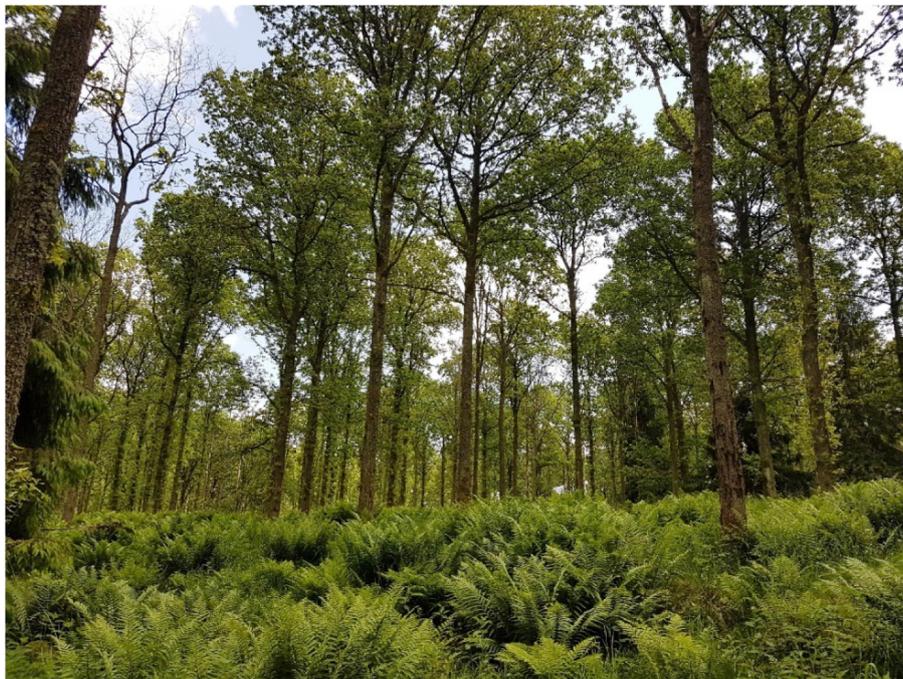




Fungal endophyte communities in declining and vital oak (*Quercus robur* L.) trees



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Master Thesis no. 293

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Abstract

The role of microbiome in tree health is receiving increasing interest in forest pathology research. This study focuses on the abundance and diversity of fungal endophytes in pedunculate oak (*Quercus robur* L.) differentiating in vitality. Earlier studies with peri-urban, young oak trees have indicated that fungal endophyte diversity in woody tissues of oaks is lower in highly vital trees. In this thesis, the hypothesis that declining trees harbour a higher fungal diversity was tested in a production forest setting, using older trees. To test the hypothesis, endophytes were isolated from bark and xylem of *Quercus robur* L., using two types of agar-media to select for a broad range of fungi. Based on the morphological characters of the colonies the isolates grouped into 38 morphotypes and a group of singletons, and the frequency and diversity of fungi in different tissue types (bark and xylem of differently vital trees) was compared. The results indicate that endophyte communities of *Quercus robur* L. xylem and bark are more diverse in trees showing some symptoms of decline in comparison to the seemingly healthy-looking trees, but that the diversity was reduced in the most strongly declined trees. In addition, the results confirmed the earlier findings showing that bark associated endophyte communities are more diverse than xylem associated communities. I also found that samples of water agar harbored different assemblage of morphotypes comparing to malt extract agar. Future studies are required to characterize fungal endophyte communities in order to evaluate the interactions that take place within the complex, understudied networks and apply them in ecosystem management.

Keywords: *endophytic fungi diversity, Quercus robur* L., *forest microbiome, morphological identification of fungi, plant-endophyte interaction.*

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1. INTRODUCTION

The plant microbiome is composed of complex archaeal, protistic, bacterial and fungal communities (Hardoim et al. 2015). The concept of the plant microbiome links microbial ecology with host's biological and functional characteristics. It can be viewed as an additional pool of genes that extends a host's ability to adapt to certain environmental conditions (Vandenkoornhuyse et al. 2015). Manipulation of plant microbiota can induce resistance (Ganley et al. 2008), increase agricultural production (Le Cocq et al. 2017) and can even contribute to the regulation of methane emission (He et al. 2015). In the recent decade there has been an increasing number of integrative studies regarding the forest microbiome. Baldrian (2017) points out that the current research on forest microbiome has been largely focused on rhizosphere, deadwood and litter, while microbiota of living above ground organs are still rather underexplored. Since many natural systems act only as temporal networks, determining a core microbiome, i.e., taxonomic members that are common to two or more microbial assemblages associated with a habitat (Shade & Handelsman 2011) would allow to recognize reliable systems that could form solid foundations of various forest management systems. Undoubtedly, the analysis of temporal and spatial variation could reveal valuable information about the stability and persistence of the core microbiome (Björk et al. 2017).

Endophytic (plant internal) fungi are an important part of the functional plant microbiome. These fungi form a taxonomically heterogeneous group that exhibit imperceptible but apparently complex associations with their hosts. Endophytic fungi reside asymptotically and may remain quiescent until favorable conditions arise through changes in either environmental conditions or host's ontogenetic state. Endophytes have been isolated from every major plant lineage to date (Rodriguez et al. 2009) and they seem to be present in all kinds of habitats, ranging from arctic to boreal (Higgins et al. 2007) to tropical (Pinnoi et al. 2006) ecosystems. During the last decades endophytic fungi have received a substantial attention due to their great potential to contribute to the natural ecosystem functioning as mediators of ecological interactions, shapers of plant communities and enhancers of host plant resistance

and resilience (Zhang et al. 2006). Several estimates suggest that there are more than a million of fungal endophyte species present on Earth, however, only around 74 thousand have been described so far (Hawksworth 2001). The appreciation of fungal endophytes is growing, yet it is still a relatively unexplored layer of biodiversity. The high taxonomic diversity (Hawksworth 2001) and ability to synthesize a great range of bioactive compounds (Schulz et al. 2002) indicate that an immense application potential is locked within the endophytic communities.

In general, two main groups of fungal endophytes based on their transmission mode can be distinguished, horizontally and vertically transmitted (White 1988; cited in Aas 2010). Vertical transmission involves a transfer of a fungus from a host to offspring through an infected seed, whereas horizontal transmission accounts for sexual or asexual spore transfer (Hartley & Gange 2009). White (1988) classified fungal endophytes into two major groups according to their phylogenetic and historical traits, especially accentuating the mode of transmission. The two described groups were: clavicipitaceous and non-clavicipitaceous endophytes (White 1988; cited in Aas 2010). Clavicipitaceous endophytes form systemic associations with most grasses, whereas non-clavicipitaceous are associated with non-vascular plants, ferns, conifers and angiosperms (Rodriguez et al. 2009).

Rodriguez et al. (2009) described four functional classes based on host range, colonization pattern, transmission, and fitness. Class 1 are the clavicipitaceous endophytes that represent a narrow range of phylogenetically related species that form highly localized infections with cool and warm season grasses (Zhang 2006; Aas 2010). Class 2, Class 3 and Class 4 belong to non-clavicipitaceous endophytes and are highly diversified. Class 2 endophytes are mostly members of Ascomycota or Basidiomycota that colonize stems and leaves. Class 3 endophytes have a broad host range and inhabit solely above ground plant organs and are horizontally transmitted. The growth of Class 4 is restricted to plant roots (Rodriguez et al. 2009) and endophytes of this class are horizontally transmitted. Their presence has been associated with increased stress in habitats (Aas 2010).

In plant-endophyte relationships, symbiosis can range from mutualism to commensalism (Schulz & Boyle 2005; Singh et al. 2011). The association is fundamentally dependent on the balance between the defense responses of the plant and the nutrient demand of the endophyte (Kogel et al. 2006). A balanced system will result in mutualism or commensalism, however, if the balance is disrupted, parasitism may manifest itself and the interaction will either become detrimental to the host or will exclude the endophyte. It used to be widely accepted that an endophyte is restricted to a certain association, however, some authors (Saikkonen et al. 1998; Faeth & Bultman 2002) suggest that this relationship is neither ecologically, evolutionary nor geographically fixed. Depending on environmental factors, physiological or genetic status of the host genotype, the association can change, meaning that at a certain point in the life cycle and under certain conditions an endophyte can switch the association, e.g. becoming from mutualistic to parasitic, or antagonistic (Müller & Krauss 2005; Schulz & Boyle 2005; Singh et al. 2011). Often endophytes of different association reside asymptotically in the same host and remain dormant until senescence (Singh et al. 2011).

Some symbiotic relationships exert beneficial effects. Mutualistic association can greatly influence plant adaptation to new habitats, alleviation of biotic (e.g. herbivory) and abiotic (e.g. drought, salinity, temperature fluctuation) stress, increase plant resource allocation, biomass and productivity (Stone et al. 2004; Rodriguez et al. 2009). Antagonistic fungal–fungal interactions can inhibit pathogen growth by secreting metabolites. For example, the endophyte *Fusarium verticillioides* limits *Ustilago maydis* growth within their shared plant host maize, *Zea mays* (Rodriguez-Estrada et al. 2012). Another important role endophytes play is that as pioneer colonizers of plant tissues they can act as catalyzers for biological degradation of the host plant, which results in nutrient recycling (Boberg et al. 2011).

Analyses of plant-pathogen interactions have been predominantly focused on a simple model of two-way interactions (host-endophyte), however, such a view excludes multiple species interactions. A more thorough consideration of multiple interactions between plants and their microbial associates could be greatly advantageous if we

want to understand the ecology of these interactions and utilize the mechanisms e.g. in sustainable plant protection. In a recent review by Abdullah et al. (2012) three pathogen co-infection strategies were described: (1) Competition - competing pathogens; (2) Cooperation - mutually beneficial pathogen interactions; (3) Coexistence - pathogen coexistence through niche specialization. For a fungus to grow asymptotically in a host plant, a balanced antagonism is required to exist between the host defense system and fungal virulence (Schulz & Boyle 2005). Studying the patterns and aspects that change the association would allow to better predict and control pathogen outbreaks. Certain fungal endophytes exhibit a degree of specificity to a certain host and Zhou & Hyde (2001) delivered evidence of 'host exclusivity' and 'host recurrence'.

Oak, *Quercus* spp., is a long lived, broad-leaved tree (Eaton et al. 2016) and the genus consists of approximately 450 species worldwide (Evans 1984). Pedunculate oak (*Quercus robur* L.) is the most common oak species in Sweden. It holds great economic and ecological importance and has taken deep roots in the Swedish culture. Owing to its outstanding hardwood properties, it was once heavily utilized for shipbuilding in Sweden during the 18th century, and nowadays is an important material in flooring industry. During the recent years, several diseases have severely affected and threatened the vitality of Pedunculate oak with oak decline attracting the greatest attention and causing a rapid decline in vitality (Denman et al. 2014).

Earlier, endophytic communities of *Q. robur* varying in vitality have been studied in young trees growing in peri-urban environment (Agostinelli 2012). In the current study, the focus is instead on endophyte communities of *Q. robur* trees in a production forest. The overall goal of my thesis was to add to our knowledge about the variation of endophyte communities in declining vs. healthy trees. The primary objective was to compare the fungal endophyte communities in oak trees differing in their vitality. Based on results of earlier studies (Agostinelli 2012; Martín et al. 2013), I hypothesized that 1) endophyte communities would be more diverse and the infections more frequent in trees that show signs of decline, as compared to trees that show high vitality; and that 2) the differences would be more pronounced in wood as

compared to bark. To test these hypotheses, I studied the culturable fraction of the fungal diversity in bark and xylem of *Q. robur* trees showing different degree of vitality (low, medium, high). I also tested whether the nutritive composition of agar medium significantly influenced the yield of fungi.

2. MATERIALS AND METHODS

2.1. Study site

The study site is located in the southern part of Sweden in Skåne County (55°30' 16.3" N and 13°25' 26.9" E). An even aged (ca. 70 years) *Quercus robur* L. stand of approximately 15 hectares at Börringekloster estate was used as study object, with kind help of the forest manager Henrik Nilsson (SUSAB). The bedrock is covered by a thick layer of glacial till mainly consisting of limestone, sandstone and marble (Sveriges Geologiska Undersökning, scale 1: 1 000 000) (Lantmäteriet) and the study area rests on a gentle north-facing slope and is situated at an elevation of approximately 95 meters above the sea level. The ground layer vegetation is dominated by *Urtica dioica*, *Dryopteris* spp., *Pteridium aquilinum* and *Poaceae* spp. in more elevated parts (Figure 1).



Figure 1. An overview of the study site representing an even aged oak stand and the underground vegetation. Photo: Johanna Witzell

2.2. Sampling procedure

Sampling of tissue samples for endophyte analyses took place during March 2017. A primary source of information about the vitality status of the trees was the inventory data acquired during the vegetation season of 2016 (surveyor: Anna Riebe) that provided information on tree crown conditions, development of epicormic and dead branches as well as of any visible insect damages (Figures 2, 3). Since crown transparency greatly reflects a tree's general health status (Metzger & Oren 2001), it was used as the main vitality indicator.



Figure 2. *Epicormic branches on oak.*

Photo: Johanna Witzell



Figure 3. *Dead and declining trees present on the site.*

Photo: Johanna Witzell

Based on the inventory data, trees were categorized into three main classes: (1) high vitality- healthy; (2) medium vitality- an early stage of decline and (3) low vitality- advanced state of decline. Dead trees were excluded from the study to avoid biased results due to the presence of saprophytic fungi.

Out of each class 10 trees were selected so that they represented the whole area, i.e., the sample trees in different classes were distributed evenly throughout the site (Figure 4). Using a cork borer, samples of each tree were acquired from the four cardinal directions (N, E, S, W), each sample containing bark and outer xylem (appr. 0.5 cm below the inner bark) tissues (Figure 5). All samples were assigned an identification number and placed in separate tubes containing sterile water to protect them from drying. The samples were kept in a refrigerator until processed (within 3 days from sampling).

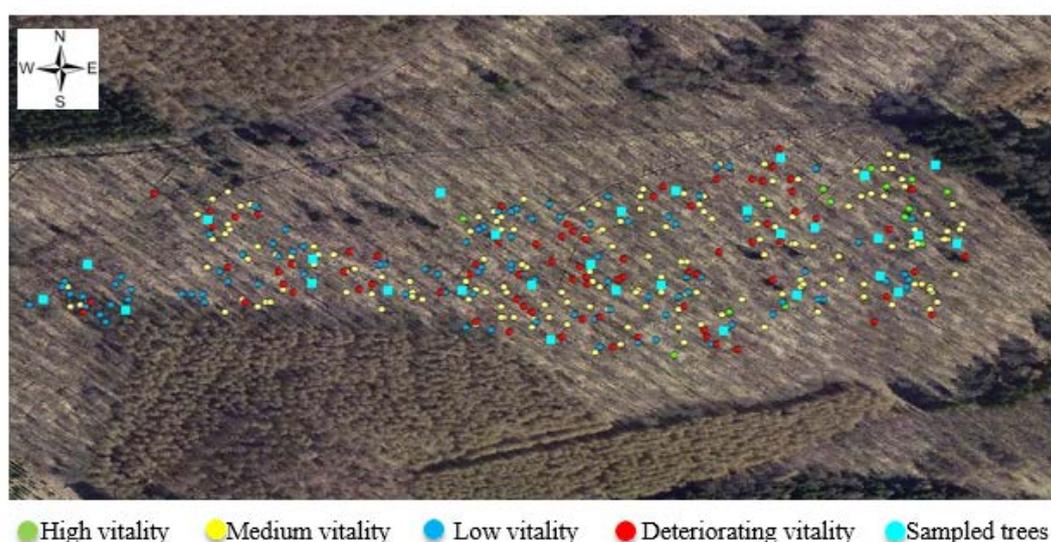


Figure 4. Satellite view of sampling with the selected trees.

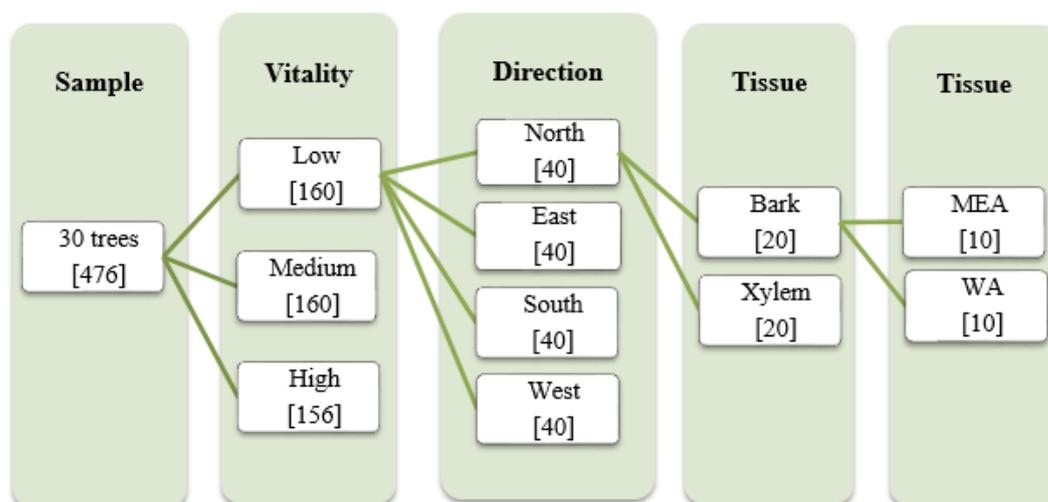


Figure 5. Schematic presentation of sampling. Numbers in brackets indicate the number of samples plated on agar.

2.3. Fungal endophyte isolation and morphotyping

Endophyte isolation protocol described by Martín et al. (2013) with minor alterations was applied. All the following procedures were carried out under the laminar hood to avoid contamination. Elimination of epiphytic organisms and other contaminants was achieved by surface sterilization. The surface sterilization solution concentrations and the treatment time frames were as follows: 75% ethanol for 30 seconds; 4% Na-hypochlorite for 5 minutes; 75% ethanol for 15 seconds. After air drying for 5 min, appr. 5x5 mm squares were aseptically cut from bark and xylem parts. The squares were then placed on agar to allow internal fungi to grow out. The effect of media can inhibit or stimulate the growth of certain fungi, thus, to be able to attain potentially greater diversity, the two following media types were selected: 2% Malt Extract Agar (MEA) for the fast-growing fungi and a nutrient poor 1.5% Water Agar (WA) to capture also the slower growing fungi (see Appendix A for media receipts). Petri dishes were sealed with Parafilm to hinder drying and incubated at room temperature in darkness. Monitoring of fungal emergence was done on a weekly basis. Subculturing of the emerging fungal hyphae to ME was carried out to obtain a pure fungal culture. A piece containing hyphal strings was taken from the growing (outer) edge of the colony, to ensure that actively growing, pure cultures were captured. Samples that were contaminated or showed growth of several fungi were re-cultured to recover pure cultures (isolates).

The fungal isolates were examined periodically and grouped to morphotypes based on their morphological characters such as colony growth, shape, elevation, mycelium color and presence or absence of aerial mycelium and droplets (see Appendix B and C). For an isolate to belong to a certain morphotype at least three typical characters needed to be distinguished.

2.4. Data analysis

In this study, **richness (S)** refers to the number of morphotypes in the endophyte community of certain sample type (bark or xylem from low, medium or high vitality trees), whereas **abundance (N)** refers to the absolute number of isolates.

Samples that were colonized (i.e., yielded at least one fungal isolate) were counted and **colonization frequency (CF)** was calculated as described by Hata & Futai (1995) in order to evaluate the suitability of the tissue type as a substrate for fungal infections as well as to determine the influence of tree vitality.

$$CF(\%) = \frac{N_{\text{colony}}}{N_{\text{total}}} * 100$$

Where N_{colony} corresponds to the number of samples colonized by at least one endophyte and N_{total} to the total number of samples plated.

The **relative abundance (RA %)** was calculated for each morphotype as the number of all isolates belonging to the morphotype in question, divided by the total number of isolates in a sample type, expressed as a percentage.

To study the fungal endophyte community diversity of examined sample types, **Shannon diversity index** was calculated (Shannon, 1948):

$$H' = \sum_{i=1}^S p_i \ln p_i$$

where S equals the number of morphotypes, p_i is the ratio of individual isolates of morphotype i divided by all isolates of all morphotypes.

Evenness (E) represents the degree to which individuals are distributed within the community. An evenly distributed fungal community is assumed when the index

value approaches 1. On the contrary, a value equal to 0 will imply a complete unevenness with only one dominant taxa. Considering that evenness is not calculated independently, rather derived from other measures, it is mostly used as a predictive measure. Evenness is determined by the formula (Hammer *et al.* 2001):

$$E = \frac{H'}{\ln S}$$

where H' is Shannon diversity index and $\ln S$ a natural logarithm of species richness (S).

To describe the similarity between endophyte communities of the different sample types, **Jaccard similarity index (J)** was calculated on the basis of presence/absence data (Jaccard, 1912):

$$J = \frac{C}{A+B-C}$$

Where A and B are the total number of morphotypes from any two hosts and C indicates the number of shared morphotypes. Concerning this study, similarity index was used to compare the morphotype communities between the xylem and bark in the three vitality classes.

Rarefaction curves were calculated to compare species richness among the tissue types and the tree vitality using PAST 3.20 software.

To evaluate temporal variation in tree condition, I used the tree vitality data acquired during the follow up inventory in 2017 (surveyor: Linn Jonsson).

Microbiome analysis was based on an approach presented in Shade & Handelsman (2012) where shared abundance and shared connectivity data were studied. Assessment of this kind may help to avoid the potential of skewed data (attributed to rare isolates) and an inherent overrepresentation of the most common morphotype.

The three previously described vitality classes were regarded as separate communities and the MTs in them were analysed for:

1. **Shared membership** - to determine which morphotype falls into which community the quantitative abundance data was transformed into presence/absence. If a morphotype was present across all three communities, it was ranked as 'core'. Presence across two communities or one community was also recorded.
2. **Shared connectivity** - members sharing an identical abundance value across all communities were ranked as 'core'. If the abundance value was shared among two communities, the relevant group was assigned.

All analyses included a combined data of samples of MEA and WA. Data was only separated for highlighting the influence of media on the overall endophyte community. 'Samples' refer to the tissue pieces that were initially plated on MEA or WA agar, whereas 'isolates' refer to fungal endophytes that were recovered from the initial plates on MEA only during the second stage.

One-way ANOVA tests were carried out in order to determine whether there are any statistically significant differences between the means of any groups. Wilcoxon Signed Rank test was used for multiple comparisons that account for a statistical difference. Species richness, diversity, evenness and similarity calculations were done using PAST 3.20 and SAS software JMP 14.

3. RESULTS

Out of the total of 476 plated tissue samples, 291 (61%) yielded at least one endophyte isolate. Samples collected from the medium vitality class yielded the highest absolute and relative number of isolates and morphotypes (Table 1).

Table 1. Distribution of endophytic fungi obtained from *Quercus robur* L. xylem and bark. Shown are the absolute counts and percentages of the total number of plated and colonized samples, recovered isolates (N) and morphotypes (S).

	Low	Medium	High	Total
Bark				
Plated	80 (33%)	80 (33%)	78 (32%)	238 (100%)
Colonized	70 (32%)	80 (36%)	71 (32%)	221 (100%)
Isolates (N)	79 (31%)	92 (36%)	85 (33%)	256 (100%)
Morphotypes (S)	25	33	25	38
Xylem				
Plated	80 (33%)	80 (33%)	78 (32%)	238 (100%)
Colonized	21 (30%)	31 (44%)	18 (26%)	70 (100%)
Isolates (N)	23 (31%)	32 (43%)	20 (27%)	75 (100%)
Morphotypes (S)	13	20	9	24
Total				
Plated	160 (33%)	160 (33%)	156 (32%)	476 (100%)
Colonized	91 (31%)	111 (38%)	89 (31%)	291 (100%)
Isolates (N)	102 (31%)	124 (37%)	105(32%)	331(100%)
Morphotypes (S)	27	37	25	39

3.1. Morphotype abundance

The abundance data revealed few frequent morphotypes, accompanied by a cohort of rarely isolated morphotypes (Figure 6). MT39 was composed of 25 singleton species. In total, there were 10 doubleton groups. The endophytic community was largely dominated by two morphotypes: MT1 and MT37, with both groups containing 34 individuals.

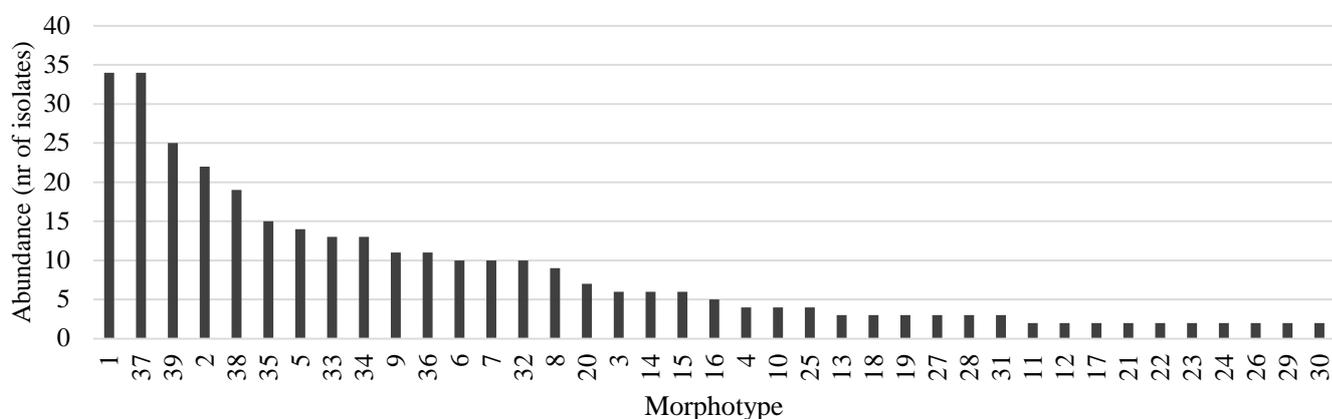


Figure 6. Morphotype abundance (of xylem and bark of three vitality classes together) in descending order.

3.2. Colonization frequency (CF)

Overall, colonization frequency (CF) of fungal endophytes was higher in bark than in xylem (92.86 % and 29.41 %, respectively; data from all vitality classes included). Colonization frequency tended to be the highest in medium vitality classes (bark 33.61 %; xylem 13.03 %) (Figure 7). In bark, both low and high vitality classes exhibited almost indistinguishable CF values (29.41% and 29.83%), while in xylem the difference between the two vitality classes was slightly more pronounced (xylem of low – 8.82 % and xylem of high – 7.56 %).

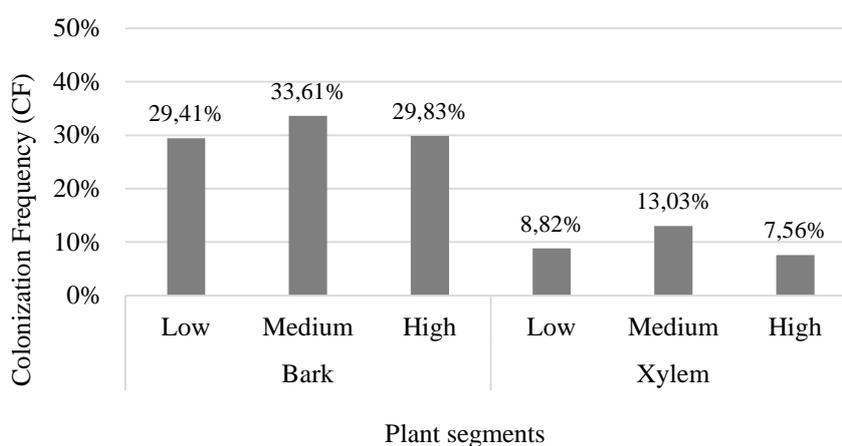


Figure 7. Colonization frequency of fungal endophytes isolated from *Quercus robur* L. xylem and bark calculated as the proportion of colonized from plated specimens in each tissue type and respective vitality class.

3.3. The effects of agar medium on the results

Slightly more positive samples (yielding at least one isolate) were found among the samples placed on MEA as compared with WA (Table 2), however the difference was not statistically significant (1-way ANOVA, $F= 0.1261$, $p= 0.7244$) (See Appendix D Table 5). The highest colonization frequency in bark was observed in medium vitality samples of MEA (18.07%), whereas in xylem the greatest value was observed in medium vitality class of WA samples (6.72%). Overall, 37 morphotypes were present in MEA samples and 32 in WA.

Table 2. The influence of WA and MEA media on fungal endophyte colonization frequency (CF) and richness (S).

		BARK			XYLEM			Total
		Low	Medium	High	Low	Medium	High	
WA	CF (%)	14.71	15.55	13.45	5.04	6.72	4.20	59.66
	S	18	23	24	9	13	9	32
MEA	CF (%)	14.71	18.07	16.39	3.78	6.30	3.36	62.61
	S	26	29	21	11	11	5	37

3.4. Species accumulation curves

Species accumulation curves show the relationship between the number of isolates and the number of taxa identified (Zabalgoitia, 2009). A non-asymptotic curve suggests that by increasing the number of isolates, the number of discovered taxa will also increase, whereas an asymptotic curve will indicate that all the associated taxa have been collected.

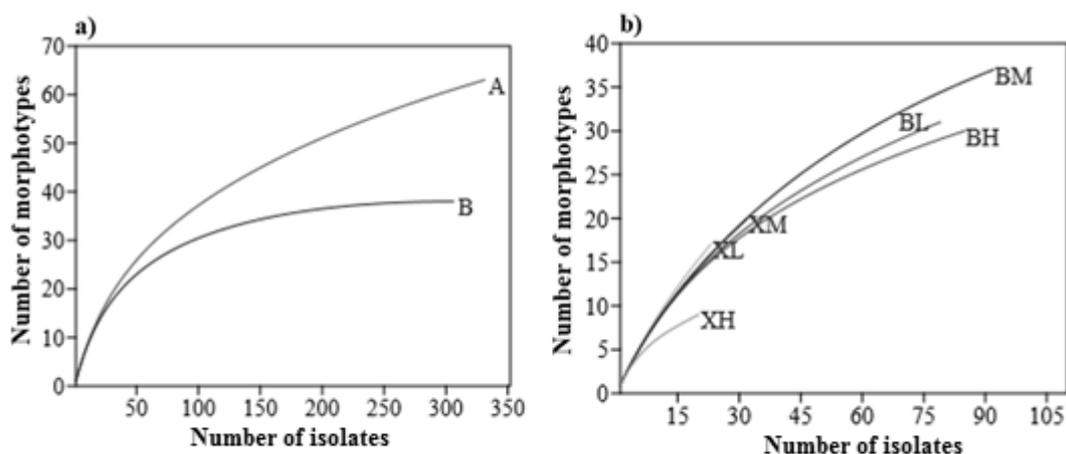


Figure 8. Species accumulation curves of fungal communities: a) A- includes singleton isolates which were regarded as separate morphotypes; B- includes only morphotypes that were represented by at least 2 isolates.; b) Species accumulation curves for xylem (X) and bark (B) isolates in different vitality classes, L=low, M=medium and H=high with without singletons.

The species curve (B) constructed without singletons, reaches an asymptote (Figure 8a), whereas the singleton curve (A) is leading towards a non-asymptotic pathway. A more detailed analysis (without singletons), revealed how communities were affected by the singleton isolate absence (Figure 8b). As it can be observed, most of the curves exhibited a continuous, non-asymptotic growth, apart from the curve representing xylem of high vitality, which steered slightly towards the asymptotic pathway.

3.5. Relative abundance (RA)

Overall, there were seven morphotypes that exhibited $RA \geq 50\%$ in xylem, while in bark 35 morphotypes had $RA \geq 50\%$. Fifteen morphotypes were solely associated with bark whereas one (MT12) was specific to xylem. (Figure 9).

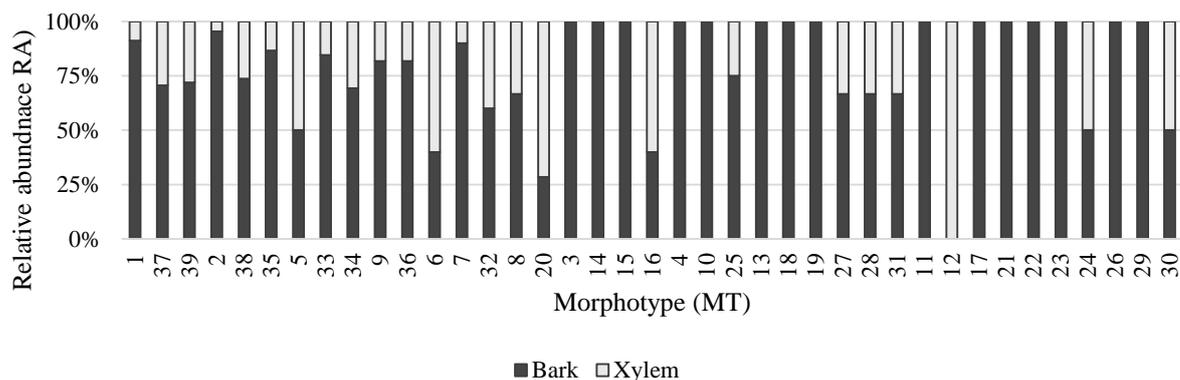


Figure 9. Relative abundance of morphotypes in xylem and bark.

Relative abundance data shows that MT15 expressed an absolute affinity to trees of high vitality, MT11, MT22, MT24, MT26, and MT28 to medium and MT17 to low vitality (Figure 10).

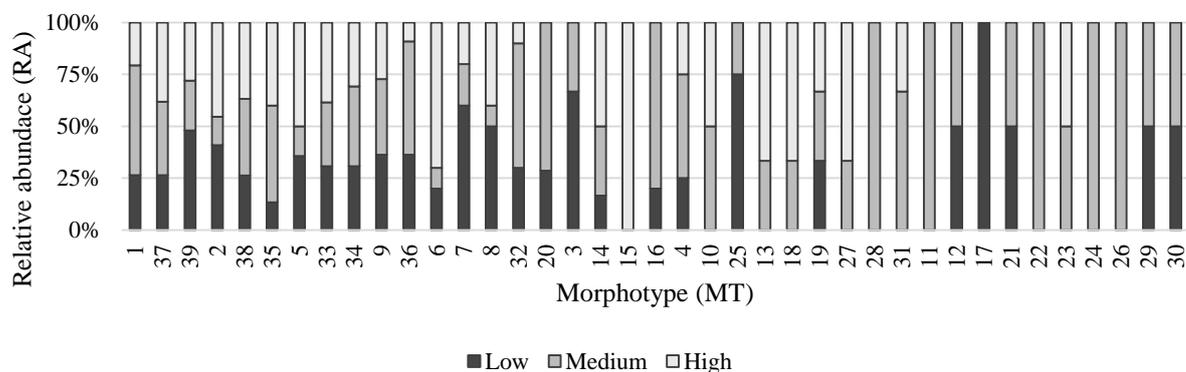


Figure 10. Relative abundance of endophytic fungi isolated from *Quercus robur* L. differing in vitality.

Different morphotypes dominated in different sample types (Figure 11). MT1 dominated the endophytic assemblage in bark tissue followed by MT37 and MT2. In bark tissue, MT1 was highly abundant in trees of medium vitality, while other morphotypes were more evenly present in all classes of vitality. In xylem tissue, MT37 appeared to be the most abundant representative, followed by MT5 and MT39 (singletons). In xylem tissue, presence of MT39 was associated with trees of low vitality and individuals belonging to MT6 associated with high vitality.

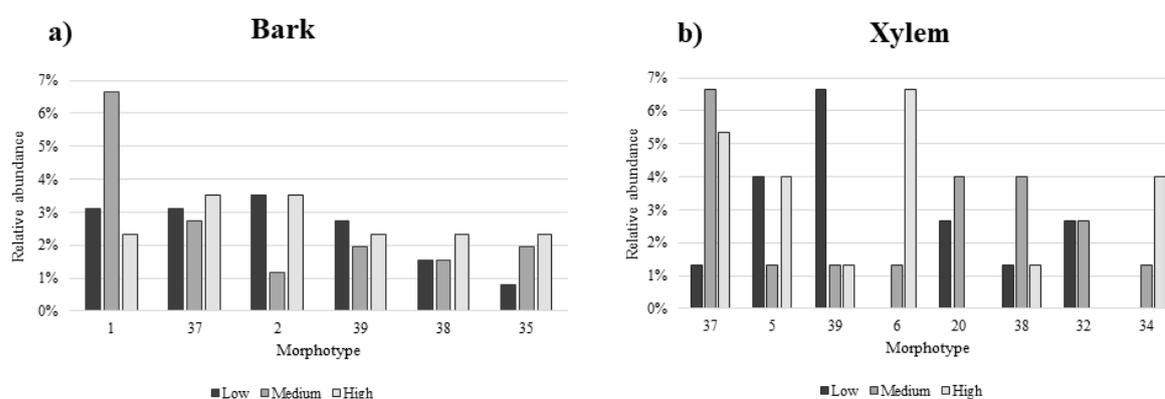


Figure 11. Relative abundance (RA%) of fungal endophytes (fungal endophytes with the overall RA \geq 5%) of a) bark and b) xylem tissue at different level of vitality.

On the whole, xylem was dominated by morphotypes which expressed more distinct affiliation to a certain class of vitality, whereas, dominant morphotypes of bark inhabited tissues of all vitality classes more uniformly.

3.6. Diversity analyses

Diversity measures confirmed that the most abundant and species rich fungal endophyte communities were found in medium vitality class (Table 3). The least diverse fungal community in bark was found in low vitality class, while in xylem fungal endophytes of high vitality accounted for the lowermost value of abundance and richness.

Table 3. Diversity indices of fungal endophytes. Letters indicate significant difference (Wilcoxon pairwise comparisons) (See Appendix D Table 4).

	BARK			XYLEM		
	Low	Medium	High	Low	Medium	High
Individuals (N)	79	92	85	23	32	20
Richness (S)	25	33	25	13	20	9
Shannon (H')	3.13 ^a	3.24 ^a	3.10 ^a	2.73 ^a	2.82 ^a	1.99 ^b
Evenness (H'/lnS)	0.910	0.896	0.912	0.963	0.941	0.904

Shannon diversity index fluctuated from 3.10 to 3.24 in bark and from 1.99 to 2.82 in xylem (Table 3). No significant variation was detected among tissue types (1-way ANOVA, $F= 2.652$, $p= 0.1085$) (See Appendix D, Table 1), nor among vitality classes (1-way ANOVA, $F=0.6617$, $p= 0.5178$) (See Appendix D, Table 2). Pairwise comparisons revealed that xylem tissue of high vitality differed the most from all the other bark segments in terms of diversity (Appendix D, Table 3).

Evenness measures in all segments ranged from 0.896 to 0.941, and the values were higher for xylem than bark, however, the distinction was not statistically significant (1-way ANOVA, $F=0.01911$, $p=0.8903$) (See Appendix D, Table 4). Bark isolates belonging to medium vitality accounted for the lowest measures in evenness (0.896), while xylem isolates of low vitality exhibited the high 0.963.

3.7. Similarity analyses

Pairwise comparisons of Jaccard similarity index suggest that the communities of different tissue possess low similarity, therefore, divergence was observed. Overall, bark communities exhibited greater within-group similarity than those of xylem (Table 4).

Table 4. Jaccard's similarity indices of different segments based on presence/absence data.

		BARK			XYLEM		
		Low	Medium	High	Low	Medium	High
BARK	Low	1.00					
	Medium	0.61	1.00				
	High	0.56	0.61	1.00			
XYLEM	Low	0.41	0.35	0.36	1.00		
	Medium	0.45	0.43	0.45	0.43	1.00	
	High	0.36	0.24	0.36	0.29	0.38	1.00

The highest (0.6<) similarity was observed between communities BM:BL and BM:BH. Similarity indices also revealed a low similarity (>0.3) between groups XH:XL and XH BM. In xylem, fungal communities of XM and XL (0.43) were more similar than communities of XM and XH (0.38).

3.8. Microbiome analysis

Shared membership analysis revealed 19 morphotypes that could be considered as core morphotypes. Based on shared connectivity data, morphotypes with the same abundance across all vitality groups were: MT9, MT19 and MT38. By compiling findings from both figures, it could be summarized that MT9, MT19 and MT38 may potentially contribute to the core microbiome of all three communities examined (Figure 12).

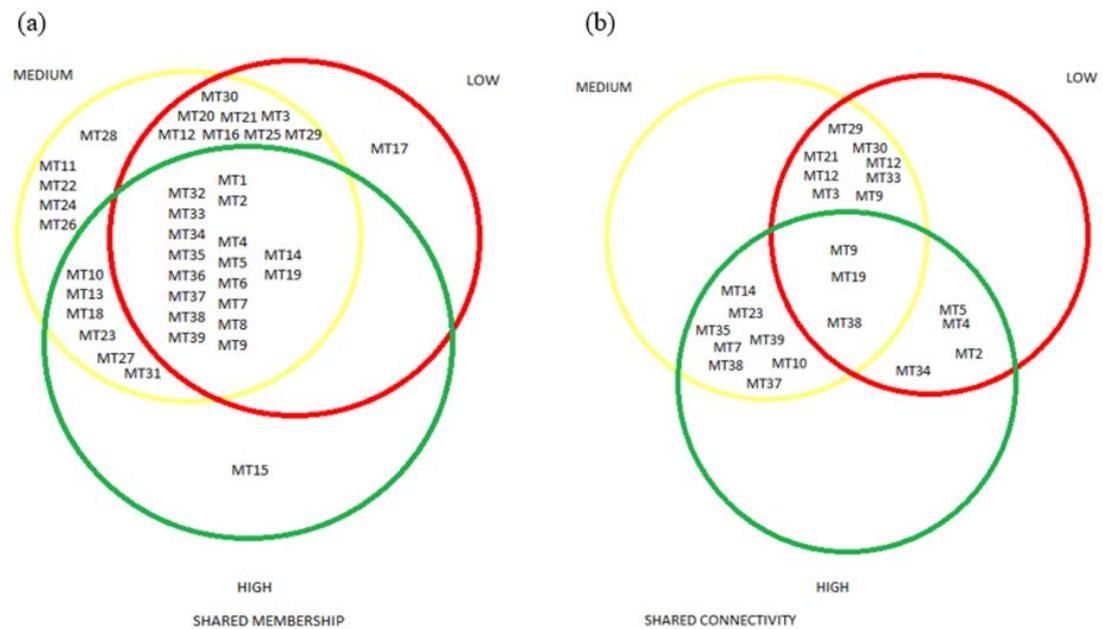


Figure 12. Venn diagram showing shared membership and shared abundance across fungal endophyte communities of *Quercus robur* L.

3.9. Changes in tree vitality over a 12-month period

The follow up inventory data of 2017 revealed that most of the sample trees declined in the vitality even further. None of the sample trees exhibited improvement, a few changed the vitality from high to medium class, however, the most common trend was the conversion from high and medium to low vitality (Table 5).

Table 5. Changes in the number of trees in low, medium and high vitality classes between June 2016 and June 2017.

	Low	Medium	High
2016 (nr of trees)	10	10	10
2017 (nr of trees)	15	9	6
Annual difference (nr of trees)	+5	-1	-6

4. DISCUSSION

Overall, my results show that the diversity and abundance of endophytic fungi of *Q. robur* were significantly dependent on type of tissue and host's vitality. By far the most diverse endophytic community was found in trees showing medium vitality. This finding did not fully meet the expectation that the most diverse community would be found in trees showing lowest vitality. The expectation was based on an earlier study by Agostinelli (2012), who investigated endophyte communities in young oaks in a peri-urban setting in southern Sweden and found that the richest fungal community was among the trees with lowest vitality.

However, my data still confirmed that there were greater richness and diversity values in declining rather than in vital trees. This result is in line with findings by Giordano et al. (2009) who detected an increased fungal diversity with more progressive symptomatic stage in *Pinus sylvestris*. Some authors (Martín et al. 2013) suggest that low diversity in high vitality trees could indicate of an induced and effective plant defense system.

Overall, colonization frequency and morphotype richness was greater in bark than in xylem. This was expected based on result of earlier studies (e.g., Agostinelli 2012). More notable, however, is the finding that endophyte communities of bark were more similar to each other than those of xylem, and the singletons were more frequent in the xylem, supporting the assumption that xylem may host more specialist species, while bark hosts more generalists with broader niche requirements. Moreover, since bark is continuously exposed to environmental inoculum it can readily receive new infections during each year, which probably supports development of a rich endophyte flora in bark.

My results also revealed more pronounced similarity between xylem-bound communities of medium and low vitality trees, as compared to those of medium and high vitality trees. Changes in species composition may signify of the likely deterioration of the stand, as changes in the endophyte species composition often

occur due to host's weakened defense system, promoting an onset of latent pathogens and switch to saprophytic mode of action (Boddy & Rayner 1983).

A few morphotypes dominated the endophyte communities of *Q. robur*. Some authors (Sieber 2007) assume that dominant endophytes have co-evolved with their hosts for a long period of time and, therefore, are unlikely to be strong pathogens. However, certain endophytes isolated from xylem have been described as latent pathogens (e.g. *Fomes fomentarius* and *Nectria coccinea* in *Fagus sylvatica*) (Sieber 2007). Such an assumption leads us to hypothesize that the morphotypes that were the least abundant in xylem (e.g. MT1, MT8, MT16, MT9, MT1) might be classified as latent pathogens and in combination with other factors may pose a strong influence of tree vitality.

Finally, I identified a number of methodological caveats that might help to achieve more comprehensive results in future. When comparing my results to similar studies (Agostinelli 2012) the difference may be attributed to a combination of factors - experimental design, microclimate, the season of sampling, host characteristics as well as depending of the age of the tree (Torres et al. 2011). Agostinelli (2012) also concluded that the number of positive isolations recovered from *Q. robur* increased later in the season. Given that sampling for my study was done in early March it could be an important aspect to consider when interpreting and comparing the results. Hyde & Soyong (2008) highlighted that many fungi will not develop growth on the routinely used media, leaving the culture-based analysis of endophyte communities biased towards faster growing fungi, while slow-growing fungi might never be detected. This may result in overall species richness underestimation. In terms of fungal identification, I settled for classification to morphotypes, as a proxy of taxonomic classification. Nonetheless, the application of DNA-based taxonomy and molecular techniques could reveal greater diversity. It would allow to identify several isolates to species or genus level, which would be useful when comparisons to other studies are done. Rarefaction curve analyses revealed that the sampling captured well the common MTs but that a more extensive sampling would retrieve even larger number of singletons. Thus, it is highly probable that the overall endophyte community in oaks is much more diverse than what could be estimated based on my analysis.

5. CONCLUSIONS

In conclusion, my study provides the evidence that bark and xylem associated fungal endophyte communities reflect the vitality of *Q. robur* trees. The results contribute to the overall knowledge of the influence of host vitality and type of tissue on fungal endophyte communities in forest ecosystem. Considering that endophytic fungi occur in all living plants, the community diversity, spatial and temporal variation have still not been documented enough and remarkably little is known about the specific drivers that shape the forest tree associated microbiome. Continued studies on bioactive microbiomes are warranted since there is already an urgent necessity to substitute chemical treatments in agriculture, forestry or medicine with biobased ones. Because the health status of oaks was apparently in dynamic state, it is plausible that the fungal communities adjust to the qualitative changes in the deteriorating trees, switching to pathogenic or saprophytic life style. An improved understanding of the fungal networks and aspects that trigger the changes in the microbial ecosystem may help to create more holistic disease management systems (Witzell and Martín 2018). Future research should focus on elucidating the functional traits of the morphological groups identified as members of core microbiome in trees.

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APPENDICES

Appendix A

Culture media for fungi

Malt Extract Agar

Agar 20 g

Distilled water 1000 ml

Malt extract 20g

Lactic acid (25%) 1 ml

Water agar

Agar 1.5 g

Distilled water 1000 ml

Appendix B

Morphological characters

Mycelium development	Absent	Intermediate	Full coverage	
Phenolic discolorations	Absent			
	Present			
Bacterial growth	Absent			
	Present			
Growth (mycelium reaching from edge of the piece)	Emerging hypha	1-5 mm		5-10 mm
	10-15 mm	15-20 mm		>20 mm
Shape	Circular, entire edge	undulate	Lobate	
	Crenate	Erose, dentate	with rhizoids	
	Radially striate with lobate edge	Fimbriate	overgrowth	
Elevation	< 1 mm	1-3 mm	>3 mm	
Aerial mycelium	Absent		Sparse	
	Dense		Aerial sporulation	
Color	White	Light grey	Beige	Light green
	Light brown	Light pink	Grey	Dark grey
	Brown	Red/grey	Dark brown	Grey/brown
	Green	Dark orange	Orange/grey	Grey/green
Droplets	Absent			
	Present			

Appendix C

Identified morphotypes

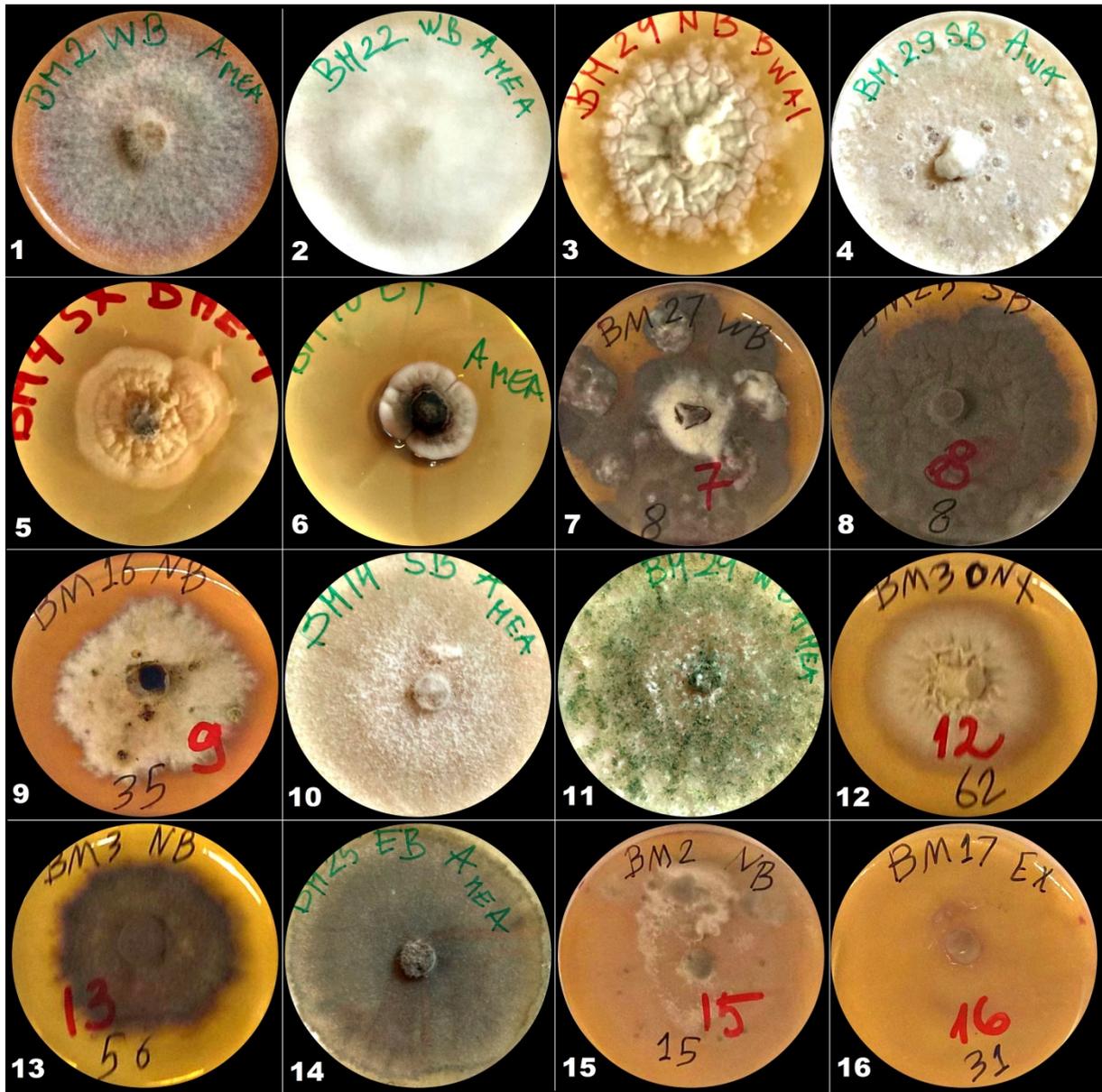


Figure 1. Representatives of identified morphotypes (MT1-MT16).

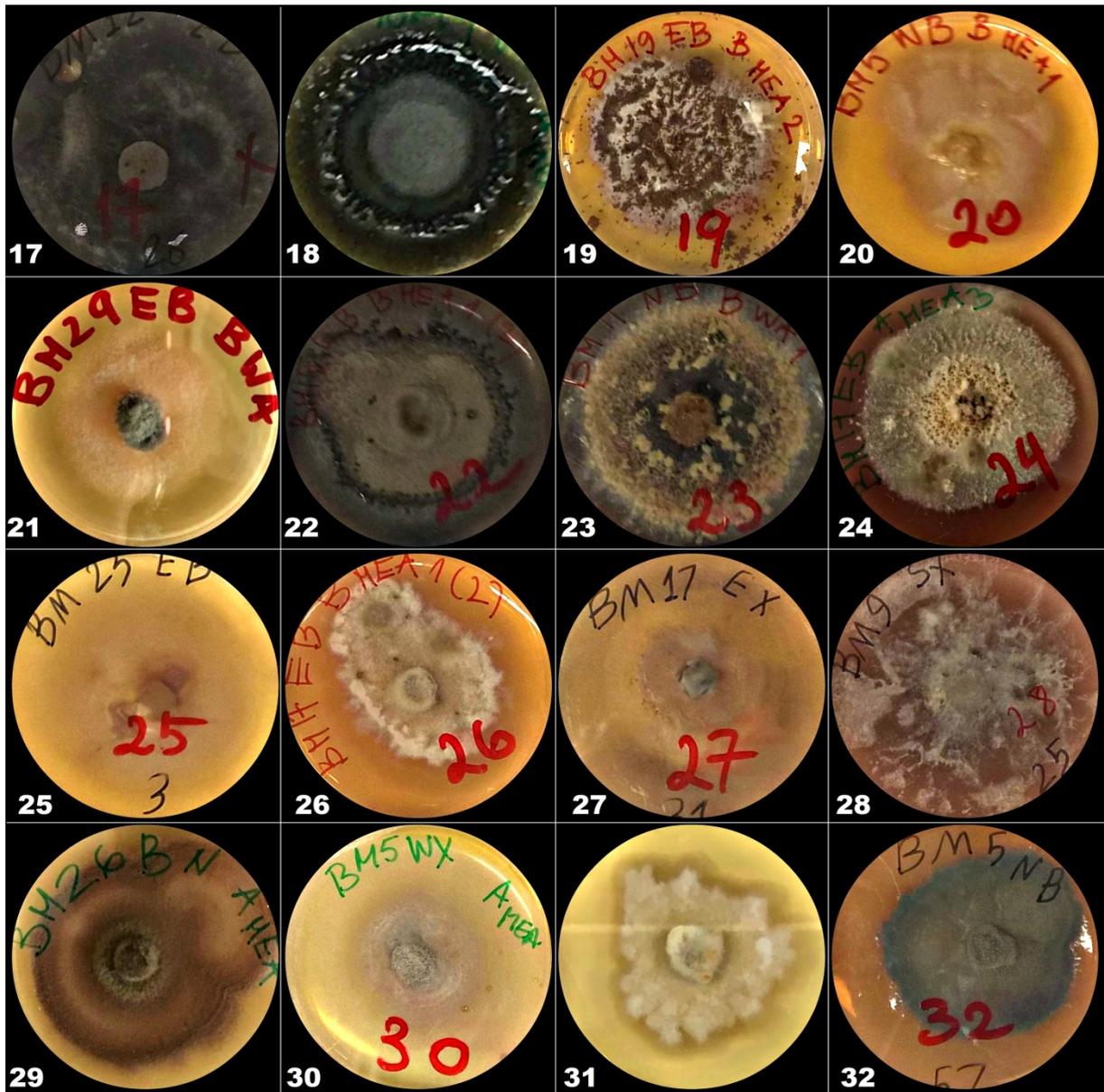


Figure 2. Representatives of identified morphotypes (MT17-MT32).



Figure 3. Representatives of identified morphotypes (MT33-MT339).

Appendix D

Supplementary data

Table 1. One-way ANOVA test evaluating differences of Shannon diversity index measures between xylem and bark tissue.

**One-way
ANOVA**

	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>P- value</i>
Between groups	1	0.0295541	0.029554	2.652	0.1085
Within groups	124	3.5335	0.028496		
Total	125	3.56305	0.05805		

Table 2. One-way ANOVA test evaluating differences of Shannon diversity index between low, medium and high tree vitality classes.

**One way-
ANOVA**

	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>P- value</i>
Between groups	2	0.008292	0.004146	0.6617	0.5178
Within groups	186	2.67186	0.014365		
Total	188	2.68015	1.85E-02		

Table 3. Wilcoxon pairwise comparisons of Shannon diversity index of the examined segments. Bold suggests a significant difference.

	BL	BM	BH	XL	XM	XH
BL		0.799	0.459	0.422	0.657	0.031
BM	0.799		0.426	0.447	0.575	0.003
BH	0.459	0.426		0.919	0.634	0.020
XL	0.422	0.447	0.919		0.819	0.611
XM	0.657	0.575	0.634	0.819		0.287
XH	0.031	0.003	0.020	0.611	0.287	

Table 4. One-way ANOVA test evaluating differences of evenness between xylem and bark.

**One-way
ANOVA**

	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>P- value</i>
Between groups	1	6.39E-05	6.39E-05	0.01911	0.8903
Within groups	124	0.41433	0.003341		
Total	125	0.414394	0.8907		

Table 5. One-way ANOVA test evaluating differences of colonization frequency between WA and MEA samples.

**One-way
ANOVA**

	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>P- value</i>
Between groups	1	0.144854	0.144854	0.1261	0.7244
Within groups	76	237.987	3.1314		
Total	77	238.131	3.276254		