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ORIGINAL ARTICLE

Inoculation of *Fraxinus excelsior* seedlings with bacterial strains to enhance the tolerance against ash dieback

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Abstract

Since ash dieback has been recognized as a major threat to Fraxinus excelsior, multiple lines of research have focused on potential management to mitigate this disease. One area of focus has been the microbiome of the trees and the application of biocontrol agents (BCAs). In this study, we aimed to find suitable BCAs that can help control *Hymenoscyphus fraxineus* and the resulting ash dieback. Therefore, we studied the health of ash seedlings under nursery conditions via inoculation with beneficial bacterial isolates and consortia to suppress the pathogen. By using offspring of two tolerant ash trees, we aimed to optimize this via synergistic effects. We found that *Schauerella fraxinea* B3P038 and *Luteimonas fraxinea* D4P002 lowered the symptoms of ash dieback. Using strain-specific qPCR assays, only the persistence of *Aureimonas altamirensis* C2P003 could be proven in the inoculated ash seedlings. Similarly, the fungal pathogen was also unable to infect the plants during the summer months. In addition, we were able to show that the inoculated bacterial strains influenced the microbiome, even without persisting on the plants. These results are encouraging, as positive effects can be taken from the greenhouse to the field and further studies should follow up on the B3P038, C2P003 and D4P002 strains. However, under field conditions, a strong variation in the effects must be considered. This strongly suggests that in addition to plant scoring, monitoring inoculation success as well as pathogen abundance is necessary.

Keywords Microbiome · Ash dieback · Biocontrol agents · Inoculation · Common ash

Introduction

Plant diseases caused by microorganisms are a major threat to agricultural and forest ecosystems and production (Wang et al. 2021). In addition to the interminable breeding of tolerant plants, the classic approach for controlling these diseases involves pesticides. The application of pesticides may lead to severe environmental problems by contaminating the environment with harmful xenobiotics. Furthermore, the application of pesticides in forest ecosystems is complicated and hardly accepted by the public due to their possible accumulation, ecosystem complexity and consequential damage to plants, animals and microorganisms that are not targeted (Kaczyński et al. 2021). The application of microorganisms as biocontrol agents (BCAs) can be a more sustainable way to suppress a pathogen and/or strengthen plant health (Raymaekers et al. 2020). New approaches are also focusing on the "optimization" of the plant microbiome and are leading to "microbiome engineering" (Syed Ab Rahman et al. 2018; Berg et al. 2020). The applied biocontrol agents are closely linked to their host metabolism (Jeyarajan and Nakkeeran 2000) and have positive effects on plant health (Syed Ab Rahman et al. 2018) as well as antagonistic effects against pathogens (Mitter et al. 2017; Müller and Behrendt 2021). Plant hormones secreted by BCAs can activate plants metabolically, increase their ability to defend themselves by activating the immune system (Rashid and Chung 2017; Romera et al. 2019; Vannier et al. 2019) and thus enable induced systemic resistance (ISR) (Pieterse et al. 2014). As another indirect effect, BCAs can lead to an alteration of the plant microbiome, causing an improvement in plant health (Berg et al. 2021). BCAs can also interact directly with pathogens as antagonists (Mavrodi et al. 2006; Ongena et al. 2007).

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Such interactions involve the production of secondary metabolites, mostly antibiotics (Moënne-Loccoz et al. 2015). Additionally, microorganisms (commensals and pathogens) compete for resources and space, which are limited. This can lead to a positive effect on the plant, as the pathogen might not be able to compete with the resident microorganisms; therefore, the residents develop colonization resistance (Zhang et al. 2022).

Host−pathogen interactions are influenced by several factors, including abiotic and biotic environmental factors, and can affect a host's attractiveness to certain pests (Brink 1962; Kearsley and Whitham 1989; MacDonald 2008; Stoffel and Bollschweiler 2008). A crucial biotic factor in host−pathogen interactions is interactions with antagonistic microorganisms. Their biocontrol activities, especially those of single members of the microbiome, have become more apparent in recent years and have positive effects on general plant vitality (Compant et al. 2019; Müller and Behrendt 2021; Syed Ab Rahman et al. 2018). For example, it is possible to control pests of tea plants by inoculating different strains of *Bacillus thuringiensis* subspecies (Unnamalai and Sekar 1995). Omoboye et al. (2019) reported the ability of different *Pseudomonas* strains to control rice blast disease caused by *Magnaporthe oryzae*. In other cases, it was possible to increase the stress tolerance of different plants by inoculating *Serratia plymuthica* MBSA-MJ1 (Nordstedt and Jones 2021). However, knowledge of plant vitality does not extend to whether the inoculated bacterial strain is the direct cause for increasing health, whether the strain is still persistent, or how abundant it is. Newer workflows allow the design of strain-specific primer−probe systems to assess the persistence of inoculated bacterial strains (Hernandez et al. 2020; Burghard et al. 2023).

The influence of pathogens and, in particular, their causes and consequences are highly important in forestry and science. Therefore, it is essential to understand their occurrence and control to minimize the sometimes severe impacts on forest ecosystems and trees. In this context, forestry is confronted with many diseases, e.g., *Phytophthora quercina*, which has caused a decline in *Quercus robur* (Jung et al. 2000), or Dutch elm disease caused by three *Ophiostoma* species, which has led to worldwide depletion of the *Ulmus* population (Brasier 1991; Harwood et al. 2011). As ash dieback has been identified as a major threat to *Fraxinus excelsior*, multiple research strategies have been pursued to treat this disease. One of the focuses was placed on the plant microbiome. Most related studies have focused on the fungal communities of leaves and shoots (Unterseher et al. 2007; Davydenko et al. 2013; Kosawang et al. 2018; Schlegel et al. 2018; Becker et al. 2020) and revealed seasonal, site-specific and vertical changes (Scholtysik et al. 2013; Cross et al. 2017; Haňáčková et al. 2017). Some fungal isolates showed in vitro the capacity for biological suppression through antifungal secondary metabolites and antagonistic abilities against *Hymenoscyphus fraxineus* (Junker 2013; Schulz et al. 2015; Schlegel et al. 2016; Haňáčková et al. 2017; Kosawang et al. 2018). Another focus was placed on the bacterial part of the ash microbiome, which revealed a relationship between the composition of the microbiome and the infection status of *H. fraxineus* (Griffiths et al. 2019; Ulrich et al. 2020). The microbiome of tolerant ash trees contains several taxa that are candidates for biocontrol. Some of these bacterial strains were already proven to have a positive effect on ash vitality under *H. fraxineus* infection in a greenhouse (Becker et al. 2022; Ulrich et al. 2022). To prove the resilience of ash trees under different environmental conditions, extended monitoring of the plants, including the persistence of the inoculated strains, can provide essential information.

In this study, we aim to find suitable BCAs that can help control *H. fraxineus* and the resulting ash dieback. Therefore, we wanted to improve the health of ash trees under nursery conditions by inoculation with beneficial bacterial isolates and consortia to increase resistance to the pathogen. By using tolerant ash genotypes, we aimed to optimize this via synergistic effects. In addition to scoring the plants and checking the establishment of selected inoculation strains, infection with *H. fraxineus* was quantified and the effect on the ash microbiome was analyzed.

Methods

Inoculation and sampling

For the pot experiment under nursery conditions, seven bacterial strains that were found to be good candidates for the biological control of ash dieback in previous studies were selected (Ulrich et al. 2020, 2022; Becker et al. 2022; Behrendt et al. 2024). *Pantoea vagans* A3K039, *Bacillus veleszensis* A4P130, *Pseudomonas coleopterorum* B2K013 and *Schaurella fraxinea* B3P038 had antagonist activity against *H. fraxineus* in vitro (Ulrich et al. 2020; Behrendt et al. 2024), whereas *Aureimonas altamirensis* C2P003, *Pseudomonas flavescens* D4P037 and *Luteimonas fraxinea* D4P002 have the potential to establish colonization resistance (Ulrich et al. 2020, 2022; Becker et al. 2022). In one consortium, the fungal strain *Papiliotrema flavescens* A3P071 was used, which had a higher abundance in tolerant trees as well as some antagonistic potential (Becker et al. 2020). The compositions of the four consortia are displayed in Table 1.

The bacterial isolates were subsequently grown in R2 broth (Merck, Germany) for two days at 22 °C. The liquid cultures were centrifuged at 5000 × g for 5 min and washed with ¼-strength Ringer's solution (VWR International, Germany), and the pellet was finally resuspended in ¼-strength

Table 1 Infestation of ash seedlings derived from two tolerant ash genotypes 14 weeks after inoculation with potentially antagonistic isolates

Inoculation strain/consortia (mix of isolates)		Proportion of damaged plants (%)**	
		Neu1	Res3
Pantoea "vagans" A3K039		41.7 (±16.7)	36.7 (±7.5)
Schauerella fraxinea B3P038		21.7 (±11.2) *	11.7 (±9.5) *
Luteimonas fraxinea D4P002		26.7 (±12.4)	16.7 (±5.9) *
Bacillus velezensis A4P130		41.7 (±27.0)	30.0 (±13.9)
Pseudomonas "coleopterorum" B2K013		38.3 (±15.1)	30.0 (±9.5)
Aureimonas altamirensis C2P003		26.7 (±13.7)	21.7 (±12.6)
Mix 1	D4P002 + C2P003	25.0 (±5.9)	25.0 (±5.9)
Mix 2	D4P002 + C2P003 + *P. flavescens* D4P037 + B3P038	28.3 (±16.2)	35.0 (±18.1)
Mix 3	*L. fraxinea* C4P040a + C2P003 + D4P037 + A4P130 + B2K013	43.3 (±10.9)	25.0 (±13.2)
Mix 4	D4P002 + C2P003 + D4P037 + A4P130 + *Papiliotrema flavescens* A3P071	43.3 (±13.7)	33.3 (±19.5)
Control		60.0 (±22.4)	40.0 (±10.9)

*Significantly different from the control, Kruskal−Wallis test and Dunn's test ($p < 0.05/2$)

**Mean values and standard deviations based on five replicates are shown

Ringer's solution. The cell density of each bacterial suspension was adjusted to 10^8 cells/ml. At the beginning of June 2021, seedlings from two tolerant ash trees (Neu1, Res3) were inoculated at the age of two years with six bacterial isolates and four consortia. For each treatment ($n = 22$), five plant containers (replicates) were planted with in total 60 seedlings. The mother trees of the progenies were selected according to their good vitality in comparison with their severely damaged neighbors. The stand where both trees grow has been heavily infected by ash dieback for 15 years. The trees have been monitored for several years as part of the joint project ResEsche (Past et al. 2021). Both trees are situated in the Pennin forest range in the Schuenhagen forest district in Mecklenburg-Vorpommern (N 54°25', E 13°01'). For inoculation, whole seedlings were dipped for 30 min into the prepared bacterial suspension, and control plants were immersed in the same way in ¼-strength Ringer's solution. Afterward, they were planted into QuickPot™ trays (QP 12 T/18) on seedling substrate (Klasmann-Deilmann, Germany), watered and kept outdoors in the nursery. High *H. fraxineus* infection pressure was simulated with petioles collected from infected ash stands in spring, which were placed between the seedlings 14 days after inoculation. The pot trial was performed at the Thünen-Institute of Forest Genetics in Waldsieversdorf. At 14 weeks after inoculation, the plants were assessed and samples from the compound leaves (two grams) of each plant were taken. Samples from 12 plants (one QuickPot) were mixed to obtain five replicates for each treatment. All the samples were stored at − 20 °C until further processing.

Before the samples were taken, all the plants were assessed. The compound leaves, including the petioles, were used for scoring. For each plant, the number of leaf spots and necrotic lesions were determined. Owing to the age of the seedlings and their resulting small size, plants were simply counted as damaged if more than approximately 10% of the leaf area showed symptoms that are associated to *H. fraxineus* infection. Twelve plants (one QuickPot) were averaged to achieve five replicates per treatment.

DNA extraction and qPCR

For DNA extraction, the plant material was ground in liquid nitrogen. An aliquot of the frozen samples (100 mg) was disrupted again in an MP Fast Prep 24 mill at 5 m*s-1 for 45 s, and total DNA was extracted via the DNeasy Plant Mini Kit (Qiagen, Germany). Deviating from the standard protocol, the incubation step at 65 °C was prolonged for 10 min. The purified DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

For strain-specific detection of the inoculated bacteria, diluted DNA samples were subjected to qPCR as described previously by Burghard et al. (2023). Briefly, Luna® Universal Probe qPCR Master Mix (New England Biolabs, Germany) was used on an ABI Prism 7500 fast thermal cycler (Thermo Fisher Scientific, Germany). Standard curves were obtained by serial dilutions of genomic DNA from A4P130, B3P038, C2P003 and D4P002. In addition to strain-specific detection, amplification capability of all DNA samples was tested via an assay with bacterial-specific 16S rRNA primers. The primer and probe sequences as well as the assay conditions for the D4P002, C2P003 and B3P038 strains were obtained from Burghard et al. (2023) and Behrendt et al. (2024), respectively. For the detection of *H. fraxineus,*

the primers and probes described by Ioos et al. (2009) were used, and the assay conditions of Nielsen et al. (2022) were used. Standard curves were generated from sixfold serial dilutions of plasmid DNA carrying the ribosomal ITS region from the *H. fraxineus* P3 isolate.

Owing to the large number of highly similar genomes in the family *Bacillaceae*, the pipeline used to design the qPCR detection system for the A4P130 strain was modified from Burghard et al. (2023) as follows: fastANI v1.3 (Jain et al. 2018) was used to identify the closest reference genome. A4P130 was split into fragments of length 500 bp with a sliding window of 100 bp via seqkit (Shen et al. 2016) and mapped to the closest reference via Bowtie2 (Langmead and Salzberg 2012) to identify unmapped or mismatched fragments as candidate sequences. After initial filtering of candidates via nucleotide BLAST, we extracted the final candidates by performing a multiple sequence alignment of 4385 genomic sequences from the family *Bacillaceae* via MUSCLE (Edgar 2004) and extracting unique subsequences via an in-house python script. The primer and probe sequences used were designed as described previously (Burghard et al. 2023). Compared with those of the three other strains, the specificity of the qPCR system of this strain was also tested via qPCR with an extended set of bacterial strains that were found as part of the ash microbiome (Ulrich et al. 2020) and type strains of various *Bacillus* species and different *B. veleszensis* strains. Only the *B. veleszensis* strains were detectable, which made this assay species specific.

Microbiome analysis

For amplicon sequencing, PCR amplification of the same DNA samples was performed as described by Ulrich et al. (2020) with bacterial primers 799F and 1115R which exclude the chloroplast and mitochondrial DNA of the host plant. The fungal part of the microbiome was amplified with the fITS7 and ITS4 primers following the protocol of Becker et al. (2020). Library preparation and 300-bp paired-end sequencing on an Illumina MiSeq platform were performed at LGC Genomics (Berlin, Germany). The raw sequence data were processed with the DADA2 R package v.1.22 (Callahan et al. 2017). The algorithm was applied for quality filtering, denoising and removing chimeric sequences. The amplicon sequence variants (ASVs) were taxonomically assigned via the naive Bayesian classifier method via the RDP reference database training set 18 or the UNITE ITS database v.9.0 (2023-07-25). In addition, the assignment was completed at the species level if the ASVs exactly matched the sequence of the reference strain of only one species. After removing residual plant DNA sequences from the dataset, 14.602.794 bacterial and 14.379.336 fungal high-quality reads were obtained. Statistical analyses were performed via the phyloseq, vegan, ape, dplyr, indicspecies and ggplot2 packages with *R* 4.2.2, (R Core Team 2023) as well as MicrobiomeAnalyst (Lu et al. 2023). All the samples were rarefied to the minimum number of sequences among all the samples. Diversity indices, such as richness, Chao 1, Shannon and Simpson, were calculated. The community structure was compared via principal coordinate analysis (PCoA) on the basis of a Bray−Curtis distance matrix of the ASVs. Significant differences between the bacterial and fungal communities were first tested via a two-factorial design via permutational multivariate analysis of variance (PERMANOVA) and subsequently via pairwise comparisons applying analysis of similarity (ANOSIM). The associations between the ASV patterns and the combination of treatments were analyzed via multilevel pattern analysis (multipatt) with correlation indices to determine the ecological preferences of the ASVs (indicator species) among a set of group combinations. Statistical significance was set at $P < 0.01$, and only ASVs that presented at least 0.1% abundance in the community were considered significant. To identify differentially abundant genera, the linear discriminant analysis effect size (LEfSe) method was used as provided by MicrobiomeAnalyst. This included the Kruskal−Wallis test to detect features with significant differences across the groups, followed by pairwise Wilcoxon tests. Finally, linear discriminant analysis (LDA) was applied to estimate the effect size of each feature. Significant genera (FDR < 0.05) with an LDA score greater than 2 were considered most likely to explain differences between inoculation treatments.

Statistical analysis

For the statistical analysis of the plant assessments and infection with *H. fraxineus,* the data were first tested for normal distribution via the Shapiro−Wilk test (Shapiro and Wilk 1965). As a result, we performed the nonparametric Kruskal−Wallis test for the plant assessments (Kruskal and Wallis 1952). As a post hoc test, Dunn's test of multiple comparisons using rank sums (Dunn 1964) was used. The *H. fraxineus* data were log-transformed to meet the assumptions of normality and homogeneity of variance. An ANOVA was performed on the linear model to assess the overall significance (Gelman 2005). As a post hoc test, the Tukey test was chosen (Keselman and Rogan 1977). Statistical significance was set at $P < 0.05$. The association between samples was tested using Kendall's nonparametric correlation coefficient. All tests were performed in *R* via the rcompanion and FSA packages, and ggplot2 was used for plotting.

Results

Plant assessments

Six bacterial strains and four consortia were applied for inoculation in a pot experiment under nursery conditions

with progenies of two different ash genotypes. To stimulate *H. fraxineus* infection, the seedlings were exposed to ascospores of *H. fraxineus* produced by apothecia formed in previous year ash leaf petioles that were placed on the ground next to the seedlings (Queloz et al. 2011) two weeks after inoculation. These petioles formed strong fruiting bodies some days after application. However, the leaf spots on the plants, representing the first symptoms, could be obtained only in September, such as leaves with dark patches and withered leaves. In symptomatic plants of both tree progenies, approximately 10 to 25% of the leaf area was infested. However, the infestation differed between the two plant genotypes. In most inoculations, Res3 showed a lower proportion of damaged plants. The relatively high variance could be due to the use of half-sib families (Table 1). There was a trend toward improved health status as a result of inoculation across all the treatments. A significantly lower proportion of damaged plants than the control was found for the *S. fraxinea* B3P038 treatment in both plus-tree progenies and for *L. fraxinea* D4P002 in Res3.

qPCR quantification

In addition, infection was quantitatively determined 14 weeks after inoculation via a qPCR assay specific for *H. fraxineus*. However, this approach does not differentiate between propagules only residing on leaf surface and those already penetrated the leaf tissues. As shown in Fig. 1, an infection was detected in all the treatment groups. The samples strongly varied, from 9.5×10^2 to 7.8×10^6 *H. fraxineus* ITS rRNA copies/100 mg. Sporadically, high values of 1.4 to 7.8×10^6 copies per 100 mg of leaf material were observed. The inoculation treatments B2K013 in Neu1 and A3K039 in Res3 resulted in the most severe infection, with 1.8×10^6 and 2.9×10^5 copies/100 mg. C2P003 presented the lowest infection rate (4×10^4 copies/100 mg) in Neu1, whereas D4P002 presented the lowest infection rate in Res3 (9.7×10^3 copies/100 mg). Significant influences were detected in the ANOVA of the inoculation treatments ($p=0.0189$) and of the plus-tree progenies ($p<0.001$). No significant interaction between inoculation and progenies was found. A post hoc test revealed no significantly lower infection in inoculated plants than in the control plants. In Neu1, the B3P038, C2P003, D4P002 strains and Mixes 1–3 presented lower

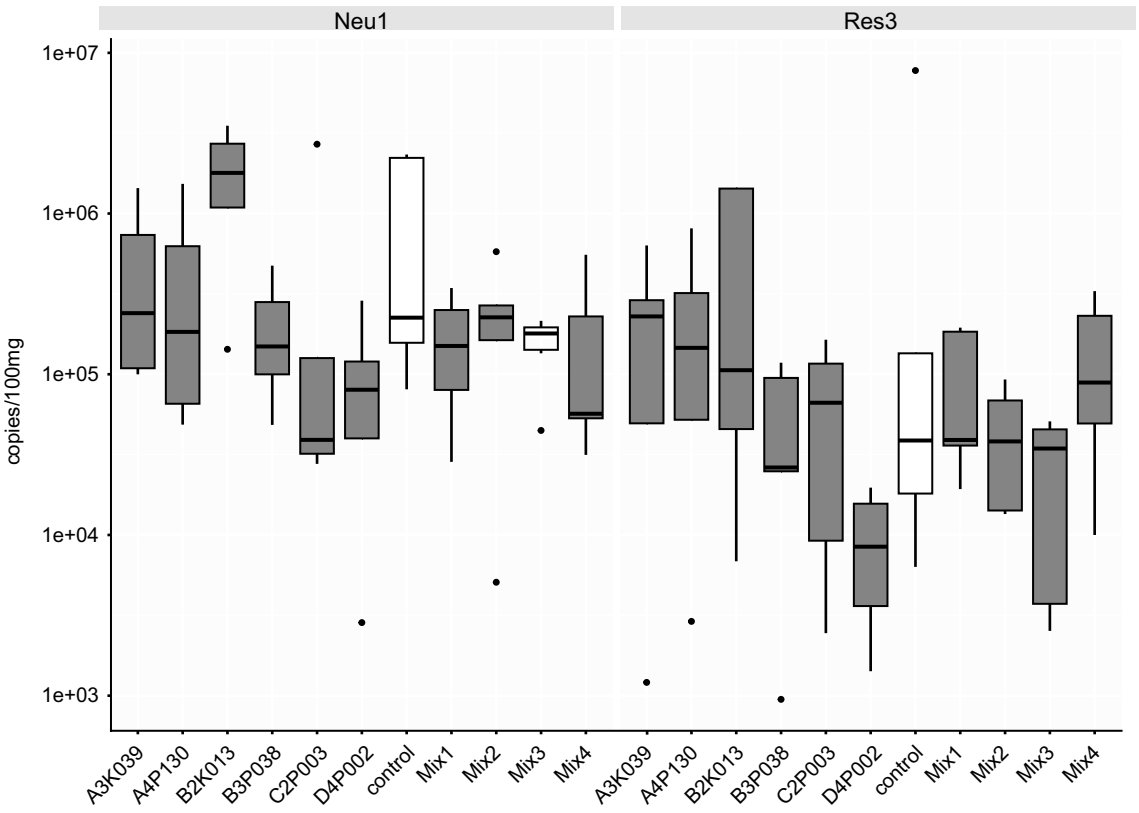

Fig. 1 Abundance of the pathogenic fungus H. fraxineus on or in the leaves of ash seedlings 14 weeks after inoculation. Quantification was performed via qPCR and represents the number of copies of the ITS rRNA region per 100 mg of leaf material. The taxonomic assignment of the strains and the composition of the consortia are shown in Table 1

infection rates, but not significantly. For Res3, a similar trend was observed for D4P002. *Hymenoscyphus fraxineus* infection was significantly lower in the Res3 ash progeny than in Neu1.

The inoculated strains were also quantified 14 weeks after inoculation by specific qPCR assays using a single copy genome region of the respective strains. A4P130 and D4P002 were no longer detectable. The strain C2P003 could be detected in both progenies close to the detection limit. The copy number in Neu1 plants ranged from 1.3×10^3 to 4.3×10^4 copies/100 mg. The strain C2P003 was found in the plants inoculated with only C2P003 alone as well as Mix2 or Mix3. In Res3, the copy number ranged from 1.9×10^3 to 1.6×10^4 copies/100 mg, and the strain C2P003 was observed in the plants inoculated with C2P003, B3P038, Mix1, Mix2 and Mix3. The strain B3P038 was found in plants inoculated with B3P038 at 1.3×10^3 copies/100 mg to 3.7×10^3 copies/100 mg in Res3.

Microbiome analysis

The analysis of the bacterial microbiome of the ash leaves was conducted via a combined approach with epiphytic and endophytic bacteria. The bacterial microbiome consisted of four main phyla. On average, *Pseudomonadota* (syn. *Proteobacteria*) accounted for 66.8% of the microbiome (Fig. S1). This was followed by the phyla *Actinobacteria* with 26.0%, *Bacteroidetes* with 3.9% and *Deinococcus-Thermus* with 3.2%. All other phyla had a proportion of less than 0.1%. At the genus level, *Methylobacterium*, *Sphingomonas* and *Klenkia* had the greatest proportions (Fig. 2). These bacteria dominated the microbiome, accounting for an average of 65% of the total bacteria. Fourth, the genus *Aureimonas* was present in an average proportion of 4.4%. At the genus level, differences between the treatments were apparent. However, *Pseudokineococcus* was the only genus that explains differences between the inoculation treatments (significant with LDA score > 2).

No clear differences were found in the α diversity between the treatments (Table S1). For the analysis of β diversity, a PERMANOVA was conducted first. These findings revealed that both the plant genotype and the inoculation had a significant influence on the bacterial microbiome (Table 2).

Overall, only 14% of the variability in the bacterial microbiome was explained by plant genotype and inoculation. However, inoculation had a significantly greater influence than did the plant genotype. The interaction effect of plant genotype * inoculation was not significant. For a better overview, the inoculation influence was nevertheless examined separately for both plus-tree progenies.

The treatment-specific differences in the structure of the bacterial microbiome for the Neu1 ash progeny are shown in Fig. 3 (left). In the ordination plot, only approximately 20% of the variability is shown by the first two axes, but it still clearly distinguishes the control from the inoculation treatments: *S. fraxinea* B3P038, *A. altamirensis* C2P003, *L. fraxinea* D4P002, Mix1 and Mix3. Accordingly, the statistical analysis via ANOSIM also revealed significant differences in these inoculation treatments (B3P038: $R = 0.36$, $p = 0.008$; C2P003: $R = 0.38$, $p = 0.009$; D4P002: $R = 0.45$, $p = 0.007$; Mix1: $R = 0.42$, $p = 0.012$; and Mix3: $R = 0.32$, $p = 0.009$). Despite partial overlap with the control in the ordination plot, the B2K013 and Mix2 treatments were also significantly different from the control ($R = 0.32$, $p = 0.01$ and $R = 0.38$, $p = 0.007$).

The composition of the microbiomes of the inoculated ash seedlings was correlated with the strength (abundance) of *H. fraxineus* infection. The direction of *H. fraxineus* abundance corresponded with the separation of the control from the inoculated treatments (along Axis 1).

The microbiome of the plus-tree Res3 progeny had only minor treatment-specific differences in community structure. No clear differences were recognizable in the ordination plot (Fig. 3 right). However, the statistical analysis likewise revealed significant differences between the two inoculation treatments and the control. This was again B3P038 ($R = 0.27$, $p = 0.01$), as well as Mix1 ($R = 0.44$, $p = 0.008$).

Analysis of the bacterial ASVs with an ecological preference for a specific inoculation variant did not reveal any taxa relevant to the Res3 progeny (abundance > 0.1%, $p < 0.01$). In the microbiome of Neu1, one ASV was found to be an indicator species for plants inoculated with B3P038. This ASV was assigned to the genus *Pseudomonas* and accounted for 0.3% of the bacterial microbiome of the B3P038-inoculated plants.

The samples were also used to examine the fungal microbiome. The analysis was based on the fungal ITS rRNA region. The reads were almost completely assigned to the fungal phyla *Ascomycota* (50.1%) and *Basidiomycota* (40.6%). The choice of fungal primers almost completely excluded the host plant DNA from the analysis. However, 0.12% of the reads were represented by the phylum *Chlorophyta*. As typical members of the plant microbiome, they were not excluded from the dataset. Approximately 9.2% of the fungal sequences were not classifiable at the phylum level according to the current UNITE database.

At the genus level, the composition of the mycobiome was highly diverse. The genera *Dioszegia*, *Cladosporium* and *Vishniacozyma* had the highest relative abundances (Fig. 4). On average, they accounted for 35% of the mycobiome. Differences between the treatments were detected, revealing the four genera *Fusicladium,* inc. sed. *Protomycetaceae, Leucosporidium* and *Didymella* that explain differences between the inoculation treatments (significant with LDA score > 2).

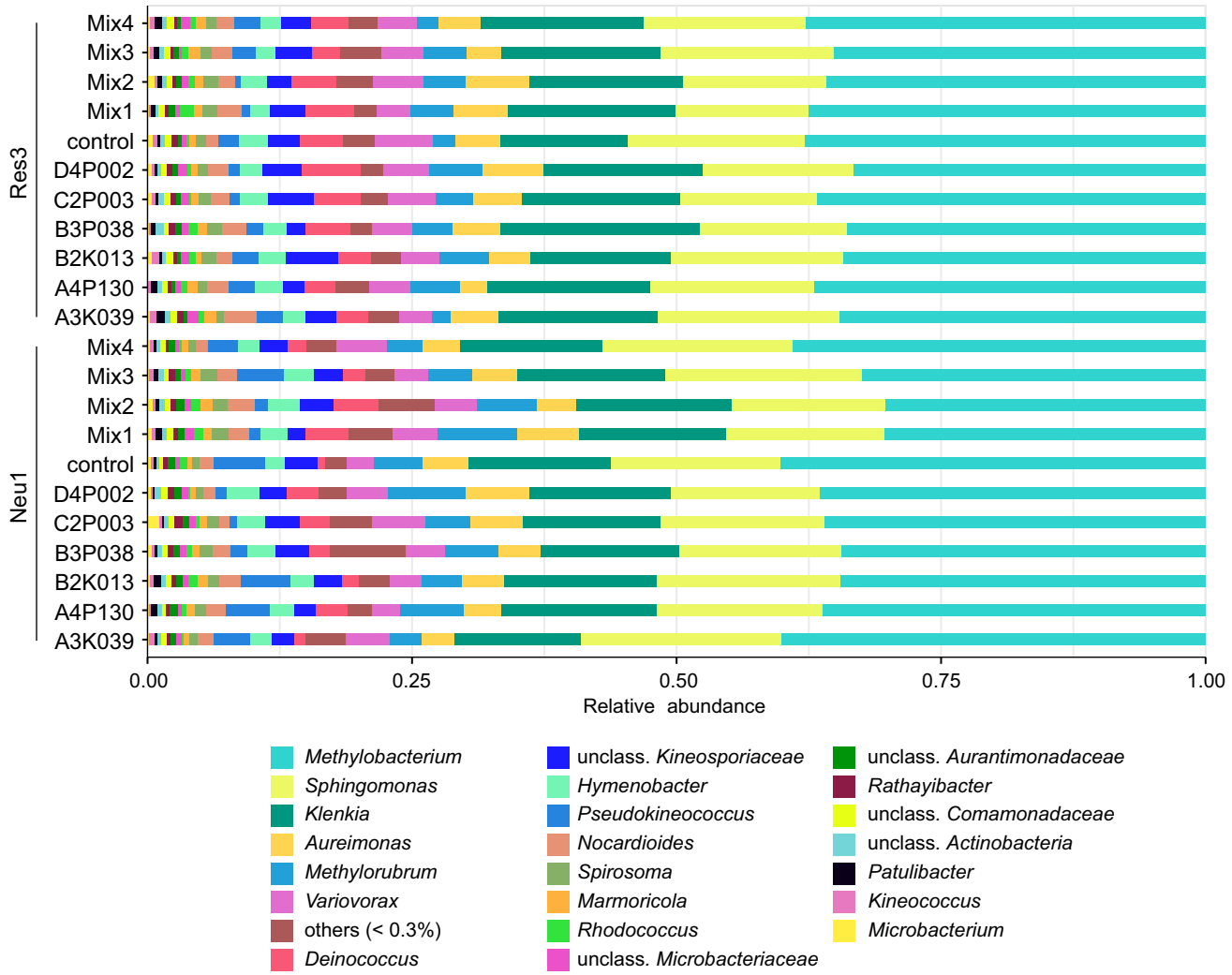

Fig. 2 Relative proportions of bacterial genera in the microbiome of ash seedlings, divided according to inoculation treatment. The taxonomic assignment of the strains and the composition of the consortia are shown in Table 1

Table 2 Relative importance of the factors of plant genotype and inoculation on the structure of the bacterial community 14 weeks after inoculation determined by PERMANOVA

	Df	R^2	F	P
Plant genotype	1	0.01344	1.5857	0.014
Inoculation	10	0.12701	1.4983	0.001
Plant genotype * Inoculation	10	0.08816	1.0401	0.266
Residual	91	0.77319		
Total	112	1.00000		

The *H. fraxineus* pathogen accounted for an average of 0.86% of the fungal sequences across all the treatments. The distribution across the inoculation treatments corresponded well to the results of quantification by qPCR, e.g., the inoculation B2K013 treatment of the Neu1 ash progeny resulted in a relative percentage of *H. fraxineus* in the fungal sequence of 4.0%. The calculation of the association between qPCR and sequencing data resulted in a correlation coefficient of 0.81.

The analysis of α diversity revealed marginal but significant differences from the control only for the Res3 ash. The Shannon diversity was significantly increased in the B3P038, C2P003, D4P002, Mix1 and Mix3 inoculation treatments (Table S1).

For the analysis of β diversity, a PERMANOVA set was again carried out in the first step. For the mycobiome, both the plant genotype and the inoculation had significant effects (Table 3). Overall, 14.8% of the variability of the fungal microbiome was explained by plant genotype and inoculation, although inoculation again explained significantly more of the variability (Factor 7), i.e., it exerted a significantly greater influence than the plant genotype. The interaction of

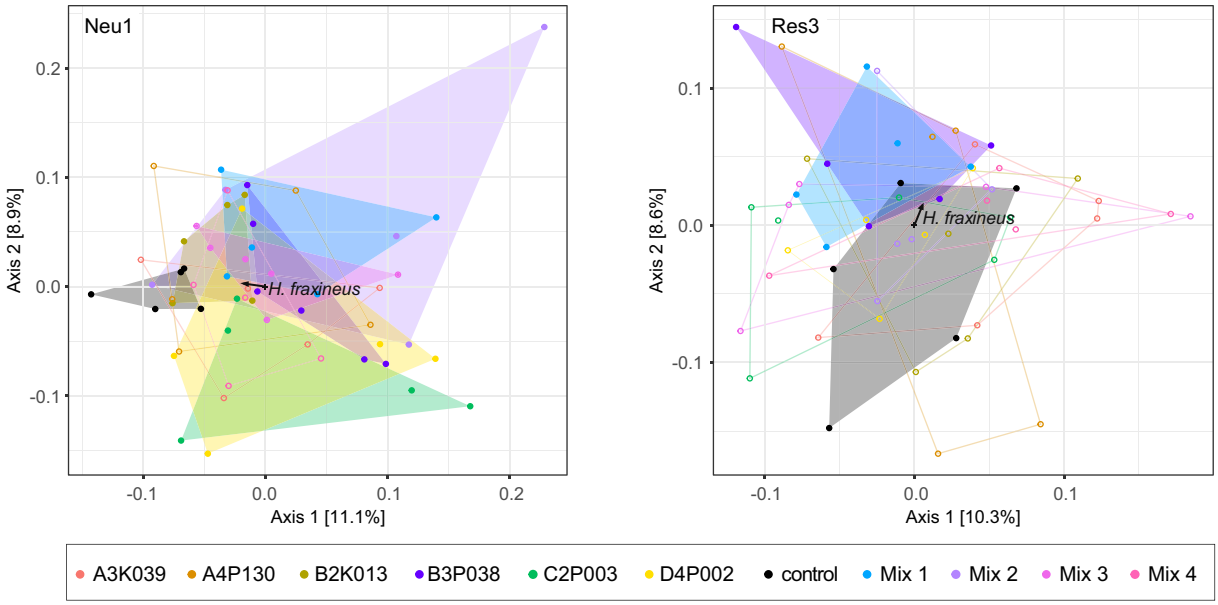

Fig. 3 Differences in the structure of bacterial communities of young ash plants of the Neu1 and Res3 single-tree progenies inoculated with six single strains and four consortia compared with the control. The ordination plot is based on a pCoA with a Bray−Curtis distance matrix. Inoculation treatments with significant differences in the structure of the bacterial communities compared with the control (after analysis with ANOSIM) are shown as filled circles and filled hulls. The taxonomic assignment of the strains and the composition of the consortia are shown in Table 1. The correlations of *H. fraxineus* abundance with both pCoA axes are indicated by arrows

plant * inoculation was not significant. As with the bacterial microbiome, the influence of inoculation was nevertheless investigated separately for both plus-tree progenies.

The ordination plot in Fig. 5 (left) visualizes the differences in the mycobiomes of the different inoculation treatments for the Neu1 ash. A clear differentiation from the control can be seen in the D4P002, C2P003, Mix1 and B3P038 treatments. The separation was almost exclusively based on Axis 2. Consistent with the plot, these treatments were significantly different from the control: ANOSIM: D4P002: $R = 0.47$, $p = 0.006$; C2P003: $R = 0.44$, $p = 0.011$; Mix1: $R = 0.29$, $p = 0.009$; and B3P038: $R = 0.31$, $p = 0.017$. As already shown for the bacterial microbiome, the *H. fraxineus* abundance also correlated with the composition of the fungal microbiome. The direction of *H. fraxineus* abundance again corresponded with the separation of the control from the inoculated treatments (along Axis 2).

Similarly, the mycobiome of the Res3 plus-tree progeny had significant differences in community structure. However, the greater heterogeneity of the control was evident in the ordination plot (Fig. 5 right). This effect was consistent for the bacterial and fungal microbiomes and appeared to be specific to individual plants. The clear separation of the B3P038, C2P003, D4P002, Mix1 and Mix3 inoculation treatments, which occurred mainly along Axis 1, was also clearly recognizable. The statistical analysis revealed a significant difference between these treatments and the control. The ANOSIM values were highly significant, with $R = 0.58$ ($p = 0.009$) for B3P038, $R = 0.52$ ($p = 0.009$) for D4P002, $R = 0.30$ ($p = 0.036$) for C2P003, $R = 0.36$ ($p = 0.029$) for Mix1 and $R = 0.24$ ($p = 0.041$) for Mix3. For this ordination, a correlation with *H. fraxineus* abundance could also be found. However, in this case, the direction did not clearly follow the differentiation between the control and the affected inoculations.

In the fungal microbiome, several ASVs with an ecological preference for a specific inoculation variant were identified. For the Neu1 progeny inoculated with B2K013, one indicator species, *Sarocladium strictum,* was detected. Furthermore, upon Mix3 inoculation, a specific ASV was identified as *Buckleyzyma aurantiaca*. In the Res3 progeny, several inoculation variants presented indicator species, e.g., in A3K039, there was a *Vishniacozyma foliicola* group, and in Mix 4, there was an ASV belonging to *Sidera vulgaris*. The B3P038-inoculated plants also contained an indicator species that was assigned to *Hannaella oryzae*. In contrast to all inoculation treatments, the control also presented two specific ASVs (a *Vararia* sp. group and a *Didymellaceae* group with a relatively high abundance of 1.06%).

Discussion

In recent decades, ash dieback has emerged as a severe threat to *Fraxinus excelsior* and the ecosystems in which it lives. A classical approach is to select more tolerant trees

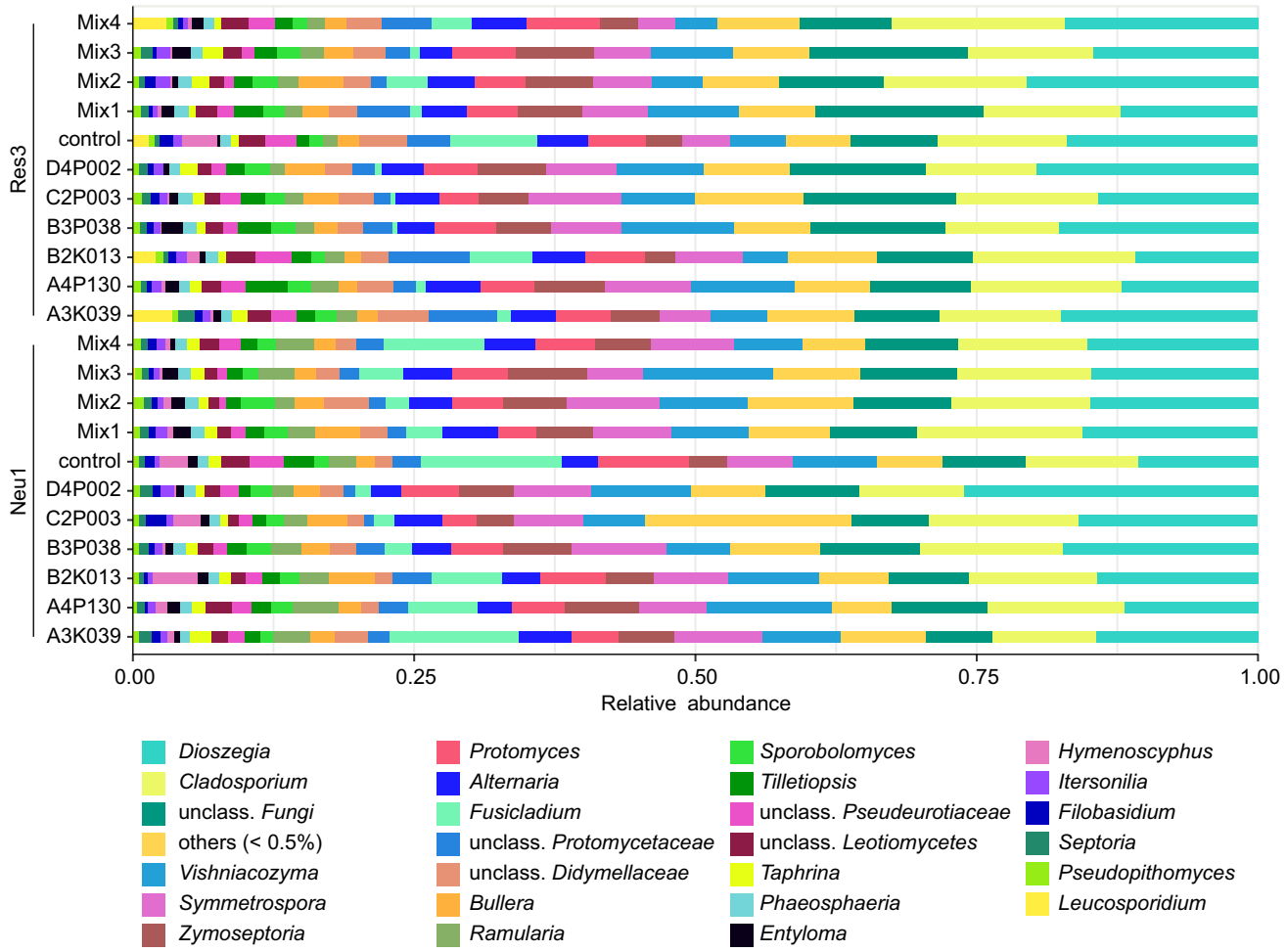

Fig. 4 Proportion of fungal genera in the mycobiome of the ash seedlings, divided according to inoculation treatments. The taxonomic assignment of the strains and the composition of the consortia are shown in Table 1

Table 3 Relative importance of the factors of plant and inoculation on the structure of the fungal community 14 weeks after inoculation determined by PERMANOVA

	Df	R^2	F	P
Plant genotype	1	0.01970	2.3416	0.001
Inoculation	10	0.12827	1.5250	0.001
Plant genotype * Inoculation	10	0.08663	1.0300	0.250
Residual	91	0.76540		
Total	112	1.00000		

and harvest seeds to establish them as plus-tree progenies. With the FraxForFuture joint project started in 2020, a holistic approach was launched to investigate the epidemiology of the disease, the pathogen and the preservation of ash (Langer et al. 2022). One focus was the microbiome of the ashes. In previous studies, its composition was already determined, in addition to the difference between tolerant and susceptible trees (Griffiths et al. 2019; Becker et al. 2020; Ulrich et al. 2020). This paved the way for finding bacterial strains that are part of the original microbiome of *F. excelsior* and that show antagonistic properties or the ability to establish colonization resistance. In this context, Compant et al. (2019) noted that single-strain applications face some setbacks, especially when the strains are taken from the greenhouse to the field. They focused on the typical approach to screen for favorable traits of the strains or the plants. This can lead to good results in the greenhouse, as the conditions are adjustable to promote the establishment of inoculated strains and to standardize plant performance as well as infection with the pathogen. These strains might lack the ability to compete with the already residing microbiome. The results of the genome analysis of C2P003 revealed properties related to the colonization of the host plant; genes for the secretion of specific polysaccharides and proteins that mediate the colonization of the host and the formation of a biofilm were detected (Becker

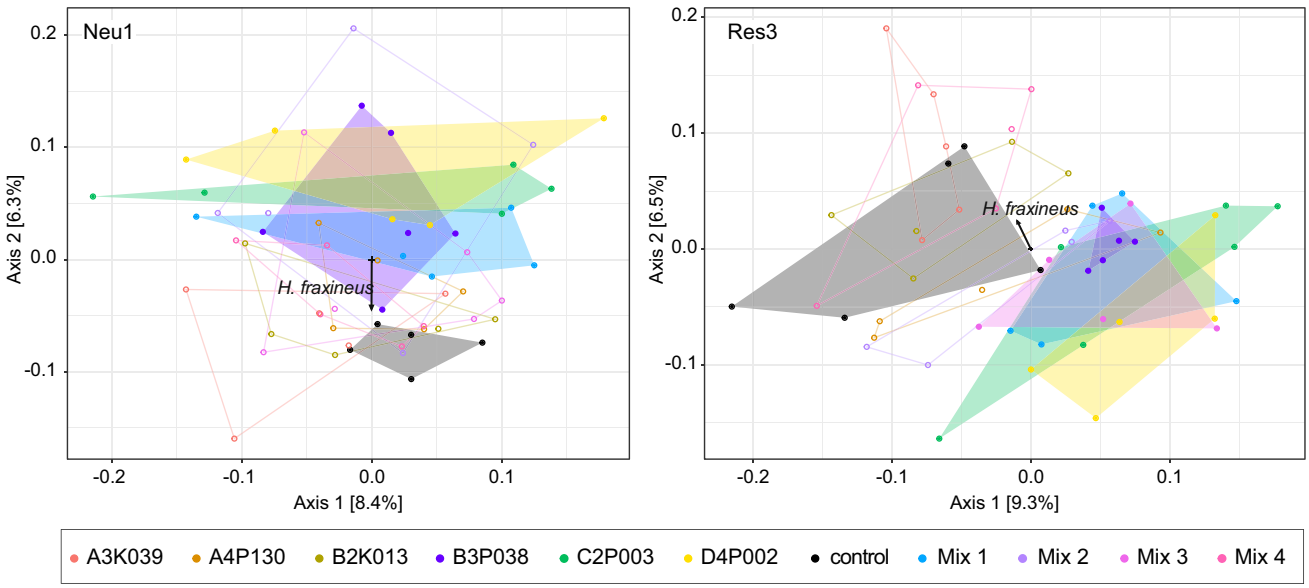

Fig. 5 Differences in the structure of the fungal communities of young ash plants of the Neu1 and Res3 single-tree progenies inoculated with six single strains and four consortia compared with the control. The ordination plot is based on a pCoA with a Bray−Curtis distance matrix. Inoculation treatments with significant differences in the structure of the fungal communities compared with the control (after analysis with ANOSIM) are shown as filled circles and filled hulls. The taxonomic assignment of the strains and the composition of the consortia are shown in Table 1. The correlations of *H. fraxineus* abundance with both pCoA axes are indicated by arrows

et al. 2022). In addition, C2P003 has genes encoding cell wall-degrading enzymes that enable colonization of the intercellular space. A potentially antagonistic activity of C2P003 is the formation of antimicrobial substances, including enzymes that degrade bacterial and fungal cell walls, such as β-N-acetylglucosaminidase (Becker et al. 2022). Owing to its resistance genes, C2P003 can also defend itself against certain antibiotics, increasing its competitiveness. The genetic makeup of C2P003 also allows adaptation to osmotic and oxidative stress (Becker et al. 2022). A genome analysis of B3P038 and D4P002 revealed properties related to biocontrol activities. Both have the ability to synthesize para-aminobenzoic acid (PABA) and phenazines, which are molecules that have antibiotic and antifungal effects (Ulrich et al. 2022; Behrendt et al. 2024). Furthermore, D4P002 can produce siderophores, which include iron. Iron plays an important role in controlling fungal infections, as microbes compete for it. The genetic makeup of D4P002 also allows adaptation to osmotic and oxidative stress (Ulrich et al. 2022). Furthermore, B3P038 has genes for the biosynthesis of p-aminobenzoic acid, and phenazine has been identified. The strain can also produce the polyamine putrescine. Polyamines play important roles in stress reduction, as well as in the formation of biofilms and the colonization of plants (Behrendt et al. 2024). The properties of the bacterial strains include their ability to establish themselves on the host plant and their ability to compete. Furthermore, in greenhouse trials, they had the ability to increase the vitality of inoculated ash seedlings (Becker et al. 2022).

The workflow developed by Burghard et al. (2023) is a useful tool that allows the design of several qPCR assays to verify the establishment of inoculated strains and their persistence. In a greenhouse trial, the inoculated strains were shown to persist on the seedlings for at least eight weeks after inoculation. The qPCR assays also allowed us to monitor their persistence in the field trial alongside the other tests. *Aureimonas altamirensis* C2P003 was observed in the plants of both progenies inoculated with the C2P003 isolate and three consortia containing this strain. Thus, C2P003 can also be established in a field trial and persist there too. As the strains were taken from 60- to 80- year-old trees, they might also be adapted to this age range and are more prone to persist. Inoculation methods, such as spraying or differing formulations, as discussed by Compant et al. (2019), can also affect inoculation success. In addition to the low establishment of strains, ash seedlings were generally not highly damaged in 2021. Owing to late *H. fraxineus* infection, the degree of damage to symptomatic individual plants was relatively low. Nevertheless, the plants inoculated with *S. fraxinea* B3P038 or *L. fraxinea* D4P002 presented significantly fewer symptoms. Although not significant, the C2P003 strain and Mix1 also presented fewer symptoms than did the control. In a greenhouse experiment, Becker et al. (2022) reported the positive influence of the A4P130, C2P003 and D4P002 strains on the health status of ash seedlings over

several growth periods. In this nursery trial, D4P002 had an effect accordingly. B3P038 was not part of the greenhouse study but was shown to be a member of the microbiome of *F. excelsior* and has antagonistic potential (Behrendt et al. 2024). In addition to examining the visible symptoms of the plants, it was crucial to investigate the abundance of *H. fraxineus*. The samples had strong variability, which is quite typical of an incipient infection. In general, we did not find a significant influence of the inoculated strains on *H. fraxineus* abundance. Nevertheless, C2P003- and D4P003-inoculated plants presented a lower abundance of *H. fraxineus*. These results are especially in line with those previously shown for D4P002. As mentioned before, the plants used were derived from plus-tree progenies, which were selected from elder ash trees and presented fewer symptoms after *H. fraxineus* infection. Thus, both progenies are generally less prone to infection, whereas Res3 shows a clear advantage. This finding is also consistent with that of Nielsen et al. (2022), who reported differences in *H. fraxineus* abundance and visible symptoms between tolerant and less tolerant progenies.

If we look at the bacterial microbiome of the ash seedlings in this study, we see that at the phylum level *Pseudomonadota*, *Bacteroidetes* and *Actinobacteria* are strongly similar to those of older trees in other studies (Griffiths et al. 2019; Ulrich et al. 2020). These phyla are generally described as phyllosphere-associated (Aydogan et al. 2018). However, at the genus level, some differences are noticeable. While *Methylobacterium*, *Sphingomonas*, *Klenkia* and *Aureimonas* dominated in ash seedlings, a clear dominance of *Sphingomonas*, *Pseudomonas*, *Curtobacterium* and *Xanthomonas* was detected in 80-year-old trees (Ulrich et al. 2020). Following the assumption of Compant et al. (2019), a visible abundance of *Aureimonas* creates a favorable environment for the establishment of C2P003. The detection of another *Aureimonas* species or strain was ruled out by ensuring the specificity of the qPCR system against a broad variety of closely related strains. Ulrich et al. (2020) described *Aureimonas* as significantly more abundant in tolerant ashes than in susceptible trees. As two plus-tree progenies of more tolerant trees were used in this study, the higher abundance of *Aureimonas* can be explained. Even though most of the bacterial strains we inoculated were not persistent, significant shifts in the beta diversity of the bacterial and fungal microbiomes were visible. After inoculation of bacterial strains, the microbiome undergoes a transition and stabilizes after several weeks (Berg et al. 2021). Additionally, inoculation increases the number of beneficial members of the microbiome while lowering the number of pathogens (Berg et al. 2021). In this study, the abundance of *H. fraxineus* was lower in certain inoculation treatments than in the control. Since the infection of the seedlings was still in its early stages, with generally low *H. fraxineus* abundance, it remains unclear whether the inoculants are the primary factor in reducing *H. fraxineus* levels. Our results at the phylum level are consistent with those of Becker et al. (2020), as *Ascomycota* and *Basidiomycota* are dominant. At the genus level, clear differences are visible again. While the mycobiome of older trees is dominated by *Aureobasidium*, *Papiliotrema* and *Vishniacozyma* with over 70% abundance, the mycobiome of the young trees in this study is dominated by genera such as *Dioszegia, Cladosporium* and *Vishniacozyma,* with an average abundance of 35%. Changes in the microbiome caused by age are often observed and are often related to growth and changes in chemical compounds exuded by the plant (Dastogeer et al. 2020). Perhaps the strong differences in the microbiomes of seedlings and older trees hampered the establishment of the inoculation strains in the seedlings, as they were isolated from tolerant trees in an ash forest (aged between 60 and 80 years). In general, the structure of the microbiome composition in every plant tissue and organ is subjected to external and dynamic biotic and abiotic factors such as climate, anthropogenic intrusions, pathogens, pH or the host genotype, to name some (Compant et al. 2019). Growing in the field without herbs or other young trees might have led to rather harsh conditions (high UV radiation, heat or dryer soil) for microbes to establish themselves on the leaves. A more favorable microclimate could have improved this. Otherwise, the conditions of the nursery trial in this specific year limited both the establishment of the inoculations and the *H. fraxineus* fungal pathogen. As a plant starts to grow in soil, its microbiome assembly is driven by microorganisms that initially live in the seed endosphere and by microorganisms enriched in the rhizosphere. The microbiome is altered not only by changes in habitat conditions as the plant develops aboveground organs but also by additional microorganisms transported to the phyllosphere through aerial transport (Compant et al. 2019). Moreover, there is a migration from the leaf surface into the endosphere of the leaves. Burghard et al. (2023) reported that inoculated bacteria were detectable on newly grown stems and leaves, indicating the mobility or transport of the bacteria. In conclusion, host plants start with an initial microbiome that is altered by environmental conditions and can be seen as constantly changing. The introduction of beneficial bacteria can lead to some problems if the bacteria used are not part of the core microbiome of the host. To avoid this problem, we used bacterial strains previously isolated from tolerant ash individuals, which have a proven effect on *H. fraxineus*, in this study. This should ease the establishment of bacterial strains especially if favorable conditions, such as shadows and moisture, are present.

Although most of the inoculated strains were no longer detectable 14 weeks after inoculation, our results showed that the inoculation with some of these strains affected the plant health as well as the microbiome. Otherwise, it highlighted the necessity of the surveillance of inoculation

success. As a next step, it would be crucial to investigate long-term persistence in field trials, especially if the bacteria can survive a winter period. Furthermore, the conditions that could further increase the persistence of the inoculated strains should be studied. Such experiments should always include the analysis of ash dieback symptoms and *H. fraxineus* abundance as well as the composition of the ash microbiome.

Conclusion

In our study, we tested the inoculation effects of a range of bacterial strains on ash seedlings under nursery conditions. We found that *S. fraxinea* B3P038 and *L. fraxinea* D4P002 lowered the symptoms of ash dieback. Using strain-specific qPCR assays, only the persistence of *A. altamirensis* C2P003 could be proven in inoculated ash seedlings. Similarly, the fungal pathogen was also unable to infect the plants during the summer months. The infection did not start until mid-September. In addition, we were able to show that the inoculated bacterial strains influenced the microbiome, even without persisting on the plants. These results are encouraging, as positive effects can be taken from the greenhouse to the field. However, under field conditions, a strong variation in the effects must be considered. This strongly suggests that in addition to plant scoring, monitoring inoculation success as well as pathogen abundance is necessary. In the context of practical use, the B3P038, C2P003 and D4P002 strains should be studied at different sites to evaluate the conditions under which the strains can persist on the plants and support their vitality.

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Author contributions VB, VS and AU conceived and designed the study. VB and SW conducted the experiments, and VB and AU visualized the results. VB wrote the manuscript with the help of AU. All the authors discussed the content, critically revised the manuscript and approved the submission of the manuscript.

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Data availability The paired sequence reads generated for the microbiome analysis were deposited in the NCBI Sequence Read Archive under Accession Number PRJNA1087259.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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