

Mycobiome diversity and structure of the Pine Wilt Disease complex in Portugal



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Background

Fungi are major ecological players in Pine Wilt Disease (PWD) complex¹. In the late stages of the disease, the pinewood nematode (PWN) *Bursaphelenchus xylophilus* feeds on fungi available in the pine tree for survival and multiplication². Previous studies have already proved a close relation between the PWN and the blue-stain fungi (Ascomycota, Ophiostomatales), necrotrophic pathogens associated with bark beetles (Coleoptera: Scolytidae). The PWN can grow densely in the presence of these fungi, which results in a higher number of nematodes transferred to the insect-vector *Monochamus* spp.³ Despite the significant progresses in understanding the mycoflora diversity in the PWD complex using NGS, few references have addressed to the diversity and abundance of blue-stain fungi communities. Here, we present the diversity and structure of *Pinus pinaster* and PWN mycobiome using ITS2 amplicon sequencing.

Methodology

Wood samples from PWN-infected and non-infected *Pinus pinaster* were collected in three locations of Continental Portugal (winter 2019-spring 2020)⁴. All samples were evaluated in terms of PWN density before being processed for NGS⁴.

A total of 30 samples of *P. pinaster* and 15 samples of PWN from PWN-infected trees were characterized using ITS2 amplicon sequencing. Total DNA was extracted with modified CTAB protocol.

Sequencing data was processed in Qiime2 (v2022.2) and visualizations were obtained in R (v 4.2.1) using Vegan, Phyloseq and MicrobiotaProcess packages. Results are presented in ASVs (average sequence variants).



Figure 1 | Images of sampling collection at Companhia das Lezírias (Jan 2021) – *Pinus pinaster* with/without PWN (a) and blue-stain fungi in PWN-infected *P. pinaster* (b).

Results & Discussion

Pinus pinaster

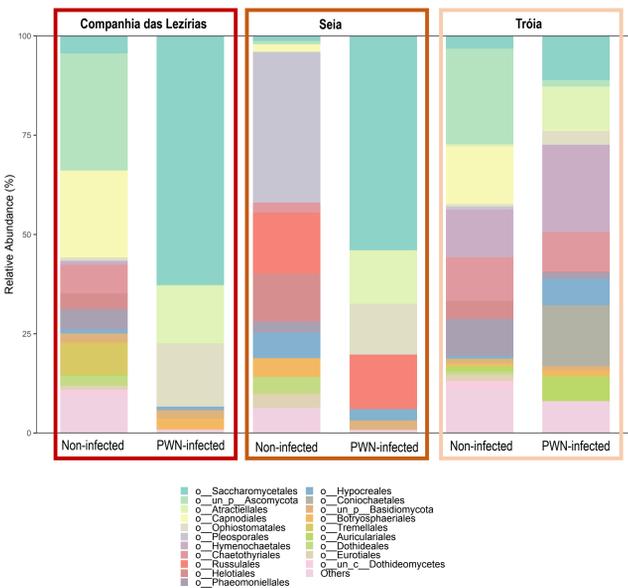


Figure 2 | Relative abundance (%) of the top 20 taxa orders presented in *P. pinaster* trees from three locations and respective conditions (Non-infected and PWN-infected).

Significant differences ($p < 0.001$, perMANOVA) were obtained between non-infected and PWN-infected *P. pinaster*. Overall, the most representative orders from phylum Ascomycota in both conditions were Saccharomycetales, Pleosporales and Ophiostomatales. However, PWN-infected pine trees recorded the highest relative abundances for Saccharomycetales (ranging from 18% in Tróia and 89% in Seia and Companhia das Lezírias) and Ophiostomatales (28% in Seia, 26% in Lezírias and 5% in Tróia (Figure 2).

The most representative genera of Saccharomycetales in PWN-infected trees were Nakazamea and Yamadazyma (ranging among sites 18-89%). In the non-infected trees, Saccharomycetales were up to 3.7% only in Companhia das Lezírias.

The most representative genera of Ophiostomatales in PWN-infected trees were: Ceratocystopsis (2-22%), Leptographium (1-17%), Ophiostoma (1.5-27%) and Graphilbum (4.6-7.4%). Ophiostomatales in non-infected *P. pinaster* was less than 1%.

Diversity indexes showed that non-infected trees were more biodiverse than the PWD affected trees (data not shown). 141 ASVs were found common between both conditions (the most representative). 775 ASVs were unique to non-infected, while 151 were only found in PWN-infected trees (Figure 3).

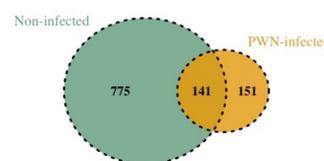


Figure 3 | Venn diagram displaying the number of common and unique ASVs between non-infected and PWN-infected *Pinus pinaster* among all three sampling locations.

Fungal communities from non-infected *P. pinaster* grouped according to the sampling site, denoting a completely different diversity and structure when comparing with the PWN-infected trees. PWN-infected trees clustered altogether, denoting more similar communities (Figure 4).

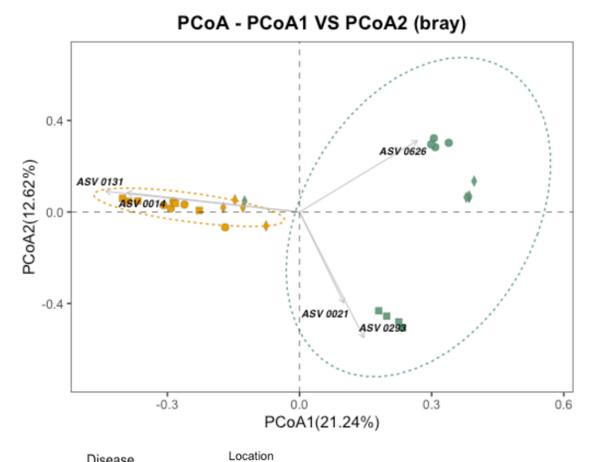


Figure 4 | Principal coordinate analysis (PCoA) at ASVs-level (based on Bray-Curtis dissimilarity matrix) comparing non-infected and PWN-infected *Pinus pinaster* mycobiome among all three sampling locations. ASVs: ASV0014 – Atractiellales; ASV0021 – Pleosporales; ASV0131 – Saccharomycetales; ASV0293 – Pleosporales (unidentified); ASV0626 – Orbilliales.

Bursaphelenchus xylophilus

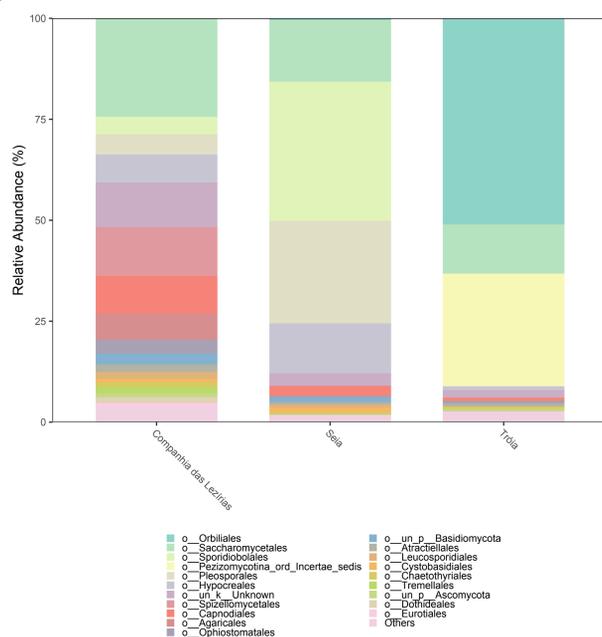


Figure 5 | Relative abundance (%) of the top 20 taxa orders presented in the PWN samples from the 3 locations.

PWN fungal communities were significantly different ($p < 0.001$, perMANOVA) between geographical locations. Overall, the most representative orders from phylum Ascomycota among locations are Orbilliales, Saccharomycetales and Sporidiobolales. Ophiostomatales were also detected in the Top 20.

The most representative genus in Orbilliales order was an unidentified_Orbilliaceae (most expressive in Tróia, 36%). The highest relative abundances for Saccharomycetales ranged between 12% (Tróia) and 25% (Companhia das Lezírias) with the most representative genera Nakazawaea and Ogataea (Figure 5). From Ophiostomatales, only Ophiostoma genus was detected in the top hits (0.4% in Seia and 2% Companhia das Lezírias).

Diversity indexes showed that PWN fungal communities from Companhia das Lezírias were more biodiverse than in the other locations (data not shown). A total of 616 ASVs were detected in PWN mycobiome. 58 ASVs found common between both conditions. 233 ASVs were unique in Companhia das Lezírias, followed by 164 in Tróia and 60 in Seia (Figure 6).

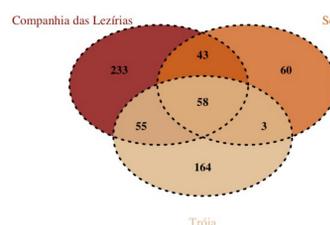


Figure 6 | Venn diagram displaying the number of common and unique ASVs between PWN samples among all three locations.

PWN mycobiomes were clustered according to location. Communities from Companhia das Lezírias and Seia were closest than Tróia (Figure 7).

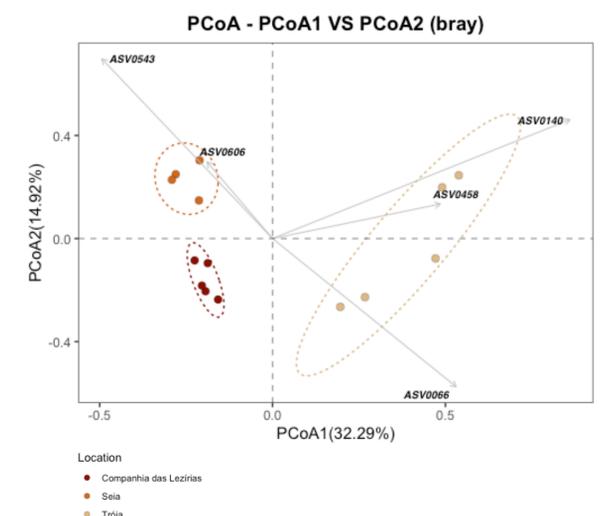


Figure 7 | Principal coordinate analysis (PCoA) at ASVs-level (based on Bray-Curtis dissimilarity matrix) comparing PWN mycobiome among all three sampling locations. ASVs: ASV0066 – Pezizomycotina_ord_incertae_sedis; ASV0140 and ASV 0458 – Orbilliales; ASV0543 – Ascomycota; ASV0606 – Unassigned.

References

- Vicente et al. (2021) J Fungi 7, 780.
- Futai (2013) Annu. Rev. Phytopathol. 51, 61-83
- Maehara and Futai (1996) Appl. Entomol. Zool. 31, 443-452.
- Vicente et al. (2022) Front. Plant Sci. 13:908308

Concluding Remarks

The diversity and structure of PWN-infected and non-infected *P. pinaster* is significantly different, suggesting that communities' shifts may be related with disease development in the pine tree and as a result of the presence of the PWN. These results are corroborated by our previous culturomics study. The high abundance of Saccharomycetales and Ophiostomatales present in PWN-infected trees are most probably related with bark beetles (Coleoptera: Scolytidae) attacks in the affected trees, since these are known vectors of both organisms. As expected, PWN mycobiome shares some taxa with the mycobiome of the pine host *P. pinaster*.