Priming of ash saplings with a low virulent Hymenoscyphus fraxineus strain as a possible disease control approach for reducing symptoms of ash dieback

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Abstract

Ash dieback is a tree disease caused by the fungal pathogen *Hymenoscyphus fraxineus.* Since its introduction into Europe, it has caused widespread and significant losses of the European ash, *Fraxinus excelsior*. Inoculations of *F. excelsior* with a low virulent *H. fraxineus* isolate was assessed as a promising method for reducing symptoms associated with ash dieback, presumably by triggering systemic induced resistance. Two strains of *H. fraxineus* were chosen based on observations of high and low *in planta* virulence. Crude extracts obtained from cultures of the highly virulent strain were more phytotoxic in a leaf puncture assay than ones obtained from the low virulent strain. UHPLC-DAD-MS/MS data identified the phytotoxin

viridiol and the potential phytotoxin hyfraxin A in both cultures. However, the production of these compounds *in vitro* did not correspond with virulence *in planta*.

To test the effects of priming, saplings of *F. excelsior* were first inoculated with the low virulent strain and subsequently with the highly virulent strain. On average, necrosis expansion on the stems was reduced by 54% in primed saplings at the end of the monitoring period of 14 weeks, thus providing proof of concept for priming. These results contribute to our understanding of a possible integrated biological disease control approach for increasing resistance in saplings and reducing potential damages associated with pathogens, particularly during nursery propagation, out-planting and through the establishment phase. We discuss the results in the context of relevant literature and summarise the limited availability of literature on priming and underlying principles in trees.

Key words

ash dieback, biocontrol, *Fraxinus excelsior*, induced resistance, priming, secondary metabolites

Introduction

Since the early 1990s, the European ash (*Fraxinus excelsior*) (Lamiales: Oleaceae) has been threatened by the invasive fungal pathogen *Hymenoscyphus fraxineus* (syn. *H. pseudoalbidus,* anamorph *Chalara fraxinea*) (T. Kowalski) Baral, Queloz and Hosoya (Kowalski et al. 2006; Gross et al. 2014). The disease has spread rapidly throughout Europe (Kowalski et al. 2006; Schumacher et al. 2007). The pathogen is associated with dieback of the crown, discolouration of the sapwood and reduced stability of standing trees due to the development of root collar necroses (Kowalski et al. 2006; Kräutler and Kirisits 2012; Gross et al. 2014; Marcais et al. 2016; Langer et al. 2017). The crown damages, specifically, have been associated with

reductions in reproductive success of individual ash trees (Semizer-Cuming et al. 2021). Furthermore, the disease is associated with considerable tree mortality (McKinney et al. 2014; Marçais et al. 2017; Cocker et al. 2019; Madsen et al. 2021). Consequently, European ash has been greatly reduced, particularly in forested areas, and it is no longer considered a commercially viable species in forestry (Pautasso et al. 2013).

Management strategies to mitigate the impact of ash dieback disease vary considerably depending on site conditions, stand characteristics and the severity of dieback (Skovgaard et al. 2017; Enderle et al. 2019). Where the impacts of ash dieback are severe, it is recommended that any remaining commercial timber is harvested and that the area is regenerated naturally or replanted with another viable tree species. Conversely, in forested areas of high conservation value, it is widely recommended to simply let natural succession proceed unhindered or replant with alternative native species (Pautasso et al. 2013; Mitchell et al. 2014; Skovgaard et al. 2017). However, ash saplings produced in nurseries are also sometimes used for replanting in certain areas (Pautasso et al. 2013). This is, in part, because it has been determined that there are no viable native species that can fully replace, alone or in combination, the ecological niche and ecosystem services of the European ash (Lévesque et al. 2023). Therefore, the preservation of the species is critical for maintaining associated biodiversity and ecosystem services (Pautasso et al. 2013; Mitchell et al. 2014; Broome et al. 2014; Hultberg et al. 2020; Turczański et al. 2022; Lévesque et al. 2023). Furthermore, replanting may be critical in some cases for maintaining genetic diversity of ash populations (Semizer-Cuming et al. 2021). However, the high rates of mortality associated with infected saplings and younger trees poses a particular challenge for forest management, particularly in areas of high conservation value (Cocker et al. 2019; Klesse et al. 2021). There are breeding programmes that aim for reduced genetic susceptibility. However, improved plant material deriving from these programmes may still require additional protection. Therefore, strategies for reducing symptom development and

92 mortality in saplings will assist in mitigating the potentially far-reaching ecosystem losses
93 associated with the ash dieback disease.

94 One approach could be to develop methods that enhance natural sapling defences. Plants can
95 employ a range of defence mechanisms to reduce damages caused by pathogens. Defence
96 mechanisms may be constitutive or induced structural and chemical defences, which reduce
97 infection success or reduce pathogen performance following infection (*e.g.,* Franceshi et al.
98 2002; Ton et al. 2002; Zeneli et al. 2006; Witzell and Martín 2008; Poland et al. 2011; Robert-
99 Seilaniantz et al. 2011). The induction of plant defences, such as the production of volatile plant
100 compounds, can result in enhanced resistance and mobilisation of plant defence in subsequent
101 interactions with the pathogen (Kuć 2001; Conrath et al. 2006). This can also be achieved
102 through the artificial treatment of plants using wounding or with pathogens of low virulence,
103 which is also known as *priming*. Priming can be induced by the secondary metabolites of the
104 pathogen, which in turn can induce changes at the physiological, transcriptional, metabolic and
105 epigenetic levels of the plants (Mauch-Mani et al. 2017). The resulting primed state can be
106 described as a physiological state in which plants have an augmented activated defence
107 response towards a pathogen (Conrath et al. 2006; Eyles et al. 2010; Pastor et al. 2013). More
108 specifically, these changes in the plant can result in locally or systemically induced defences,
109 where signalling and transportation of defence compounds can lead to an increased resistance
110 response (Eyles et al. 2010). The induction of defences may also prime the host plant
111 epigenetically against repeated pathogen challenges in the future, including in other tissues on
112 distal parts of a plant (Eyles et al. 2010; Pastor et al. 2013; Prospero et al. 2021).

113 Investigations on the underlying mechanisms of resistance and priming have been restricted to
114 just a few species of plants (Bonello and Blodgett 2003; van Hulten et al. 2006; Wilson et al.
115 2023). Besides conifers, the majority of studies of defence mechanisms has been conducted in
116 short-lived plants, primarily agriculture crops and *Arabidopsis thaliana* (Vallad and Goodman

2004; van Hulten et al. 2006; Pieterse et al. 2014; Telford et al. 2015; Westman et al. 2019). There are several examples of systemic acquired resistance (SAR) and induced systemic resistance (ISR) in trees. While SAR is defined as reduced susceptibility in plants as a result of previous local infection with a pathogen predominantly mediated by salicylic acid (SA) and pathogenesis-related (PR) proteins, ISR is phenotypically similar but instead mediated by jasmonate (JA) and ethylene (ET) pathways (de Kesel et al. 2021). However, trees also exhibit a unique type of systemic resistance, referred to here as systemic induced resistance (SIR). SIR is considered separately from SAR or ISR because the signalling mechanism as part of the systemic induced response is not currently understood (Bonello et al. 2001; Wilson et al. 2023). SIR has been less extensively studied, but it is typically associated with necrotising pathogens, where their presence can result in enhanced tree defences (Eyles et al. 2010; Wilson et al. 2023).

Whereas there have been numerous studies on SIR in conifers, there have been fewer studies investigating SIR in deciduous trees (Wilson et al. 2023). For example, in *Salix* spp. (Ruuhola et al. 2001; Ollerstam and Larsson 2003)*, Populus* spp. (Constabel et al. 2000; Babst et al. 2005; Li et al. 2018), *Castanea* spp. (Cooper and Rieske 2008), *Eucalyptus* spp. (Henery et al. 2008; Naidoo et al. 2013) and *Fraxinus* spp. (Whitehill et al. 2014; Villari et al. 2016). Genes encoding for abscisic acid- or jasmonate-signalling pathways have been identified (Sahraei et al. 2020) and their potential role in induced resistance in ash trees is indicated. For example, Whitehill et al. (2014) found that the application of methyl jasmonate (MeJA) on the bark of *Fraxinus mandshuria* and *Fraxinus americana* can induce phenolic compounds in the phloem of treated stems. In another study, induced resistance in *Fraxinus nigra* and *Fraxinus manchuria* in response to exogenous treatment with methyl jasmonate has been associated with increased concentrations of verbascoside, lignin and/or trypsin inhibitors in the bark, which decreased survival and/or growth of Emerald Ash Borer larvae (*Agrilus planipennis*) (Whitehall et al. 2014; Villari et al. 2016). To the authors' knowledge, however, no studies have considered the

use of priming with a low virulent necrotising pathogen to induce resistance against future pathogen infections in a deciduous tree species.

The efficacy of priming with an isolate of major pathogens has been successfully demonstrated for conifers. There are several studies where priming against a pathogen was conducted with non-lethal isolates of the same species of pathogen. For example, priming with different inoculum densities has been demonstrated in *Pinus radiata* (Monterey pine) against the causal agent of pitch canker disease (*Fusarium circinatum*) (Bonello et al. 2001; Swett and Gordon 2016). Its efficacy has also been shown in *Pinus nigra* (Austrian pine) against the causal agent of Diplodia shoot dieback (*Diplodia pinea,* synomyn: *Sphaeropsis sapinea*) when the pathogen was used to prime saplings against secondary challenges with the same isolate (Eyles et al. 2007; Blodgett et al. 2007). It has also been demonstrated in *Picea abies* (Norway spruce) against the root rot pathogen *Heterobasidion parviporum,* when the same isolate was used to prime and challenge the saplings (Swedjemark et al. 2007), as well as priming with sub-lethal doses of the necrotrophic fungal pathogen *Ceratocystis polonica* (syn. *Endoconidiophora polonica*) ahead of challenge with lethal doses of the pathogen (Krokene et al. 2003). The fact that these studies were all conducted on conifers is likely due to their commercial importance internationally as well as the dominance of *Picea abies* and *Pinus nigra* globally (Cooper 2003; Farjon et al. 2018; Felton et al. 2020). As a consequence, *Pinus nigra* has even been proposed as a model pathosystem for investigating local and systemic effects of fungal infections in pines, which will undoubtedly further promote investigations in conifers (Bonello and Blodgett 2003).

Priming of deciduous saplings might be just as effective. This may be used to increase resistance of ash genotypes derived from nurseries thus giving them a high degree of tolerance during the critical first years of establishment. The application of priming in nurseries during seedling and sapling propagation could reduce the risk of out-planting losses and during the establishment phase in forests (Mitchell et al. 2012). Even if mortality does not occur, infections can cause

deformation that impairs production of high-quality timber and priming could be a way to reduce this risk. Induced defences in trees can be associated with reduced resource allocation costs compared with constitutive defences (Heil and Baldwin 2002; Bostock 2005; Walters and Heil, 2007; Bolton et al. 2009). Therefore, priming does not have significant impacts on tree fecundity (Mitchell et al. 2012; Swett and Gordon 2016). Induced resistance also does not involve the manipulation of plant genes. Therefore, there is no concern for genetic modifications as is often associated with genetically engineering for resistance (Eyles et al. 2010). Furthermore, in comparison to the production of resistant material or application of chemical pesticides, biological controls offer an alternative approach that could be more economically and environmentally viable (Eyles et al. 2010; Westman et al. 2019).

In this study, the potential efficacy of priming with a low virulent isolate of *H. fraxineus* is investigated in *F. excelsior* against a highly virulent isolate of *H. fraxineus,* the causal agent of ash dieback. The investigation falls within the framework of the "FraxForFuture" project (Langer et al. 2022). Two isolates of *H. fraxineus* were selected based on known virulence: one highly virulent strain and one with a low virulence. The phytotoxicity of these strains was evaluated, as well as their secondary metabolite production. Subsequently, the efficacy of priming with an isolate of *H. fraxineus* with low virulence as a method for reducing symptom development and mortality in saplings was assessed. Overall, this investigation aims to contribute to a better understanding of general principles regarding priming with a low virulent pathogen in trees. Furthermore, this investigation aims to improve current knowledge of additional strategies for managing and maintaining ash tree resistance despite the enduring threat of the ash dieback pathogen.

Methodology

Selection and laboratory evaluation of *H. fraxineus* strains

Isolation of *H. fraxineus*

Isolates of *H. fraxineus* were obtained from a mature common ash stand (*F. excelsior*) near Rhüden (Lower Saxony, Germany; N 51° 57' 32.2092 E 10° 9' 46.89), which was sampled as part of the FraxForFuture project (Langer et al. 2022; Peters et al. 2023). These isolates where obtained from branch samples with visible signs of a necrosis, typically associated with *H. fraxineus*. The identity of the isolates was confirmed morphologically and by PCR with species-specific primers for *H. fraxineus* Johansson et al. (2010). Subsequently, virulence was determined based on observed infection rate and severity of symptom development in a greenhouse virulence assay (Ridley et al. manuscript in preparation). Two strains were selected for further investigation: the most virulent strain (DSM 116307; Strain 7; FraxForFuture reference strain) and the least virulent strain (DSM 116306; Strain 13) (Table 1).

Fermentation and extraction of metabolites

H. fraxineus Strain 7 and *H. fraxineus* Strain 13 were grown on malt extract agar [20 g/L malt extract, 0.1 g/L yeast extract, 12 g/L agar (HiMedia, Mumbai, India) /L H_2O] in Petri dishes (9 cm in diameter). These cultures were incubated for four weeks at 23 °C in darkness. Four weeks later, cultures were cut into pieces and extracted twice with ethyl acetate for 30 min on a magnetic stirrer. After filtration, ethyl acetate was removed under reduced pressure at 40 °C to obtain the crude extracts. The crude extracts were subjected to analytical HPLC-UV/Vis-MS analyses.

Metabolomics

The crude extracts from Strain 7 and Strain 13 were dissolved to a concentration of 4.5 mg/mL in acetone methanol (1:1) before LC/MS analyses (Charria-Giron et al. 2023a). Then, electrospray ionisation mass (ESI-MS) spectra were recorded on an UltiMate 3000 Series uHPLC (Thermo Fischer Scientific, Waltman, MA, USA) using a C18 column (Acquity UPLC BEH 1.7 µm, 2.1 × 50 mm; Waters, Milford, MO, USA) with an injection volume of 2 µL, and connected to an amaZon speed ESI-Iontrap-MS (Bruker Daltonics, Bremen, Germany). The mobile phase consisted of A (H_2O + 0.1% formic acid) and B (ACN + 0.1% formic acid) with constant flow rate of 0.6 mL/min. The gradient started with 5% B for 0.5 min, increasing to 100% B in 20 min and holding at 100% B for 10 min. The temperature of the column was maintained at 40 °C and UV/Vis data were recorded with a DAD at 190–600 nm.

For metabolomic analyses, each sample was measured at a concentration of 450 µg/mL on an ultrahigh performance liquid chromatography system (Dionex Ultimate3000RS, Thermo Scientific, Dreieich, Germany), using a C18 column (Kinetex 1.7 µm, 2.1 × 150 mm, 100 Å; Phenomenex, Aschaffenburg, Germany) with an injection volume of 2 µL. The mobile phase consisted of A (H_2O + 0.1% formic acid) and B (ACN + 0.1% formic acid) with constant flow rate of 0.3 mL/min. The gradient started with 1% B for 0.5 min, increasing to 5% B in 1 min, and increasing to 100% B in 19 min and then holding at 100% B for 5 min. The temperature of the column was maintained at 40 °C and UV/Vis data were collected with a DAD at 190–600 nm. MS spectra were recorded using a trapped ion mobility quadrupole time-of-flight mass spectrometer (timsTOF Pro, Bruker Daltonics, Bremen, Germany) with the following settings: tims ramp time 100 ms, spectra rate 9.52 Hz, PASEF on, cycle time 320 ms, MS/MS scans 2, scan range (*m/z*, 100–1800 Da; $1/k_0$, 0.55–2.0 V·s/cm^2).

MS spectra were acquired in positive ion mode. Raw data were pre-processed with MetaboScape 2022 (Bruker Daltonics, Bremen, Germany) in the retention time range of 1.0 to 20 min (Charria-Girón et al. 2023b). The obtained features were dereplicated based on their accurate molecular weight and MS/MS spectra against the compounds as previously reported for *H. fraxineus* in the Natural Product Atlas (NP Atlas) database (van Santen et al. 2019). For this purpose, MetaboScape performed automatic in silico MS/MS matching based on the InChI-encoded structures using the MetFrag algorithm in the absence of MS/MS reference data (Ruttkies et al. 2016).

Leaf puncture assay

The phytotoxicity of Strain 7 and Strain 13 was determined using the leaf puncture assay on *F. excelsior*, as previously described (Andolfi et al. 2014; Evidente et al. 2014; Demir et al. 2023, Demir et al. 2024). The crude extracts were dissolved at 5 mg/mL in MeOH. Test samples (20 µL) were applied to the adaxial sides of previously needle-punctured leaves. Droplets (20 µL) of MeOH were applied to leaves as a control. No positive control was used. Each treatment was repeated three times and the leaves were then placed on moistened paper filters in Petri dishes (diameter 9 cm) to keep droplets from drying out. In total, six leaves were used to test crude extracts from two *H. fraxineus* strains. Symptoms were evaluated 5 days after droplet application, and ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to quantify the percentage necrotic leaf area caused by the extracts.

Greenhouse trial and evaluation of priming with *H. fraxineus* strains

Establishment

In summer 2022, an infection study was initiated using two-year-old ash saplings. The saplings were established from seed by FraxForFuture project partners at the Thünen Institute (Waldsieversdorf, Brandenburg, Germany). The seeds were collected in 2011 from a seed orchard with tree provenance classified as "81108" in Schorndorf (Baden-Württemberg, Germany). They were not selected for ash dieback tolerance. By 2013, the seed plantation was already heavily affected (Enderle et al. 2015). Seed was collected and germinated *in vitro* to produce multiple genotypes. In total, 70 clonal saplings of the F001_3 genotype were produced for use in this greenhouse trial. The susceptibility of these genotypes has not been tested, but it appears F001_3 is somewhat tolerant to *H. fraxineus* (Ridley et al. unpublished). Height of the saplings was measured from the soil surface at the start of the experiment.

The effect of priming was tested with the two strains of *H. fraxineus,* namely the low virulent strain of *H. fraxineus* Strain 13 and the highly virulent Strain 7 (Table 1). The infection study was established in the greenhouse (15 °C night - 20 °C daytime temperatures and natural light conditions). The study ran from 29.06.2022 to 28.09.2022. Strain 13 was used to prime saplings with artificial inoculations. Four treatments were established for this purpose; [1] saplings inoculated with the low virulent strain (Strain 13) alone (10 saplings); [2] dual-inoculated saplings, first with Strain 13 and subsequently with Strain 7 (40 saplings), [3] saplings inoculated with the highly virulent strain (Strain 7) alone (10 saplings), [4] 10 mock-inoculated saplings. The inoculations were conducted on the stem by creating one small, superficial cut down to the cambium in a proximal direction, approximately 1.0 cm in length, using a sterile scalpel. Then, a 5mm plug of fresh mycelial culture was placed into the wound between phloem and xylem, which was subsequently gently pressed and sealed with Parafilm. First, 50 saplings were inoculated with Strain 13. Strain 7 was inoculated three weeks later into 40 of these plants. It was decided to inoculate Strain 7 three weeks later based on the results described by Krokene et al. (2003). The highly virulent strain was inoculated approximately 2.0 cm above the original

inoculation site of Strain 13. This left 10 saplings inoculated with only Strain 13 as positive controls. Ten additional plants were inoculated as positive controls with Strain 7. The remaining 10 saplings were mock inoculated with sterile agar plugs using the same protocol to act as control saplings. The first five mock-inoculated control plants were established at the same time as the inoculations with Strain 13 and the remaining five mock-inoculated control plants were established three weeks later with the inoculations of Strain 7.

Evaluation

Following inoculations with *H. fraxineus,* the saplings were monitored once weekly for visible symptoms and severity of disease development. Disease severity was evaluated as the occurrence and expansion rate of necroses from the secondary infection sites with Strain 7, girdling of the main stem and wilting of leaves. The effects of priming on disease development were evaluated by assessing differences in the expressions of observable symptoms in plants with single inoculations (Strain 7 or Strain 13) versus those with the dual inoculations (Strain 13 and Strain 7). In total, saplings were monitored for a period of 18 weeks.

Following the monitoring period, all asymptomatic sapling stems were sampled and the inner stem was macroscopically evaluated for discolouration of the inner stem typically associated with *H. fraxineus* infection. DNA was extracted to further ascertain if the asymptomatic samples had been colonised by *H. fraxineus.* To check if the final of Koch's postulates was fulfilled, ten symptomatic samples from primed saplings were also taken to confirm colonisation by the pathogen, macroscopically and using molecular techniques. In addition, two symptomatic stem samples inoculated with only the highly virulent strain of *H. fraxineus* were assessed with molecular analyses. The stem samples were cut vertically along the phloem and small pieces were removed from the inner stem with sterile equipment. The stem samples were

taken from the inner stem beneath the inoculation site, as well as a couple of centimetres above and below the inoculation site. These were milled with steel beads to break up the tissue material before completing a DNA extraction using a DNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. PCR was conducted with species-specific primers following Johansson et al. (2010).

Data analysis

For metabolomic analysis, the annotated feature table was exported and analysed using the R packages-stats (version 4.3.1). The threshold for significant features was p-value < 0.05 and an absolute value of fold change > 1.0. For visualisation of data, the R package ggplot2 (version 3.4.4) was used.

For the greenhouse experiment, data analysis was conducted in RStudio, namely for calculations of growth rate and inhibition of symptom development. The inhibition of lesion expansion due to priming was calculated as 1 – average lesion length of primed saplings / average lesion length of unprimed saplings x 100. Kruskal-Wallis and Mann Witney U tests were conducted to assess differences in symptom development over the monitoring period and between the treatments. Spearman correlations were also conducted to access relationship between symptom development and saplings' height. Figures to visualise results were created using 'tidyverse' package in RStudio.

Table 1 *H. fraxineus* strains used for the study. Strain 7 is highly virulent and Strain 13 is low virulent. Strains have been deposited in the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures.

Strain ID	Species name	Source	Other studies
Strain 7 DSM: 116307 Accession form ID: 12213	*Hymenoscyphus fraxineus*	M. Ridley and V. Reckemeyer, Julius Kuehn Institute (01.03.2021) symptomatic ash trees near Rhüden, Germany (N 51° 57' 32.2092 E 10° 9' 46.89)	FraxForFuture reference strain Lutz at al. (2023) Callegari Ferrari et al. (2024)
Strain 13 DSM: 116306 Accession form ID: 12211	*Hymenoscyphus fraxineus*	M. Ridley and V. Reckemeyer, Julius Kuehn Institute (01.03.2021) symptomatic ash trees near Rhüden, Germany (N 51° 57' 32.2092 E 10° 9' 46.89)	Lutz at al. (2023)

Results

Laboratory evaluation of *H. fraxineus* strains

Metabolome comparison

After examination of the HPLC-UV/Vis chromatograms of crude extracts from both Strain 7 and Strain 13 of *H. fraxineus*, a diverse array of metabolites was observed. These included viridiol (**1**), viridin (**2**), 1-deoxy-2-demethylviridiol (**3**), 3-dihydrovirone (**4**), and hyfraxin A (**5**), with viridiol being the major metabolite for both strains (Fig. 1a and 1b). Subsequent analyses were conducted using UHPLC-DAD-IM-MS/MS to identify any significant differences in the metabolomes of Strain 7 and Strain 13 and to ascertain whether the production of these identified metabolites relates to their virulence.

After preprocessing, the resulting data set comprised 2912 features detected at the MS level and 2024 at the MS/MS level. Among these, 1918 were differentially produced (p-value < 0.05 and absolute Log_2 FC > 1) between Strain 7 and Strain 13 metabolomes (Fig. 1b, Table SI1). Interestingly, our analysis revealed that all viridiol-like molecules were significantly reduced in Strain 7 compared to Strain 13 and that hyfraxin A was produced in a similar level in both strains (not significantly changed). This suggests that these metabolites are not positively associated to virulence in their respective strains.

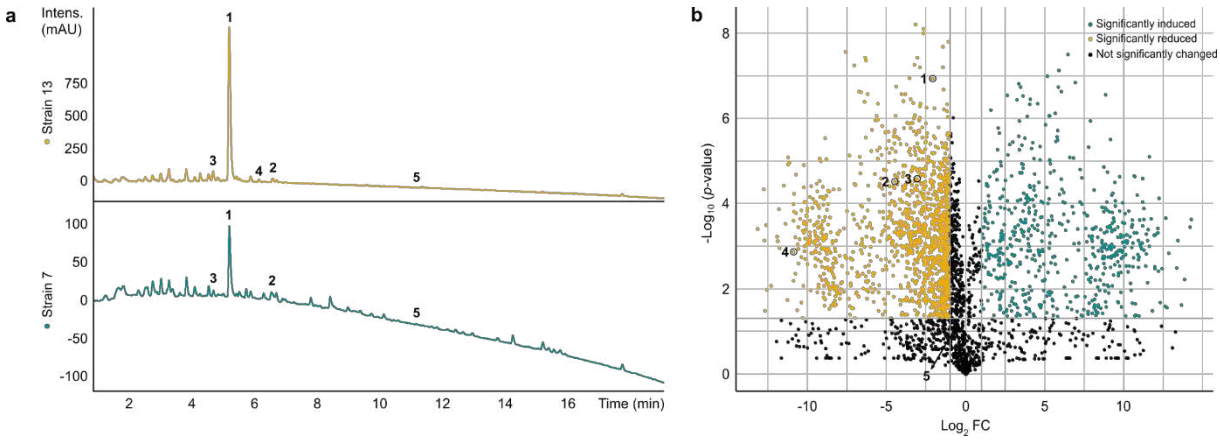
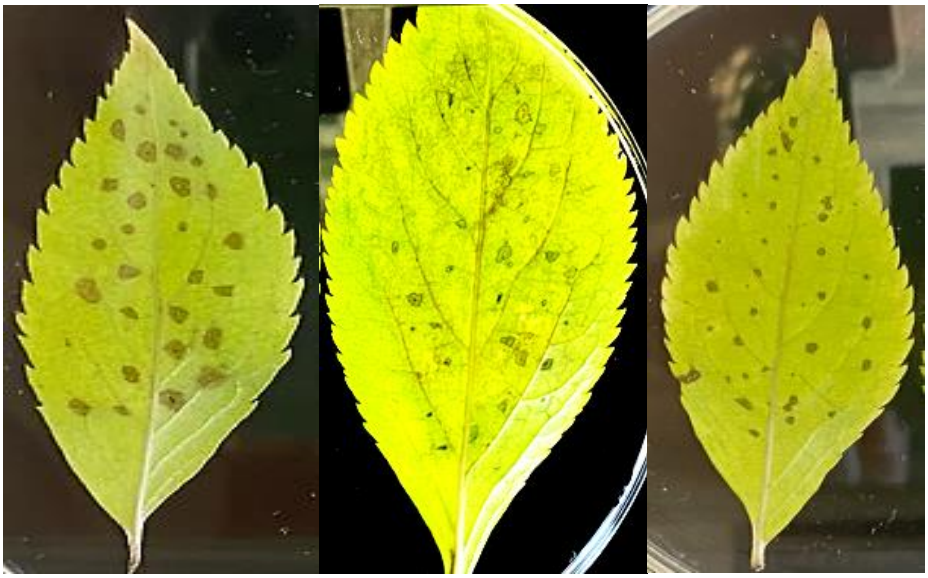


Fig. 1 (a) Comparison of the HPLC-UV/Vis chromatograms (210 nm) of the crude extracts obtained from Strain 7 and Strain 13 with peaks of dereplicated metabolites indicated by bold numbers. Dereplicated metabolites: viridiol (**1**), viridin (**2**), 1-deoxy-2-demethylviridiol (**3**), 3-dihydrovirone (**4**), and hyfraxin A (**5**). (b) Volcano plot of differentially produced metabolites from the crude extract of Strain 7 vs the crude extracts from Strain 13. Differentially produced metabolites were obtained by subtracting the log_2-mean abundance values of the highly virulent strain (Strain 7) from the log_2-mean abundance values of the low virulent strain (Strain 13). Thresholds for significantly induced or reduced metabolites depicted in light blue and orange respectively are a $-log_{10}$ p-value < 1.3 and an absolute value of log_2 FC > 1. Dereplicated metabolites except for hyfraxin A (not significantly changed) are circled, as they represent metabolites significantly reduced in Strain 7 when compared with Strain 13.

366

367 **Leaf puncture assay**

368 The crude extracts from both strains caused brown lesions on *F. excelsior* leaves (Fig. 2). The
369 leaf puncture assay indicated differences in the phytotoxic activity of extracts from Strain 7 and
370 Strain 13. Strain 7 exhibited higher phytotoxicity compared to Strain 13, causing the
371 development of larger leaf lesions, whereby the phytotoxicity of Strain 13 was barely more than
372 that of the solvent methanol (Fig. 2). As computed using ImageJ, 8% of leaf area was necrotic
373 after application of crude extract of Strain 7, but only 2.3% after application of that of Strain
374 13 and 2.1% with the solvent (Fig. SI2, Fig. SI3).



375

376 **Fig. 2** Leaves seven days after application of the crude extracts (from left to right; Strain 7,
377 Strain 13 and negative control).

378

379 **Greenhouse trial**

380

381 **Inoculation success**

382

383 **1. Unprimed saplings**

384 All saplings inoculated with Strain 7 alone (n=10) were successfully infected by *H. fraxineus,*
385 as determined by a necrosis developing on every inoculated stem during the monitoring period.
386 The cause of these necroses was confirmed as *H. fraxineus* with species-specific primers.

387 In comparison, none of the saplings inoculated with only Strain 13 (n=10) resulted in obvious
388 necroses on the outer stem. Further investigation of the inner stem of asymptomatic saplings
389 inoculated with Strain 13 revealed inner stem discolouration in three of the ten saplings, and
390 sampled wood material of four out of ten tested stems tested positive for *H. fraxineus* following
391 DNA extraction and PCR with species-specific primers. Therefore, based on the infection trial,
392 Strain 13 showed a lower colonisation success than Strain 7, and the virulence of Strain 13 was
393 confirmed to be lower than Strain 7.

394

395 **2. Primed saplings**

396 The primed saplings were inoculated twice, first with Strain 13 to prime the sapling and
397 subsequently with the highly virulent Strain 7. Inoculation success was evaluated at the end of
398 the experiment.

399 Following the monitoring period, 60% (n=24) of the primed saplings were randomly sampled.
400 Both inoculation sites were macroscopically evaluated for inner stem discolouration. Further
401 analysis of both inoculation sites was conducted for 10 randomly selected samples (25%) where
402 there were two distinct infection sites (Table 3). These samples were analysed using DNA
403 extraction and PCR with species-specific primers. From these ten samples assessed for
404 colonisation of the upper inoculation site by Strain 7, all tested positive for *H. fraxineus.* In
405 comparison, only 3 of the same 10 samples assessed for colonisation of the lower inoculation

406 site by Strain 13 tested positive for *H. fraxineus*. There were no observable differences in the
407 necrosis expansion of Strain 7 between the saplings that tested negative and those that tested
408 positive at the lower inoculation site.

409 In the nine cases where the inner stem discolouration from Strain 7 overlapped with the
410 inoculation site of Strain 13, only the upper inoculation site of Strain 7 was tested for
411 colonisation of *H. fraxineus* with molecular techniques. In each case, the tissue material tested
412 positive for colonisation with *H. fraxineus.*

413 There were five saplings that had been primed but that developed no visible symptoms within
414 the monitoring period. All five saplings were macroscopically assessed for inner stem necroses
415 and tested for *H. fraxineus* colonisation with species-specific primers. All samples were
416 associated with inner stem discolouration typically associated with infection by *H. fraxineus.*
417 However, *H. fraxineus* was only successfully identified in two out of five saplings with species-
418 specific primers. These two saplings were considered as successfully colonised by *H. fraxineus*
419 when analysed. No further analysis was conducted for saplings inoculated with Strain 13 alone.

420

421 **3. Control saplings**

422 The control saplings were all visibly negative for necrosis on the outer and discolouration of
423 the inner stem. DNA extraction and PCR with species-specific primers was conducted for each
424 control sapling. The wood material from all control saplings tested negative for *H. fraxineus*
425 with PCR using species-specific primers at the end of the experiment. It was concluded that the
426 control saplings were negative for *H. fraxineus.*

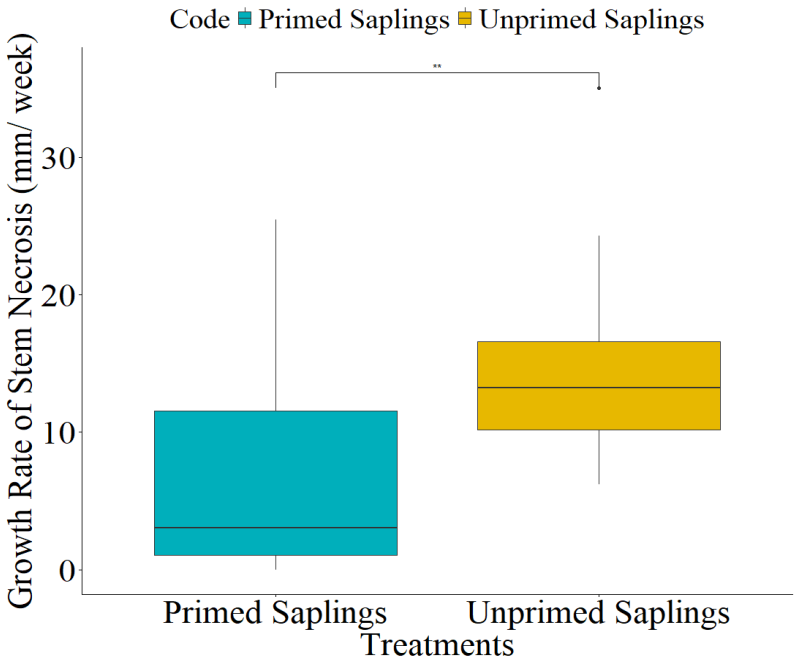
427

428 **Symptom development**

Strain 7 was inoculated into the saplings three weeks after Strain 13 and saplings were subsequently monitored for 14 weeks. The length of stem necroses that developed from inoculations with Strain 7 were measured weekly in primed and unprimed saplings. Regardless of priming, necrotic lesions were first observed four weeks after inoculation with Strain 7. The first observable symptom was a stem necrosis near the inoculation site. Furthermore, the necrotic lesions in primed saplings were smaller and the subsequent growth rate was lower in unprimed plants (Fig. 3, Fig. 4, Table 2). Girdling of the stem and wilting of the crown were observed for some of the saplings as a consequence of advanced symptom development (results below).

1. Growth rate of necrotic lesion

The active growth rate (mm/ week) of Strain 7 necroses was 15.41 mm ± 8.54 standard deviation per week in unprimed saplings (n=10) (Fig. 3). In comparison, the active growth rate (mm/ week) of Strain 7 necroses was 6.4 mm ± 6.9 standard deviation per week in primed saplings (n=40). The minimum value was 0 and the maximum is 25.4 mm. There was a significant difference between the active growth rate of Strain 7 necroses in unprimed saplings and Strain 7 in primed saplings (p-value = 0.002517), based on a Mann-Witney test (Fig. 3).



446

447 **Fig. 3** Mean active growth rate (mm/ week) of Strain 7 necrotic expansion in primed and
448 unprimed saplings, where statistical significance is indicated according to a Mann-Witney test
449 (p-value = 0.002517).

450

451 **2. Expansion of necrotic lesions**

452 The necrosis lesion length was consistently longer for Strain 7 in unprimed saplings compared
453 to the primed saplings over the monitoring period (Fig. 4). The expansion of necrosis length
454 during the monitoring period was not linear in either treatment (Fig. 4). The growth of Strain 7
455 in unprimed saplings (Adjusted R-squared = 0.7275, p-value = 0.03953) and the growth of
456 Strain 7 primed saplings (Adjusted R-squared = 0.8383, p-value = 0.01862) followed a diverse
457 pattern of increment over time based on the package 'growthrates', resembling a sigmoid
458 function.

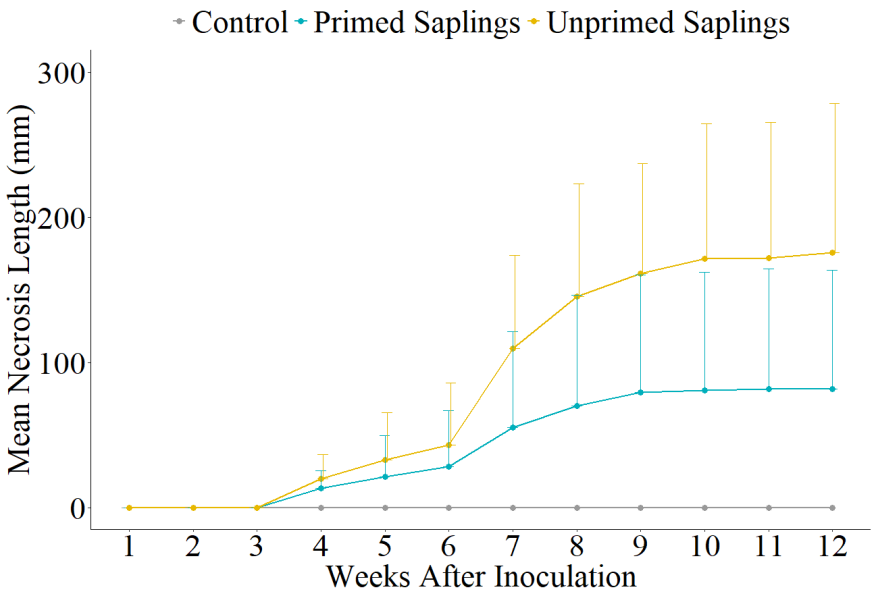


Fig. 4 The mean necrosis length (mm) of Strain 7 in primed and unprimed saplings over the monitoring period + standard deviation (SD).

There was high amount of variation between length of necrosis resulting from inoculation of Strain 7 in primed and unprimed treatments. Specifically, necrosis length varied significantly between primed and unprimed saplings from the seventh week of monitoring, based on a Mann-Witney test (Table 2).

3. Inhibition of *H. fraxineus* growth

Inhibition of necrosis lesion expansion can be used as an indicator that the growth of *H. fraxineus* is reduced. Necrotic lesion expansion associated with *H. fraxineus* was compared with primed and unprimed saplings. There was between 32% and 54% inhibition over the monitoring period (Table 2). Since no symptoms were observed before the fourth week, inhibition started at week 4 and increased with time during the monitoring period.

475 **4. Mortality of saplings**

476 Mortality was assigned to saplings with fully necrotic stems or saplings that had become girdled
477 during the monitoring period. Mortality was observed in primed and unprimed saplings (Table
478 3). However, there was no significant difference between the mortality in primed and unprimed
479 saplings inoculated with Strain 7 (p-value = 0.5502), based on a Pearson's Chi-squared test.

480

481 **5. Height of saplings**

482 The mean sapling height was 21.4 cm +/- 6.8 standard deviation at the beginning of the
483 greenhouse experiment (Table 3). Based on a Kruskal-Wallis Test and Dunn test (BH adj.),
484 there was no significant difference between treatments for sapling height at the beginning of
485 the experiment (p-value = 0.34). Based on a Spearman's correlation, there was also no
486 correlation found between necrosis length and sapling height (R = 0.074, p-value = 0.54).
487 Further analysis suggested starting height did not have a significant influence on the
488 development of girdling or wilting, based on a Kruskal-Wallis test with a Dunn post-hoc test
489 (p-value > 0.05).

490

491 **Table 2** Percent decrease in necrotic lesion length (cm) in primed in comparison to unprimed
492 saplings each week (W). No symptoms were observable prior to the fourth week of monitoring.
493 Significant differences between the necrosis length of Strain 7 in primed and unprimed saplings
494 are also provided, based on a Mann-Witney test. Non-significant (n.s.) results (p-value > 0.05)
495 are not shown.

	W 4	W 5	W 6	W 7	W 8	W 9	W 10	W 11	W 12
Decrease in necrotic lesion length	32%	35%	34%	50%	52%	51%	53%	53%	54%

Difference between primed and unprimed treatments (p-value < 0.05)	n.s.	n.s.	n.s.	0.021	0.008	0.010	0.007	0.008	0.008

Table 3 Overview of treatments, symptom development by final week of monitoring (week 14) and samples taken for further investigation. Control: mock inoculation, unprimed saplings: only inoculated with *H. fraxineus* Strain 7 or Strain 13, primed saplings: first inoculated with *H. fraxineus* Strain 13 and 21 days later with *H. fraxineus* Strain 7. Asymptomatic saplings were sampled for further analysis and colonisations for these saplings were confirmed by observations of inner stem discolouration and PCR analysis.

	Treatment			
	Control	Unprimed saplings – Strain 7	Unprimed saplings – Strain 13	Primed saplings
Number of saplings	10	10	10	40
Mean height of saplings (cm)	20.7	24.1	23.3	20.4
Percentage of saplings with a stem necroses	0%	100%	0	87.5%
Mean necrosis length (cm)	0.0	207.4	0.0	85.4
Percentage of dead saplings	0%	10%	0%	25%
Asymptomatic saplings sampled for further analysis	10	2	10	10
Percentage of confirmed colonisations (inner discolouration) in asymptomatic stems	N/a	100%	30%	100%
Percentage of confirmed colonisations (PCR) in asymptomatic stems	N/a	100%	40%	90%

Discussion

A primed state can be achieved through the artificial inoculation of saplings with a low virulent strain, thus decreasing symptom development in a subsequent challenge with a highly virulent strain (Conrath et al. 2006; Eyles et al. 2010; Pastor et al. 2013). In this study, smaller necroses

developed in primed saplings and the growth rate of necrotic lesions associated with a highly virulent *H. fraxineus* infection was significantly lower in the primed saplings compared to unprimed saplings. This corresponded with a mean of between a 32% and 54% reduction in lesion expansion on the stems, which increased consistently over 14 weeks of monitoring. The necrosis length continued to differ significantly in the final weeks of monitoring despite an increasing number of necrotic saplings. This result is similar to those in a previous study of Austrian pine (*Pinus nigra*), where there was a 42% reduction of symptoms associated with *S. sapinea* following priming with the same isolate (Eyles et al. 2007). Other studies have also observed statistically significant reductions in disease development in *Picea abies*, but with higher reductions of 63-90% in symptoms after priming with a sub-lethal dose of *Ceratocystis polonica* compared to a lethal dose (Krokene et al. 2003). Interestingly, the rate of inhibition in our study was observed increasing each week to be higher than the previous, despite increasing rates of mortality. Therefore, it is possible that the monitoring period in this present study was too short to fully evaluate the rate of symptom inhibition and efficacy of priming in *F. excelsior* with *H. fraxineus.*

Priming has also been successfully conducted using endophytic fungi and bacteria, both against fungal pathogens of trees and shorter-lived plants, including agricultural crops and the model plant species *Arabidopsis thaliana* (Pierterse et al. 2014). Priming with endophytes is analogous to employing low virulent pathogens, especially considering that many endophytes are latent pathogens (Schulz and Boyle 2005). For example, priming with fungal endophytes enhanced tolerance of *Populus alba* to the pathogen *Venturia tremulae* (Martinez-Arias et al. 2019) and in the field elm (*Ulmus minor*) to the pathogen *Ophiostoma-novo-ulmi* (Martinez-Arias et al. 2021). Similarly, the bacterial endophyte *Pseudomonas fluorescens* primed carnations against the pathogen *Fusarium oxysporum*, which was shown to induce systemic resistance (Pieterse et al. 2014). Another recent study evaluated the use of plant-associated beneficial bacteria for

533 increasing resistance against *H. fraxineus.* The results showed that the bacterial priming resulted
534 in a significantly higher concentration of plant defence related secondary metabolites in *F.*
535 *excelsior*, which is often linked to less plant stress (Striganaviciute et al. 2021).

536 The evaluated secondary metabolites produced by Strain 7 and Strain 13 indicate significant
537 differences in the metabolomic profiles between the strains, as shown by volcano plot analysis.
538 However, these differences may only reflect differences between strains of the same species.
539 *H. fraxineus* is known to produce a variety of different secondary metabolites, including several
540 furano-steroids (Andersson et al. 2010; Andersson et al. 2012; Andersson et al. 2013). Among
541 these metabolites, viridiol (Fig. 5) was identified in the crude extracts from both Strain 7 and
542 Strain 13 as the most prominent compound, whereby all viridiol-like molecules were
543 significantly reduced in the more virulent Strain 7 compared to Strain 13. Besides viridiol, other
544 furano-steroid congeners have been reported from *H. fraxineus* (Andersson et al. 2010;
545 Andersson et al. 2012; Andersson et al. 2013); of which viridin, 1-deoxy-2-demethylviridiol,
546 and 3-dihydrovirone (Fig. 5) were also detected in the crude extracts of these strains. Viridin,
547 the type compound of this class of furano-steroids, was initially discovered in 1945 as a strong
548 anti-fungal agent produced by *Trichoderma virens* (earlier described as *Trichoderma viride*/
549 *Gliocladium virens*) (Brian and McGowan 1945). This may explain the antifungal activity of
550 the *H. fraxineus* isolates observed in previous studies (*e.g.,* Halecker et al. 2020; Kowalski &
551 Bilanski 2021; Demir et al. 2023).

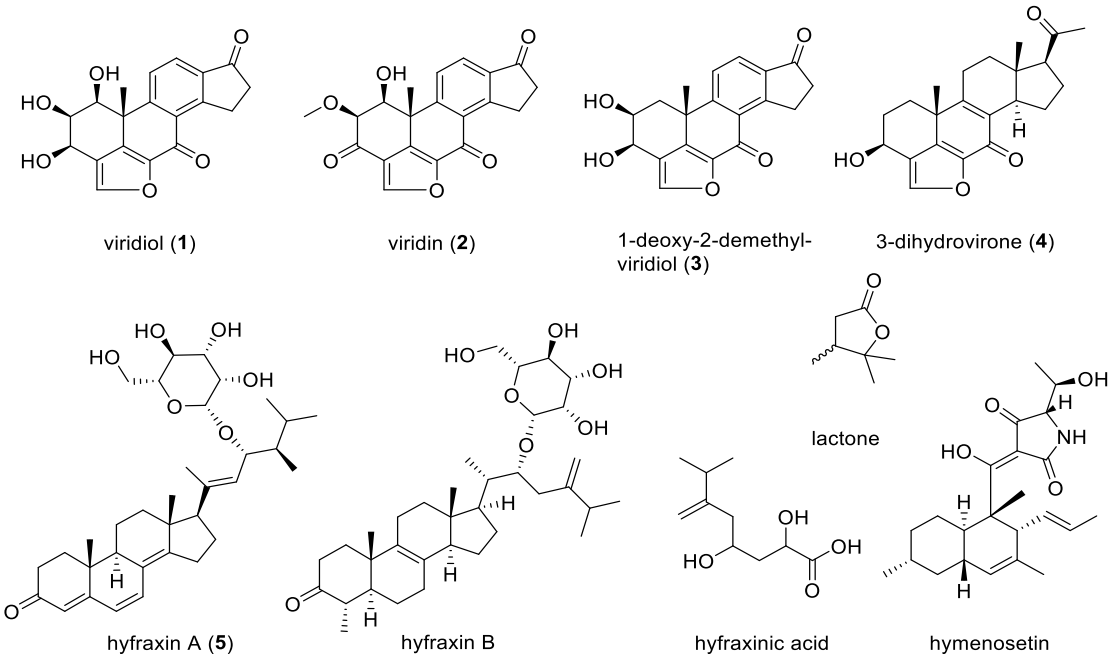


Fig. 5 Overview of the secondary metabolites known from *Hymenoscyphus fraxineus;* 1 – 5 were identified in this study. Hyfraxin B, hyfraxinic acid, the depicted lactone and hymenosetin were described by Citron et al. (2014), Halecker et al. (2014), Masi et al. (2019) and Surup et al. (2019).

Viridiol has been found to be phytotoxic not only to all tested genotypes of *F. excelsior* (Cleary et al. 2014), but also to *F. angustifolia* and other tree species (Masi et al. 2019). Nevertheless, as with Strains 7 and Strain 13, the concentration of viridiol, which is produced by both *H. fraxineus* and the closely related endophyte *H. albidus,* has been shown to not correlate with the virulence of the respective strains (Cleary et al. 2014; Junker et al. 2014). The phytotoxic lactone, (rac)-3,4-dimethylpentan-4-olide (Fig. 5), which is likewise found in both *H. fraxineus* and *H. albidus,* suggests that it is also not responsible for the virulence of *H. fraxineus* (Citron et al. 2014).

Masi et al. (2019) also found hyfraxinic acid, isolated in parallel to viridiol, to be a phytotoxic agent. However, our analyses using UHPLC-DAD-MS/MS could not detect the production of hyfraxinic acid either by Strain 7 or by Strain 13. Unlike viridiol-like molecules, hyfraxin A

568 was detected in similar levels in the crude extracts of Strain 7 and Strain 13. Since hyfraxins
569 were found to be active on eukaryotic cell lines, a phytotoxic activity seems probable, although
570 this has not yet been confirmed experimentally (Surup et al. 2018). Further studies are required
571 to uncover the biological role of this metabolite, as well as its potential link to the virulence of
572 this fungal pathogen. Herein, our results revealed significant differences in production between
573 Strain 7 and Strain 13. Since other metabolites of *H. fraxineus, e.g.,* the phytotoxic small
574 volatile lactone, (rac)-3,4-dimethylpentan-4-olide (Citron et al. 2014), and the antibiotic,
575 hymenosetin (Halecker et al. 2014), were not detected in the extracts of Strain 7 and Strain 13,
576 the metabolite spectrum of *H. fraxineus* is likely highly strain specific. The spectrum could also
577 vary considerably under different culture conditions and/ or methods of metabolite isolation
578 and extraction (Citron et al. 2014; Halecker et al. 2014). Albiducins, known from the sister
579 species *H. albidus* (Halecker et al. 2018), were also not detected. It is also possible that the
580 metabolite profile of strains *in vitro* may not be representative of the metabolite profile *in*
581 *planta*, and additional studies will be necessary to determine these differences.

582 Inoculations with fungal pathogens have been linked to enhanced production of secondary
583 metabolites and induced resistance of plants. While most studies on priming have focused on
584 evaluating the stimuli, attempts to elucidate molecular or cellular mechanisms that drive
585 priming have only recently begun receiving attention (Wallis et al. 2008; Conrath, 2011; Pastor
586 et al. 2013; Mageroy et al. 2020; Nair et al. 2022; Wilson et al. 2023). In a previous study it
587 was shown that the application of viridiol results in the production of secoiridoid and ABA-
588 related compounds by plants, proposed to have a role in the plant defences of Oleaceae and
589 resistance to pathogens (Cleary et al 2014; Nemesio-Gorriz 2020). More specifically, low
590 susceptibility in ash to *H. fraxineus* has been previously linked with having higher amounts of
591 secoiridoids and coumarins (Cleary et al 2014; Nemesio-Gorriz 2020). Therefore, the
592 metabolome of *F. excelsior* saplings following treatment with viridiol shows increases in the

593 production of putative defence compounds, which are involved in sapling resistance against *H.*
594 *fraxineus* (Cleary et al 2014). More recently, a study confirmed biotic stress can increase the
595 chlorophyll a/b ratio, caroteroid content, total phenol (TPC) and total flavonoid (TFC) in
596 seedlings (Striganavičiūtė et al. 2021). Increases in production of phenolic compounds and
597 secondary metabolites is often linked to enhanced resistance against biotic stress, such as
598 pathogen challenges (Wallis et al. 2008; Sherwood et al. 2013; Hu et al. 2021; Striganaviciute
599 et al. 2021). Whereas, we have elucidated structures of metabolites of *H. fraxineus* and found
600 that they may be involved in the phytotoxicity and antifungal activities of *H. fraxineus,* it is also
601 possible that these metabolites are involved in a molecular mechanism to induce priming. This
602 will have to be the subject of further investigations, as well as whether it is locally or
603 systemically upregulated.

604 The effect of priming with a low virulent strain was significant, despite considerable variation
605 (SD) in the length of necrosis. This variation was observed regardless of treatment. This
606 variation is considered normal because the growth characteristics of mycelium is known to vary
607 between cultures (Kowalski and Bartnik 2010; Kirisits et al. 2013; Botella et al. 2016).
608 Furthermore, previous studies have also observed considerable variation in necrosis expansion
609 from a single isolate (*e.g.,* Lygis et al. 2017). Nevertheless, studies have suggested the effects
610 of priming are density dependent, whereby induced resistance increases with inoculum load
611 (Christiansen 1985; Swett and Gordon 2016). Despite efforts to standardise the plugs of
612 inoculum, differences in inoculum concentrations could help explain the high rates of variation
613 in necrosis length development within each treatment. These discrepancies may also help
614 explain why some inoculated saplings tested negative for *H. fraxineus,* as inoculum plugs with
615 lower concentrations of inoculum may not have successfully colonised the saplings after
616 inoculation. It is likely for this reason that previous priming studies with *F. circinatum* have
617 used spores as inoculum to control for inoculum concentrations (*e.g.,* Bonello et al. 2001; Swett

618 and Gordon 2016). Unfortunately, there is until now no standard protocol for the mass
619 production of *H. fraxineus* spores for *in planta* trials.

620 To evaluate the efficacy of priming as a possible integrated disease control approach,
621 observations of reduced mortality compared to unprimed plants are as important as reduced
622 symptom development. The results of this study demonstrate that mortality was still possible
623 despite priming, but that rates of mortality did not differ significantly between primed and
624 unprimed saplings. Nevertheless, these results should be considered in the context that mortality
625 of infected European ash is understood to be highest in saplings and younger trees compared to
626 mature trees (Klesse et al. 2021). It has been proposed that these higher rates of mortality are
627 related to the smaller diameter of stems that increase susceptibility to stem girdling, which
628 results in the catastrophic disruption of the xylem (Madsen et al. 2021). Moreover, wound
629 inoculation at the stem is likely to lead to much higher rates of girdling and mortality than
630 natural infections. Priming in saplings of other tree species has, however, been shown to
631 decrease mortality. For example, Swett and Gordon (2016) observed that priming reduced the
632 incidences of mortality in *Pinus radiata* seedlings, however no further analysis was conducted
633 in that study. Therefore, further investigations with low virulent strains of *H. fraxineus* are
634 needed to more thoroughly examine the impact of priming on rates of mortality. Swett and
635 Gordon (2016) postulated that rate of mortality may also be dependent on the timing and
636 location of infection, suggesting that additional investigation into the impacts of different
637 inoculation protocols is also necessary to more accurately assess the potential for priming to
638 decrease sapling mortality.

639 Timing between application of priming and challenges is an important consideration in the
640 evaluation of priming. Priming can only be effective if the augmented plant defence response
641 can be expressed before the invading pathogen is able to overcome host plant defences (Ahmad
642 et al. 2010). Time between inoculations of *H. fraxineus* was based on previous priming studies

643 with *Pinus nigra* and *Picea abies* (Krokene et al. 2003; Eyles et al. 2007). Critically, previous
644 studies found that the effectiveness changes over time. For *Picea abies*, pretreated with
645 sublethal doses of *C. polonica*, protection decreased with time against lethal doses of *C.*
646 *polonica*, with pre-treatment 3-9 weeks before mass inoculation being found to be optimal
647 (Krokene et al. 2003). Furthermore, no significant difference was observed between conducting
648 pre-treatment at 3, 6 or 9 weeks before mass inoculation (Krokene et al. 2003). However, we
649 assume that the effect of timing varies strongly among pathosystems and suggest that future
650 trials investigate whether the priming effect is still observable with longer periods of time
651 between the pre-treatment and the inoculation with a highly virulent strain.

652 Evaluating different timings between pre-treatment and challenge by the pathogen will also
653 bring into question the durability of priming. As trees are perennial plants, it is proposed that
654 biological control measures should be effective for several years. The evaluation of inhibition
655 in this study was conducted for 14 weeks after inoculation with the pathogen. While the
656 observed rate of inhibition was still increasing at the end of the monitoring period, it was not
657 possible to continue the experiment beyond 14 weeks due to increasing mortality of saplings.
658 Moreover, it is not possible to discuss the long-term durability of priming over such a short
659 timeframe. One study with *Picea abies* and *C. polonica* still observed an intermediate reduction
660 in symptoms (44-71%) when pre-treatment with a sub-lethal dose of the pathogen was applied
661 52 weeks before mass inoculation (Krokene et al. 2003). For another study with *Picea abies*
662 and *H. parviporum,* the priming effect could still be observed after at least 78 weeks
663 (Swedjemark et al. 2007). Therefore, Krokene et al. (2003) and Swedjemark et al. (2007)
664 indicate that priming with the pathogen, or at least the resulting induced resistance *in planta*,
665 may persist for relatively long periods of time. Further studies will be necessary to determine
666 if priming of ash is durable and effective over time, if a single pre-treatment is adequate or if
667 multiple applications are required (Vallad and Goodman, 2004).

In the forest, ash trees are exposed to infections by multiple *H. fraxineus* individuals (Landolt et al. 2016). Some of those have low virulence and some have high (Landolt et al. 2016; Lygis et al. 2017). Hence one may argue that a potential priming effect of low virulent individuals would manifest anyway naturally in the forests. However, it is likely that such small infections by low virulent individuals are often restricted to the leaves (Gross et al. 2012). Host response in the leaves is different from xylem infections as applied in our study, which aims at developing a priming method to protect the xylem, not the leaves. Infections in the xylem are associated with significant damages, including loss of stem structure, dysfunction of the sapwood and development of root collar necroses, which reduce commercial value and cause public safety risks (Gross et al. 2014; Enderle et al. 2017; Langer et al. 2017).

Arguably, the trade-offs between augmented disease resistance and costs of activation involved in induced resistance must be considered (van Hulten et al. 2006). In this study no clear indications of trade-offs were recorded as a consequence of priming. In comparison, primed defences have been observed reducing radial sapwood growth in *P. radiata* that was primed with the same isolate of *S. sapinea* (Gould et al. 2008). However, in this example, the seedlings recovered and growth rate exceeded that of the control seedlings by the end of the experiment. Future assessments of priming should also consider possible reductions and recovery in growth, especially in deciduous species. In another study of priming in *Pinus radiata* against *F. circinatum*, recovery of saplings was observed, where the lesion length associated with *F. circinatum* seemingly decreased over the monitoring period (Bonello et al. 2001). No recovery of the inoculated saplings was observed in this study, but perhaps this is because the monitoring period in our study was too short to reveal such tendencies.

Another possible trade-off associated with priming may be increases in susceptibility to other pests or pathogens, such as *Armillaria* spp. (Blodgett et al. 2007; Bonello et al. 2008). In European ash, it is known that *Armillaria* spp. can interact with *H. fraxineus* at the root collar,

693 which can lead to increased tree mortality (Marcais et al. 2016; Heinzelmann et al. 2019;
694 Madsen et al. 2021). Interactions with other pathogens were not investigated in this study, but
695 future studies should consider the potential occurrence of systemic induced susceptibility (SIS)
696 as a consequence of priming. Previous studies have indicated interactions between fungal
697 pathogens, as well as pathogens and insect pests, can result in greater damages (Blodgett et al.
698 2007; Bonello et al. 2008; Puentes et al. 2021). For example, Bonello et al. (2008) found that
699 stem inoculations of *Pinus pinea* with *Heterobasidion annosum* resulted in *S. sapinea* causing
700 larger necrotic lesions in the shoots. Similarly, Blodgett et al. (2007) tested if inoculation of
701 *Pinus nigra* with *S. sapinea* results in SIR or SIS to subsequent infection by *S. sapinea*. The
702 study found that the expression of resistance can be organ-dependent, where inoculations at the
703 stem base resulted in SIS in shoot tips. Nevertheless, unlike constitutive resistance, inducible
704 resistance in most cases seem to outweigh the costs of infection with a pathogen as found in
705 agricultural examples and with the model organism *Arabidopsis* spp. (Heidel and Dong 2006;
706 Van Hulten et al. 2006).

707 One critical limitation of this study is the lack of a negative control, where saplings would be
708 mock inoculated prior to inoculation with the virulent pathogen to assess the impact of
709 wounding. SIR can also be triggered by wounding, but it has been less extensively studied
710 (Luchi et al. 2005; Chassot et al. 2008). Wounding leads to increased synthesis of defence
711 metabolites, for example (+)-catechin concentration increased in *Picea abies* after inoculation
712 with both fungal material and sterile malt agar plugs (Brignolas et al. 1995). However, the study
713 also found that this was higher after inoculation with fungal material and the increase was
714 localised to the site of inoculation (Brignolas et al. 1995). Nevertheless, mock induction
715 (wounding with sterile plugs of culture media) in *Pinus nigra* did not have any effects on
716 pathogen responses (Eyles et al. 2007). A similar result was found by Christiansen et al. (1999)
717 and Bonello and Blodgett (2003), where sterile inoculations did not induce significant plant

responses. In a previous study in which the causal agent of pitch canker disease (*F. circinatum*) was inoculated in *Pinus radiata* (Bonello et al. 2001), there was also no significant difference between the lesion length developing in trees with no history prior to inoculation and wounding of the trees prior to inoculation with the pathogen.

Inoculations with Strain 7 resulted in infections that were traceable with species-specific primers in almost all cases. However, only 3 out of ten inoculations with Strain 13 could be verified with species specific primers. One may argue that many infections with Strain 13 were not successful, and therefore Strain 13 cannot be the reason for the priming effect. The relative susceptibility of this genotype is not known, but this could provide a possible explanation for how the contrary may be true, *i.e.*, induced plant defence may have inhibited traceable infections by Strain 13 in most cases. As observed in this study, inoculations may not necessarily have to result in traceable infections in order to cause a priming effect.

This investigation was a small-scale study, composed of only two strains and clonal saplings of one genotype. Studies testing interaction with more strains are necessary. Furthermore, some variation with regard to plant age and genetic susceptibility of the plants is needed. This study also did not test if different environmental conditions can influence the efficacy of priming. Primed defences have been found to result in reduced plant growth, especially under low nutrient conditions (Bostock 2005; Walters and Heil, 2007; Madsen et al. 2021). Conversely, high fertility can also alter host susceptibility to pathogens (*e.g.,* Blodgett et al. 2005; Nybakken et al. 2018). However, some studies have indicated the positive effects of priming remain stable with fertility changes (Eyles et al. 2007). These environmental interactions must be further investigated with special consideration of variation in field conditions, including varying pathogen pressure. Sherwood and Bonello (2016) postulated that if enough damage is inflicted on the host, examples of SIR can result in SIS over time. The point at which systemic resistance can become systemic susceptibility as well as the mechanisms involved are not yet fully

743 understood. This may well be due to the fact that both the priming organism and the pathogen
744 are costly for the host because they require assimilates from the host, which under
745 environmentally stressful conditions could result in SIS instead of SIR. These interactions
746 should be carefully studied if priming should be recommended as a management option for
747 increasing resistance in saplings, especially when saplings can be exposed to varying
748 environmental conditions and pathogen pressures in the first years of establishment in the forest.

749 In conclusion, the two strains of *H. fraxineus* varied both in their secondary metabolite profiles
750 and in virulence, although the individual roles and interplay of the various phytotoxic
751 metabolites are not yet fully understood. The study also provides proof of concept for the
752 efficacy of priming to reduce symptom development associated with highly virulent *H.*
753 *fraxineus* infections. To the best of our knowledge, this is the first study to consider the efficacy
754 of priming with a pathogen to induce resistance against future pathogen infections in deciduous
755 tree species. Since no viable alternative species has been found that can fully replace the
756 ecological niche of *F. excelsior* or associated ecosystem services, minimising the losses of *F.*
757 *excelsior* saplings for use in replanting is critical. Subsequent studies should investigate the
758 most appropriate protocol for application of priming and how long the priming effect can
759 meaningfully reduce damages associated with pathogen infection. Another important
760 consideration should be determining any potentially negative effects, or trade-offs, as a
761 consequence of priming saplings, such as a reduction in sapling growth or increased
762 susceptibility to other pathogens (*e.g., Armillaria* spp.), as well as pests (*e.g., Agrilus*
763 *planipennis*). These trials can begin under greenhouse conditions, but must be also conducted
764 in field trials to confirm true efficacy as a management option. If priming can be further
765 investigated and better understood, it could become a recommended disease management
766 option applied during seedling propagation in nurseries to effectively reduce out-planting losses

and support establishment in the critical first years in the forest, especially in areas of high conservation value, without significant impacts on tree fecundity.

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Ethics declarations

Conflict of interest

The authors declare there are no conflicts of interest.

Ethical approval

The authors did not conduct any studies with human participants or animal for this publication.

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Supplementary information

Table SI1. Statistical analysis of dereplicated metabolites: viridiol (**1**), viridin (**2**), 1-deoxy-2-demethylviridiol (**3**), 3-dihydrovirone (**4**), and hyfraxin A (**5**). Status was defined as having *p*-value < 0.05 and absolute Log_2 FC >1.

Name	Strain 7 – Mean	Strain 13 – Mean	*p*-value	-log10 (*p*-value)	Fold change	Log_2FC	Status
1-Deoxyviridiol	114156.185	241965.813	< 0.001	4.627	0.472	-1.084	Reduced
Viridiol	3099127.333	13163933.000	< 0.001	6.937	0.235	-2.087	Reduced
1-Deoxy-2-demethylviridiol	72344.516	689059.563	< 0.001	4.526	0.105	-3.252	Reduced
Viridin	41438.219	574534.521	< 0.001	4.461	0.072	-3.793	Reduced
3-Dihydrovirone	36.343	57088.615	< 0.001	3.603	0.001	-10.617	Reduced
Hyfraxin A	77689.318	66273.589	0.04	1.355	1.172	0.229	No change

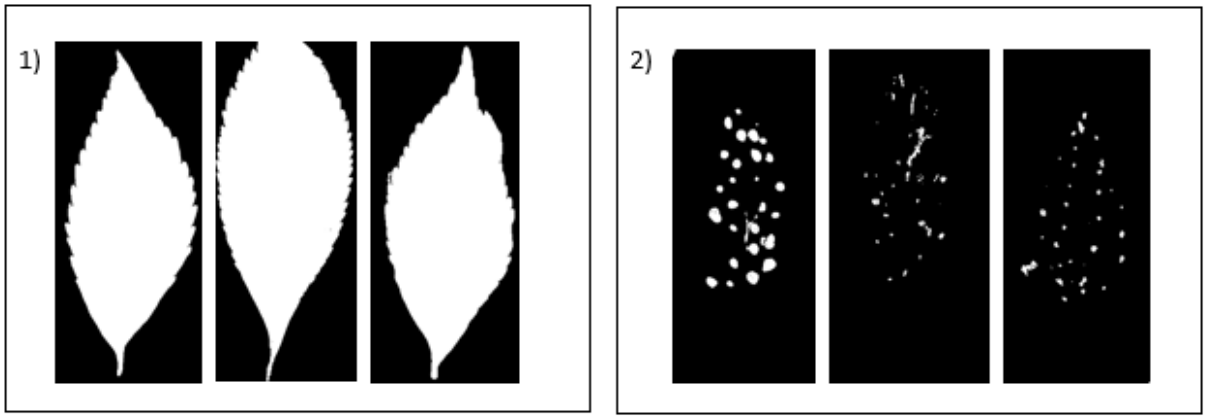

Fig. SI2. Measurement of the 1) total leaf area and 2) diseased area by using ImageJ (from left to right; Strain 7, Strain 13 and negative control).

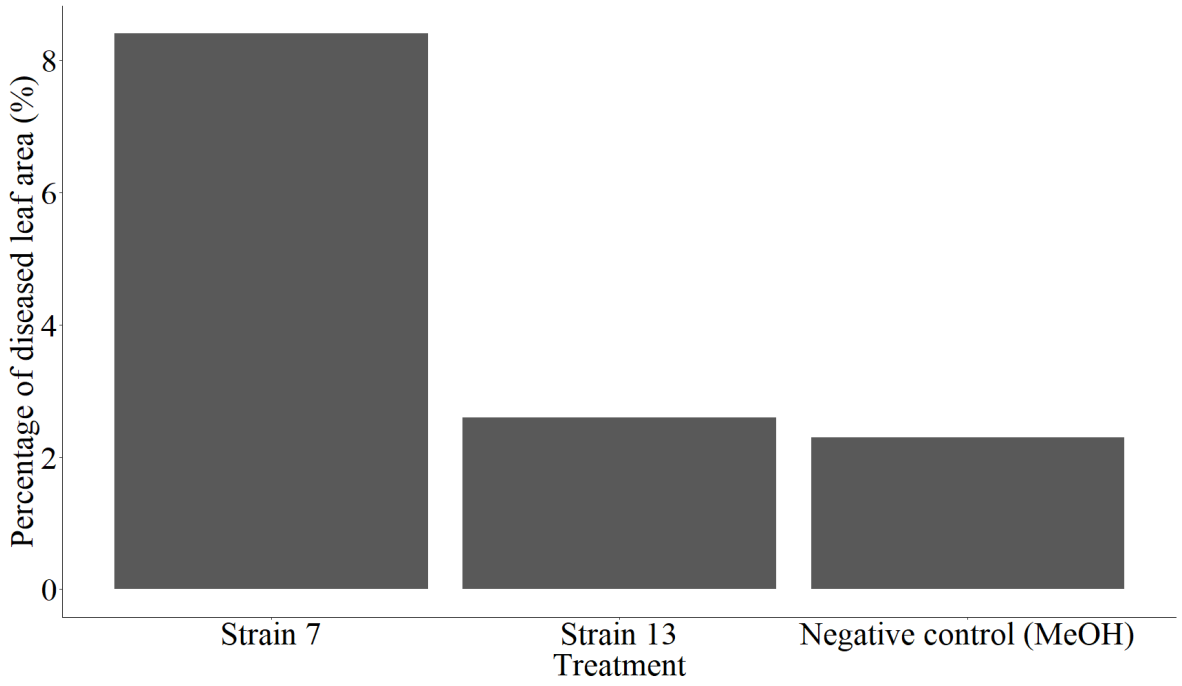

Fig. SI3. Percentage of diseased area/ total leaf area after application of crude extracts to ash leaves as measured by the ImageJ software.