Plant, Cell & Environment

WILEY

Plant, Cell & Environment

ORIGINAL ARTICLE OPEN ACCESS

The Ash Dieback Pathogen Resists the Chemical Defences of Its Host Tree, European Ash, by Depleting Them From the Growth Medium

Björn Lichnock[1] | Niklas Gentsch[1] | Antonia Papadopulos[1] | Natalia Rauch[1] | Michael Reichelt[1] | Gitta Langer[2] | Susanne Jochner-Oette[3] | Jonathan Gershenzon[1,4] | Maximilian Lehenberger[1]

[1]Department of Biochemistry, Max Planck Institute for Chemical Ecology, Jena, Germany | [2]Northwest German Forest Research Institute (NW-FVA), Göttingen, Germany | [3]Catholic University Eichstätt-Ingolstadt, Landscape Ecology and Sustainable Ecosystem Development, Eichstätt, Germany | [4]Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Prague, Czech Republic

Correspondence: Maximilian Lehenberger (mlehenberger@ice.mpg.de)

Received: 4 August 2026 | **Revised:** 1 October 2026 | **Accepted:** 9 October 2026

Funding: German Research Foundation, Grant/Award Number: 520486374; Max-Planck Society

Keywords: ash dieback | chemical defences | fungi | *Hymenoscyphus fraxineus* | pathogen

ABSTRACT

Plant pathogenic fungi are major drivers of disease in forest trees, often causing substantial ecological and economic damage. Ash dieback, caused by the ascomycete *Hymenoscyphus fraxineus*, has resulted in the widespread decline of *Fraxinus excelsior* populations throughout Europe. As infection is initiated in leaves containing diverse plant defensive metabolites, the ability of *H. fraxineus* to cope with host chemistry may determine successful host colonisation. We therefore investigated the responses of *H. fraxineus* to crude ash leaf extracts and individual defensive compounds. In contrast to the non-ash associated fungi tested, *H. fraxineus* was not inhibited by crude methanolic leaf extracts or most plant defence compounds tested. In addition, *H. fraxineus* depleted ash compounds extensively from the growth medium. Consistent with these findings, untargeted metabolomics revealed extensive alterations of the ash leaf-based medium by *H. fraxineus*, with 48% of detected chemical features being significantly reduced following fungal growth. Moreover, the growth of *H. fraxineus* was promoted by nearly all tested ash metabolites in minimal medium, indicating that these compounds can support fungal growth and may serve as carbon sources. Together, these results suggest that successful colonisation of ash tissues by *H. fraxineus* depends on its resistance to host defences, facilitated by its ability to deplete defensive compounds from colonised tissue.

1 | Introduction

Plants possess many modes of defence against fungal pathogens: the waxy cuticle and cell walls form physical barriers to fungal penetration, the hypersensitive response keeps infections from spreading by localised cell death, and a large variety of defensive proteins and low molecular weight antifungal compounds inhibit pathogen growth (Bednarek and Osbourn 2009; Serrano et al. 2014; Jorgensen et al. 2017; Tripathi et al. 2024). However, some pathogens overcome these defensive measures to successfully invade plants and cause disease. Unfortunately, we know very little about how pathogens resist plant chemical defences, especially in woody plants. They may rapidly excrete toxic compounds from their cells, metabolise them to non-toxic products or be resistant by lacking the target site for toxin action (Westrick et al. 2021).

Fungal infections of trees are increasing worldwide (Loo 2009; Gomdola et al. 2022), contributing to large-scale declines in many

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2026 The Author(s). *Plant, Cell & Environment* published by John Wiley & Sons Ltd.

forest tree species, both conifers and deciduous trees. The spread of numerous forest pathogens has been strongly facilitated by international trade, and ongoing climate change (Evans and Waller 2010; Singh et al. 2023) as well as their efficient dispersal capacities and ecological flexibility (Ghelardini et al. 2017). However, some pathogens may also be well-adapted to resist the defences that they encounter in new host species.

Among the invasive fungal diseases affecting forest trees, the ash dieback caused by *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya (Helotiaceae, Helotiales, Leotiomycetidae, Leotiomycetes, Ascomycota) represents one of the most severe and widespread in Europe (Carroll and Boa 2024). Since its introduction from East Asia since at least the 1990s, *H. fraxineus* has caused extensive mortality and decline of European ash (*Fraxinus excelsior* L.) populations throughout large parts of Europe (Carroll and Boa 2024). In its native Asian range, *H. fraxineus* largely occurs asymptomatically as a harmless leaf associate on native *Fraxinus* species found there, such as *F. mandshurica* Rupr., for example. In contrast, the European ash *F. excelsior* appears highly susceptible to infection (Nielsen et al. 2017; Lévesque et al. 2023). Nevertheless, substantial variation in disease severity exists among individual European ash trees, and a small proportion of trees consistently exhibits reduced susceptibility or partial resistance to ash dieback (Seidel et al. 2024; Schertler et al. 2026). Such variation has stimulated considerable interest in identifying the mechanisms underlying host resistance, including the potential role of defensive chemistry (Nemesio-Gorriz et al. 2020; Sidda et al. 2020; Schertler et al. 2026), but the ability of the pathogen to overcome host defences has not been investigated. The infection cycle of *H. fraxineus* normally begins on ash leaves, where airborne ascospores commonly infect leaf tissues before the fungus spreads into shoots and woody tissues, ultimately causing characteristic shoot dieback and crown decline (Carroll and Boa 2024). *H. fraxineus* also infects the trunk, leading to stem and stem collar necrosis (Langer 2017; Peters et al. 2023; Peters et al. 2024). However, because infection is commonly initiated in ash leaves, understanding the interaction between *H. fraxineus* and ash leaf chemistry may provide important insights into the mechanisms underlying host colonisation and disease development.

In this study, we hypothesise that the high colonisation success of *H. fraxineus* is linked to its ability to resist the chemical defence repertoire of European ash. First, we investigated the effects of both crude ash leaf extracts and individual ash defensive metabolites on the fungus using growth inhibition assays. Next, we examined the ability of *H. fraxineus* to deplete defences in its growth medium by targeted and untargeted metabolite analyses. Finally, we assessed whether selected ash metabolites have the potential to promote fungal growth as an indication for their utilisation as carbon sources. To place these responses into a broader ecological context, we compared *H. fraxineus* with other fungal species associated with woody plants but differing in host tree and lifestyle. Together, our results provide new insights into how *H. fraxineus* interacts with the defensive chemistry of ash and identify potential mechanisms that may contribute to its success in colonising *Fraxinus excelsior* tissues.

2 | Materials and Methods

2.1 | Fungal Species and Culture Media

We studied three strains of *Hymenoscyphus fraxineus* (P343, P359, P360) together with three additional fungal species for comparison, the ambrosia beetle mutualist *Dryadomyces sulphureus* (L.R. Batra) Procter & Z.W. de Beer (Ophiostomataceae, Ophiostomatales, Diaporthomycetidae, Sordariomycetes, Ascomycota), associated with *Xyleborinus saxesenii* (Ratzeburg, 1837) primarily colonising *Fagus sylvatica* L; the bark beetle mutualist *Endoconidiophora polonica* (Siemaszko) Z.W. de Beer, T.A. Duong & M.J. Wingf. (Incertae sedis, Microascales, Hypocreomycetidae, Sordariomycetes, Ascomycota), associated with *Ips typographus* (Linnaeus, 1758) and colonising *Picea abies* (L.) H. Karst., but also known to act as a pathogen of mature Norway spruce (Kirisits and Offenthaler 2002), and the wood-degrading basidiomycete *Pleurotus ostreatus* (Jacq.) P. Kumm. (Pleurotaceae, Agaricales, Agaricomycetidae, Agaricomycetes, Basidiomycota), which colonises a broad range of hardwood species (see also Table S1 for further strain information). All fungal species were maintained as glycerol/water (40/60, v/v) stocks at −80°C at the Max Planck Institute for Chemical Ecology (Jena, Germany). Prior to each experiment, fungi were revived on 4% malt extract agar (MEA; ROTH, Germany) and stored at 4°C until subcultured onto fresh MEA plates at 26°C and 65% humidity before experimental use. Depending on the experiment, fungi were cultivated on either MEA (e.g., inhibition experiments), an 1% ash leaf-based minimal medium (e.g., depletion experiments), or a yeast nitrogen base with amino acids (YNB, Sigma Aldrich, Germany; e.g., carbon source experiments). The pH of all culture media was adjusted to approximately 5.5 prior to use. To prepare the ash leaf medium, fresh leaves were collected from mature, asymptomatic *Fraxinus excelsior* trees growing at a forest edge site near Jena, Germany. The trees were not phenotyped for resistance to *H. fraxineus*, and their resistance status was therefore unknown. Leaves were immediately frozen and subsequently freeze-dried and finely milled in the laboratory. The resulting powder was added at 4% to agar medium supplemented with 2% agar (Roth, Germany) and sterilised by autoclaving. For carbon source experiments, YNB served as the basal minimal medium and was individually supplemented with the respective test compounds or with an ash leaf extract, in addition to 2% agar.

2.2 | Inhibitory Effects of a Crude Ash Leaf Extract

Potential inhibitory effects of a crude ash leaf extract on all three strains of *H. fraxineus* (P343, P359, P360), as well as on *E. polonica*, *D. sulphureus*, and *P. ostreatus*, were tested by supplementing nutrient-rich malt extract agar (MEA) with 1% freshly prepared ash leaf extract. This concentration was chosen based on our previous experience with fungal growth on media supplemented with plant extracts. For ash leaf extract preparation, 20 g of freeze-dried ash leaves (see above) were finely milled and extracted with 200 mL methanol under gentle constant shaking at room temperature for 30 min. The extract was subsequently filtered to remove solid leaf material, and the methanol was completely evaporated under a continuous nitrogen flow. The remaining residue was re-dissolved in 10 mL dimethyl sulfoxide (DMSO; Sigma-Aldrich, Germany). Following autoclaving, MEA

13653040, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pce.70989 by Katholische Universitat Eichstatt-Ingolstadt, Wiley Online Library on [27/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

was supplemented with an appropriate volume of the extract to achieve a final concentration of 1% (v/v). Pure fungal mycelium (Ø 6 mm) was inoculated onto sterilised cellophane discs placed on the supplemented medium. Control plates contained the same concentration of DMSO but lacked the ash leaf extract. Plates ($N = 5$–6 per fungus and control) were incubated at 26°C and 65% relative humidity until at least one treatment per fungus reached complete plate coverage. Pure fungal biomass was then harvested from the cellophane, freeze-dried, and weighed to determine dry biomass. Fungal growth on ash leaf extract-supplemented medium was compared with growth on the corresponding DMSO control medium. Data were visualised using boxplots while normality was assessed using the Shapiro-Wilk test, and homogeneity of variances was evaluated using Levene's test. Depending on whether model assumptions were met, comparisons between treatment (fungi on ash leaf extract) and control groups were conducted using Student's *t*-tests (normally distributed data with homogeneous variances), Welch's *t*-tests (unequal variances), or Wilcoxon rank-sum tests (non-normally distributed data). To account for multiple testing across fungal species, *p* values were adjusted using the Benjamini-Hochberg procedure. All statistical analyses were performed in R (version 4.4.3) using the packages "ggplot2" (Wickham 2016), "tidyr" (Wickham and Vaughan 2024), and "dplyr" (Wickham et al. 2023). All figures presented in this study were subsequently refined for publication using Adobe Illustrator (CS5).

2.3 | Inhibitory Effects of Individual Ash Chemical Defences

To assess the effects of individual ash chemical defensive compounds on fungal growth and to compare the findings for *H. fraxineus* with those of other fungi, one strain of *H. fraxineus* (P343), together with *D. sulphureus* and *P. ostreatus*, was inoculated using actively growing mycelial plugs (Ø 6 mm) onto sterilised cellophane discs placed on MEA in small petri dishes (50 × 9 mm; ROTH, Germany). Following autoclaving, the medium was individually supplemented with one of 14 test compounds: the coumarins esculin, esculetin, fraxin, fraxetin, and scopoletin; the flavonoids catechin, isoquercitrin, naringin, naringenin, quercetin, and rutin; and the other compounds chlorogenic acid, ferulic acid, and quinic acid ($N = 5$ per compound and control; see Table S2 for further information on commercial origin). The selection of compounds was based on our preliminary analyses of ash leaf chemistry as well as findings reported by (Nemesio-Gorriz et al. 2020). As concentrations of defensive metabolites in leaf tissue can vary substantially among individual trees and developmental stages (Häuser et al. 2025), and similar variation was observed in our own metabolite analyses (data not shown), all compounds were tested at a standardised final concentration of 100 μg/mL in MEA. This concentration was chosen to represent the upper range of concentrations observed in our chemical analyses for most compounds and to maximise the likelihood of detecting potential antifungal activity while ensuring comparability across treatments. Although concentration-dependent effects may occur, this initial screening approach enabled the identification of compounds with potential antifungal activity under standardised conditions. Test compounds were dissolved in DMSO, and fungal growth was compared with control treatments containing the same concentration of DMSO but lacking any test compound. Fungi were incubated at 26°C and 65% humidity until either the treatment or control plates were fully colonised, or for a maximum of 28 days in the case of the slow-growing *H. fraxineus*. Subsequently, fungal biomass was harvested separately for each fungal species, freeze-dried, and weighed to determine dry biomass. In addition, targeted LC-MS analyses were conducted to quantify the remaining concentrations of the 14 supplemented compounds in fungus-colonised medium relative to fungus-free controls (see method description for the depletion experiments).

Differences in fungal biomass production were analyzed using fitted generalised linear models followed by pairwise contrasts with Dunnett correction, specifically comparing fungal growth on control medium with growth on each individual compound treatment for each fungal species separately (see also [Lehenberger et al. 2025]). Results were visualised as boxplots showing raw data, as well as a summary table in which fungal growth was expressed relative to the respective control treatment in percentage. Data visualisation was performed using the R package "ggplot2" (Wickham 2016).

2.4 | Depletion of Ash Chemical Defences From Ash Leaf-Based Medium

To assess the depletion capacity of *H. fraxineus* (strain P343) in comparison with *E. polonica*, *D. sulphureus*, and *P. ostreatus*, fungi were inoculated onto freshly prepared ash leaf-based medium as described above. Cultures were grown on standard petri dishes with cellophane ($N = 5$–8 per fungal species) until complete coverage of the medium or a maximum incubation time of 14 days was reached. Subsequently, medium as well as fungal biomass samples were collected, freeze-dried, and methanolic extracts were prepared as described previously. The analysis focused on a targeted set of ten ash compounds that were found to be abundant in our analyses (but see also (Nemesio-Gorriz et al. 2020): esculin, esculetin, fraxin, fraxetin, scopoletin, isoquercitrin, rutin, chlorogenic acid, quinic acid, and ferulic acid. These compounds were quantified in fungus-colonised medium relative to fungus-free controls using the targeted LC-MS approach described below. Further, the capacity of *H. fraxineus* to reduce the concentration of four selected ash chemical defences over time was evaluated. Therefore, strain P343 was pre-cultured by inoculating actively growing mycelium (Ø 6 mm) onto cellophane discs placed on malt extract agar (MEA) in small petri dishes (50 × 9 mm). Plates were incubated at 26°C and 65% relative humidity for 21 days until the medium was mostly covered by fungal mycelium. Subsequently, the colonised cellophane discs were transferred onto freshly prepared MEA individually supplemented with 100 μg/mL of either scopoletin, esculetin, chlorogenic acid, or fraxetin ($N = 4$–6 per compound, time point, and control; dissolved in DMSO and added after autoclaving). Plates were incubated under the same conditions as mentioned above and medium samples were collected at three time points: 1, 2, and 4 days after transfer. Afterwards, medium samples were freeze-dried, and methanolic extracts were prepared as described above. Targeted LC-MS analyses as described below were performed. Concentrations of chemical defence compounds in *H. fraxineus*-colonised medium were compared with those in fungus-free control plates to which the same chemicals were added.

13653040, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pce.70989 by Katholische Universitat Eichstatt-Ingolstadt, Wiley Online Library on [27/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Chromatographic separation was performed using an Agilent 1200 HPLC system (Agilent Technologies, Böblingen, Germany). Separation was achieved on an Agilent Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 μM; Agilent Technologies, Santa Clara, CA, USA). Mobile phases consisted of 0.05% formic acid in water (A) and acetonitrile (B). The elution gradient was as follows: 0.0–0.5 min, 5% B; 0.5–6.0 min, 5%–37.4% B; 6.0–6.02 min, 37.4–80% B; 6.02–7.5 min, 80%–100% B; 7.5–9.5 min, 100% B; and 9.5–12 min, 5% B. The flow rate was set to 1.1 mL/min, and the column temperature was maintained at 25°C. The HPLC system was coupled to an API 3200 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a turbo ion spray source operating in negative ionisation mode. Instrument parameters were optimised by direct infusion of authentic standards. The ion spray voltage was set to −4500 V, and the turbo gas temperature was 500°C. Nebulising gas was set to 60 psi, curtain gas to 30 psi, heating gas to 60 psi, and collision gas to 4 psi. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Detailed MRM transitions and compound-specific parameters are provided in Table S2. Data acquisition and processing were performed using Analyst 1.5 software (Applied Biosystems, Darmstadt, Germany). Quantification was based on external calibration curves generated from serial dilutions of commercial standards (see Table S2 for suppliers). All concentrations were normalised to the dry weight of the respective samples.

To evaluate overall metabolic differences across fungal species, principal component analysis and permutational multivariate analysis of variance (999 permutations) were performed based on the ten quantified compounds. The same approach described below was used to analyze the chemical profiles of medium samples from the inhibition experiment. Prior to PCA, data were log1p-transformed and Z-score-scaled to account for differences in variance among features. For statistical testing, global and pairwise PERMANOVAs were conducted using Bray-Curtis dissimilarities calculated from log1p-transformed but non-scaled data. p values were adjusted for multiple comparisons using the Benjamini-Hochberg procedure. In addition, individual mean compound concentrations per fungus/control from medium and fungal biomass samples were visualised in heatmaps. Further, total depletion from medium samples was assessed by summing the abundances of all ten compounds per sample and comparing these totals across fungal species relative to the fungus-free control. Differences in overall depletion from medium samples were tested using fitted general linear models (GLMs) with adjusted pairwise contrasts (see (Lehenberger et al. 2025)). For the time-dependent depletion assays, statistical differences were assessed using fitted generalised linear models followed by pairwise contrasts with Dunnett correction, specifically comparing compound concentrations in fungus-free control medium with those in *H. fraxineus*-colonised medium across the different time points (see above). All analyses were performed in R using the packages "vegan" (Oksanen et al. 2017), "ggplot2" (Wickham 2016), and "pheatmap" (Kolde 2019). Chemical structures of the individual compounds were generated using ChemDraw (version 23.1.1) and incorporated into the plot.

2.5 | Metabolomic Analyses of Ash-Leaf-Based Medium Colonised by *H. fraxineus*

A plug of pure mycelium of *H. fraxineus* (strain P343, $N = 5$) was inoculated onto freshly prepared ash leaf medium (see above) and additionally placed on a sterilised cellophane disk (Pure Nature, Germany) to enable separation of fungal biomass from the growth medium. Plates were incubated at 26°C and 65% relative humidity until the plates were almost fully colonised. Cellophane discs were then removed to separate fungal biomass from the medium. Medium samples were collected from areas previously colonised by the fungus. As controls, medium samples were taken from uninoculated ash leaf medium plates incubated under identical conditions. All samples were subsequently freeze-dried, and dry weights were recorded.

For metabolomic analyses, methanolic extracts (Honeywell, Germany) were prepared. Freeze-dried medium samples were homogenised by adding 3–4 metal beads per samples (Ø 3 mm, Askubal, ASK Kugellagerfabrik Artur Seyfert GmbH, D-70825 Korntal-Münchingen, Deutschland) and processing them in a paint shaker (Skandex SO-10M, Fluid Management Europe, The Netherlands) at full speed for 5 min. Samples were then centrifuged at 9400 rcf for 2 min, and the supernatant was collected for further analysis. Injection volumes were adjusted based on the dry weight of the freeze-dried samples to normalise metabolite measurements across samples. To monitor analytical quality, a pooled quality control sample was prepared by combining 10 μL aliquots from each sample and injected periodically throughout the randomised sequence of samples.

For non-targeted metabolite profiling, ultra-high-performance liquid chromatography coupled to electrospray ionisation high-resolution mass spectrometry (UHPLC-ESI-HRMS) was performed using a Dionex Ultimate 3000 UHPLC system (Thermo Scientific) coupled to a Bruker timsTOF mass spectrometer (Bruker Daltonik, Bremen, Germany), as described in (Medina-van Berkum et al. 2024). Chromatographic separation was achieved on a reversed-phase Zorbax Eclipse XDB-C18 column (100 mm × 2.1 mm, 1.8 μM; Agilent Technologies, Waldbronn, Germany) using a solvent system consisting of 0.1% formic acid in water (A) and acetonitrile (B) at a flow rate of 0.3 mL/min. The elution gradient was as follows: 0–0.5 min, 5% B; 0.5–11.0 min, 5%–60% B; 11.0–11.1min, 60%–100% B; 11.1–12.0 min, 100% B; 12.1–15.0 min, 5% B. HRMS data were acquired in negative ionisation mode with data-dependent acquisition ("autoMS") enabled. Instrument parameters were set as follows: capillary voltage −3.5 kV, end plate offset 500 V, nebuliser pressure 2.8 bar, and nitrogen drying gas at 280°C with a flow rate of 8 L/min. Data were acquired at 12 Hz over an m/z range of 50–1500. Data-dependent MS/MS was triggered with an active exclusion window of 0.1 min, a reconsideration threshold of 1.8-fold change, and exclusion after five spectra. Fragmentation was triggered at an intensity threshold of 50 counts and applied to the two most intense precursor ions. Collision energies were alternated between 20 and 50 V. At the beginning of each chromatographic run, 10 μL of a sodium formate-isopropanol calibration solution (10 mM NaOH in 50/50 (v/v) isopropanol/water containing 0.2% formic acid) was injected to recalibrate the mass spectrometer using known cluster ions. Peak detection was performed using MetaboScape

13653040, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pce.70989 by Katholische Universitat Eichstatt-Ingolstadt, Wiley Online Library on [27/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

(Bruker Daltonik, Bremen, Germany) with the T-Rex 3D algorithm for qTOF data. The following parameters were applied: intensity threshold of 1000, minimum of ten spectra per feature, and a retention time window of 0.4–12 min. Features were retained only if detected in at least 60% of replicates within a sample group. Adducts, including $[M-H]^-$, $[M+Cl]^-$, and $[M+COOH\text{-}H]^-$ (negative mode), were grouped into a single feature if showing an extracted ion chromatogram (EIC) correlation ≥ 0.8.

The resulting feature table was used for statistical analysis in R (version 4.5.1). Data were first visualised using principal component analysis (PCA) based on cube-root transformed and Z-score scaled intensities. To test for significant differences between treatments, a permutational multivariate analysis of variance (PERMANOVA; 999 permutations) was performed on Bray-Curtis dissimilarities calculated from cube-root transformed but unscaled data. The metabolomic dataset was further visualised using a heatmap generated from cube-root transformed and Z-score normalised intensities. To highlight group-specific patterns, non-scaled cube-root transformed feature intensities were ordered according to differences in mean peak intensity between treatments. Feature-wise differences between fungus-free control medium and *H. fraxineus*-colonised medium were assessed using Wilcoxon rank-sum tests followed by Benjamini-Hochberg correction for multiple testing. Features were classified as significantly reduced, significantly higher in abundance, or not significantly different based on adjusted p values (< 0.05) and the direction of the difference in mean feature intensity between treatments. All analyses were conducted using the R packages "dplyr" (Wickham et al. 2023), "e1071" (Meyer et al. 2025), "ggplot2" (Wickham 2016), "vegan" (Oksanen et al. 2017), and "pheatmap" (Kolde 2019).

2.6 | Growth Promotion Experiments

2.6.1 | *Growth on Ash Leaf Crude Extract*

To test the ability of *H. fraxineus* to utilise ash defence compounds as potential growth promoters, experiments were first conducted using a crude ash leaf extract prepared as described above. The extract was added to a final concentration of 1% to a sterilised minimal medium consisting of yeast nitrogen base (YNB) with amino acids and 2% agar, without any additional carbon source. To avoid thermal degradation, YNB was sterile-filtered prior to use and added to the water agar after autoclaving. Actively growing mycelium (Ø 6 mm; $N = 5$–6) of strain P343 was inoculated onto the experimental medium as well as on the control and incubated at 26°C and 65% humidity until fungal growth was sufficient for quantification. As *H. fraxineus* degrades cellulose when grown on nutrient-poor media, biomass-based quantification using cellulose-based cellophane was not feasible. Therefore, fungal growth on YNB supplemented with ash leaf extract was quantified as surface area (mm^2) using ImageJ (version 1.54 g) and compared to control plates containing the same amount of DMSO but lacking extract.

Data were visualised using boxplots and statistically analyzed in R using either Student's *t*-test, Welch's t-test, or Wilcoxon rank-sum tests, depending on data distribution and homogeneity of variances. Analyses were performed using the packages "ggplot2" (Wickham 2016), "dplyr" (Wickham et al. 2023), and "tidyr" (Wickham and Vaughan 2024).

2.6.2 | *Growth on Single Ash Chemical Defences*

To further resolve whether individual ash metabolites promote the growth of *H. fraxineus*, strain P343 was tested on a set of 15 compounds: esculin, esculetin, fraxin, fraxetin, scopoletin, quercetin, isoquercitrin, catechin, naringin, naringenin, rutin, chlorogenic acid, quinic acid, caffeic acid, and ferulic acid. Actively growing mycelium (Ø 6 mm; $N = 5$–7) was inoculated onto YNB medium supplemented with 2% agar, omitting cellophane, and individually amended with each compound (dissolved in DMSO) at a final concentration of 100 µg/mL (see also inhibition assays for concentration rationale). Fungal growth was monitored for each tested compound separately until surface area (mm^2; ImageJ) was sufficient for quantification. Growth on supplemented media was compared to growth on control plates lacking additional compounds but containing the same amount of DMSO.

Data were visualised using boxplots and the R package "ggplot" (Wickham 2016). Statistical analyses were performed using fitted generalised linear models followed by pairwise contrasts with Dunnett correction, comparing growth on each compound-supplemented medium against the control (see also above).

2.6.3 | *Growth on Alternative Plant-Derived Carbon Sources*

To examine whether the growth of *H. fraxineus* (strain P343) is further enhanced by the presence of common plant polymers, additional substrates including cellulose (Avicel, Sigma Aldrich, Germany), xylan (ROTH, Germany), galactomannan (ROTH, Germany), pectin (ROTH, Germany), amylopectin (MP Biomedicals, USA), and lignin (TCI, Japan) were tested. All substrates were added to a YNB supplemented with 2% agar. Cellulose, xylan, galactomannan, and lignin were individually added at a final concentration of 1% to the water agar component prior to autoclaving. As pectin and amylopectin form highly viscous or gel-like suspensions during autoclaving, they were sterilised separately by suspension in absolute ethanol (VWR, Germany) under sterile conditions. Ethanol was subsequently evaporated under a laminar flow hood, and the remaining material was finely homogenised using a sterile mortar and pestle. The resulting sterile substrate powder was then weighed and added to the YNB to obtain a final concentration of 1%. Fungal inoculation, quantification of growth as well as statistical analyses followed the description above ($N = 5$–6).

3 | Results

3.1 | The Growth of the Ash Pathogen *H. fraxineus* Is Unaffected by Ash Leaf Extract and Most Individual Ash Chemical Defences

The growth of three tested *H. fraxineus* strains on malt extract agar (MEA) was not significantly affected by supplementation with ash leaf methanol extract at 1% ($p > 0.1$, Student's *t*-test or Wilcoxon rank-sum test; Figure 1; Figure S1; see also Table S3a). In contrast, the ash extract caused strong growth inhibition of *E. polonica* ($p < 0.001$, Student's *t*-test) and slight inhibition of

13653040, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pce.70989 by Katholische Universitat Eichstatt-Ingolstadt, Wiley Online Library on [27/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

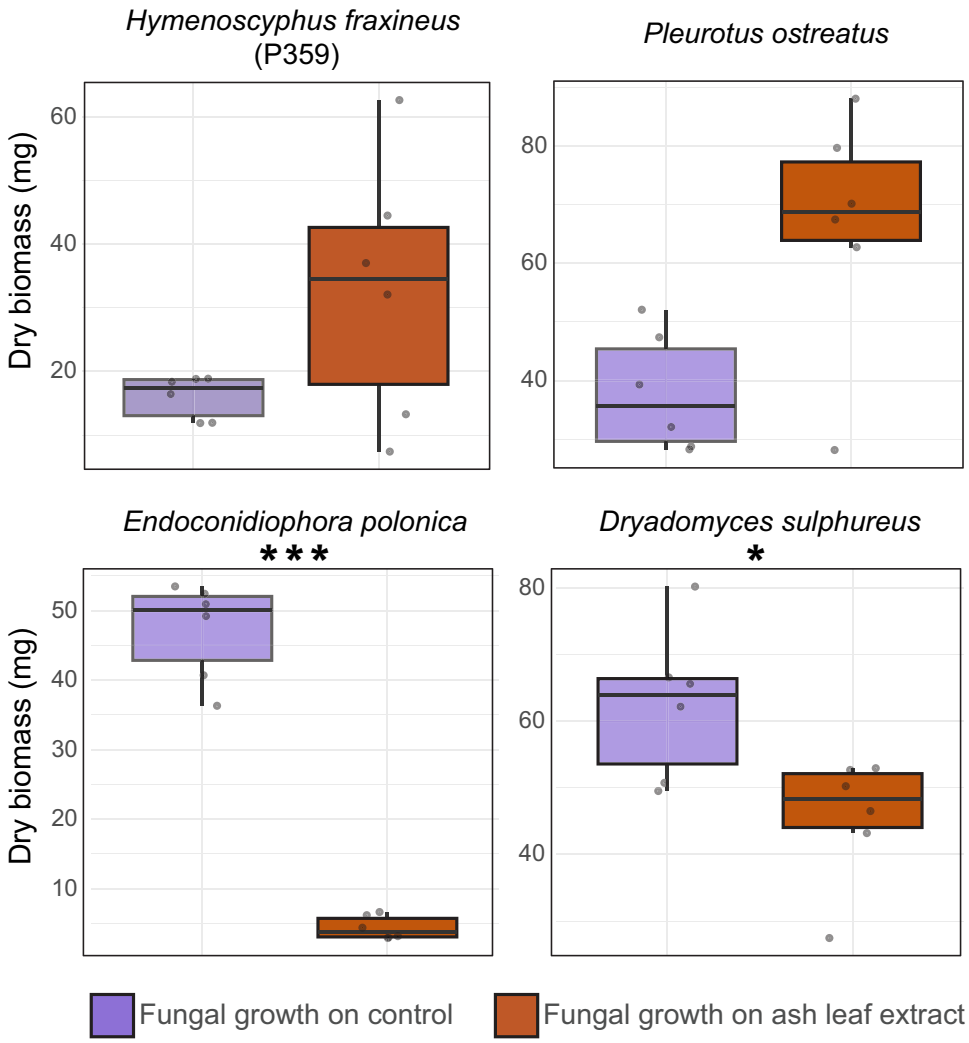

FIGURE 1 | *H. fraxineus* is not affected by a methanol extract of ash leaves, in contrast to other fungi. Freeze-dried ash leaf powder was extracted with methanol, which was subsequently evaporated and the extract re-dissolved in dimethyl sulfoxide (DMSO). This extract was added to MEA at a final concentration of 1% and was inoculated with three strains of *H. fraxineus* and three additional fungal species. Fungal growth (mg of dry biomass) on the ash leaf extract was compared to growth on ME medium supplemented with 1% DMSO. Statistical differences were analyzed using either Student's *t*-test, Welch's *t*-test, or a Wilcoxon rank-sum test with Benjamini-Hochberg correction, depending on the normality of data and homogeneity of variances. Asterisks indicate significant differences between the two treatments for each fungus ($N = 5$-6; $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; see Suppl. Table S3a for individual *p* values as well as Figure S1 for the remaining two *H. fraxineus* strains). [Color figure can be viewed at wileyonlinelibrary.com]

D. sulphureus ($p < 0.04$, Student's *t*-test), whereas growth of *P. ostreatus* was significantly promoted ($p < 0.04$, Student's *t*-test).

Next, MEA was individually supplemented with 14 ash defensive compounds. Of these, nine compounds had no significant effect on the growth of *H. fraxineus*, whereas five compounds significantly reduced fungal growth ($p < 0.042$, GLM with adjusted pairwise contrasts; Figure 2; Figure S2; further statistical details, biomass data, and individual incubation times are provided in Tables S3a and S3b). The coumarin esculetin and the flavonoid isoquercitrin showed the strongest inhibitory effects ($p < 0.001$), while chlorogenic acid ($p < 0.002$), and the flavonoid glycosides naringin ($p < 0.03$), and rutin ($p < 0.042$) caused weaker inhibition. However, none of the tested compounds completely suppressed the growth of *H. fraxineus*. *D. sulphureus* was inhibited by seven of the 14 tested compounds ($p < 0.043$), with the coumarin scopoletin exerting the strongest inhibitory effect ($p < 0.001$). In contrast, *P. ostreatus* was not inhibited by any tested compound and even exhibited enhanced growth in response to scopoletin ($p < 0.001$) and ferulic acid ($p < 0.015$).

3.2 | *H. fraxineus* Efficiently Depletes Ash Chemical Defences From Medium

As most ash defensive compounds tested showed little or no inhibitory activity against *H. fraxineus*, we examined whether the fungus could deplete these compounds from colonised ash medium. The depletion capacity of *H. fraxineus* was significantly different from that of the three additional fungal species with different host tree associations (*E. polonica*, *D. sulphureus*, and *P. ostreatus*) using targeted LC-MS analyses focused on ten abundant ash defensive compounds (PERMANOVA: $F = 57.3$,

13653040, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pce.70989 by Katholische Universitat Eichstatt-Ingolstadt, Wiley Online Library on [27/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

	Chemicals	H. fraxineus	D. sulphureus	P. ostreatus
Coumarins	Esculin	83	51	75
	Esculetin	36	37	90
	Fraxin	64	48	113
	Fraxetin	61	57	106
	Scopoletin	69	17	216
Flavonoids	Catechin	64	73	138
	Isoquercitrin	42	51	100
	Naringin	52	45	119
	Naringenin	122	82	109
	Quercetin	81	81	87
	Rutin	59	44	102
Others	Chlorogenic acid	42	34	145
	Ferulic acid	122	38	178
	Quinic acid	69	50	152

No effect | Enhanced growth | Inhibition

FIGURE 2 | *H. fraxineus* tolerates most tested ash defensive compounds without growth inhibition. The potential antimicrobial activity of 14 individual ash chemical defences (see also (Nemesio-Gorriz et al. 2020) was determined in bioassays with three different fungi. The effect of test chemicals dissolved in DMSO was measured on fungal growth measured relative to a control (MEA + DMSO at the same concentration). Due to the high natural variation in concentrations of the individual compounds in plant tissue, a uniform concentration of 100 μg/mL was applied for all chemicals. Chemicals were individually supplemented in the MEA, and fungi were inoculated on cellophane discs until petri dishes of one treatment were fully covered or for a maximum of 28 days. The table shows fungal growth as a percentage of the respective control treatment for each fungus, grouped by three chemical classes (coumarins, flavonoids, and others), with significant effects on fungal growth indicated by colour. Statistical differences were identified using raw data and fitted GLM models and pairwise contrasts including Dunnett's correction ($N = 5$; see also Figure S2 as well as Table S3a for individual p values). [Color figure can be viewed at wileyonlinelibrary.com]

$R^2 = 0.90$, $p = 0.001$; pairwise PERMANOVAs: $p < 0.003$; Figure 3a; further statistical details, biomass data, and individual incubation times are provided in Tables S3a and S3b). The heatmap visualised the stronger depletion of the tested ash defensive compounds from medium by *H. fraxineus*, with *P. ostreatus* showing weaker depletion capacities and the other two species being much weaker (Figure 3b). Analysis of the summed concentrations of all ten ash defensive compounds (total ash defences) indicated that only *H. fraxineus* and *P. ostreatus* significantly reduced the total concentration of ash defensive compounds in the medium ($p < 0.001$, GLM with adjusted pairwise contrasts; Figure S3; see also Table S3a), whereas no significant differences were observed for *E. polonica* or *D. sulphureus* compared with the fungus-free control. Moreover, *H. fraxineus* reduced the total concentration of ash defensive compounds significantly more strongly than *P. ostreatus* ($p < 0.001$). In these experiments, the fungal biomass was also chemically analyzed (Figure S4). Most ash defensive compounds were only present in trace amounts in the mycelia of *H. fraxineus* and *P. ostreatus*, although *P. ostreatus* accumulated high amounts of quinic acid in its mycelia as well as lower levels of rutin and scopoletin. In contrast, *E. polonica* and *D. sulphureus* were found to accumulate high levels of several of the tested compounds in their mycelial biomass.

LC-MS analyses of medium samples obtained from the individual compound inhibition assays (Figure 2) revealed similar depletion patterns under nutrient-rich conditions (Figure S5). Significant differences among fungal taxa were detected (PERMANOVA: $F = 59.04$, $R^2 = 0.92$, $p = 0.001$; Figure S5a; further statistical details, biomass data, and individual incubation times are provided in Tables S3a and S3b), and pairwise comparisons revealed significant differences between *H. fraxineus* and both *D. sulphureus* and *P. ostreatus* (pairwise PERMANOVAs: $p = 0.009$; see Table S3a). Heatmap visualisation confirmed the strong depletion capacities of *H. fraxineus* compared with the other tested fungi (Figure 5b).

To determine whether depletion represents a broader metabolic pattern, untargeted metabolomic analyses were performed comparing ash leaf-based medium colonised by *H. fraxineus* with an uncolonized medium. The pathogen *H. fraxineus* was found to extensively alter the chemical composition of the medium. PCA revealed a clear separation between fungus-free ash leaf medium and medium colonised by *H. fraxineus* (PERMANOVA: $F = 66.5$, $R^2 = 0.89$, $p = 0.009$; Figure 4a; see also Table S3a). In total, 1,631 metabolomic features were detected in the culture medium. Comparisons showed that 783 features (48%) were significantly reduced in *H. fraxineus*-colonised medium, whereas 277 features (17%) showed significantly higher abundance and 571 features (35%) did not differ significantly between treatments ($p < 0.05$, Wilcoxon rank-sum test with BH correction; Figure 4b; see also Table S3c).

Time-dependent depletion assays further demonstrated that *H. fraxineus* rapidly reduced the concentrations of all four tested ash defensive compounds (esculetin, fraxetin, scopoletin and chlorogenic acid) in colonised medium. Significant depletion was already observed after only 1 day of incubation, with all four compounds showing strongly reduced concentrations at the earliest sampling point ($p < 0.001$, GLM with adjusted pairwise contrasts; Figure 5).

Taken together, these results demonstrate that *H. fraxineus* efficiently depletes a broad range of ash defensive compounds, including coumarins, flavonoids and other phenolic compounds, both glycosides and aglycones. Compared with the other tested fungi, *H. fraxineus* exhibited the strongest depletion capacity, whereas *P. ostreatus* also depleted several compounds, although less efficiently than *H. fraxineus*.

3.3 | The Growth of *H. Fraxineus* Is Promoted by a Broad Range of Ash Chemical Defences

The ability of *H. fraxineus* to show enhanced growth in the presence of ash chemicals was examined by cultivating the pathogen on a minimal medium supplemented either with an

13653040, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pce.70989 by Katholische Universitat Eichstatt-Ingolstadt, Wiley Online Library on [27/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

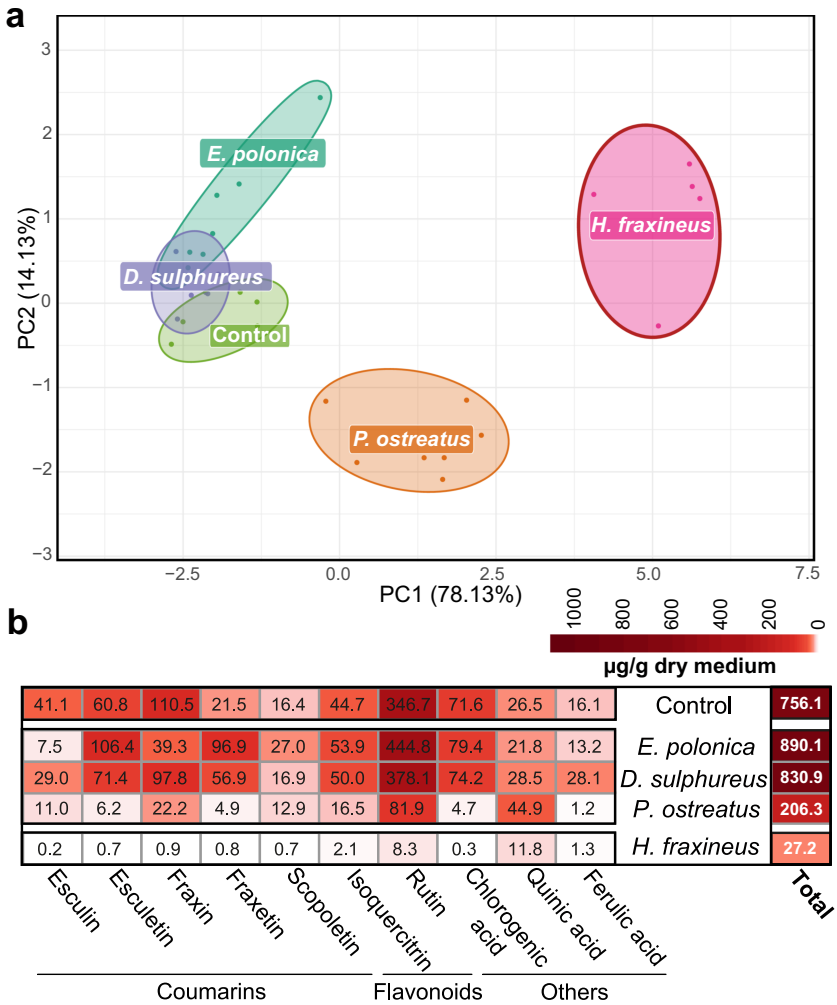

FIGURE 3 | *H. fraxineus* more efficiently depletes ash chemical defences from ash leaf-based medium than other fungi. The capacity of different fungi to deplete a set of 10 ash chemical defence compounds from ash leaf-based medium was assessed by LC-MS following fungal growth on 4% ash leaf medium until full colonisation of the petri dishes or for a maximum of 14 days. Biomass was separated from the colonised medium, and concentrations of individual compounds were quantified relative to a fungus-free control. (a) PCA of log1p-transformed and Z-score-scaled metabolite data from media colonised by different fungi and a fungus-free control ($N = 5$–8; see Suppl. Table S3 for results of pairwise PERMANOVAs with Benjamini-Hochberg correction). (b) Heatmap showing mean concentrations (in µg/g dry medium) of the analyzed ash chemical defence compounds in media colonised by different fungi and in the fungus-free control ($N = 5$–8; see also Suppl. Fig. S3 for total depletion from the sum of all compounds and Suppl. Table S3a for statistical analyses). [Color figure can be viewed at wileyonlinelibrary.com]

ash leaf methanol extract or with each of 15 individual ash chemical defence compounds (Figure 6). Growth on minimal medium supplemented with 1% ash leaf extract was significantly greater than on the control ($p < 0.0044$, Wilcoxon rank test; see Suppl. Table S3a; Figure 6a), suggesting the ability to utilise components of the extract as carbon sources. Consistent with this result, growth of *H. fraxineus* on medium supplemented with almost all defence compounds was significantly higher compared to the control lacking any additional carbon source ($p < 0.001$, GLM with adjusted pairwise contrasts; see Suppl. Table S3a; Figure 6b). The only tested compound for which no significant difference from the control was observed was fraxetin. Overall, growth of *H. fraxineus* was particularly enhanced on scopoletin. Among several plant polymers tested, *H. fraxineus* produced the largest surface area when grown on the polysaccharide galactomannan ($p < 0.001$, GLM with adjusted pairwise contrasts; see Table S3a; Figure S6), followed by the polysaccharides cellulose, amylopectin, and xylan. No difference in surface area relative to the control was observed for lignin or pectin.

Taken together, *H. fraxineus* showed a strong capacity to grow in the presence of a broad range of ash chemical defences, with scopoletin and several other compounds promoting the highest growth rates. In addition, certain plant polymers, particularly galactomannan, strongly promoted fungal growth as well.

4 | Discussion

The success of plant pathogens is often closely linked to their ability to resist host defence mechanisms, such as chemical defences (van der Does and Rep 2017; Lichnock et al. 2026). The degree of resistance may depend on the host range of the pathogen with those specialising on a single or closely-related plant species being especially resistant to host defence

13653040, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pce.70989 by Katholische Universitat Eichstatt-Ingolstadt, Wiley Online Library on [27/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

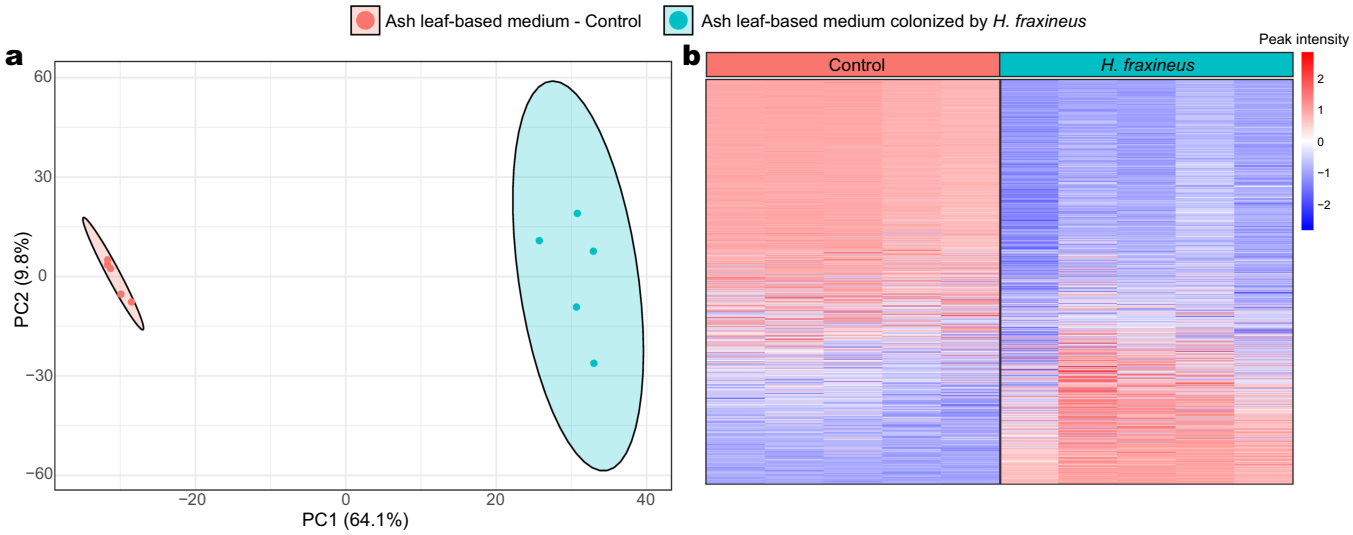

FIGURE 4 | *H. fraxineus* depletes a wide range of chemical features from ash leaf-based medium. Metabolomic analyses were performed using LC-ESI-QTOF-MS with *H. fraxineus* inoculated on 4% ash leaf medium until the petri dishes were completely covered. Biomass and culture medium were separated by a previously added cellophane disk, and medium colonised by *H. fraxineus* was compared to a fungus-free control. (a) PCA of cube-root-transformed and Z-score-scaled metabolomic data from fungus-free control and *H. fraxineus*-colonised medium samples ($N = 5$; see Table S3a for PERMANOVA results). (b) Heatmap visualising feature-level differences between *H. fraxineus*-colonised media and fungus-free controls. Features were cube-root-transformed and Z-score-scaled across samples to allow comparison of relative abundance patterns and were ordered by the difference in mean transformed (non-Z-score-scaled) feature intensities between control and *H. fraxineus* samples to enhance visual separation of group-specific patterns ($N = 5$; see Table S3c for results of feature-wise comparisons using Wilcoxon rank-sum tests with Benjamini-Hochberg correction). [Color figure can be viewed at wileyonlinelibrary.com]

compounds (Vanderplank 2012; Newman and Derbyshire 2020; Westrick et al. 2021). The ash dieback fungus *H. fraxineus* represents a highly specialised pathogen that is restricted to ash (*Fraxinus*) species at all stages of its life cycle (Carroll and Boa 2024). Because ash tissues, especially leaves, contain a broad diversity of defensive metabolites (Sidda et al. 2020; Schertler et al. 2026), and leaf infection represents the initial stage of *H. fraxineus* invasion (Carroll and Boa 2024), the ability to cope with ash metabolites may represent an important prerequisite for successful host colonisation.

Here, we demonstrate that *H. fraxineus* resists a broad range of European ash- metabolites, many of which have previously been described as chemical defences (Nemesio-Gorriz et al. 2020). Most of the tested chemicals had no inhibitory effect on fungal growth and some even promoted growth. The most likely mechanism of resistance appeared to be metabolism of defences since *H. fraxineus* was able to deplete nearly all of the measured ash defensive compounds in its colonised medium down to much lower levels than the other fungi investigated without any observable accumulation in its mycelia. These results suggest the uptake of defence compounds from the medium followed by metabolic transformation. This transformation may involve further breakdown to intermediate metabolites in some cases since *H. fraxineus* showed strong growth in the presence of nearly all tested European ash defence compounds. On the other hand, resistance is unlikely to arise from excretion of defence compounds by the fungus since these substances did not accumulate in the fungal medium.

The ability of *H. fraxineus* to resist European ash defences was demonstrated in several ways. First, its growth was not inhibited by the methanolic crude extract of ash leaves nor by the majority of ash defensive compounds tested. Interestingly, several compounds previously suggested to contribute to European ash resistance against *H. fraxineus* (Nemesio-Gorriz et al. 2020) showed only limited inhibition here. However, the coumarin esculetin, the flavonoid isoquercitrin, and the widespread caffeoyl quinate ester, chlorogenic acid, showed strong inhibitory effects, whereas the flavonoids rutin and naringin and the other coumarins and flavonoids tested caused only weak reductions in growth if any. These findings suggest that ash metabolites with related chemical structures can differ strongly in their effects on *H. fraxineus*. For instance, the coumarin monoglucoside, esculin, did not inhibit fungal growth, whereas its aglycone esculetin caused the strongest inhibition observed in this study. In contrast, the dihydroflavone diglycoside, naringin, slightly reduced fungal growth, while the corresponding aglycone naringenin did not. In fact, among the six flavonoids tested, the three glycosides all inhibited growth and the three aglycones did not. The glycosides constituted the most abundant of the defence compounds in our methanol extracts. Despite the diverse activities of the individual defence compounds in our tests, resistance *in planta* may actually depend on the entire mixture of compounds present in a specific tissue, which may change after pathogen infection. Mixtures may be more active than the equivalent amounts of single compounds because of synergistic effects (Gershenzon et al. 2012; Kant et al. 2015; Richards et al. 2015; Lehenberger et al. 2025; Zaman et al. 2025). Moreover, although all individual compounds were tested at a single concentration in this study, their effects may vary depending on the actual concentration *in planta*.

When we assayed ash extracts and pure compounds on additional fungal species, the results varied among the fungi tested.

13653040, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pce.70989 by Katholische Universitat Eichstatt-Ingolstadt, Wiley Online Library on [27/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

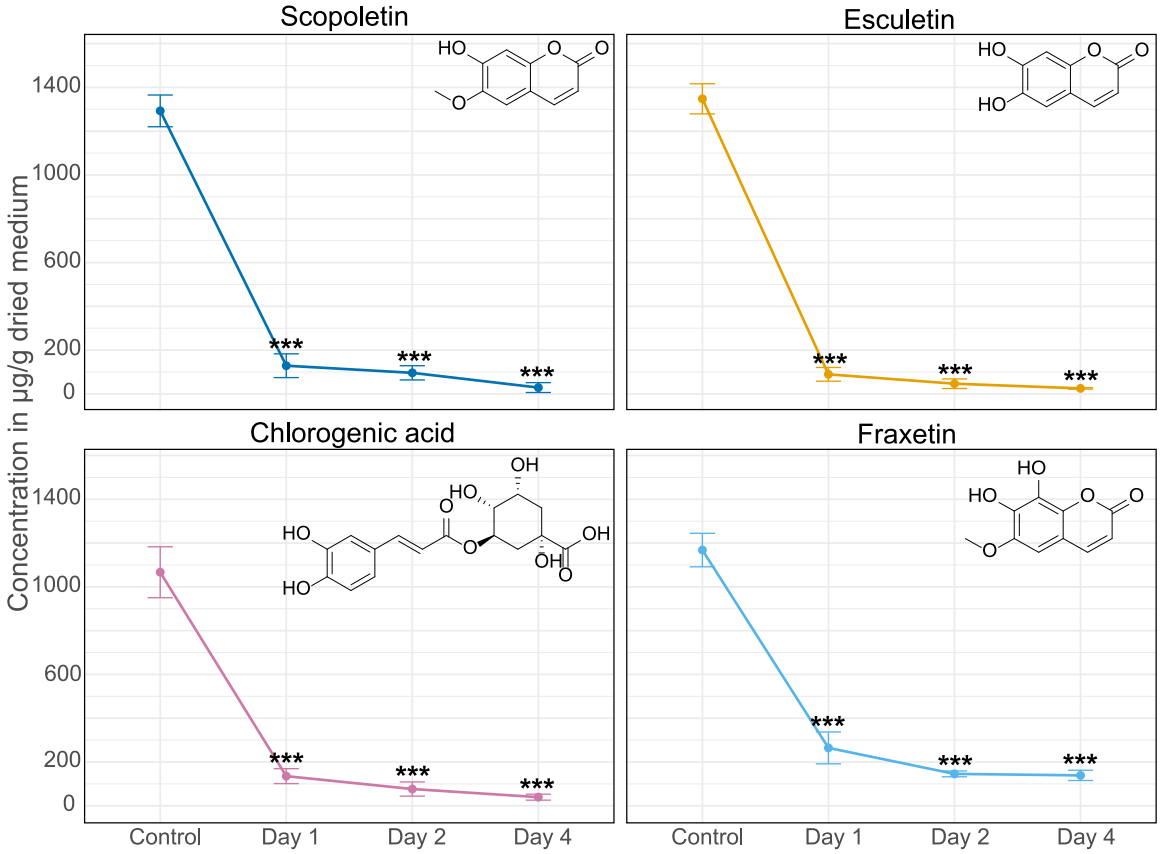

FIGURE 5 | *H. fraxineus* rapidly reduces the content of ash chemical defences in medium within 24 h. To assess the capability of *H. fraxineus* to reduce the content of four tested ash chemical defences over time, MEA was individually supplemented with the four test compounds (100 µg/mL dissolved in DMSO). *H. fraxineus* was pre-cultured on separate MEA medium on cellophane for 21 days prior to this experiment to allow transfer of the fungus-colonised cellophane disk onto new MEA plates containing the test compounds. The concentrations of the individual test compounds in medium (µg/g dry medium) colonised by *H. fraxineus* were compared across three time points (1, 2, and 4 days) and against a fungus-free control containing only the test compound. Line plots show the decrease in the individual compounds over time. Asterisks indicate significant differences between concentrations in medium at each time point relative to the control for each analyzed compound. Fitted GLM models and pairwise contrasts including Dunnett's correction were used to identify significant differences ($N = 4$–6; $^{ns}p > 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; see Table S3a for individual p values). [Color figure can be viewed at wileyonlinelibrary.com]

Although the growth of the ash pathogen *H. fraxineus* was not inhibited by the crude European ash leaf extract nor by most of the pure ash defence compounds tested, the beech-associated fungus *D. sulphureus* and especially the spruce-associated bark beetle symbiont *E. polonica* were both inhibited by the ash extract and, in the case of *D. sulphureus*, by half of the ash compounds tested. Meanwhile, *P. ostreatus*, a decomposer of many hardwood trees, was not inhibited by the European ash extract, nor by any of the ash compounds, even showing enhanced growth on some of them. These comparisons demonstrate that the fungi tested differed substantially in their responses to ash metabolites. However, because the studied fungi differ in phylogeny, lifestyle, and host association, the observed differences cannot be directly attributed to host specialisation or pathogenicity. Still, the particularly broad tolerance of *H. fraxineus* to the tested ash compounds provides evidence for its ability to withstand the chemical environment of its host and highlight the need to better understand how fungi are able to resist these substances.

Our results indicate that *H. fraxineus* resistance to European ash metabolites is associated with its ability to rapidly remove a broad range of these compounds from colonised tissue (Pedras and Abdoli 2017; Westrick et al. 2021; Lichnock et al. 2026). The pathogen readily depleted European ash compounds from the medium to a much greater extent than any of the other fungi tested under the conditions of our assays without any accumulation in fungal biomass. Although differences in incubation time and fungal biomass may have contributed to the observed differences in depletion, these factors alone do not fully explain the particularly strong depletion observed for *H. fraxineus*. The extensive chemical changes identified in the untargeted metabolomic analyses further demonstrate that *H. fraxineus* strongly alters the chemical composition of ash leaf medium as nearly half of all detected metabolomic features in ash leaf medium were significantly reduced following *H. fraxineus* growth. The numerous features showing higher abundance may represent transformation products of ash defences, other metabolites involved in defence compound breakdown, or *de novo* products of fungal metabolism, such as antibiotics (Surup et al. 2018; Masi et al. 2019). Together, these metabolomic shifts suggest that the large-scale reduction of ash metabolites is a central element of host colonisation by *H. fraxineus*. The capacity of other pathogens to metabolise antifungal defences has been shown to be an important virulence factor for pathogens such as

13653040, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pce.70989 by Katholische Universitat Eichstatt-Ingolstadt, Wiley Online Library on [27/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

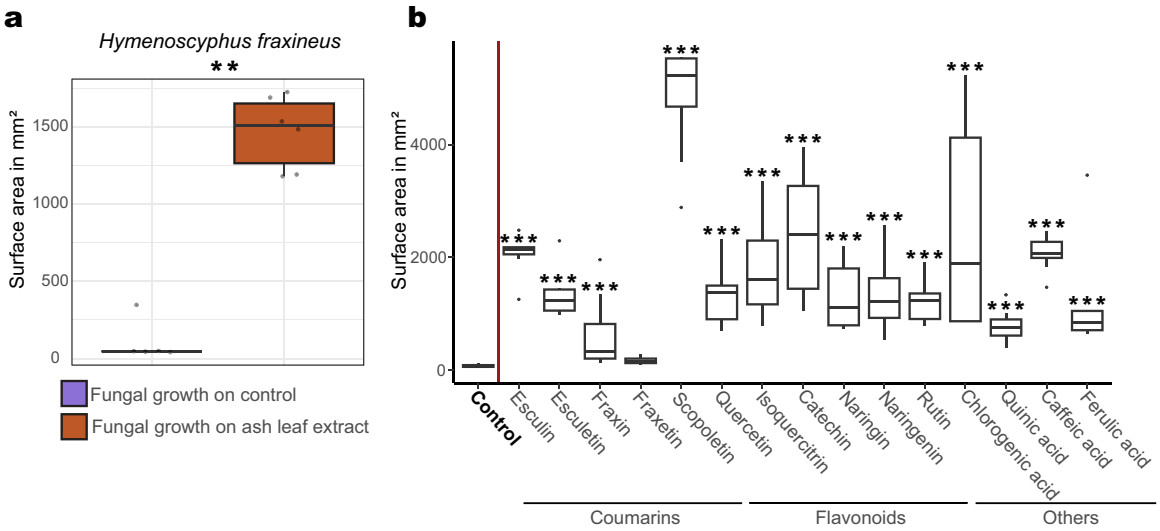

FIGURE 6 | The growth of *H. fraxineus* is promoted by a broad range of ash chemical defencesThe ability of *H. fraxineus* (strain P343) to grow in the presence of ash chemicals was assessed using a minimal yeast nitrogen base (YNB) medium, which was either supplemented (a) with an ash leaf extract obtained from freeze-dried ash leaf powder using methanol, which was subsequently evaporated and re-dissolved in DMSO (final concentration in medium = 1%), or (b) with 15 individually tested ash chemical defences (100 μg/mL for each compound, dissolved in DMSO). Asterisks indicate significant differences in fungal growth (surface area, mm^2) for the tested extract and each compound relative to a control (DMSO at the same concentration) without any additionally provided carbon source ($^{ns}p > 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). (a) Boxplot showing fungal growth on YNB containing 1% ash leaf extract relative to a control without extract ($N = 5$–6; Wilcoxon rank-sum test, see Table S4 for further details). (b) Boxplots showing fungal growth on the different tested ash chemical defences ($N = 5$–7; fitted GLM models and pairwise contrasts including Dunnett's correction, see Suppl. Table S3a for individual p values and Figure S6). [Color figure can be viewed at wileyonlinelibrary.com]

Nectria haematococca Berk. & Broome (Nectriaceae, Hypocreales, Hypocreomycetidae, Sordariomycetes, Ascomycota) on pea or *Botrytis cinerea* Pers. (Sclerotiniaceae, Helotiales, Leotiomycetidae, Leotiomycetes, Ascomycota) on grape (Sbaghi et al. 1996; Delserone et al. 1999). The depletion of European ash compounds by *H. fraxineus* occurred rapidly, with strongly reduced concentrations already observed within 24 h after exposure. Comparable rapid metabolic transformations have been described in other plant-pathogenic fungi, where they are associated with successful infection and colonisation of plant tissue (Pedras and Ahiahonu 2005; Westrick et al. 2021). Indeed, as leaf infection represents the initial stage of ash dieback development (Carroll and Boa 2024), the rapid removal of host defensive metabolites may provide a substantial advantage during early establishment in chemically well-defended ash leaves. However, depletion from the medium alone does not directly demonstrate that these compounds are metabolised by *H. fraxineus*, as this would require identification of transformation products or isotopic labelling. Nevertheless, the depletion of a broad diversity of plant defence compounds from colonised medium demonstrates the ability of *H. fraxineus* to alter its chemical environment and suggests a strong adaptation to the chemical conditions encountered in its host tree. The extensive depletion of ash defence compounds by *H. fraxineus* may be associated with their ability to support fungal growth. Our experiments revealed that *H. fraxineus* grew more rapidly when medium was supplied with various ash metabolites suggesting that these substances were likely broken down to form products that then enter intermediary metabolism. However, increased growth alone, without isotope tracing, does not demonstrate that these compounds were directly utilised as carbon sources or broken down into products entering intermediary metabolism. Still, similar metabolic transformations have been reported for other plant pathogens. For example, the citrus pathogen *Penicillium digitatum* (Pers.) Sacc. (Aspergillaceae, Eurotiales, Eurotiomycetidae, Eurotiomycetes, Ascomycota) transforms the monoterpene (+)-limonene into other terpenoids and has been suggested to utilise this host defence compound as a carbon source (Tan et al. 1998; Duetz et al. 2003; Hammerbacher et al. 2019). Likewise, the bark beetle-associated fungus *Grosmannia clavigera* (Rob.-Jeffr. & R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf. (Ceratocystidaceae, Microascales, Hypocreomycetidae, Sordariomycetes, Ascomycota) can grow on the monoterpenes of pine resin as a sole carbon source (DiGuistini et al. 2011). These examples demonstrate that the ability to metabolise host defensive compounds can contribute to the growth of some specialised fungi, although such capacities have not been widely documented.

Among the compounds tested as potential *H. fraxineus* growth promoters, there were large differences in effect. Particularly strong growth was observed on the coumarin scopoletin, whereas compounds such as the coumarin fraxetin, the cyclic polyol quinic acid, and the phenolic acid, ferulic acid, supported more moderate growth. The ability of some ash metabolites to promote fungal growthmay help explain why *H. fraxineus* grows substantially better on ash leaf-based medium than on artificial culture media (Kowalski and Bartnik 2010; Carrari et al. 2015; Ridley et al. 2026). However, not all compounds depleted from the medium by *H. fraxineus* necessarily contribute directly to fungal growth. For example, the coumarin aglycone fraxetin was strongly depleted from colonised medium but did not support fungal growth, suggesting that its removal may instead involve a short transformation pathway rather than

13653040, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pce.70989 by Katholische Universitat Eichstatt-Ingolstadt, Wiley Online Library on [27/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

one leading to full degradation to primary metabolites or other processes (Westrick et al. 2021; Sun et al. 2026).

Plant polymers may also support the growth of *H. fraxineus*. We showed that the pathogen exhibited increased growth on medium supplemented with both structural and storage polysaccharides, including cellulose, amylopectin, galactomannan and xylan. The ability to degrade plant polysaccharides is widespread among fungal plant pathogens and is mediated by diverse carbohydrate-active enzymes that facilitate both nutrient acquisition and host colonisation (Kubicek et al. 2014; Berlemont 2017). In addition to supporting pathogen nutrition, degradation of plant polysaccharides also generates oligosaccharides that can activate plant immune responses (Carrasco-López et al. 2026).

Our findings on *H. fraxineus* resistance to ash leaf chemicals may help to explain the patterns of resistance of different ash species to this pathogen. While invasive to Europe, *H. fraxineus* is native to East Asia, so the resistance to European ash defence metabolites observed in this study may reflect adaptations acquired during long-term association with Asian *Fraxinus* species (Carroll and Boa 2024). Differences among ash species in the composition and abundance of defensive metabolites may contribute to the contrasting susceptibility observed across *Fraxinus* taxa. Similarly, variation in the composition and abundance of defensive metabolites among individual European ash trees may contribute to differences in their susceptibility to *H. fraxineus*. The trees used to prepare the ash leaf medium were asymptomatic but had not been phenotyped for resistance to *H. fraxineus*, so their resistance status was unknown. Therefore, variation in the chemical composition associated with differences in resistance or susceptibility cannot be excluded and may have influenced the composition of the leaf medium. Resistant ash species, such as *F. mandshurica* or *F. chinensis* Roxb., may contain compounds or metabolite combinations that are more resistant to *H. fraxineus* (Eyles et al. 2007; Nielsen et al. 2017; Carroll and Boa 2024). Future studies should therefore compare defensive chemistry between resistant and susceptible ash species and examine whether this influences fungal establishment. Although three *H. fraxineus* strains were included in the crude extract assay, most experiments were conducted with strain P343, but the extent to which these findings are representative of the species remains to be established. All three strains used in this study were able to cause mortality of young ash saplings in climate chamber experiments conducted in our laboratory (data not shown). However, their relative virulence was not assessed in the present study, and future studies should investigate whether variation in virulence is associated with differences in resistance to host-derived metabolites. Comparing strain-specific differences in resistance within *H. fraxineus* populations (Junker et al. 2017) and studying the native non-pathogenic fungal species *H. albidus* may provide further insights into the evolution of host resistance in these interactions. Overall, our results suggest that the colonisation success of *H. fraxineus* is likely linked to its resistance to a broad range of host-derived metabolites, including compounds with demonstrated antifungal activity. The observed growth-promoting effects of several ash compounds indicates that some host defences may serve as carbon sources for *H. fraxineus*.

Acknowledgements

The authors are thankful to Beate Rothe and Bettina Raguschke for their technical support in the laboratory. This project was funded by the German Research Foundation (DFG: Grant number 520486374 to M.L.) and the Max-Planck Society. Open Access funding enabled and organized by Projekt DEAL.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data generated or analyzed during this study are included in this published article (and its Supporting Information files).

References

Bednarek, P., and A. Osbourn. 2009. "Plant-Microbe Interactions: Chemical Diversity in Plant Defense." *Science* 324, no. 5928: 746–748.

Berlemont, R. 2017. "Distribution and Diversity of Enzymes for Polysaccharide Degradation in Fungi." *Scientific Reports* 7, no. 1: 222.

Carrari, E., P. Capretti, A. Santini, and N. Luchi. 2015. "*Hymenoscyphus fraxineus* Mycelial Growth on Media Containing Leaf Extracts of Different Oleaceae." *Forest Pathology* 45, no. 6: 540–543.

Carrasco-López, C., S. López-Cobos, and A. Sánchez-Vallet. 2026. "Plant Cell Walls as a Key Driver of Plant–Pathogen Coevolution." *FEMS Microbiology Reviews* 50: fuag023.

Carroll, D., and E. Boa. 2024. "Ash Dieback: From Asia to Europe." *Plant Pathology* 73, no. 4: 741–759.

Delserone, L. M., K. McCluskey, D. E. Matthews, and H. D. VanEtten. 1999. "Pisatin Demethylation by Fungal Pathogens and Nonpathogens of Pea: Association With Pisatin Tolerance and Virulence." *Physiological and Molecular Plant Pathology* 55, no. 6: 317–326.

DiGuistini, S., Y. Wang, N. Y. Liao, et al. 2011. "Genome and Transcriptome Analyses of the Mountain Pine Beetle-Fungal Symbiont *Grosmannia Clavigera*, a Lodgepole Pine Pathogen." *Proceedings of the National Academy of Sciences* 108, no. 6: 2504–2509.

Meyer, D., E. Dimitriadou, K. Hornik, A. Weingessel, and F. Leisch. 2025. "e1071: Misc Functions of the Department of Statistics, Probability Theory Group (Formerly: E1071), TU Wien." R package version 1.7-17. https://CRAN.R-project.org/package=e1071.

van der Does, H. C., and M. Rep. 2017. "Adaptation to the Host Environment by Plant-Pathogenic Fungi." *Annual Review of Phytopathology* 55: 427–450.

Duetz, W. A., H. Bouwmeester, J. B. Beilen, and B. Witholt. 2003. "Biotransformation of Limonene by Bacteria, Fungi, Yeasts, and Plants." *Applied Microbiology and Biotechnology* 61, no. 4: 269–277.

Evans, H. C., and J. M. Waller. 2010. *Globalisation and the Threat to Biosecurity. The Role of Plant Pathology in Food Safety and Food Security*, edited by R. N. Strange, M. L. Gullino, and Dordrecht, 53–71. Springer Netherlands.

Eyles, A., W. Jones, K. Riedl, et al. 2007. "Comparative Phloem Chemistry of Manchurian (*Fraxinus mandshurica*) and Two North American Ash Species (*Fraxinus americana* and *Fraxinus pennsylvanica*)." *Journal of Chemical Ecology* 33, no. 7: 1430–1448.

Gershenzon, J., A. Fontana, M. Burow, U. Wittstock, and J. Degenhardt. 2012. "Mixtures of Plant Secondary Metabolites: Metabolic Origins and Ecological Benefits." In *The Ecology of Plant Secondary Metabolites: From Genes to Global Processes*, edited by G. R. Iason, M. Dicke, and S. E. Hartley, 56–77. Cambridge University Press. http://www.cambridge.org/aus/catalogue/catalogue.asp?isbn=9780521193269.

13653040, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pce.70989 by Katholische Universitat Eichstatt-Ingolstadt, Wiley Online Library on [27/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Ghelardini, L., N. Luchi, F. Pecori, et al. 2017. "Ecology of Invasive Forest Pathogens." *Biological Invasions* 19, no. 11: 3183–3200.

Gomdola, D., C. Bhunjun, K. Hyde, R. Jeewon, D. Pem, and R. Jayawardena. 2022. "Ten Important Forest Fungal Pathogens: A Review on Their Emergence and Biology." *Mycosphere* 13, no. 1: 612–671.

Hammerbacher, A., T. A. Coutinho, and J. Gershenzon. 2019. "Roles of Plant Volatiles in Defence Against Microbial Pathogens and Microbial Exploitation of Volatiles." *Plant, Cell & Environment* 42, no. 10: 2827–2843.

Häuser, H., A. Pilger, C. Ulrichs, and R. Kätzel. 2025. "Phenolic Leaf Compounds in Ash Trees (*Fraxinus excelsior* L.) in the Context of Ash Dieback." *Forests* 16, no. 9: 1387.

Jorgensen, I., M. Rayamajhi, and E. A. Miao. 2017. "Programmed Cell Death as a Defence Against Infection." *Nature Reviews Immunology* 17, no. 3: 151–164.

Junker, C., J. de Vries, C. Eickhorst, and B. Schulz. 2017. "Each Isolate of *Hymenoscyphus fraxineus* Is Unique as Shown by Exoenzyme and Growth Rate Profiles." *Baltic Forestry* 23, no. 1: 25–40.

Kant, M. R., W. Jonckheere, B. Knegt, et al. 2015. "Mechanisms and Ecological Consequences of Plant Defence Induction and Suppression in Herbivore Communities." *Annals of Botany* 115, no. 7: 1015–1051. https://doi.org/10.1093/aob/mcv054.

Kirisits, T., and I. Offenthaler. 2002. "Xylem Sap Flow of Norway Spruce After Inoculation With the Blue-Stain Fungus *Ceratocystis polonica*." *Plant Pathology* 51, no. 3: 359–364.

Kolde, R. 2019. "Pheatmap: Pretty Heatmaps." *R Package Version* 1, no. 0: 12.

Kowalski, T., and C. Bartnik. 2010. "Morphologial Variation in Colonies of *Chalara fraxinea* Isolated From Ash (*Fraxinus excelsior* L.) Stems With Symptoms of Dieback and Effects of Temperature on Colony Growth and Structure." *Acta Agrobotanica* 63, no. 1: 99–106.

Kubicek, C. P., T. L. Starr, and N. L. Glass. 2014. "Plant Cell Wall–Degrading Enzymes and Their Secretion in Plant-Pathogenic Fungi." *Annual Review of Phytopathology* 52: 427–451.

Langer, G. 2017. "Collar Rots in Forests of Northwest Germany Affected by Ash Dieback." *Baltic Forestry* 23, no. 1: 4–19.

Lehenberger, M., Y. Pan, S. Ungerer, et al. 2025. "Fungal Symbiont of an Ambrosia Beetle Possesses High Nutrient Content and Suppresses Competing Fungi With Antimicrobial Compounds." *ISME Journal* 19, no. 1: wraf258.

Lévesque, M., J. I. Bustamante Eduardo, and V. Queloz. 2023. "Potential Alternative Tree Species to *Fraxinus excelsior* in European Forests." *Frontiers in Forests and Global Change* 6: 2023.

Lichnock, B., S. M. Tanin, M. Reichelt, P. Biedermann, J. Gershenzon, and M. Lehenberger. 2026. "The Mutualistic Fungi of the Bark Beetle Pityokteines Vorontzowi Are Nutrient-Rich and Efficiently Deplete Their Medium of Fir Chemical Defenses." *ISME Communications* 6: ycag131.

Loo, J. A. 2009. "Ecological Impacts of Non-Indigenous Invasive Fungi as Forest Pathogens." *Biological Invasions* 11, no. 1: 81–96.

Masi, M., R. Di Lecce, A. Tuzi, et al. 2019. "Hyfraxinic Acid, a Phytotoxic Tetrasubstituted Octanoic Acid, Produced by the Ash (*Fraxinus Excelsior* L.) Pathogen *Hymenoscyphus fraxineus* Together With Viridiol and Some of Its Analogues." *Journal of Agricultural and Food Chemistry* 67, no. 49: 13617–13623.

Medina-van Berkum, P., E. Schmöckel, A. Bischoff, et al. 2024. "Plant Geographic Distribution Influences Chemical Defences in Native and Introduced *Plantago lanceolata* Populations." *Functional Ecology* 38, no. 4: 883–896.

Nemesio-Gorriz, M., R. C. Menezes, C. Paetz, et al. 2020. "Canditate Metabolites for Ash Dieback Tolerance in *Fraxinus excelsior*." *Journal of Experimental Botany* 71, no. 19: 6074–6083.

Newman, T. E., and M. C. Derbyshire. 2020. "The Evolutionary and Molecular Features of Broad Host-Range Necrotrophy in Plant Pathogenic Fungi." *Frontiers in Plant Science* 11: 2020.

Nielsen, L. R., L. V. McKinney, A. M. Hietala, and E. D. Kjær. 2017. "The Susceptibility of Asian, European and North American *Fraxinus* Species to the Ash Dieback Pathogen *Hymenoscyphus fraxineus* Reflects Their Phylogenetic History." *European Journal of Forest Research* 136, no. 1: 59–73.

Oksanen, J., R. Kindt, and G. L. Simpson. 2017. "vegan3d: Static and Dynamic 3D Plots for the 'Vegan' package." R package version 1: 0. https://CRAN.R-project.org/package=vegan3d.

Pedras, M. S. C., and A. Abdoli. 2017. "Pathogen Inactivation of Cruciferous Phytoalexins: Detoxification Reactions, Enzymes and Inhibitors." *RSC Advances* 7, no. 38: 23633–23646.

Pedras, M. S. C., and P. W. K. Ahiahonu. 2005. "Metabolism and Detoxification of Phytoalexins and Analogs by Phytopathogenic Fungi." *Phytochemistry* 66, no. 4: 391–411.

Peters, S., S. Fuchs, S. Bien, J. Bußkamp, G. J. Langer, and E. J. Langer. 2023. "Fungi Associated With Stem Collar Necroses of *Fraxinus Eexcelsior* Affected by Ash Dieback." *Mycological Progress* 22, no. 7: 52.

Peters, S., N. Gruschwitz, S. Bien, et al. 2024. "The Fungal Predominance in Stem Collar Necroses of *Fraxinus Excelsior*: a Study on *Hymenoscyphus fraxineus* Multilocus Genotypes." *Journal of Plant Diseases and Protection* 131, no. 5: 1341–1353.

Richards, L. A., L. A. Dyer, M. L. Forister, et al. 2015. "Phytochemical Diversity Drives Plant–Insect Community Diversity." *Proceedings of the National Academy of Sciences* 112, no. 35: 10973–10978.

Ridley, M., V. Reckemeyer, and R. Enderle. 2026. "Opportunities for Maintaining and Rejuvenating Strain Collections of *Hymenoscyphus fraxineus*." *Mycologia* 118, no. 3: 512–523.

Sbaghi, M., P. Jeandet, R. Bessis, and P. Leroux. 1996. "Degradation of Stilbene-Type Phytoalexins in Relation to the Pathogenicity of *Botrytis cinerea* to Grapevines." *Plant Pathology* 45, no. 1: 139–144.

Schertler, E., V. Queloz, F. Monteleone, M. Eisenring, and R. Heinzelmann. 2026. "Resistance and Tolerance Mechanisms of Common Ash (*Fraxinus excelsior*) Against the Ash Dieback Pathogen *Hymenoscyphus fraxineus*: Latest Advances and Knowledge Gaps." *Forestry: An International Journal of Forest Research* 99, no. 2: cpaf038.

Seidel, H., M. Šeho, and B. Fussi. 2024. "Hope for Ash Conservation and Propagation—Single Individuals Can Be Highly Resistant to an Invasive Pathogen." *Journal of Plant Diseases and Protection* 132, no. 1: 18.

Serrano, M., F. Coluccia, M. Torres, and F. L'Haridon. 2014. "and Métraux, J.-P. The Cuticle and Plant Defense to Pathogens." *Frontiers in Plant Science* 5: 274.

Sidda, J. D., L. Song, J. L. Parker, D. J. Studholme, C. Sambles, and M. Grant. 2020. "Diversity of Secoiridoid Glycosides in Leaves of UK and Danish Ash Provide New Insight for Ash Dieback Management." *Scientific Reports* 10, no. 1: 19566.

Singh, B. K., M. Delgado-Baquerizo, E. Egidi, et al. 2023. "Climate Change Impacts on Plant Pathogens, Food Security and Paths Forward." *Nature Reviews Microbiology* 21, no. 10: 640–656.

Sun, R., B. Hu, Y. Nakamura, et al. 2026. "Detoxification of Conifer Antimicrobial Defenses Promotes Entomopathogenic Fungus Infection of Bark Beetles." *Proceedings of the National Academy of Sciences* 123, no. 1: e2525513122.

Surup, F., S. Halecker, M. Nimtz, et al. 2018. "Hyfraxins A and B, Cytotoxic Ergostane-Type Steroid and Lanostane Triterpenoid Glycosides From the Invasive Ash Dieback Ascomycete *Hymenoscyphus fraxineus*." *Steroids* 135: 92–97.

Tan, Q., D. F. Day, and K. R. Cadwallader. 1998. "Bioconversion of (R)-(+)-Limonene by *P. digitatum* (NRRL 1202)." *Process Biochemistry* 33, no. 1: 29–37.

13653040, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pce.70989 by Katholische Universitat Eichstatt-Ingolstadt, Wiley Online Library on [27/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Tripathi, A., V. K. Pandey, A. K. Jha, et al. 2024. "Intricacies of Plants' Innate Immune Responses and Their Dynamic Relationship With Fungi: A Review." *Microbiological Research* 285: 127758.

Vanderplank, J. E. 2012. *Host-Pathogen Interactions in Plant Disease*. Elsevier.

Westrick, N. M., D. L. Smith, and M. Kabbage. 2021. "Disarming the Host: Detoxification of Plant Defense Compounds During Fungal Necrotrophy." *Frontiers in Plant Science* 12: 651716.

Wickham, H. 2016. "Data Analysis." In *ggplot2: Elegant Graphics for*. Springer-Verlag New York.

Wickham, H., and D. Vaughan. 2024. "Girlich, M. tidyr: tidy Messy Data." *R Package Version* 1, no. 3: 1.

Wickham, H., R. François, L. Henry, K. Müller, and D. Vaughan. 2023. dplyr: A Grammar of Data Manipulation. https://dplyr.tidyverse.org.

Zaman, R., A. Jain, A. Hammerbacher, J. Gershenzon, and D. Kandasamy. 2025. "Both Synergism and Interaction Diversity Explain the Mixtures of Defensive Monoterpenes in Spruce Oleoresin." *Functional Ecology* 39, no. 9: 2539–2551.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.
Supporting File 1: pce70989-sup-0001-Suppl_Table_S1.xlsx.
Supporting File 2: pce70989-sup-0002-Suppl_Table_S2.xlsx.
Supporting File 3: pce70989-sup-0003-Suppl_Table_S3a.docx.
Supporting File 4: pce70989-sup-0004-Suppl_Table_S3b.xlsx.
Supporting File 5: pce70989-sup-0005-Suppl_Table_S3c.xlsx.
Supporting File 6: pce70989-sup-0006-Supplementary_Information_Clean_version.docx.

13653040, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pce.70989 by Katholische Universitat Eichstatt-Ingolstadt, Wiley Online Library on [27/10/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License