ 1.25
Isobutanal	2.016	-	4.08 $\pm$ 0.97	-	4.17 $\pm$ 0.34
3-Methylbutanal	2.670	-	-	-	9.74 $\pm$ 0.88
2-Methylbutanal	2.771	-	-	-	5.10 $\pm$ 1.46
Pentanal	3.156	-	1.05 $\pm$ 0.41	-	-
Hexanal	4.902	-	2.30 $\pm$ 0.16	-	1.55 $\pm$ 0.29
Octanal	10.321	-	1.41 $\pm$ 0.26	-	-
(E)-Oct-2-enal	11.968	-	0.00–0.43	-	-
Nonanal	13.334	0.61 $\pm$ 0.07	1.61 $\pm$ 0.10	-	0.92 $\pm$ 0.08
Aromatic aldehydes, including:	-	-	-	-	2.41 $\pm$ 0.16
Benzaldehyde	9.067	-	-	-	0.00–1.50
Benzeneacetaldehyde	11.536	-	-	-	0.91 $\pm$ 0.07
Aliphatic ketones, including:	-	5.17 $\pm$ 0.66	19.15 $\pm$ 0.82	-	-
3-Methyl-2-butanone	2.744	0.35 $\pm$ 0.23	-	-	-
3-Octanone	9.844	4.82 $\pm$ 0.75	19.15 $\pm$ 0.82	-	-
Aromatic ketones, including:	-	0.00–0.22	-	49.46 $\pm$ 2.87	-
Acetophenone	12.219	0.00–0.22	-	-	-
4-Hydroxy-3-methyl-2-phenyl-2-cyclopenten-1-one	27.354	-	-	49.46 $\pm$ 2.87	-
Carboxylic acids, including:	-	0.46 $\pm$ 0.06	10.60 $\pm$ 1.53	-	18.04 $\pm$ 1.75
Acetic acid	2.322	-	10.60 $\pm$ 1.53	-	15.80 $\pm$ 1.83
2-Methylpropanoic acid	4.178	-	-	-	1.06 $\pm$ 0.23
Butanoic acid	4.732	-	-	-	1.17 $\pm$ 0.05
Nonanoic acid	18.046	0.46 $\pm$ 0.06	-	-	-
Esters, including:	-	11.97 $\pm$ 0.83	2.27 $\pm$ 0.33	-	-
Methylbenzoate	13.074	0.80 $\pm$ 0.07	-	-	-
cis-2-tert-butyl-Cyclohexanol acetate	18.767	1.32 $\pm$ 0.12	2.27 $\pm$ 0.33	-	-
trans-2-tert-butyl-Cyclohexanol acetate	19.324	0.00–0.12	-	-	-
α,α -Dimethylphenethyl acetate	19.455	0.00–0.12	-	-	-
4-tert-butyl-Cyclohexyl acetate	19.826	0.00–0.09	-	-	-
1-(2-Hydroxy-1-methylethyl)-2,2-dimethylpropyl 2-methylpropanoate	20.342	6.27 $\pm$ 0.66	-	-	-
3-Hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate	20.888	3.47 $\pm$ 0.49	-	-	-
Furan derivatives, including:	-	11.06 $\pm$ 2.96	-	0.59 $\pm$ 0.20	2.54 $\pm$ 0.25
2-Methylfuran	2.321	8.24 $\pm$ 2.95	-	0.59 $\pm$ 0.20	-
Furfural	5.678	-	-	-	1.45 $\pm$ 0.36
2-Furanmethanol	6.243	-	-	-	1.10 $\pm$ 0.11
2-(2-Furanyl)-1-propanol	7.967	0.65 $\pm$ 0.10	-	-	-
Methyl 2-furoate	9.537	1.33 $\pm$ 0.13	-	-	-
2-Pentylfuran	9.980	0.84 $\pm$ 0.20	-	-	-

Table 1. Cont.

Group of Compounds	Retention Time (min)	Relative Abundance (%TIC): Mean $\pm$ Standard Deviation or Range			
		<i>H. annosum</i> -Colonized Pine Wood	Uncolonized Pine Wood	<i>H. annosum</i> Culture on MEA	Uninoculated MEA
Nitrogen-containing and chlorinated compounds, including:	-	2.60 $\pm$ 0.34	4.72 $\pm$ 0.63	5.25 $\pm$ 0.58	6.94 $\pm$ 2.00
Pyridine	3.918	0.79 $\pm$ 0.13	-	-	-
Tetrachloroethylene	5.102	1.82 $\pm$ 0.31	4.72 $\pm$ 0.63	5.25 $\pm$ 0.58	6.94 $\pm$ 2.00
Monoterpene hydrocarbons and oxygenated monoterpenoids, including:	-	13.26 $\pm$ 1.17	13.28 $\pm$ 0.83	-	-
α -Pinene	8.292	1.96 $\pm$ 0.41	2.38 $\pm$ 0.55	-	-
Camphene	8.703	0.00–0.17	-	-	-
Thuja-2,4(10)-diene	8.874	0.00–0.27	-	-	-
delta-3-Carene	10.528	-	1.18 $\pm$ 0.19	-	-
p-Cymene	10.955	0.94 $\pm$ 0.07	-	-	-
Eucalyptol	11.166	0.00–0.39	-	-	-
Dihydromyrcenol	12.405	5.57 $\pm$ 0.80	4.95 $\pm$ 0.45	-	-
Fenchone	12.895	0.00–0.23	-	-	-
Tetrahydrolinalool	13.159	1.17 $\pm$ 0.15	1.46 $\pm$ 0.22	-	-
Camphor	14.547	0.93 $\pm$ 0.23	1.26 $\pm$ 0.19	-	-
Borneol	15.164	0.00–0.11	-	-	-
iso-Menthol	15.355	0.47 $\pm$ 0.06	-	-	-
iso-Pinocamphone	15.426	0.41 $\pm$ 0.05	-	-	-
p-Cymen-8-ol	15.714	0.33 $\pm$ 0.04	-	-	-
Bornyl acetate	18.604	1.10 $\pm$ 0.10	2.04 $\pm$ 0.29	-	-
Sesquiterpenes, including:	-	11.42 $\pm$ 2.05	4.50 $\pm$ 0.68	7.67 $\pm$ 0.30	-
Silphin-1-ene	20.026	-	-	0.74 $\pm$ 0.19	-
α -Cubebene	20.279	-	-	0.00–0.20	-
α -Copaene	20.997	-	-	0.00–0.13	-
iso-Longifolene	21.065	1.98 $\pm$ 0.18	-	1.45 $\pm$ 0.40	-
β -Elemene	21.395	-	-	0.00–0.22	-
Sativene	21.531	-	-	0.00–0.27	-
Longifolene	21.793	0.35 $\pm$ 0.01	-	0.00–0.38	-
α -Barbatene	21.902	0.68 $\pm$ 0.07	-	0.00–0.40	-
β -Cedrene	22.178	0.00–0.10	-	-	-
α -trans-Bergamotene	22.485	-	-	0.00–0.15	-
Isobazzanene	22.559	0.23 $\pm$ 0.02	-	-	-
β -Barbatene	22.757	6.67 $\pm$ 1.81	4.50 $\pm$ 0.68	4.40 $\pm$ 1.05	-
epi- β -Santalene	22.797	-	-	0.00–0.34	-
α -Acoradiene	23.307	-	-	0.00–0.16	-
γ -Muurolene	23.529	0.86 $\pm$ 0.09	-	0.00–0.33	-
α -Muurolene	24.104	0.62 $\pm$ 0.05	-	0.00–0.27	-
β -Bisabolene	24.280	-	-	0.00–0.15	-
δ -Cadinene	24.669	-	-	0.00–0.23	-
Unidentified compounds, including:	-	22.71 $\pm$ 1.63	7.56 $\pm$ 0.87	28.62 $\pm$ 3.13	7.85 $\pm$ 1.48
NN	3.232	20.16 $\pm$ 1.20	7.56 $\pm$ 0.87	25.01 $\pm$ 2.99	-
NN	7.518	2.55 $\pm$ 0.53	-	2.07 $\pm$ 0.34	7.85 $\pm$ 1.48
NN	26.230	-	-	1.54 $\pm$ 0.14	-

Table 2. Summary of VOC profiles detected in the four prepared sample types.

Sample Type	Number of Detected VOCs	Prominent Chemical Groups
Colonized pine wood	64	Furans, esters, terpenoids
Uncolonized pine wood	24	Aldehydes, ketones, monoterpenoids
<i>H. annosum</i> culture on MEA	27	Sesquiterpenes, aromatic compounds
Uninoculated MEA	25	Aldehydes, acids, alcohols

Multivariate and Univariate Differentiation of VOC Profiles

Replicate-level PCA of the CLR-transformed profiles showed compact grouping of the three analyzed samples within each prepared sample type (Figure 1). PC1 explained 59.8% and PC2 explained 23.7% of the CLR variance (83.5% cumulatively). Colonized wood was separated mainly along PC1, while the fungal-culture samples were differentiated primarily along PC2. These coordinates provide a descriptive summary of the supplied samples.

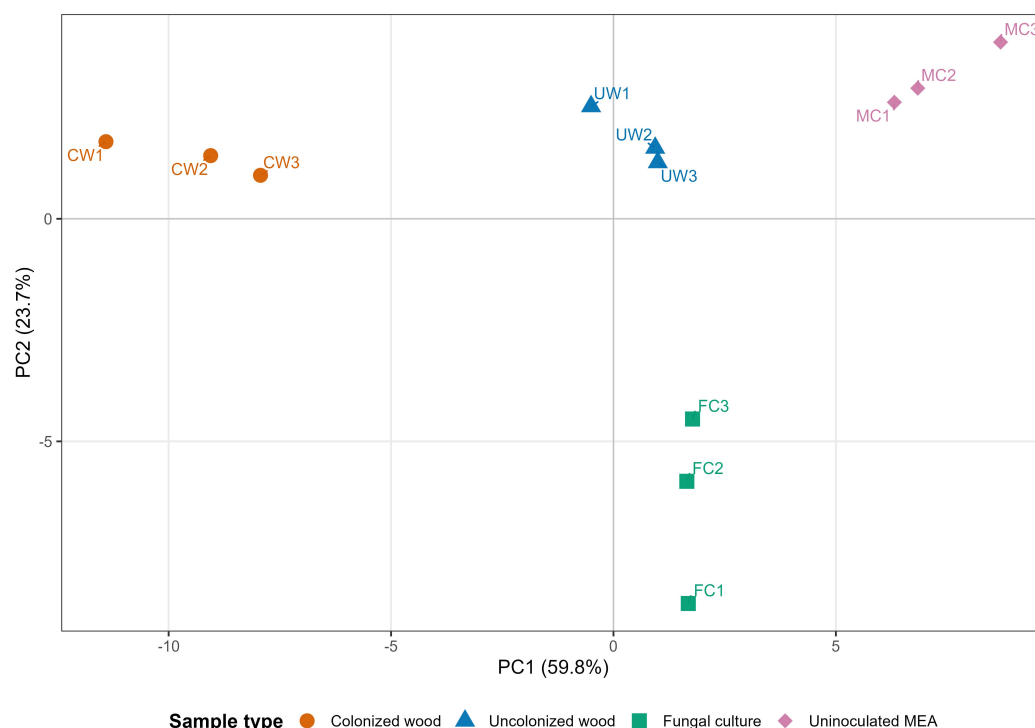


Figure 1. Replicate-level PCA of CLR-transformed relative VOC compositions after exclusion of styrene and tetrachloroethylene. All 12 analyzed samples are shown. PC1 and PC2 explain 59.8% and 23.7% of the CLR variance, respectively. Sample points represent separately analyzed headspace units; the ordination displays within- and between-type variation after zero replacement.

Replicate-level hierarchical clustering was consistent with the PCA (Figure 2). The three samples within each type clustered together; uncolonized wood and fungal culture were the closest treatment groups at the higher branch level, while colonized wood formed the most distinct branch. The heatmap displays the 30 features with the largest CLR variance and summarizes standardized relative-abundance differences.

In the replicate-level Welch comparisons, 41 of 99 tested features had globally adjusted $q < 0.05$; 30 had a higher and 11 a lower mean relative abundance in colonized than in uncolonized wood. The Welch results are reported as exploratory estimates from the replicate-level data.

The exact permutation sensitivity analysis yielded a minimum attainable two-sided $p = 0.10$, with all global q -values ≥ 0.05 , reflecting the resolution of six-sample label permutations.



Figure 2. Hierarchically clustered heatmap of the 30 VOC features with the highest CLR variance across the 12 analyzed samples. Rows represent feature-wise z-scores calculated from CLR-transformed relative abundances; columns represent individual samples. Rows and columns were clustered using Euclidean distance and Ward’s minimum-variance method. Red and blue indicate comparatively high and low standardized relative abundances, respectively.

Five VOCs form an exploratory display panel (Table 3; Figure 3). Statistical interpretation uses the single global correction across all 99 features.

All five displayed features had positive colonized-minus-uncolonized wood mean differences (Table 3; Figure 3). Global FDR-adjusted *q*-values were 0.0242 for methyl 2-furoate, 0.0242 for iso-longifolene, 0.0293 for 2-(2-furanyl)-1-propanol, 0.0885 for 2-methylfuran, and 0.320 for β-barbatene.

Table 3. Exploratory replicate-level evaluation of five displayed VOC signals.

Compound	Colonized Wood (%TIC)	Uncolonized Wood (%TIC)	Difference in Percentage Points (95% CI)	Welch <i>t</i> (df)	Raw <i>p</i>	Global BH <i>q</i>
2-Methylfuran	8.24 ± 2.95	ND	8.24 (0.91–15.57)	4.84 (2.00)	0.0402	0.0885
Methyl 2-furoate	1.33 ± 0.13	ND	1.33 (1.02–1.64)	18.39 (2.00)	0.00294	0.0242
iso-Longifolene	1.98 ± 0.18	ND	1.98 (1.54–2.43)	19.12 (2.00)	0.00273	0.0242
β-Barbatene	6.67 ± 1.81	4.50 ± 0.68	2.17 (−1.75–6.10)	1.95 (2.55)	0.162	0.320
2-(2-Furanyl)-1-propanol	0.65 ± 0.10	ND	0.65 (0.40–0.90)	11.11 (2.00)	0.00800	0.0293

Values are mean ± standard deviation for three separately analyzed samples per type. Differences were calculated as colonized minus uncolonized wood. Welch tests used replicate-level %TIC values; global BH *q*-values were adjusted once across all 99 tested features. ND, not detected under the applied conditions; CI, confidence interval; df, approximate degrees of freedom; BH, Benjamini–Hochberg.

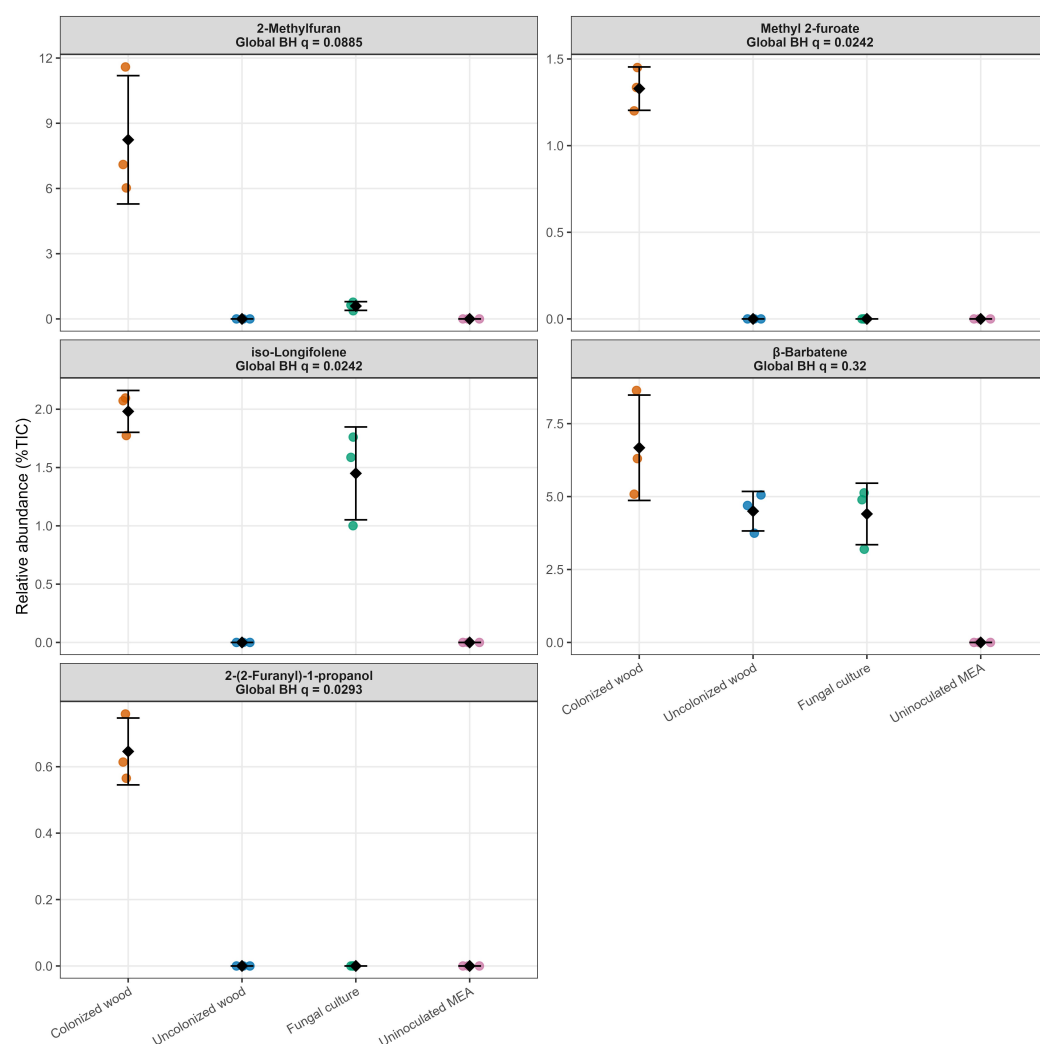


Figure 3. Replicate-level relative abundances (%TIC) of the five displayed exploratory VOC signals across the four prepared sample types. Individual points represent separately analyzed samples; larger points and error bars represent means $\pm$ one standard deviation. Panel labels report the global BH q -value for the colonized-versus-uncolonized wood Welch comparison, adjusted across all 99 tested features.

The differential screen contained features with both higher and lower relative abundance in colonized wood (Figure 4). For example, 3-octanone represented $4.82 \pm 0.75\%$ TIC in colonized wood and $19.15 \pm 0.82\%$ TIC in uncolonized wood, a mean difference of -14.33 percentage points (Welch $t = -22.37$, $df = 3.97$, $p = 2.50 \times 10^{-5}$, global $q = 0.00247$). The bidirectional relative differences emphasize the complete compositional pattern.

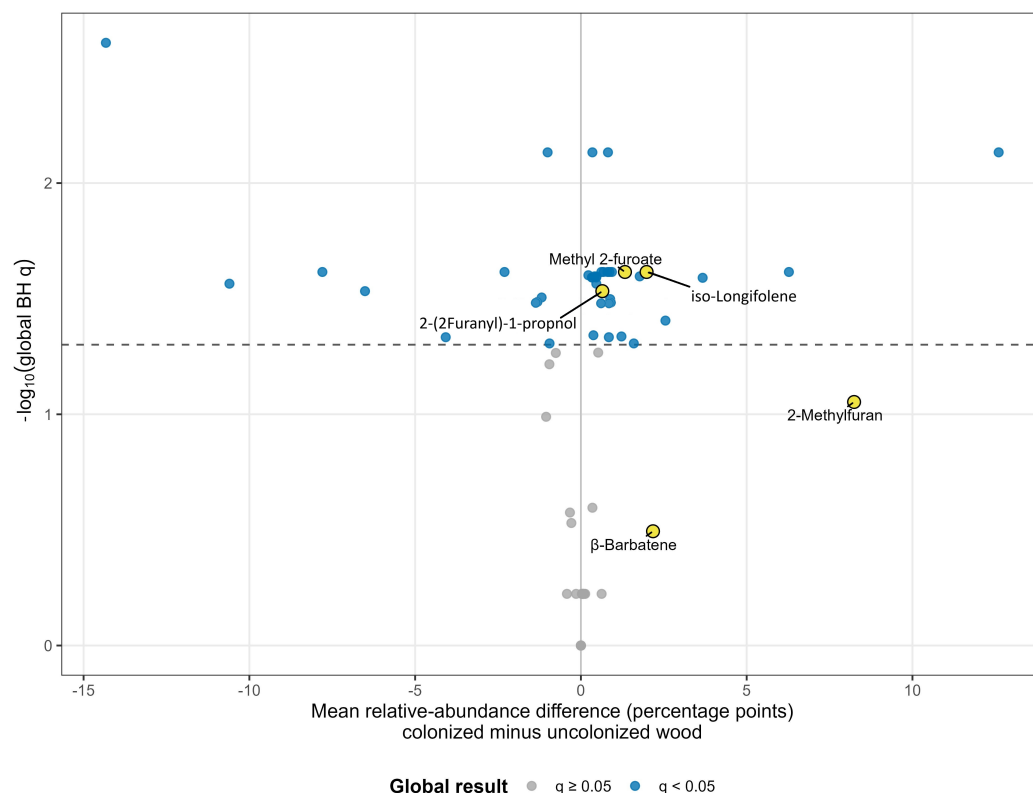


Figure 4. Exploratory differential screen comparing *H. annosum*-colonized and uncolonized pine wood. The horizontal axis is the mean relative-abundance difference in percentage points (colonized minus uncolonized), and the vertical axis is $-\log_{10}$ of the global BH q -value from replicate-level Welch tests. Adjustment was performed once across all 99 tested features. The dashed line denotes $q = 0.05$; the five displayed VOC signals are labelled.

4. Discussion

Colonized wood contained a more diverse headspace profile than the uncolonized-wood reference. The comparatively high relative contributions of several furan derivatives, esters, and terpenoids are compatible with fungal metabolism and chemical transformation of a lignocellulosic substrate. The headspace profile reflects the combined chemistry of fungal activity, heat-treated wood, culture medium transferred with the inoculum, and the sampling environment. Related studies of thermally processed and valorized biomass show that feedstock composition and processing conditions modify material properties [44–46].

The replicate-level PCA and clustering summarized the complete compositional differences, and the Welch comparisons identified individual features contributing to the colonized-versus-uncolonized contrast. Global FDR-adjusted q -values were 0.0242 for methyl 2-furoate and iso-longifolene, 0.0293 for 2-(2-furanyl)-1-propanol, 0.0885 for 2-methylfuran, and 0.320 for β -barbatene. The exact-permutation sensitivity analysis provides complementary information on the resolution of the six-sample comparison. Together, these results define exploratory hypotheses from the replicate-level profiles.

The %TIC values quantify each compound's relative contribution within a closed profile. The lower 3-octanone share may reflect its own abundance and redistribution among other components. Calibrated internal standards would enable future absolute quantification. ND denotes a signal below detection under the applied conditions.

Background evaluation assigned styrene and tetrachloroethylene to the analytical background and *n*-butane to a tentative annotation. Biological comparisons therefore used the remaining 99 features, whose annotations are putative based on spectral and retention-index comparisons. Published work on living spruce responses to *Heterobasidion* [7] and on

VOC effects from other microorganisms [47] supports evaluation of biological origin and specificity across living tissue and comparator organisms.

4.1. Comparison with Existing Detection Methods

Current approaches to detecting *Heterobasidion* include visual assessment, isolation of the pathogen from sampled tissues, and molecular analysis of internal wood or root samples [6,48]. These approaches provide complementary evidence and differ in their sampling and analytical requirements.

Visual assessment provides rapid field triage after symptom development [49]. Molecular analysis provides taxonomic resolution and detects fungal DNA in sampled tissues, with performance shaped by assay and sampling design [6,48]. Culture and molecular workflows use root or wood samples, laboratory processing, and trained personnel [50].

In contrast, VOC-based diagnostics have the potential to provide rapid and non-destructive detection of infection-associated metabolic processes. Volatile compounds can be sampled directly from the surrounding air without damaging host tissues, creating opportunities for future deployment of portable instruments and sensor-based monitoring systems. Current research is directed toward field specificity, calibration, and portable-sensor workflows [51].

VOC profiling is positioned as a research-stage complement to molecular and culture-based diagnostics. External validation can establish its value for screening and prioritizing specimens for confirmatory testing.

4.2. Exploratory Candidate VOC Signals of *Heterobasidion annosum* Colonization

The exploratory five-feature panel summarizes compounds selected for closer inspection. Under the single global correction across 99 features, the q -values were 0.0242 for methyl 2-furoate and iso-longifolene, 0.0293 for 2-(2-furanyl)-1-propanol, 0.0885 for 2-methylfuran, and 0.320 for β -barbatene.

Methyl 2-furoate and 2-(2-furanyl)-1-propanol were confined to colonized wood among the two wood sample types, and iso-longifolene occurred in colonized wood and fungal culture. These patterns are consistent with colonization-associated transformation of the dead wood substrate. Their occurrence across comparator fungi, plant material, and relevant background matrices can be evaluated in subsequent specificity studies.

The multicomponent relative-abundance pattern provides the primary candidate signature for validation. Subsequent studies can quantify specificity, sensitivity, reproducibility, and robustness to medium and vessel conditions.

This proof-of-concept evaluates replicate-level relative headspace composition across the four prepared sample types. The analytical unit is the individual vessel/chromatogram, and the resulting profile provides exploratory candidates for broader calibrated validation.

4.3. Potential Applications in Forest Management

The practical value of VOC-based diagnostics extends beyond pathogen detection itself. Early identification of infected trees may support forest managers in locating disease centres before visible symptoms become apparent and before extensive root-to-root spread occurs within the stand. Following validation, VOC profiling could serve as an initial screen for prioritizing trees or wood samples for confirmatory molecular or culture-based testing.

A separate potential application concerns integration with tree-risk assessment near roads, recreation sites, and urban–forest interfaces. Prospective studies combining VOC profiles with pathogen-confirmed status and independent mechanical or arboricultural assessments could evaluate integration into tree-risk workflows.

4.4. Future Research Directions

The preliminary relative VOC differences provide a basis for validation across independent isolates, donor trees, comparator fungi, and colonization time points. Calibrated measurements and standardized analytical quality control can extend the relative profile toward quantitative comparison.

Field studies on naturally colonized living trees can quantify diagnostic sensitivity and specificity and evaluate models using nested cross-validation and an independent validation dataset. These studies can also establish operating conditions for portable sensors and routine forest-health screening.

5. Conclusions

A total of 101 VOC signals were detected in *Heterobasidion annosum*-colonized Scots pine wood, uncolonized pine wood, fungal culture, and uninoculated culture medium. Of these signals, 98 received putative or tentative assignments and three remained unidentified. The colonized-wood headspace showed the greatest feature diversity. Replicate-level PCA and clustering described distinct relative compositions among the supplied samples. In the exploratory colonized-versus-uncolonized wood comparison, 41 of 99 features had global $q < 0.05$; among the five displayed signals, methyl 2-furoate, iso-longifolene, and 2-(2-furanyl)-1-propanol were FDR-supported, while 2-methylfuran and β -barbatene were not. These results concern relative %TIC composition and do not establish absolute production or species-specific biomarkers.

The study should be regarded as a preliminary characterization of one fungal culture colonizing dead, sterilized pine substrate. The unmatched wood reference, uncertain biological independence, small sample size, different vessel types, putative identifications, background signals, and compositional normalization prevent causal or diagnostic claims. Validation with matched controls, additional isolates and donor trees, comparator fungi, authentic standards, quantitative calibration, and naturally colonized living trees is required before the profile can be evaluated as a screening tool.

The replicate-level relative-composition analysis provides a foundation for broader calibrated validation in naturally colonized living trees.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/f17091088/s1>.

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