Profiling mycobiota communities associated with the Pine Wilt Disease

C. Vicente[1,2], J. Faria[2], F. Nóbrega[2], H. Bragança[2,3], L. Bonifácio[2,3], P. Naves[2], E. Sousa[2,3], M. Espada[1], M. Mota[4], M.L. Inácio[2,3]

[1]MED - Mediterranean Institute for Agriculture, Environment and Development, Institute for Advances Studies and Research, University of Évora, Évora, Portugal; [2]INIAV, Instituto Nacional de Investigação Agrária e Veterinária, Oeiras, Portugal; [3]GREEN-IT Bioresources for Sustainability, ITQB NOVA, Av. da República, 2780-157 Oeiras, Portugal; [4]NemaLab, MED – Mediterranean Institute for Agriculture, Environment and Development, Dpt of Biology, University of Évora, Évora, Portugal.

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https://twitter.com/EnemyPine

Instituto Nacional de Investigação Agrária e Veterinária, I.P.

MED

Universidade de Évora

Pine Wilt Disease (PWD) is one of the most damaging diseases for conifer forests worldwide. This complex disease involves the interaction between three primary biological elements - the pinewood nematode (PWN) *Bursaphelenchus xylophilus*, the insect-vector *Monochamus* sp., and the host tree *Pinus* spp. – and other secondary elements such as endophytic bacteria and fungi. The development of *B. xylophilus* is strongly associated with fungi that colonize the declining trees, with special impact in their reproduction and number of individuals carried by the vector.

In light of previous knowledge, we are focused in obtaining a **detailed characterization of the structure and dynamics of the nematode-fungi interactions.** Using the ITS2 amplicon-based metagenomic approach, we compared the fungal communities from PWN infected and non-infected *P. pinaster* trees collected in two distint study sites, Tróia (location where PWN was first detected in 1999) and Seia (northwestern of Portugal).

Sampling Survey

Pinus pinaster trees (with and without PWD symptoms) were sampled on October 2019 at Seia (Guarda, Portugal) and Tróia (May 2020). All trees were checked for the presence of the PWN. Our preliminary results are based on 6 samples (Troia, n=4; Seia n=2).

DNA Extraction

Total DNA extraction of wood sawdust was conducted using a modified CTAB method (REF). DNA samples were sequenced by EUROFINS (Germany) with Illumina MiSeq 2x300bp paired-end.

Preprocessing and analysis of ITS2 sequences

Data processing was conducted using QIIME2[1] ITS2 region was extracted using ITSxpress[2]. Samples were dereplicated and chimera checked using USEARCH 6.1. Resulting reads were clustered to operational taxonomic units (OTUs) by close-reference clustering using UNITE reference database with 97% similarity threshold.

[1]Bolyen et al. 2019. Nature Biotechnology 37, 852-857.
[2]Rivers et al. 2017. F1000Research 7, 1417

Results & Discussion

Sequencing output

Final data set contained ca. 158,325 demultiplexed and quality-filtered ITS2 reads from 6 samples ranging between 8545 (sample T8_1) to 75629 reads (S2_2). All accumulation curves tended to approach the saturation plateau (Fig.1) indicating that the number of reads in each sample was suficient for recovering most of the diversity present.

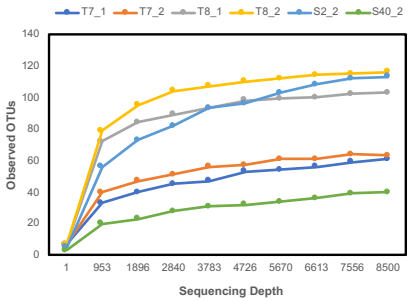

FIGURE 1 | Accumulation curves of fungal mycobiomes per sample. Sequencing depth was normalized at 8500 features per sample.

Fungal communites Diversity

Close-reference clustering (at 97% sequence similarity cutoff) produced a range of 40-65 operational taxonomic units (OTUs) for *P. pinaster* trees infected with PWN and 103-116 OTUs for *P. pinaster* without PWN (Table 1). Fungal communities from Seia showed higher diversity than fungal communities of Troia. Core metrics also suggest that the presence of PWN may alter the fungal community structure.

Samples of *P. pinaster* infected with PWN (PWD symptoms) clustered opposite of trees without PWN indicating contrasting fungal communities (Fig.2).

TABLE 1 | Core metrics (diversity indices) calculated for each sample from collection sites Troia (T) and Seia (S).

Core Metrics	Presence of PWN			Absence of PWN		
	T7_1	T7_2	S40_2	T8_1	T8_2	S2_2
Chao1	70	68	53	106	118	135
Observed OTU	62	65	40	103	116	112
Simpson index	0.93	0.92	0.60	0.90	0.91	0.74
Shannon Entropy	4.06	4.06	1.84	4.42	4.51	3.12
Pielous J	0.68	0.68	0.34	0.66	0.66	0.46
Good Coverage	0.99	0.99	0.99	0.99	0.99	0.99

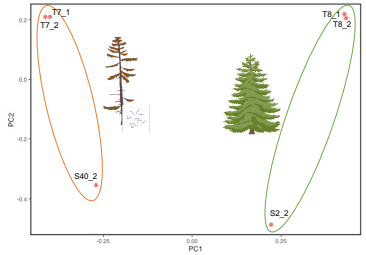

FIGURE 2 | Principal coordinate analysis (PCoA) based on Bray-Curtis distance matrix of 6 samples (2 *Pinus pinaster* from Seia and 4 *P. pinaster* from Troia.

Fungal communities Composition

Figure 3 presents the contrasting fungal communities (order level) of *P. pinaster* with and without PWN infection. Not only the communities differ between study sites, which are biogeographically distinct, but also due to the presence of the nematode.

Our results show that fungal communities of trees infected with PWN (S40_2; T7_1 and T7_2) are dominated by fungi of the Ophiostomatales order (Ascomycota, Sordariomycetes). In terms of genus within this order, S40_2 presents 55% of *Ophiostoma*, 31% of *Leptographium* and 0.56% of *Sporothrix*, while T7_1 and T7_2 present ca. 6% of *Ophiostoma* and 4-6% of *Sporothrix*. Trees without the presence of PWN (S2_2, T8_1 and T8_2) were dominated by Helotiales (Ascomycota, Letiomycetes), followed by Pleosporales (Ascomycota, Dothideomycetes) and Phaeomononiellales (Ascomycota, Eurotiomycetes). In these trees, Ophiostomatales were almost undetected with relative frequencies lower than 1.4%.

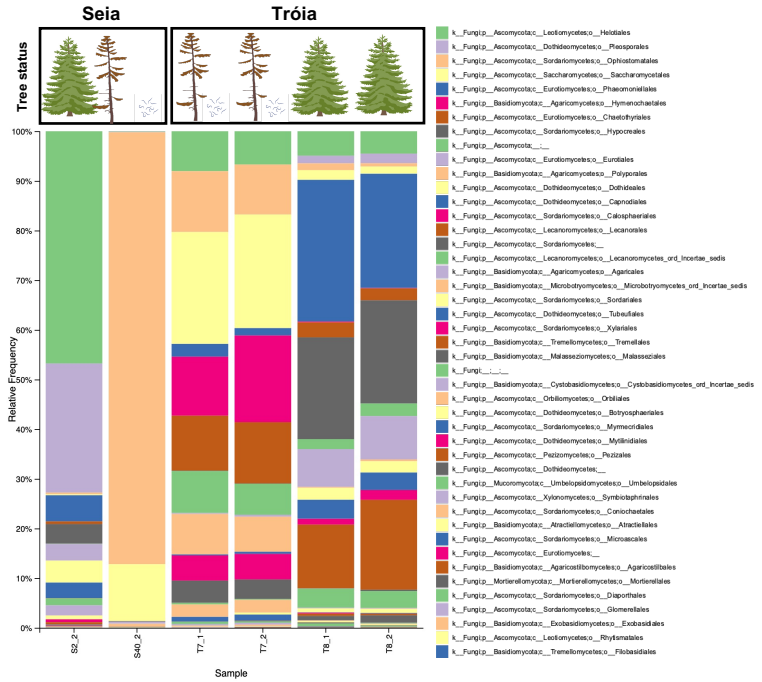

FIGURE 3 | Relative frequency of fungal communities (level order) of *Pinus pinaster* trees infected with PWN (showing visible PWD symptoms) and without PWN.

CONCLUDING REMARKS

In declining pine trees, PWN is able to feed on the fungal communities to increase their population. These communities are mostly dominated by ophiostomatoid fungi carried by secondary bark beetles. Our preliminary results corroborate previous studies on cultivable mycobiota, and describe for the first time, the complex fungal communities associated with PWD and the PWN.

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