Applied Soil Ecology 164 (2021) 103929

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Bacterial microbiome in *Armillaria ostoyae* rhizomorphs inhabiting the root zone during progressively dying Scots pine

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ARTICLE INFO

Keywords:
Armillaria mellea sensu *lato* rhizomorphs
Root parasite
Tree dieback
Bacterial microbiome
Enzymatic activity

ABSTRACT

The aim of the work was to describe the bacterial microbiome inhabiting *Armillaria ostoyae* rhizomorphs collected from roots of progressively dying scots pine trees and explain the relationship between bacteria and fungi during the pathogenesis process. Differences between phases of tree dieback were characterized, and the potential roles of the dominant bacterial groups hypothesized. Illumina 16S rRNA sequencing, as well as qPCR and API tests were used. The microbiome was composed mainly of Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria phyla, which constituted about 80% of the community. The most abundant bacteria belonged to the eudominant Parabacterioides genus (~14.5%). Particular bacterial genera from rhizomorphs collected from trees in phases I, II, and III of dieback varied in frequency. Interestingly, Bacilliales showed an increase in abundance with progressive tree dieback. Analysis of the cellulolytic potential (and other hydrolytic enzymes) of rhizomorphs confirmed the involvement of bacteria in each of the examined stages of tree dieback. Additionally, the rhizomorphs were inhabited by N-utilizing bacteria. The results obtained indicate that rhizomorphs are inhabited by numerous bacteria. These bacteria are able to hydrolyze compounds containing esters, phosphorus bonds, proteins, and sugars; including cellulose and hemicellulose, which support the rhizomorphs' enzymes in the decomposition of wood. They can also share nitrogen.

1. Introduction

Globally, Armillaria root rot and annosus root rot are infectious forest diseases of major economic importance. *Armillaria* and *Heterobasidion* above all cause losses in current growth and increase tree mortality. In Poland, in 2018, these diseases occurred over a total surface area of 135.8 thousand ha, corresponding to around 1.5% of the total forest area (Zajączkowski et al., 2019). Data concerning only Armillaria root rot from 2017 indicates that it occurred over an area of 56.4 thousand ha. Małecka (2019) found that Armillaria root rot accounts for over 30% of all infectious forest diseases in Poland. Żółciak (2007), studying species composition for *Armillaria* species in Polish coniferous forests found that only the *A. ostoyae* species occurs in the Scots pine stands, affecting class I stands (1–20 years of age) in fresh mixed coniferous forest types of habitat. Research conducted in 2019 also proved that main threat to the studied pine crops in forest districts of the Regional Directorate of State Forests in Olsztyn is Armillaria root rot, whose share in more fertile habitats exceeds 5% of the number of trees, and locally, together with annosus root rot, as much as 20% (Sierota et al., 2020). This indicates that Armillaria root rot is of major significance for Scots pines in young coniferous forests in Poland.

Coniferous forests account for 68.4% of stands in Poland, the dominant species being pine (58.2%). There has been a considerable increase in the occurrence of damage caused by *Armillaria* from the 1950s (40–60 thousand ha) to the start of the 21st century (over 200 thousand ha). After 2005, the area where the disease occurs started to decrease gradually due to improvements in the cultivation of stands. In the forests in Poland that are the most at threat, Armillaria root rot may

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https://doi.org/10.1016/j.apsoil.2021.103929
Received 5 September 2020; Received in revised form 16 December 2020; Accepted 5 February 2021
Available online 18 February 2021

cause losses in production of wood grain at 100 years of age of 400 m3/ha valued at up to 1000 EURO/ha/year (Lech and Żółciak, 2017). The *Armillaria mellea* sensu *lato* complex, which also includes *A. ostoyae*, is also described as a major factor in forest dieback in the northern hemisphere, mostly in harvested stands (Cleary et al., 2013; Kubiak et al., 2017b; Labbé et al., 2017; Heinzelmann et al., 2018).

The *Armillaria* persists in its dormant form in soil as rhizomorphs and mycelial mats. In its active state, it exists as hyphae, infecting roots of trees and decaying the timber (La Porta et al., 2008; Baumgartner et al., 2011; Labbé et al., 2015; Heinzelmann et al., 2019; Devkota and Hammerschmidt, 2020; Jayawardena et al., 2020). Rhizomorphs are a special type of hyphae consisting of an outer preservative cortex and inner soft sub-cortex with an aerial core. Because these develop in the root rhizosphere, they are commonly inhabited by diverse soil organisms including mites, nematodes, fungi, and bacteria (Schulz, 2006; Mamiya and Shoji, 2009; Tomalak, 2017).

The multifaceted roles of microorganisms and parasites and their metabolites, as well as their interactions in soil environments are still being investigated (Artursson et al., 2006; Song et al., 2016; Wrzosek et al., 2017; Kubiak et al., 2017a; Heinzelmann et al., 2018). Tomalak (2017) has described *Armillaria* rhizomorphs as a 'subway channel' for some nematode species. Percival et al. (2011), Pellegrini et al. (2012) and Chen et al. (2019) point to the roles of some inhabiting fungi such as *Trichoderma* spp. in the growth of *Armillaria* rhizomorphs in soils. Also, harmless *Armillaria* species, such as *A. altimontana*, may limit the development of pathogenic species, such as *A. solidipes*, which cause root disease (Warwell et al., 2019). Other antagonistic fungi e.g. *Rhizoctonia lamellifera*, *Scytalidium lignicola*, *Phlebiopsis gigantea*, *Pleurotus ostreatus*, *Coriolus versicolor*, *Stereum hirsutum*, and *Xylaria hypoxylon* can inhibit the development of *Armillaria* in soils (Mercado-Blanco et al., 2018). Bacteria could be expected to play a major role in functioning of the rhizosphere (Lladó et al., 2017). The study conducted by Lalande et al. (2019) on *A. solidipes* (high virulence) and *A. altimontana* (low virulence) made it possible to identify 712 unique bacterial OTUs. More Pseudomonadaceae and Spartobacteria were associated with healthy trees, while more Acidobacteria were associated with dead trees. With respect to *Armillaria* spp., more Pseudomonadaceae and Rhizobiales were associated with *A. altimontana*; whereas, more Acidobacteria and Enterobacteriaceae were associated with *A. solidipes*. Mesanza et al. (2016) found strong antagonistic in vitro and in vivo effects of *P. fluorescens* and *Bacillus simplex* strains towards *A. mellea*. de Vasconcellos and Cardoso (2009) reported similar findings with regard to the effects of *Streptomyces* towards an unidentified strain of *Armillaria*.

Previous studies point to a symbiosis between bacteria and fungi in the decomposition of wood, and describe the benefits of these relationships (e.g. nitrogen fixation), but, to date, bacterial interactions with *A. ostoyae* have not been described (Johnston et al., 2016). The above examples indicate that microbial, and mainly bacterial communities, can stimulate or decrease the growth of rhizomorphs and that pathogen-host relationships can have an influence on resistance reaction levels (Baumgartner and Warnock, 2006; Dintner et al., 2011). Bacteria co-operate with mycorrhizal fungi in forming fruiting bodies (Duponnois and Plenchette, 2003; Aspray et al., 2006; Kumari et al., 2013), and participate in the transfer of many nutrients, either directly or indirectly within the hyphae via roots to the stem and crown of trees (Bending et al., 2006; Calvaruso et al., 2006; Barbato et al., 2019). Specifically, nitrogen-fixing bacteria play an important role in stimulating the growth of trees (Hodge et al., 2000; Lladó et al., 2017).

It has been assumed that the structure of the bacterial microbiome of rhizomorphs depends on the phase of tree dieback, or is representative of a given phase of tree dieback. Its size and diversity is reflected in differential enzymatic activity, relative to the temperature of growth and interactions with microbiota components of rhizomorphs (Mercado-Blanco et al., 2018).

The presence and abundance of active bacteria and fungi inside rhizomorphs expressing enzymes can rapidly accelerate wood tissue death. This is a global problem in the forestry and timber industry (DeLong et al., 2002; Mercado-Blanco et al., 2018). However, the importance of microbiome for rhizosphere development is still unclear. Therefore, in this work, our aim was to examine the structure of bacterial communities inhabiting rhizomorphs collected from progressively dying Scots Pine trees. The functions (antagonists, saprotrophs, and stimulants) of the dominant bacterial groups were characterized. Additionally, the relationships between enzymatic activity, temperature, and phase of tree dieback were investigated. The hypothesis assumes that the known structure of the microbiome and the relationships with enzymatic activity and phase of tree dieback will make it possible to identify bacterial taxa that play an essential role in the course of infection of pine roots by *Armillaria* (e.g. as bioindicators of tree dieback).

2. Material and methods

2.1. Rhizomorphs preparation

The experiment was performed in the Nowe Ramuki Forest District, north-east Poland, in a managed nine-year-old Scots pine plantation, planted in 2009 (subdivision 288 h; 53°38′51 N: 20°33′42E). In the summer of 2016, trees with *A. ostoyae* (Romagnesi) Herink symptoms indicative of Armillaria root disease (Fox, 2003; Isidorov et al., 2008; Eichhorn et al., 2016), and two trees representing three different stages of disease (phases I, II, and III of tree dieback) were chosen at random. Rhizomorphs were carefully cut out, removed and placed in sterile tubes, transported to the laboratory and refrigerated. *A. ostoyae* was identified as the root rot pathogen in the dying Scots pines, as well as in the roots of the remaining stumps. The fungus was determined using molecular techniques and deposited in GenBank under Nos: MH793515–MH793522.

2.2. Quantitative PCR and metagenome analysis

For DNA isolation, each of the rhizomorphs was cut, then 1 g from each of the samples was crushed in a TissueLyser LT homogenizer (Qiagen, Germany). For extraction and purification, we used the GeneMATRIX Soil DNA Purification Kit (EURx Ltd. Gdansk, Poland) according to the manufacturer's recommendations. 250 mg of material from the rhizomorphs initially homogenized was put aside for extraction. Quantification of microbiota was performed from directly isolated DNA in two technical replicates and compared to the standard curve. The number of 16S rRNA gene copies was calculated per gram of rhizomorph. Quantification of bacterial 16S rRNA gene copies was performed by qPCR. Primers and a probe developed by Yu et al. (2005) were used. Prokaryotic domains were amplified with the Maxima Probe qPCR Master Mix (2×) including ROX solution (Thermo Fisher Scientific, USA). The PCR reactions for bacteria and archaea started with an initial denaturing step at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and at 60 °C for 1 min.

Microbial communities were examined by sequencing the V3-V4 region of the 16S rRNA gene. DNA libraries were created using PCR primers recommended for the Illumina technique – the Forward PCR primer: 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG3′ and the Reverse PCR primer: 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC 3′. These primers were developed by adding Illumina adapter overhang nucleotide sequences to the PCR primers specified by Klindworth et al. (2013). Amplicons were indexed using the Nextera® XT Index Kit according to the manufacturer's protocol. DNA was sequenced in Illumina MiSeq in 2 × 250 paired-end mode. Demultiplexing and FASTQ file generation was performed using Miseq Reporter v.2.4. Sequencing results were uploaded to the MetaGenome Rapid Annotation Subsystems Technology (MG-RAST) server as FASTQ files for analysis (Meyer et al., 2008). Each file underwent quality control

(QC) including quality filtering (removing sequences with ≥5 ambiguous base pairs) and length filtering (removing sequences with a length ≥ 2 standard deviations from the mean). The UCLUST algorithm was used to cluster identified rRNA sequences. The representative sequence of each cluster was used to assign taxonomy using the Greengenes reference database. The Illumina metagenomic datasets are available at MG-RAST under access numbers 4,791,502.3 (phase I), 4,791,501.3 (phase II) and 4,791,500.3 (phase III).

2.3. Biochemical analysis of isolated bacteria

The enzymatic strip tests and enumeration of the count of cellulose-degrading bacteria were measured for the whole rhizomorph and the internal part of the rhizomorph. Half of obtained material was first sterilized for 15 s with 70% ethanol, then for 1 min with 2% sodium hypochlorite, and washed three times with sterile water. Both the 30 sterilized and the 30 unsterilized rhizomorphs were then cut into 0.5–1 cm fragments and placed on Congo Red Agar (K_2HPO_4 1.2 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, carboxymethylcellulose (CMC) 5 g, gelatin 2 g and agar 15 g^{-1} diluted in 1 L of demineralized H_2O (Hankin and Anagnostakis, 1977). The Petri dishes with growing cultures were incubated for 48 h in darkness: at 28 °C for saprotrophs, 22 °C for psychrophiles and 11 °C for psychrotrophs. For biochemical activity, overnight preincubation at 28, 22 and 11 °C, in peptone water was performed for both variants of rhizomorphs. Next the activity of whole microbial community was measured using API® ZYM and API® 20NE kits (Biomerieux, France). Index values of 0–5 were assigned according to the manufacturer's guidance for semi-quantitative assays: 0-inactivity, 1-weakest activity (5 n moles; turbidity or color intensity), 5-strongest activity (> 40 n moles; turbidity or high color intensity).

2.4. Statistics

Indices used in the ecological assessment were calculated according to the following standards: Simpson's (λ) dominance index: $\lambda = \Sigma pi^2$ (Simpson, 1949), the Shannon-Wiener diversity index $= H' - \Sigma\, pi \times \ln pi$, Pielou's evenness index $= H'\, Hmax^{-1}$, where p*i* is the relative coverage of species *i* (Pielou, 1974; Neumann and Starlinger, 2001).

Taxonomic differences between microbiomes were analyzed using Statistical Analysis of Metagenomic Profiles (STAMP v. 2.1.3) (Parks and Beiko, 2010).

The significance of the relative proportion difference in taxonomic distribution of samples was performed using the two-sided Fisher's exact test, with the Newcombe-Wilson confidence interval method. Results with $q < 0.05$ were considered significant and the unclassified readings were removed from analyses. The biological relevance of the statistic taxa was determined applying a difference between the proportions of at least 1% and a twofold ratio between the proportions.

Real-time PCR results were calculated by Statistica 12, using ANOVA and Tukey's HSD test ($p = 0.05$). Principal Component Analysis (PCA) and Agglomerative Hierarchical Clustering (AHC) were calculated in the XLSTAT program. For AHC, the Bray-Curtis test was used with Ward's agglomerative method. Correlations were calculated in SigmaPlot v.12.

3. Results

3.1. Bacteria abundance and dominance

The bacterial load values (log_{10} of 16S rRNA gene copies per 100 mg^{-1}) quantified from the qPCR were 7.95 ± 6.09, 7.91 ± 6.46 and 8.53 ± 6.24 (significantly highest) for phases I, II and III respectively. The results of the hierarchical grouping showed large differences in the number of bacterial cells, depending on the level of grouping. In the AHC analysis, automatic truncation was located below all the nodes, indicating that each stage of development has its own unique microbiome. This implies dynamic microbial communities, undergoing significant change during the process of tree dieback (Fig. 1).

The microbiome analysis of the examined rhizomorphs allowed us to identify bacteria representing 24 phyla. The most numerous were Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria, constituting about 80% of the community in total. The relative composition of communities associated with different phases of tree decline varied, although most of the bacterial phyla were recorded as present across all phases of dieback. We detected an increase in abundance of Acidobacteriales between phases I and II, simultaneous with an apparent disappearance of Rhodospirillales. Between phases II and III, we noted an increase in the abundance of Bdellovibrionales (Fig. 2). This is also reflected in the proportion of bacterial species. We noted an abundance of *Parabacterioides goldsteinii* (>10%), along with constant levels of *Clostridium disporicum* (~4%) and *C. glycolicum* (~3%), and members of the Flavobacteriaceae, which decreased in proportion to the progression of dieback (from 7.1% to 3.9%). Conversely, other taxa increased in abundance as dieback progressed, such as *Bacillus circulas* (from 0 to 6%), *B. horikoshii* (from <0.1 to 3.1%).

Significant differences were found at the level of phylum and order of bacteria (Table 1, Fig. 3). At the phylum level, between phases I and II of tree dieback, significant differences were observed for three high-abundance taxa; Bacteroides ($p < 0.001$) were more frequent in phase I, while Firmicutes and Actinobacteria ($p < 0.001$) were more frequent in phase II. The highest abundance was observed in phase I in the case of Proteobacteria and Actinobacteria ($p < 0.001$), and for Bacteroides ($p < 0.00$). Between the second and third phases (both characterized by 4 high-abundance phyla), in the later stages of tree decline, there was a marked increase in the abundance of Firmicutes and Bacteroides and a decrease ($p < 0.001$) for Proteobacteria and Actinobacteria. In phase III, we observed a significant and very large increase in Firmicutes ($p < 0.001$).

At the order level, more fluctuations in abundance were observed. Both Bacillales and Actinomycetales showed an increased abundance in phase II, whereas Bacteroides, Clostridiales, Rhodobacterales, Flavobacteriales, and Rhizobiales ($p < 1{\cdot}10^{-15}$) all showed decreasing abundance in phase II. Increased abundance in the final phase of dieback compared to phase I was noted for Bacilliales ($p < 1{\cdot}10^{-15}$), alongside a reduction in Actinomycetales, Rhodobacterales ($p < 1{\cdot}10^{-15}$), Clostridiales, Bacteroides, Flavobacteriales and Lactobacilliales ($p < 1{\cdot}10^{-5}$). An increase in abundance in phase III was observed, particularly in the following orders: Bacilliales, Bacteroides ($p < 1{\cdot}10^{-15}$) and Rhizobiales, Clostridiales and Flavobacteriales ($p = 3.8{\cdot}10^{-15}$ to $4.7{\cdot}10^{-7}$).

Among the most frequent taxa, only the increase in *Bacillus* spp. was correlated with progress in tree dieback. A positive correlation of 0.98 was found at both the levels of order and genus. A clear positive correlation, corresponding to the progressive phases of dieback, was demonstrated by the Bacilli (phase I 6%, phase II 13%, phase III 16%; $R^2 = 0.98$, Table 1).

Analysis of the structure of the microbiome at the level of the genus allowed clear changes in the structure of the community to be detected between the various phases of tree decline, which largely confirmed the results of the hierarchical cluster analysis (Fig. 1, Fig. 2). The most numerous group was the eudominant *Parabacterioides* genus (14.5% average). Although the overall abundance of this genus was relatively constant, the species composition of *Parabacteroides* sp. members differed significantly between all the analyzed phases (Fig. 3).

Another genus present in high abundance (11.2% average) was *Clostridium* spp., whose presence in communities associated with phases I and III of dieback exceeded 12 and 11% respectively. The genus demonstrated eudominance in these phases, yet, in phase II, the population constituted a dominant group (9.8%). The differences between phases I and II, as well as between phases II and III, were significant (Fig. 4). The abundance of *Bacillus* spp. increased as the tree died down, from occasional microbiota in phase I (~1%), to dominating in phase II (5.9%), and eudominating in dead wood (13.7%). The composition of

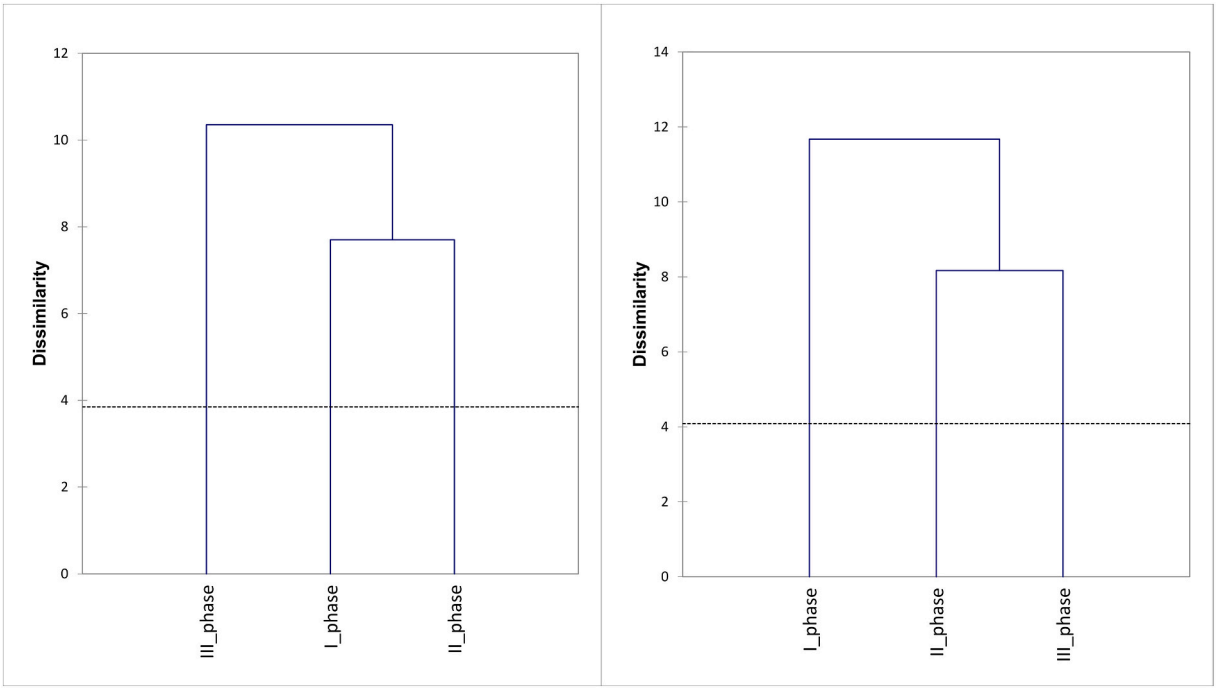

Fig. 1. Agglomerative Hierarchy Clustering analysis (Bray and Curtis method) of genetic distance between phylum, and order-representing microbiomes inhabiting *Armillaria ostoyae* rhizomorphs collected from phases I, II and III of tree dieback (phylum on the left, order on the right).

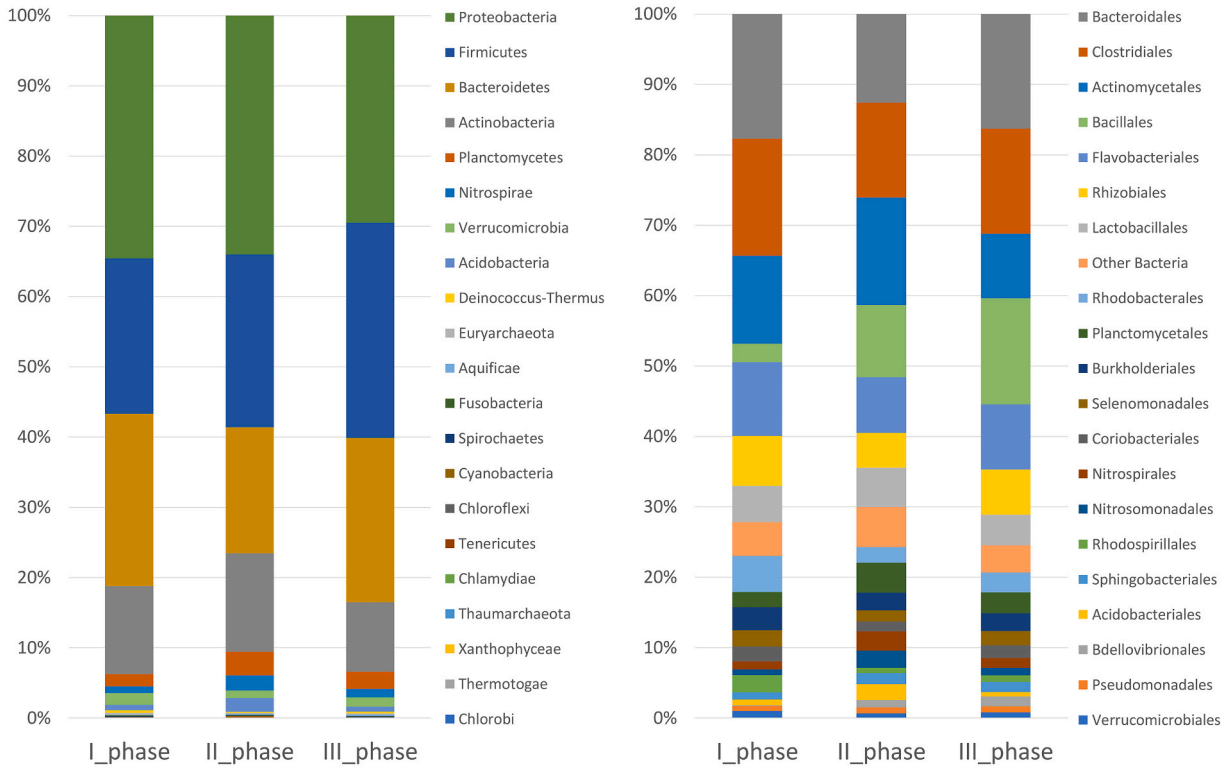

Fig. 2. The structure of microbial communities inhabiting *Armillaria ostoyae* rhizomorphs collected from phases I, II and III of tree dieback on the level of phylum (left) and order (right).

bacilli members was significantly different across all the analyzed phases, and was directly proportional to progression in the phase of tree dieback.

Of the remaining families, a noticeable proportion consisted of the genera *Agrobacterium*, *Lactobacillus*, *Paracoccus* and *Ruminococcus* as subdominates. It is worth noting that the genera *Actinomadura*, *Paenibacillus*, *Trichococcus*, *Nitrospira*, *Mycobacterium*, *Nitrosomonas* and *Acidobacterium* accounted for a larger share of the population in phase II, also constituting subdominants, while, in other phases, they were rare or occasional microbiota (Table 1).

Table 1
Frequency (%) bacteria genus identified in *Armillaria ostoyae* rhizomorphs collected from phases I, II and III of tree dieback.

Genus	I Phase	II Phase	III Phase	Mean
Parabacteroides	16.8	12.3	14.5	14.5
Clostridium	12.5	9.8	11.3	11.2
Bacillus	1.0	5.9	13.7	6.8
Agrobacterium	4.6	2.3	4.2	3.7
Lactobacillus	3.8	2.7	3.0	3.2
Paracoccus	4.9	1.9	2.5	3.1
Ruminococcus	3.6	2.9	2.7	3.1
Actinomadura	1.8	4.0	1.7	2.5
Paenibacillus	1.2	4.1	1.4	2.2
Selenomonas	2.3	1.6	2.0	2.0
Trichococcus	1.4	3.1	1.2	1.9
Nitrospira	1.2	3.0	1.4	1.9
Mycobacterium	1.9	2.7	0.8	1.8
Nitrosomonas	0.8	2.7	1.1	1.6
Leucobacter	1.9	1.3	1.4	1.5
Rhizobium	1.6	1.5	1.4	1.5
Flavobacterium	1.8	1.2	1.5	1.5
Acidobacterium	0.9	2.6	0.7	1.4
Chryseobacterium	0.7	0.4	3.0	1.4
Bacteroides	1.7	1.0	1.4	1.4
Rothia	1.3	1.0	0.9	1.1
Collinsella	0.2	1.2	1.7	1.0
Porphyromonas	1.0	0.7	1.2	1.0
Arthrobacter	1.2	0.7	1.0	1.0
Swaminathania	1.8	0.2	0.7	0.9
Bdellovibrio	0.1	1.2	1.5	0.9
Comamonas	1.4	0.5	0.6	0.8
Bordetella	0.8	0.9	0.7	0.8
Terrimonas	0.9	0.6	0.8	0.8
Eggerthella	1.9	0.3	0.1	0.8
Elizabethkingia	0.7	0.6	1.1	0.8
Burkholderia	0.8	0.6	0.8	0.7
Microbacterium	0.6	1.0	0.5	0.7
Acinetobacter	0.8	0.7	0.6	0.7
Actinoallomurus	0.6	1.2	0.2	0.6

Eudominant	Dominant	Subdominant	Rare	Occasional

All identified classes of bacteria were found to be moderately differentiated in relation to groups, with an episodic turnout (>5%). The largest shares in each community were occupied by the classes Alphaproteobacteria, Bacteroidia, Clostridia, Actinobacteria, Bacilli, Flavobacteria and Betaproteobacteria. The Alphaproteobacteria clearly showed a higher share in the first phase of tree dieback (27%) than in phases II and III (ca. 21% each). In contrast to the other variants, the Bacteroidia, Clostridia and Flavobacteria were characterized by a lower abundance in phase II of tree dieback, and a reverse relationship was observed with regard to the proportion of Actinobacteria and Betaproteobacteria (Table 1, Fig. 4).

3.2. Ecological indices

It was noted that the values of the Simpson's dominance and Shannon-Weiner (SW) ecological diversity indices for the communities were similar, which proves a high stability of collective diversity (Table 2). However, their significant difference in composition as tree dieback progresses (Fig. 1) indicates the dynamic nature of community structure. Very low values of the dominance index (average $\lambda = 0.030$) and accompanied simultaneously by high diversity on the SW index (H'= 4.64) indicate a very large pool of species, of which almost all taxa belong to occasional microbiota with very small (<1%) community shares (Table 2, Appendix). The Pielou's J evenness index was above 0.7 in phase II, implying that, despite a lack of clear dominance in the microenvironment, there were small differences between the number of individuals within each taxa present. The occurrence of single dominant forms or clearly higher frequencies in some genera was confirmed on the basis of dominance classification (Table 2).

3.3. Biochemical characteristics of isolated strains

Analyzing the results of the enzymatic activity of the cultured bacteria (Fig. 5), a high variability in enzymatic activity was observed depending on the phase of development, and an association with temperature was demonstrated. The heat map visualization and accompanying dendrograms allowed us to distinguish four groups of enzyme activities (top dendrogram). The largest clade containing almost half of the enzymes included those that were not active or were negligible in some cases. The remaining 3 clades were separated due to large

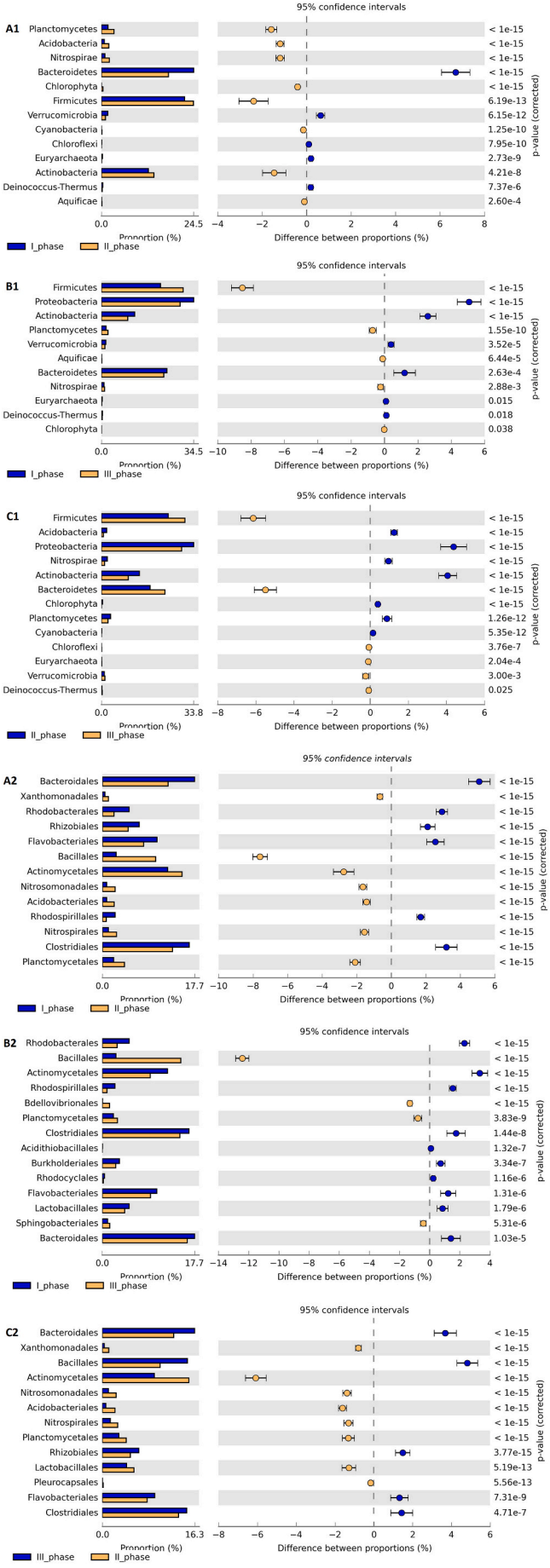

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Fig. 3. Comparative taxonomic profile of *Armillaria ostoyae* rhizomorphs collected from phases I, II, and III of tree dieback at phylum (A1, A2, A3) and order (B1, B2, B3) level, computed by MG-RAST. Differences in the microbial abundance of samples were calculated with the two-sided Fisher's exact test for analyses of two samples in STAMP. Only genera with significant biological differences ($P < 0.05$) are shown.

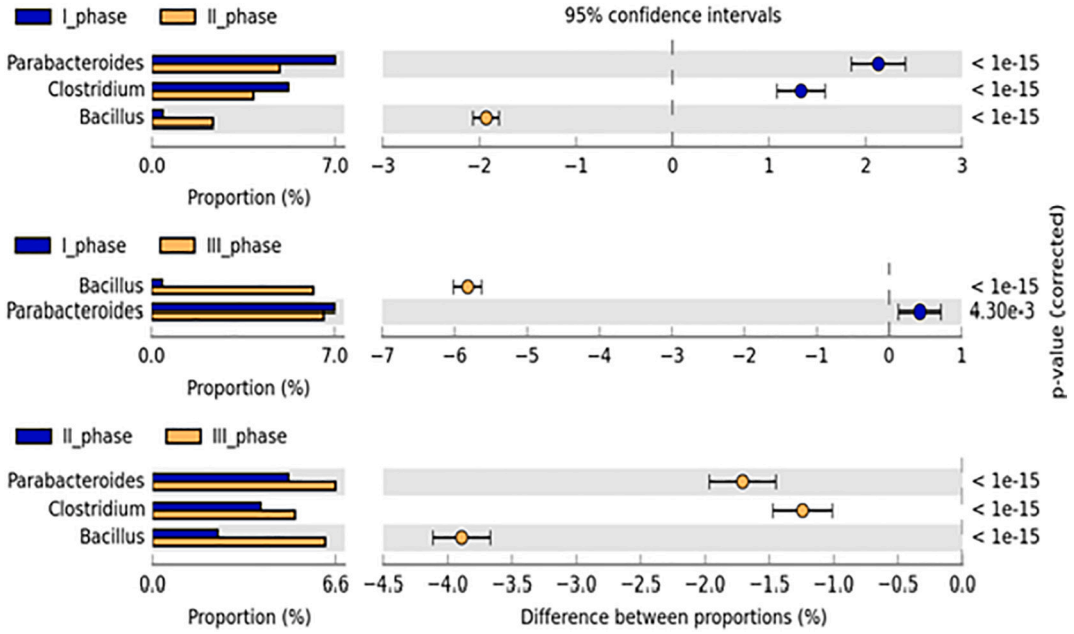

Fig. 4. Comparative taxonomic profile of *Armillaria ostoyae* rhizomorphs collected from phases I, II and III of tree dieback at genus level, computed by MG-RAST. Differences in the microbial abundance of samples were calculated with the two-sided Fisher's exact test for analyses of two samples in STAMP. Only genera with significant biological differences ($P < 0.05$) are shown.

differences in activity depending on the temperature. Nevertheless, the smallest clade included 3 enzymes responsible for nitrogen and phosphate transformation, which were strongly active regardless of the temperature. Some differences were observed between the activity of the surfaces of colonizing microbiota and the inner part of the rhizomorph. Esterases were active in all phases but in the third phase, the activity of enzymes produced by psychrotrophs (11 °C) was lost. In the case of protein degradation, there was significant activity loss in α-chymotrypsin. α-chymotrypsin activity was observed if the enzyme was produced at 28 °C by bacteria living outside of rhizomorphs, while only internal bacteria obtained from rhizomorphs in phase I showed a positive result for α-chymotrypsin activity. The hydrolysis activity of melibiose, lactose and hyaluronic acid was confirmed at 28 °C in each case. Some saprotrophs exhibited utilization of simple compounds, including: D-glucose, *N*-acetylglucosamine, D-maltose and malic acid. At every temperature and in every phase of dieback, bacteria carried out denitrification.

The results of the cellulolytic potential analysis confirmed the presence of cellulolytic bacteria in each of the analyzed stages of dieback (Fig. 5). Regardless of the dieback phase (progress of the starching process), there were microorganisms within the community metabolizing maltose (product of starch hydrolysis), which is a primary predictor of cellulolytic activity. We confirmed that, for *Bacillus* spp., a positive test of arabinose activity at 37 °C acts a hallmark of cellulolysis. The average number of colonies increased with the progress of root degradation in the subsequent stages of tree dieback. On the medium with CMC, cell number at 28 °C was 2.9 10^3, 9.2 10^3, and 9.9 10^4 cfu g^{-1} of rhizomorph in subsequent stages of dieback, while at 22 °C it was lower, at 1.1 10^3, 7.1 10^3, and 2.3 10^4 cfu g^{-1}. Cellulolytic activity at 11 °C was found only in single colonies.

The highest enzymatic activity of cellulase (β-glucosidase) was observed at 28 °C, in all samples. High activity was also noted at 22 °C, though only in the outer zone of rhizomorphs. In the inner zone, there was no activity at this temperature, with the highest potential observed in the first phase of dieback. These results indicate, for example, colonization of the inner rhizomorph channel by cellulolytic strains during rhizomorph growth and subsequent transfer of bacterial strains to its external part in subsequent phases of the disease process.

Chitinase activity was observed only in phases I and II of tree dieback. A high potential of esterases at 22 °C and 28 °C was also diagnosed. Naphthol-AS-BI-PH-ase, which is responsible for the dephosphorylation of complex aromatic compounds (phosphorus acquisition), denitrification, and the ability to absorb potassium gluconate and organic and inorganic acids as carbon sources and electron acceptors, was recorded in rhizomorphs collected from all phases of tree dieback.

The heat map (Fig. 5) and the left hierarchical dendrogram describing activity at variable temperature for each community collected from the surface of both sterilized and unsterilized rhizomorphs, allowed us to separate three significantly different groups. Within the range of temperatures, the enzymatic activity of bacteria at 28 °C was clearly different to that observed at 22 and 11 °C. Between 22 and 11 °C, no significant differences were observed. This is indicative of

Table 2
Diversity indices of microbiota inhabiting the *Armillaria ostoyae* rhizomorphs collected from phases I, II and III of tree dieback.

Indices	Phase I	Phase II	Phase III	Mean
Simpson's dominance (λ)	0.037	0.023	0.031	0.030
S-W diversity (H′)	4.478	4.847	4.605	4.643
Pielou's evenness (J′)	0.668	0.705	0.671	0.681

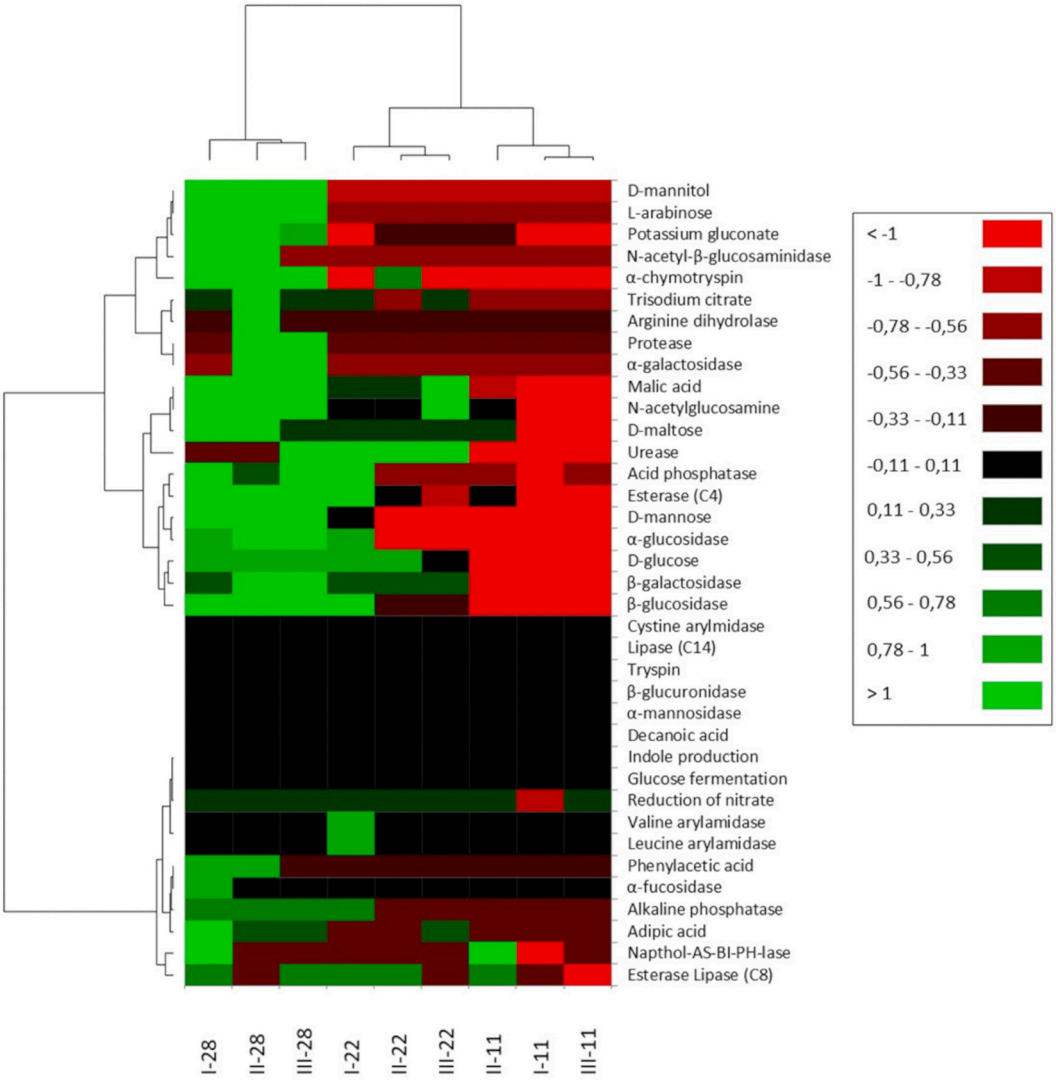

Fig. 5. Heatmap Cluster Analysis of the normalized data: potential biochemical activity expressed by microbiomes at 11, 22 and 28 °C in *Armillaria ostoyae* rhizomorphs collected from phases I, II and III of tree dieback.

the key influence of temperature on the enzymatic activity of the bacterial communities which accompany Armillaria root rot infections. In addition, at a temperature of 28 °C, there was a clear difference in activities between phases I, II and III. At 22 °C, only phase I differed in this respect, while at 11 °C phase II differed (whereby, in this case, only with respect to the low activity of three enzymes, without any clear overall effect on the enzymatic system). It is to be expected that progress in dieback of the host plant may be slowed with decreases in ambient temperature.

3.4. Cooperation and competition

Microbiomes inhabiting rhizomorphs collected from all phases of tree dieback were found to differ. Comparing the orders of microbiome with the phase of dieback of trees indicates strong relationships between many microorganisms and the first and second phases of dieback. A smaller number of orders were characteristic of the third phase of tree dieback. The first phase was related with high abundance of the Verrucomicrobiales, Clostridiales, Flavobacteriales, Selenomonadales, Coriobacteriales, Rhodospirillales and Burkholderiales.

The orders most frequently associated with phase II of dieback were the Planctomycetales, Nitrospirales, Nitrosomonadales, Acidobacteriales and Xanthomonadales. In phase III, there was no unique link to any order, however, the strongest relationships were observed with the Bacillales, Bdellovibrionales and Pseudomonadales. The orders Rhizobiales and Bacteroidales were commonly observed during phases II and III, while the Actinomycetales and Lactobacilliales were detected during phases I and II.

The distances between the first and second principal components identified by PCA (Fig. 6), which are associated with the phases, allowed us to state that each phase was unique in terms of the quality and quantity of its associated metabolic activity. Results regarding the physiology demonstrated at the optimal growth temperatures for saprotrophs (including the *A. ostoyae*) showed more features related to phases I and II than to phase III (7 in phase I versus 9 in phases II and III). Results indicate that enzyme activity was highest in phases I and II. A reverse-proportional correlation was observed between the group of enzymes with the highest activity in phase I (leucine arylamidase, valine arylamidase, α-glucosidase, β-glucosidase, α-fucosidase, D-mannose) and the groups of features associated with phases II and III: α-galactosidase and malic acid uptake ability. The group of metabolic features (α-chymotryspin, arginine dihydrolase, D-glucose, L-arabinose, uptake of potassium gluconate and trisodium citrate) with the highest activity in phase II was inversely correlated with the ability to utilize adipic acid. On the other hand, the ability to utilize D-mannitol and phenylacetic acid, as well as esterase lipase and *N*-acetyl-β-glucosaminidase

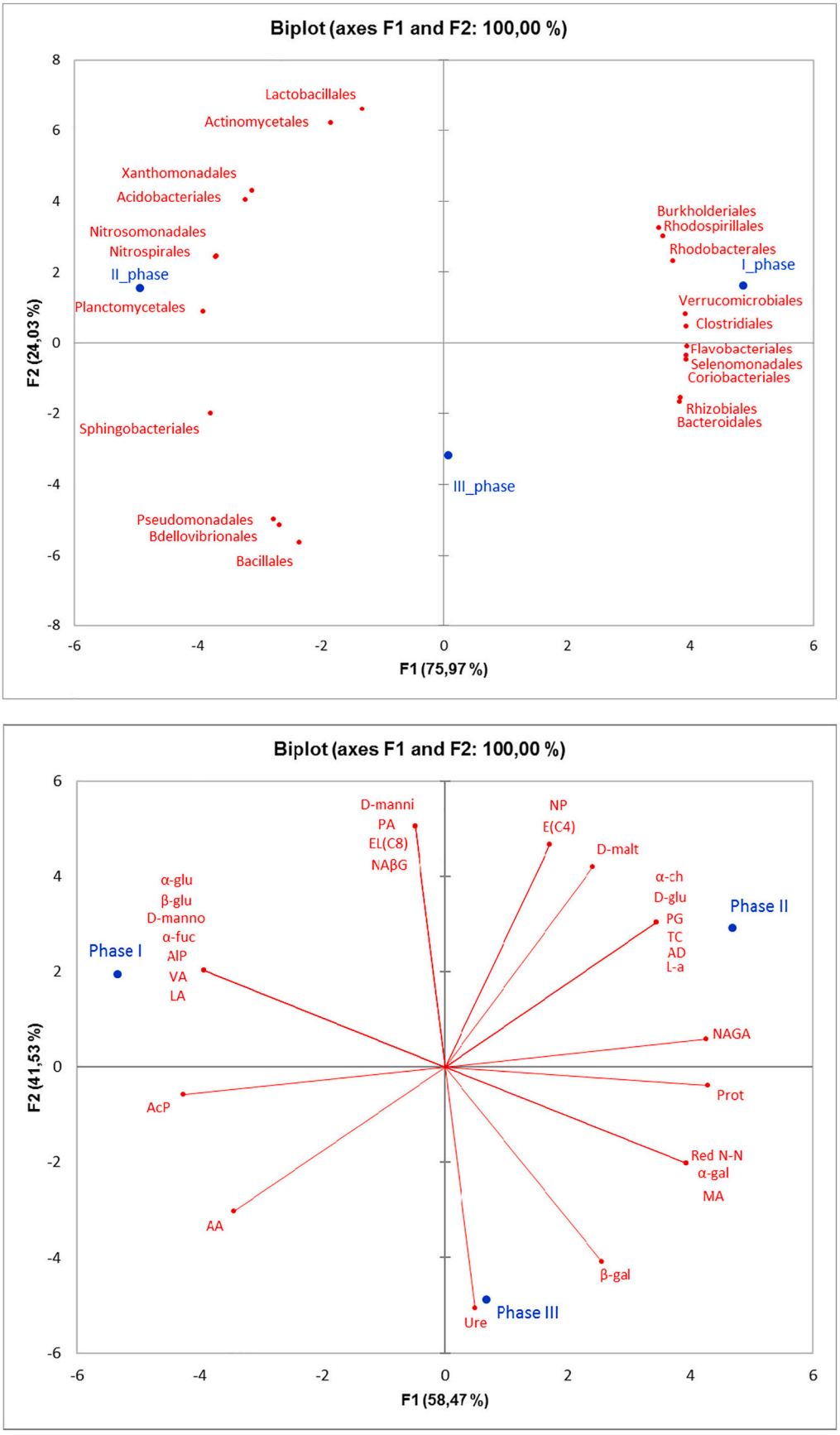

Fig. 6. Relation between the most abundant orders of bacteria (top) and metabolism properties (bottom) and their influence on phases of tree dieback, according to PCA.

(chitinase), was inversely correlated with urease activity, which was at its highest in phase III.

4. Discussion

The quantitative analysis, based on qPCR results, shows a significant increase in the 16S rRNA gene copy number in phase III compared to phase I and II. This indicates an increase in the size of the bacterial community in the rhizomorph for phase III of tree dieback, possibly due to a sharp increase in the availability of nutrients or to other factors related to the wood decay. Between phase I and II, a significant change was observed in the community though without any significant change yet being observed in quantity. Rinta-Kanto et al. (2016) attribute a similar number of copies of the 16 s rRNA gene (total bacteria) to wood in the state of degradation characterized by high density, and correlated quantitative changes with decreasing wood density. Analyzing genomic similarity and differences between the microbiomes of *Armillaria* rhizomorphs collected from particular phases of tree dieback caused by the pathogenic complex, we noted one fact of particular interest. This was that the level of differences, or the ability to detect them, is dependent on the level of taxonomic grouping specified due to uneven distributions of particular groups of bacteria in the microbiome. This influenced measures of differentiation in phases I and III of dieback. Among the dominant taxa, there was high homogeneity in quality. However, in terms of quantity, abundance varied significantly between phases, which explains the large differences seen in the AHC.

The high values of both Shannon's diversity index (H′) (usually evaluated in the range from 1.5 to 5), and Simpson's diversity index (λ), which was closer to 1, suggest communities are inherently variable and strongly adaptive to changes in the analyzed environment. This plasticity may enable communities to expand their range, and points to adaptation to a new environment and environmental pressure to adapt to changing factors. The values of the indexes used here indicate that the greatest variation and taxonomic diversity occurred in phase II of dieback, i.e. with the progressive degradation of the tree, and that these rates were lower at the beginning and end of tree dieback.

The infection process of *Armillaria* starts with an epiphytic phase, after which penetration occurs – by means of mechanical pressure together with some toxic action (Devkota and Hammerschmidt, 2020). Antibiotic toxins produced by the fungi, which may limit the bacterial community, are responsible for breaking down the protective barriers of the host plant (Dörfer et al., 2019). The actual phase of pathogenesis occurs after the enzymatic degradation of the cell walls of the host plant. Progressive degradation of the roots gives rise to symptoms which are visible on parts of the host above ground, which we refer to as phase II of dieback. In this phase, microorganisms showing the highest enzymatic activity were isolated. Similar observations were made by Proença et al. (2010) in the case of pine wilting caused by *Bursaphelenchus xylophilus*. The authors analyzed pines at varying degrees of infection, and from different areas. Despite different microbiomes varying by observation territory, they observed a constant tendency to modify the bacterial community of endophytes as the pathogenesis progressed. Bacteria and Actinobacteria living in roots of trees play an important role both as endophytes and saprotrophs in wood decay, metabolizing nutrients (e.g. pectin, cellulose, carbon and nitrogen) (Burke et al., 2006; Barriuso et al., 2008; Mallik and Williams, 2008; Izumi, 2011).

The diversity of the endophytes detected increased as dieback progressed, which was explained as a gradual activation of defense mechanisms and increased penetration of bacteria into the wood. Many *Bacillus* species (e.g. *B. cereus*, *B. subtilis*, *B. pumilus*) and Actinobacteria are known not only as promotors of plant growth, but also promote soil fertilization, and can be active in protecting against pathogens in forest trees or in reducing environmental stress (Gaiero et al., 2013; Nongkhlaw and Joshi, 2014; Kubiak et al., 2018). Pointing to the properties of the bacilli, it can be concluded that these cosmopolitan bacteria have very diverse metabolic traits. Bacteria of this type may have the potential to break down hard-to-decompose substances, and may engage in proteolytic activity, lipolytic activity, or breaking down chitin and cellulose. They also have the ability to form spores. Kubiak et al. (2017a) confirmed the cellulolytic potential of *Bacillus* spp. in post-arable soil with pine sawdust containing lignin, hemicellulose, and cellulose, which increased the population of *Bacillus* spp., such as *B. flexus*, *B. megaterium*, *B. cereus* and *Paenibacillus* spp. The same treatment with sawdust added to forest soil increased the share of *B. muralis* and *B. simplex*, as previously demonstrated by Wright and Cornelius (2012). They easily colonize habitats even at the expense of other microorganisms. Their properties have been used commercially, among other things, for controlling fungal pathogens of plants (Przemieniecki et al., 2018) and energy production (Kumar et al., 2017).

The bacilli were observed as subdominant in phase II. *Burkholderia* spp. were an occasionally detected species in all phases of dieback. Both genera were found in an endophytic community of roots of healthy pine stumps, while *B. sediminicola*, *P. telluris* were found in roots of dying pine stumps infected with *A. ostoyae*, and *B. cereus* and *P. pini* in dying stumps infected by *H. annosum* (Kubiak et al., 2016). This indicates their important role in wood decomposition.

Due to the limited number of reports regarding the *Parabacteroides* sensu stricto, and their decomposition of living wood or role in the pathogenesis of plants, Toczyłowska-Mamińska et al. (2018) studied the alleged co-metabolism of this genus with a microbiota typical of decomposing wood. In this study, they observed the natural growth processes of Parabacteroides after completing the decomposition process by testing energy acquisition via "cellulose fed" bacteria. In contrast, another cellulolytic genus *Clostridium*, added as a control inoculant to support the process, did not change its share in the total population of bacteria. It should be noted that these bacteria exhibit cellulolytic activity under anaerobic conditions. In this work, the Clostridiales and Bacteroides orders were present in the same abundance in each phase of tree dieback. Their presence indicates the presence of anaerobic conditions under which wood decay occurs.

In studies by Fang et al. (2013), the authors presented correlations between the mass of N- and C-fixing microorganisms and enzymatic activity. Their research showed that the activity of proteolytic enzymes increases along with the diversity of the population of microorganisms. In contrast, Okur et al. (2009) observed a positive correlation between the population of microorganisms and an increase in the activity of dehydrogenase, protease and urease and alkaline phosphatase. In the light of these assumptions, our work notes that there is a relatively small increase in total enzymatic activity. However, the highest enzymatic activity and the emergence of new enzymes relating to nitrogen demand (e.g. urease, α-chymotrypsin) point to the colonization or proliferation of groups of bacteria during dieback. This indicates the occurrence of more intense rhizomorph growth and the development of pathogenesis with a simultaneous increase in the quantity and activity of the bacterial community after breaking down the host plant's defense mechanisms. Regardless of the phase of tree dieback, the presence of maltose (starch)-utilizing microorganisms was constantly observed in the community, which is the main predictor of cellulolytic activity.

Talbot and Treseder (2012) have pointed to the role of nitrogen in cellulose and lignin decomposition, increasing *N*-acetyl-β-glucosaminidase (chitinase) activity in the early stages of decay, and described competition among microbial community decomposers over time. Aruwajoye et al. (2014) identified the largest population and a high cellulolytic potential for *Bacillus circulans*, a bacterial species whose population increased along with the progression of tree dieback. Other analyzes have shown that these bacteria can use carbon from CMC, starch, lactose and maltose for the production of cellulases. Bacteria were able to produce many enzymes involved in the degradation of plants (wood) cells: α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, and protease. They must also have had the ability to obtain substances resulting from the decomposition of organic matter in order to provide energy, electron acceptors and nutritional

possibilities at low pH or limited oxygen availability (e.g. malic acid). One important observation made in this experiment and confirmed by other authors (Álvarez et al., 2016; Singh et al., 2016) is the lack of ability to produce α-mannosidase, a typical fungal enzyme which participates in the distribution of lignin together with, among other things, α-galactosidase and β-glucosidase produced by bacteria.

In the present paper, we show there to be a high abundance of bacteria involved in the nitrogen cycle and dealing with nitrification, such as Rhizobiales (n-fixation), and also anammox bacteria. This is another mechanism by which bacteria interact with *Armillaria* and bacteria involved in the disease process. This perpetuates a constant supply of nitrogen, which is dependent on the growth of the entire community living in a rhizomorph. These dependencies have been described recently and the connection between nitrogen and pathogenic disease, as well as the link to the direct degradation of tree tissue, has been established in other previous studies (Johnston et al., 2016; Rinta-Kanto et al., 2016).

The results obtained indicate the flexibility and wide range of enzymatic activity, utilization of hemicellulose, lignin proteins and nitrogen, enabled by a microbiota that can flexibly adapt to environmental conditions, condition and type of wood. The inability to activate α-mannosidase indicates necessity of cooperation between 'rhizomorphs' and inhabiting microbiota used for parasitizing. Bacteroides, Clostridiales and Actinomycetes are associated with rhizomorphs regardless of the stage of development, while representatives of the Bacillales order increase the size of their population as tree dieback progresses.

Another important observation of this study relates to the synthesis of alkaline and acid phosphatases at a broad range of temperatures. Margalef et al. (2017) have already shown in a review paper that phosphatases in soil environments are produced more frequently and are more stable at high temperatures. The low temperature-tolerant bacteria may play a role in the sharing of phosphorus at the lowest temperatures. This mechanism required energy layers (e.g. ATP) and the synthesis of nucleic acids. In terms of our findings relating to the season of sample collection, it is probable that this symbiosis is a protection mechanism against a sudden decrease in temperature, allowing rhizomorphs to survive and function, while inhibiting microorganisms.

5. Conclusions

In summary, this study presents a new perspective on the role of the *Bacillus* genus in the pathogenesis of rhizome-producing fungi, which requires more detailed research to explain the mechanism behind this involvement. The total bacterial loads measured (qPCR) showed a constant abundance of bacteria linked with rhizomorphs in the first and second phases of tree dieback. In *A. ostoyae*, the whole process of pathogenesis, from infection to the end of life of the infected tree, was characterized by anaerobic bacteria coexisting with rhizomorphs. The Bacteroides and Clostridiales orders are two of the bacteria groups which are most effective at decaying under anaerobic conditions. The total community share of Bacilliales increases together in a linear relationship with progress in dieback. This may be related to the colonization of the rhizomorph by bacteria (the canal or its external part), or to the transport of bacteria through the rhizomorph channel. This is largely confirmed by the results of PCA, which indicate an inverse relation between the frequencies of specific groups of microorganisms and dieback of trees. This suggests that symbiotic relationships with different bacteria may be beneficial to rhizomorphs in certain given phases of pathogenesis (tree dieback), in association with the changing availability of simple energetic and building materials resulting from decomposition or co-metabolism. A very wide range of enzymes produced by bacteria points to the possibility of hydrolysis of compounds containing esters, phosphorus bonds, proteins and sugars, including cellulose and hemicellulose, which, combined with the activity of the rhizomorph's own enzymes, creates an excellent mechanism for wood decay. The bacterial taxa identified as playing an essential role in the particular phases of infection of pine roots by *Armillaria* could have a useful application in bio-monitoring as indicators of tree dieback in managed forests.

Data availability

The raw Illumina sequencing data generated in this study is available in the MG-RAST database (accession number: 4791502.3 phase I, 4791501.3 phase II and 4,791,500.3 phase III, https://www.mg-rast.org/mgmain.html?mgpage=project&project=mgp85259).

Funding

This work was supported by statutory funds from the University of Warmia and Mazury in Olsztyn Nos. 20.610.029-300; 20.610.016-300 and from the University of Agriculture in Krakow No. 010014-D011.

CRediT authorship contribution statement

Sebastian Wojciech Przemieniecki: Conceptualization, Methodology, Software, Validation, Formal analysis, Data curation, Writing – original draft, Visualization, Supervision, Project administration. **Marta Damszel:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Funding acquisition. **Sławomir Ciesielski:** Software, Formal analysis, Data curation, Writing – original draft. **Katarzyna Kubiak-Siwińska:** Formal analysis, Data curation, Writing – original draft. **Jędrzej Mastalerz:** Writing – review & editing. **Zbigniew Sierota:** Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition. **Anna Gorczyca:** Validation, Writing – review & editing, Funding acquisition.

Declaration of competing 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.

Acknowledgements

The authors would like to thank the anonymous reviewers for their valuable comments and suggestions.

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