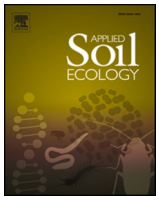
Applied Soil Ecology 193 (2024) 105142

Contents lists available at ScienceDirect

Applied Soil Ecology

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



Relationships between rhizosphere microbiota and forest health conditions in *Pinus pinaster* stands at the Iberian Peninsula

Carmen Morales-Rodríguez [a], Jorge Martín-García [b,c], Francisco J. Ruiz-Gómez [d], Jorge Poveda [b,c,*], Julio J. Diez [b,c,*]

[a] Department for Innovation in Biological, Agro-food and Forest Systems (DIBAF), Agriculture and Forestry Campus, University of Tuscia, Via S. Camillo de Lellis, 01100 Viterbo, Italy
[b] University Institute for Research in Sustainable Forest Management (iuFOR), University of Valladolid, 34004 Palencia, Spain
[c] Department of Vegetal Production and Forest Resources, University of Valladolid, 34004 Palencia, Spain
[d] Evaluation and Restoration of AgroForestry Ecosystems, Forest Engineering Department, University of Córdoba, Ed. Leonardo Da Vinci P1. Campus Universitario de Rabanales, CT. N IV Km 396, 14001 Córdoba, Spain

ARTICLE INFO

Keywords:
Maritime pine
Forest decline
Metabarcoding
Umbelopsis
Paenibacillus

ABSTRACT

Maritime pine (*Pinus pinaster*) is a Mediterranean forest tree species of great ecological importance within the European Union and the Iberian Peninsula in particular, whose presence is seriously threatened by forest decline. Knowledge of the diversity, abundance, and functionality of rhizospheric microorganisms can provide important information in the design of new strategies for sustainable forest management against forest decline. In this work, rhizospheric samples were collected from representative pine forests in the region of Castilla y León (Northwestern Spain) (in 10 municipalities of 5 different provinces), analyzing different physicochemical parameters and bacterial and fungal biodiversity (by metabarcoding). In addition, different variables of tree health and climatic conditions were analyzed. The main phylum of microorganisms found in the rhizosphere of *P. pinaster* were the Ascomycota (54.5 %) and Acidobacteria (16.4 %) in fungi and bacteria, respectively. A clear relationship was found between the presence/absence of certain bacterial and fungal groups (taxonomic and functional) and the presence/absence of healthy/sick trees. Specifically, the fungal genus *Umbelopsis* and the bacterial genus *Paenibacillus* were thought as possible control agents of decline, as their presence was related to the absence of disease. Understanding the relationships between rhizosphere microbiota and forest health parameters in *Pinus pinaster* can be only achieved by exploring the complex 'ecosystem microbiome' and its functioning using focused, integrative microbiological and ecological research performed across multiple habitats.

1. Introduction

Maritime pine (*Pinus pinaster* Ait.) is a Mediterranean forest tree species distributed in Western Europe and northern Africa, where it grows in a wide range of habitats with contrasting gene pools (Hurel et al., 2021). The ecological relevance of these Mediterranean pine forests is well known because of their role in conservation and maintaining biodiversity (Prieto-Recio et al., 2015). Mediterranean *P. pinaster* forests are classified as natural habitats of community interest and designed as special areas of conservation referred to as "Mediterranean pine forests with endemic Mesogean pines" (EU Council Directive 92/43/EEC on the conservation of natural habitats and wild fauna and flora).

Recently it has been pointed out that the decline of Mediterranean pine forests is associated with increasing temperatures (El Khoury et al., 2021), drought, lack of fire, intensive grazing (Connor et al., 2021), and/or pathogen and pest action (Rubio-Cuadrado et al., 2021), which has triggered the mortality and decline of these forests. *P. pinaster* decline is a serious problem causing the disappearance of the Maritime pine (the more extended pine in the Iberian Peninsula) in several areas all around Northern Spain (Ribeiro et al., 2022). Forest decline can be defined as a complex disease caused by the interaction of several interchangeable factors, both abiotic and biotic (Thomas et al., 2002), leading to the gradual deterioration of the forest and, eventually, the death of trees (Manion, 1981). These factors have been classified as predisposing, inciting or contributing factors. Among the first, historical climate, site

* Corresponding authors at: University Institute for Research in Sustainable Forest Management (iuFOR), University of Valladolid, 34004 Palencia, Spain.
E-mail addresses: jorge.poveda@uva.es (J. Poveda), juliojavier.diez@uva.es (J.J. Diez).

https://doi.org/10.1016/j.apsoil.2023.105142
Received 5 May 2023; Received in revised form 27 August 2023; Accepted 22 September 2023
Available online 30 September 2023

management, stand age and genetic predisposition may predispose the trees to decline (Hennon et al., 2012). Inciting factors such as extreme climatic events or the detriment produced by competition for resources may cause trees to enter into a state of decline at any given time; after, the trees may recover or enter a more progressive state of decline (Hennon et al., 2012). Finally, contributing factors, such as pathogenic fungi, parasitic plants and opportunistic insects may also contribute to the further weakening and subsequent death of the trees (Hennon and McWilliams, 1999).

Plants harbor a wide variety of microorganisms in nearly all tissues, both inside and outside their surfaces. The portion of soil influenced by plant roots, the rhizosphere, is a niche of great microbial diversity, strongly determined by plant metabolism through root exudates (Pantigoso et al., 2022). At the same time, plant-associated microorganisms, especially those found in the rhizosphere, play an important role in plant nutrition and adaptation to different types of stress (Kumawat et al., 2022). Indeed, the plant microbiome could represent an additional source of genes and functions to its host, expanding plants' ability to adapt to several environmental changes (Poveda et al., 2022). Consequently, plant-associated microorganisms, specifically bacteria and fungi, might improve their host's growth and survival abilities, that is, the overall plant fitness. Thus, plants should be considered as a 'holobiont' instead of standalone organisms (Lloyd and Wade, 2019). Fungi that affect trees can be classified according to feeding type: symbionts, parasites and saprophytes (Kavanagh, 2017). Fungal communities in forests are formed different functional guilds: endophytes, saprotrophs, pathogens, etc. Knowing the species composition and the factors influencing the presence of different fungal communities is important for unraveling the role that fungi play in regulating other organisms (Gomes et al., 2023). Similar classification can be adapted to the bacterial communities in forest soils (Schlatter et al., 2017). In addition, the role of bacteria associated with trees in the forest as growth promoters and inducers of tolerance and resistance is widely known (Lloyd and Wade, 2019).

There is a strong relationship between soil biota, soil fertility and plant health (Tahat et al., 2020). The contribution of soil biota to plant pests and pathogen protection can occur through direct interactions with the plant (e.g. improved nutritional status or activation of the plant 'immune' system) or through other interactions within the rhizosphere (e.g. competition, predation and parasitism) (Díaz-Urbano et al., 2023). The activity and effects of beneficial rhizosphere microorganisms on plant health are well documented for bacteria belonging to the Proteobacteria (noticeably *Pseudomonas* and *Burkholderia*) and Firmicutes (*Bacillus* and related genera) (Sarker et al., 2021), and for fungi from the anamorphic Ascomycetes (e.g. *Trichoderma*, *Gliocladium* or non-pathogenic *Fusarium oxysporum* strains) (Poveda et al., 2020).

Unraveling the structure of rhizosphere bacterial and fungal assemblages associated with woody species could represent the first step towards the development of an effective integrated management strategy. For example, by using soil amendments that modify the fungal and bacterial assemblages or the use of bioinoculants with biological control capacities. Therefore, the aim of this work is to analyze the soil microbiota diversity of *P. pinaster* ssp. *mesogeensis* rhizosphere in northwestern Spain to unmask their associations with several tree health parameters.

2. Materials and methods

2.1. Site description and sampling procedure

The present study was carried out in the center of the Iberian Peninsula (Castile and Leon, NW Spain). Ten circular plots of radius 15 m were selected and installed in ten natural stands of *Pinus pinaster* ssp. *mesogeensis* covering most of the distribution of the provenance regions in the center of the Iberian Peninsula. The sites were chosen among a selection of 27 stands (see Prieto-Recio et al., 2015) with the aim to have stands with different levels of damages in the more important provenances of *P. pinaster* we have in the Iberian Peninsula. The stands belong to the Permanent Sample Plots Network of the Spanish National Forest Inventory (NFI) network. There was one–two zone(s) per each provenance depending of their representativity, except for Meseta Castellana where four zones were included. This provenance region is widespread throughout three provinces with different climatic and soil conditions (Table 1).

2.2. Site characterization

Soil characteristics and tree health status were determined in all the studied plots. Mineral soil samples were collected from the upper 30-cm soil layer in each stand (four samples per plot). The samples were collected from the bulk soil. At each sampling site, three soil samples were collected approximately 4–5 m apart from each other from the top 10 cm of soil, using a hand-held metal corer of 4.4 cm diameter × 10 cm length. A total of 40 soil samples were collected and kept in individual 250 ml containers which were transported to the laboratory shortly after sampling and stored at −20 °C until further processing. The samples were mixed and homogenized to produce one composite sample per plot. Soil pH was determined potentiometrically with a pH meter in a soil solution (1:2.5, soil/water) (Faria et al., 2023). Organic matter was determined by the $K_2Cr_2O_7$ method (Gerenfes et al., 2022). Total N was determined by Kjeldahl digestion (Amin and Flowers, 2004), by Auto Kjeldahl Nitrogen Analyzer OLKN-9830 (LABOA, Henan, China). Soil available P was extracted by the Olsen procedure and determined photometrically by the molybdenum-blue method (Hurtado et al., 2008). Soil exchangeable cations (K^+, Mg^{2+}, Na^+, Ca^{2+}) were extracted with ammonium acetate and determined by atomic absorption/emission spectrometry (Goyal, 2002). Particle-size distribution was determined by the Bouyoucos method (hydrometer method), and the ISSS (International Society of Soil Science) classification was applied (Ashworth et al., 2001). The Cation Exchange Capacity (CEC) was determined by Bascomb's method (i.e., the exchange cations were displaced by Ba ions, which were then displaced by Mg ions and the remaining concentration of Mg was determined by titration against EDTA) (Aprile and Lorandi, 2012).

Forest health variables were assessed in 24 trees per plot (a total of 240 trees) by visual assessment of crown conditions, such as crown defoliation, discoloration, and live crown ratio. The variables were visually estimated, based on an absolute reference tree (Ferreti and Peña, 1994), according to Level I of the European network methodology (Eichhorn et al., 2010). Crown defoliation has been widely used as an indicator of forest health (Dobbertin and Brang, 2001). Furthermore, considering that factors involved in mortality may differ between sites, the use of different vitality indicators is highly recommended (Cailleret et al., 2014). Hence, other phytosanitary variables were also assessed, such as the presence of cankers, insects exit holes, fungi presence on trunk and needles, and the percentage of healthy, damaged, and dead trees (Table S1).

2.3. Environmental data selection

For the characterization of environmental conditions in the location of plots, 81 variables were obtained from Digital Climatic Atlas of the Iberian Peninsula, including topographic (n = 4), precipitation (n = 17), temperature (n = 43) and solar radiation (n = 17) variables (Table S1). The initial dataset with a total of 134 environmental variables, including the previous 81 plus the 20 soil characteristics obtained from soil sample analysis, and the 33 variables describing stand characteristics and competence indices (Table S1) were firstly analyzed searching for data noise. Variables that did not present Pearson's correlation coefficients (r) higher than 0.3 with any of the other variables of the dataset were eliminated (Navarro-Cerrillo et al., 2018). Then, selected variables were analyzed for collinearity, and variables with Variable Inflation Factor

Table 1
Identification and location of the study sites.

Sample ID	Location	Province	X coordinate	Y coordinate	Altitude	Orientation	Texture	Oxidizable organic matter (g/100 g)	pH	Anual precipitation (mm)	Anual Tm (°C)	Anual Tmax (°C)
A38007	Quintana del Pidio	Burgos	3°44′48″	41°46′18″	878	NE	Sandy	0.25	6.59	490	11	17
A38006	La Horra	Burgos	3°51′18″	41°44′44″	846	S	Sandy	0.22	6.53	488	11	18
A38004	Iscar	Segovia	4°29′06″	41°22′29″	653	N	Sandy clay loam	0.66	6.33	619	12	17
A3800A	Bayubas de Arriba	Soria	2°55′22″	41°33′40″	971	–	Sandy	1.17	8.78	662	10	17
A38009	Gete	Burgos	3°17′02″	41°56′48″	998	–	Sandy	0.54	5.63	547	11	17
A38008	Huerta del Rey	Burgos	3°21′36″	41°50′22″	964	S	Sandy	0.51	6.44	544	11	17
A38005	El Arenal	Ávila	5°4′45″	40°14′54″	852	W	Sandy	0.54	6.27	527	11	18
A38003	Aguas Cánidas	Burgos	3°29′11″	43°43′10″	1059	W	Sandy clayey-sandy	0.85	5.51	619	10	16
A38002	Pinilla de la Valdería	León	6°5′36″	42°13′10″	1046	S	Sandy	2.09	6.05	615	11	17
A38001	Nogarejas	León	6°9′55″	42°12′13″	469	–	Sandy	0.57	8.34	477	12	19

(VIF), higher or equal to 10 were also eliminated (Duque-Lazo et al., 2016). Through this methodology a total of 56 variables were discarded for redundancy or collinearity problems, being selected 78 (Table S1).

The environmental dataset was used in a multivariate and discriminant analysis following a methodology adapted from the described in Navarro-Cerrillo et al. (2018). All variables satisfying conditions of normality and homoscedasticity, previously confirmed the presence of bivariate correlations >0.3 ($p < 0.05$) and the absence of collinearity, were included in a Principal Component Analysis (PCA) to select the most representative ones, according to Sleighter et al. (2010). Selected variables were identified by an optimal PCA solution that included Bartlett's sphericity test ($p > 0.05$), a Kaiser-Meyer-Olkin test (KMO) ($p < 0.05$), and communalities ($r > 0.6$), correlations with principal components ($r > 0.5$), and maximum explained variance. With the variables selected from the optimal PCA solution, plots were subjected to an agglomerative Hierarchical Cluster Analysis (unsupervised HCA clustering) using typified values (z punctuations) with the Ward's method based upon a squared Euclidean distance matrix. The original k blocks corresponding to each observation as a separate cluster, were merged to form successively larger and fewer clusters. At each iteration, squared Euclidean distance was calculated between the resulting clusters. The abrupt change (i.e. the maximum slope derivative) in the sequential difference in squared Euclidean distance (ΔSED) provided the stopping criterion for determining the optimal cluster number. We tested the separation of the groups identified in the cluster analysis using PCA bidimensional representation.

The same procedure described above for the environmental data set (PCA and HCA clustering) was applied to the dataset of biodiversity variables for fungal and bacterial communities and for a mixed database, including the 78 filtered environmental and phytosanitary variables and the 3 α-diversity variables (Shannon, Observed OTUs and Pielou evenness) for both communities.

2.4. DNA extraction, PCR amplification and sequencing

DNA extraction was performed using the DNeasy PowerLyzer PowerSoil kit (Qiagen). The V4 domain of bacterial 16S rRNA genes was amplified using primers F515 (5′-NNNNNNNNGTGTGCCAGCMGCCGCGGTAA-3′) and R806 (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2011), with the forward primer modified to contain a unique 8-nt barcode and a 2-nt linker sequence at the 5′ terminus. Fungal internal transcribed spacer (ITS) 1 loci were amplified with primers BITS (5′-NNNNNNNNCTACCTGCGGARGGATCA-3′) and B58S3 (5′-GAGATCCRTTGYTRAAAGTT-3′) (Bokulich and Mills, 2013), with a unique 8-nt barcode and linker sequence (bold portion) incorporated in each forward primer. The samples were sent to the company Biome Markers (Valladolid, Spain) for processing and sequencing by Illumina MiSeq 2 × 300.

2.5. Operational taxonomic units identification and functional guilds classification

After the quality filtering, paired-end assembly and demultiplexing the sequences were processed and a similarity clustering was done based on the UPARSE pipeline of the USEARCH v8 (Edgar, 2013) with a 97 % of clustering threshold (Lindahl et al., 2013). Sequences failing alignment or identified as chimeric were removed before downstream analysis. The consensus OTUs were identified using the BLAST tool in the Genbank database with the algorithm parameters word size = 11, match/mismatch scores = 2,−3, gap cost existence = 5 and gap cost extension = 2 (Morales-Rodríguez et al., 2021). The xml file from the BLAST and the blasted fasta file were imported into MEGAN (Huson et al., 2011) to compute and explore the taxonomical content of the data set, employing the NCBI taxonomy to summarize and order the results. The lowest common ancestor parameters were: Min score = 170; Max. expected = 0.01; Top percent = 2.0, Min support percent = 0.3; Min support = 1 and LCA percent = 40) and with the following minimum requirements of similarity to accept the proposed taxonomy: Species 99 %, Genus 97 %, Family 95 %, Order 90 %, Class 85 %, and Phylum 80 %.

Finally, the OTU abundance table was generated with USEARCH v8 (Edgar, 2013; Bálint et al., 2014). For both fungi and bacteria, any OTU representing <0.001 % of the total filtered sequences were removed to avoid the inclusion of erroneous reads, leading to inflated estimates of diversity (Parks et al., 2013).

Functional guilds of the identified taxa were characterized using FunGuild database (Nguyen et al., 2016). Only OTUs classified with a confidence level of “Probable” or “Highly Probable” were used. The functional guilds of the remaining OTUs, classified as “Unassigned” or “Possible”, were revised based on the literature; those confidently classified were added to the analysis. The rest of the unassigned OTUs and those assigned with low confidence were placed in the “Unclassified” category. Taxa classified as soil saprotrophs, plant saprotrophs, litter saprotrophs or wood saprotrophs were grouped in a unique Soil-Plant Saprotroph category (Ruiz-Gómez et al., 2019). Those taxa confidently classified in more than two guilds were assigned to the class >2 Guild.

2.6. Statistical analysis

For numerical parameters, prior to the analysis of correlations, the

normality of variable residues was checked through the normal Q-Q graph and the D'Agostino–Pearson test. If the data did not satisfy the assumptions of homoscedasticity and normality, the logarithmic transformation was used and then normality was checked again. The Pearson or Spearman correlation test was used, depending on whether the two variables in each pair were distributed normally or not. Null hypotheses were rejected in all cases when $p \leq 0.05$. Differences between alpha diversity indices and categorical parameters were analyzed through Kruskal-Wallis test.

Prior to the analysis of the OTUs abundance matrices, rarefaction curves were evaluated (Supplementary Material, Fig. S1) and Good's Coverage was calculated to estimate the adequate depth of sampling using QIIME2 2018.2 (Caporaso et al., 2010). Then, the core biome OTUs (OTUs present in at least 70 % of samples) was obtained using the Feature Table plugin of QIIME2 (McDonald et al., 2012). This core biome was used as independent variables matrix in a Non-metric Multidimensional Scaling analysis (NMDS) based on the Bray-Curtis dissimilarity matrix together with Analysis of Similarity (ANOSIM, 999 permutations) to evaluate differences in β-diversity between plots, for both fungal and bacterial datasets.

Except for the treatment of OTUs abundance matrices, the statistical analyses were performed in the environment R Studio V 1.1.463 (RStudio Inc. 2015. Boston, MA, USA) running under R 3.5.2 (R Foundation for Statistical Computing, 2014. Vienna, Austria). NMDS and ANOSIM analyses were carried out using the package *vegan: Community Ecology Package* (Oksanen et al., 2018). Other packages used were *ape*, *cxar*, *dendextend*, *devtools*, *dunn.test*, *ggplot2*, *ggpurb*, *ggrepel*, *Hclust*, *MASS*, *nortest*, *pvclust*, *relaimpo* and *usdm* (https://CRAN.R-project.org/).

Frequency matrices of metabarcoding does not allow to carry out parametric tests, mainly due to the high proportion of zeroes in the matrix column, so most of the literature published on this field use this approach to carry out the multivariate analysis of samples and taxonomic groups. Non-metric multidimensional scaling is a multivariate method adequate for analysis of non-normal data, as it uses a non-parametric approach based on a distance matrix. By its way, ANOSIM is a non-parametric multivariate test to determine the statistical significance of grouping. Most of the literature published on this field use this approach to carry out the multivariate analysis of samples and taxonomic groups, for example, Ruiz-Gómez et al. (2019).

3. Results

3.1. Forest health and stand characteristics

The multivariate analysis for plot characterization results in the selection of the 8 most relevant characteristics which represented the maximum variability among plots, including altitude, dominance, codominance, suppressed trees, basimetric area factor (BAF), Mean height of trees (from 24 trees per plot), Maximum temperature in December and Maximum temperature at year (from Digital Climatic Atlas of the Iberian Peninsula). The HCA (Hierarchical Cluster Analysis) based on those variables discriminated the plots in 4 different groups (Fig. 1B), clearly differentiated, especially in the case of A38001 (Nogarejas). Principal component analysis showed the greatest distance of this plot from the rest (Fig. 1A), mostly influenced by altitude and temperatures from plots A38009 and A38003 (Grete and Aguas Cándidas). The separation between the rest of the plots was mostly influenced by stand characteristics (dominance, codominance, suppressed trees, and BAF).

When the relationship between the selected environmental variables and the health status variables (summarized in Table 2) was evaluated, a relevant positive correlation was found between the presence of fungi in needles and the main characteristics of the stands, including Dominance ($r = -0.635$, $p < 0.01$), Codominance ($r = 0.637$, $p < 0.05$), Suppressed ($r = 0.798$, $p < 0.01$) and Mean height of trees ($r = -0.763$, $p < 0.05$), but no other relevant relationships were identified among health status

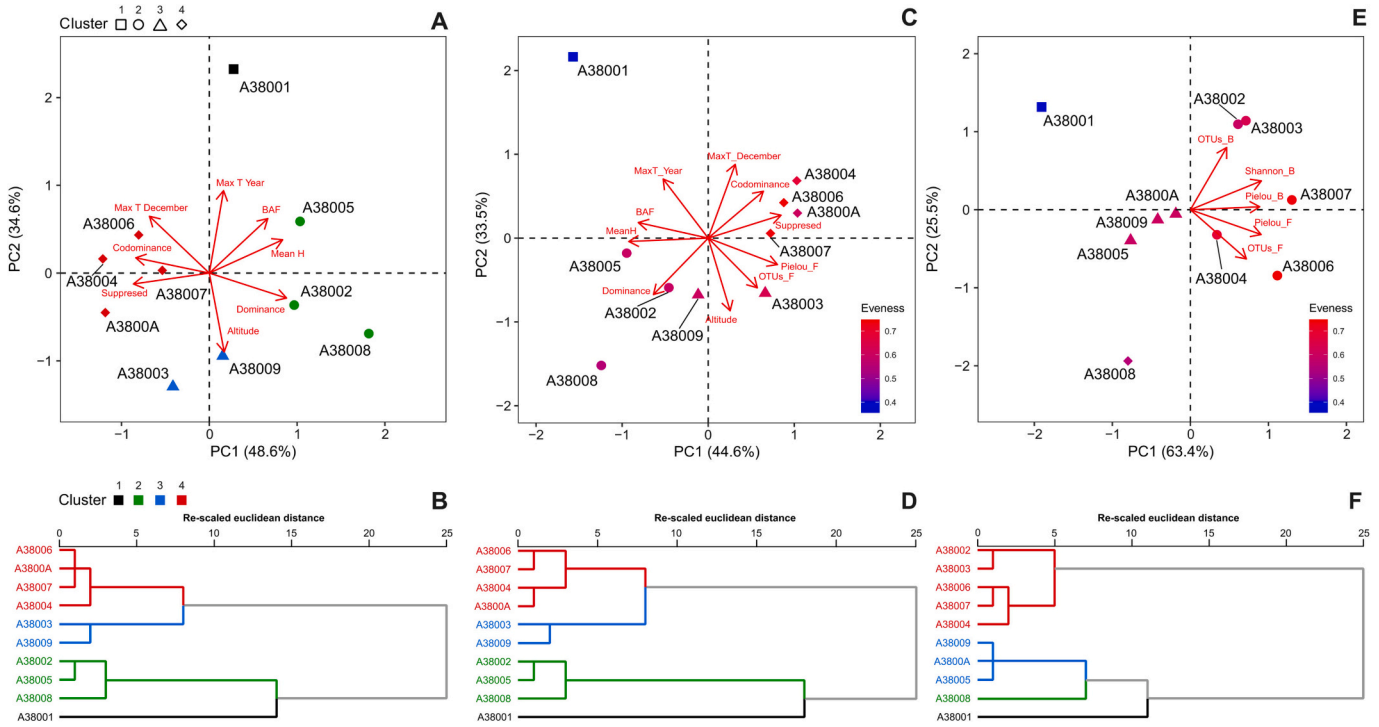


Fig. 1. Multivariable and discriminant analysis of environmental characteristics of the studied plots. A: PCA scores (points) and loads (arrows) of environmental variables. B: HCA dendrogram of environmental variables. C: Scores and loads of PCA analysis for a mixed environmental and biodiversity dataset. D: HCA dendrogram for mixed environmental and biodiversity dataset. E: PCA scores and loads for biodiversity variables. F: HCA dendrogram for biodiversity variables. Clusters are represented by colours and shapes. The colour gradient of plots C and E represent the average of Pielou's Evenness vector for each plot. F: fungi and B: bacteria.

Table 2
Summary of forest health data and stand characteristics of the 10 plots sampled.

Sample ID	Location	Kind	Sp2[a]	Density[b]	Age tree	Insect (%)[c]	Fungi (%)[d]	Fungi trunk[e]	Fungi needles[f]	Abiotic[g]	Crown[h]	Health status[i]	Defol (%)[j]	Discol (%)[k]	Cankers[l]	Death (%)[m]	Sick (%)[n]	Healthy (%)[o]
A38007	Quintana del Pidio	Mixed	*Quercus ilex*	Normal	70	21	8	0	1	25	86	2	37	38	17	12.50	41.67	45.83
A38006	La Horra	Mixed	*Pinus pinea*	Deficient	50	67	8	0	1	4	79	2	44	35	8	20.83	45.83	33.33
A38004	Iscar	Mixed	*Juniperus communis*	Excessive	50	63	71	0	1	33	97	1	18	25	0	16.67	37.50	45.83
A3800A	Bayubas de Arriba	Pure		Excessive	50	33	17	0	1	4	93	1	32	28	0	0.00	25.00	75.00
A38009	Gete	Pure		Excessive	70	8	0	0	0	21	89	2	38	33	0	4.17	16.67	79.17
A38008	Huerta del Rey	Pure		Deficient	70	4	0	0	0	0	100	1	22	13	0	8.33	33.33	58.33
A38005	El Arenal	Pure		Normal	70	33	0	0	0	0	98	1	25	16	0	0.00	12.50	87.50
A38003	Aguas Cánidas	Mixed	*Quercus pyrenaica*	Excessive	50	13	13	13	0	0	88	1	30	22	8	0.00	16.67	83.33
A38002	Pinilla de la Valdería	Mixed	*Juniperus thurifera*	Deficient	85	0	0	0	0	0	100	1	30	23	8	12.50	20.83	66.67
A38001	Nogarejas	Pure		Deficient	70	17	0	0	0	0	100	1	25	20	0	0.00	29.17	70.83

[a] Secondary specie in mixed forest.
[b] For the study we organize in thee different intervals: Normal (from 450 to 650 trees/ha), Deficient (<450 trees/ha) and Excesive (more that 650 trees/ha).
[c] Percentage of the tress with insect presence.
[d] Percentage of the tress with fungal presence.
[e] Percentage of the tress with fungal presence on the trunk (as *Phomitopsis* sp., *Phellinus* sp. …).
[f] Percentage of the tress with fungal presence on the needles (as *Lophodermiun* sp., *Dothistroma pini*).
[g] Percentage of trees with abiotic damage.
[h] Percentage of evaluable crown.
[i] Average health status (0 = healthy to 6 = dead > 5 years).
[j] Percentage of medium defoliation.
[k] Percentage of medium discoloration.
[l] Percentage of trees evaluated with cankers.
[m] Percentage of trees evaluated dead.
[n] Percentage of trees evaluated with disease symptoms.
[o] Percentage of trees evaluated healthy.

and environmental variables.

3.2. Microbial community diversity and composition related to forest health

3.2.1. Taxonomy and community composition

After quality filtering and reads processing, a total of 747.825 fungal ITS sequences and 115.941 bacterial V4 sequences remained for community analyses, with an average number of approximately 75.355 ± 34.348 and 11.937 ± 7.918 reads/sample for each dataset. From these, 449 OTUs for fungi and 335 OTUs for bacteria resulted (97 % clustering similarity), 442 (98.44 %) and 335 (100 %) were finally assigned to kingdom Fungi and domain Bacteria, respectively. The analysis of the rarefaction curves indicated that the total diversity was covered for both communities (Fig. S1).

The number of OTUs and their relative abundance belonging to fungal phyla were as follows: Ascomycota (241 OTUs; 54.5 %) Basidiomycota (140 OTUs; 31.67 %), Mucoromycota (15 OTUs; 33.9 %), *incertae sedis* corresponding to the Mortierellales order (18 OTUs; 4.07 %) and unknown or not assigned (28 OTUs; 6.33 %) due to the low percentage of similarity (Fig. 2A). The number of OTUs and their relative abundance belonging to bacterial phyla were as follows: Actinobacteria (53 OTUs; 16 %) Bacteroidetes (19 OTUs; 5.7 %), Acidobacteria (55 OTUs; 16.4 %), Chloroflexi (4 OTUs; 1.2 %), Cyanobacteria (1 OTUs; 0.3 %), Euryarchaeota (1 OTUs; 0.3 %), Firmicutes (17 OTUs; 5.1 %), Gemmatimonadetes (3 OTUs; 0.9 %), Planctomycetes (22 OTUs; 6.6 %),

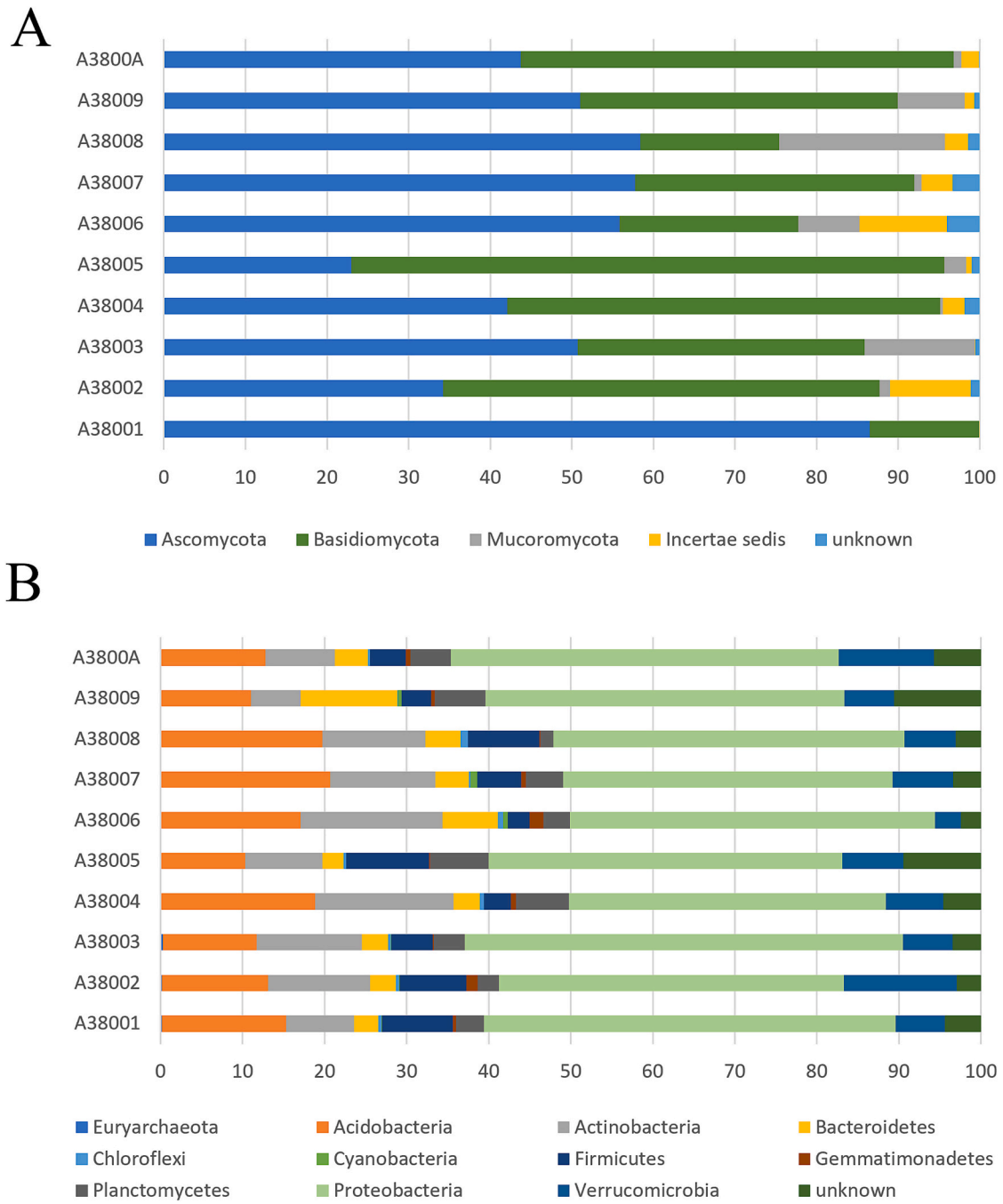


Fig. 2. Relative abundances of fungal (A) and bacterial (B) phyla from the ten natural stands of *P. pinaster* ssp. *mesogeensis* studied. *Incertae sedis* correspond to the Mortierellales order. Unknown grouped OTUs with <80 % to similarity using the BLAST tool in the Genbank database.

Proteobacteria (123 OTUs; 36.7 %), Verrucomicrobia (12 OTUs; 3.6 %) and unknown (25 OTUs; 7.46 %) due to the low percentage of identity (Fig. 2B).

Within the phylum Ascomycota, the most abundant orders were Helotiales, Pleosporales and Eurotiales (Fig. 3). In the case of Basidiomycota, Agaricales, Thelephorales and Atheliales were those with a higher relative abundance and the orders Mucorales and Umbelopsidales order for Mucoromycota phylum (Fig. 3).

Regarding relations between fungal and bacterial phyla, positive correlations were found between Basidiomycota and Planctomycetes ($r = 0.70$; $p < 0.05$), *Incertae sedis* (Mortierellaes order) and Gemmatimonadetes ($r = 0.94$; $p < 0.001$), Proteobacteria and Euryarchaeota ($r = 0.75$; $p < 0.01$). Negative correlations were found between Planctomycetes and Choroflexi ($r = -0.65$; $p < 0.05$) and Basidiomycota and Ascomycota ($r = -0.90$; $p < 0.001$) (Fig. 4).

3.2.2. *Microbial diversity and forest health*

The fungal community presents a greater variety in the alpha diversity with diversity and species richness indices that vary in each sampling site (Table 3). Only 14 OTUs (3.12 %) were present in the 10 plots.

Although a low level of variability was present in the bacterial community, differences were enough to present a relevant contribution to the variability of the biodiversity dataset of the microbial community. The multivariate discriminant analysis differentiated 4 different clusters, showing good consistency with the environmental characterization of plots. Once eliminated the Shannon index of the fungal community to avoid collinearity problems, the PCA analysis and the HCA (Fig. 1E and F) showed again a clear separation of the A38001 plot (Nogarejas) from the rest, being most of the plots aggregated in the further cluster (Cluster 4) classified similarly by environmental characteristics. Moreover, the analysis of biodiversity indices separated the A38008 plot (Huerta del Rey) from the rest in a transitional position.

The correlation matrix evaluation for the biodiversity indices from fungal community showed positives correlations between Shannon's diversity index and the percentage of trees with insect and fungi presence ($r = 0.65$ and $r = 0.72$; $p < 0.05$), percentage of the trees with fungal presence on the needles ($r = 0.84$; $p < 0.01$), trees with abiotic damage ($r = 0.84$; $p < 0.05$), discoloration ($r = 0.84$; $p < 0.01$), dead trees ($r = 0.75$; $p < 0.01$) and trees evaluated with disease symptoms ($r = 0.69$; $p < 0.05$). Negative correlations with the age of the tree ($r = -0.67$; $p < 0.05$), percentage of valuable crown and healthy trees ($r = -0.77$; $p < 0.01$). The evenness vector (Pielou-e) of the fungal community presented significant correlation with trees with fungal presence ($r = 0.692$, $p < 0.05$), fungi in needles ($r = 0.751$, $p < 0.05$), abiotic disturbances ($r = 0.708$, $p < 0.05$), crown ($r = -0.901$, $p < 0.01$) and discolouration ($r = 0–799$, $p < 0.01$), with significant differences between mixed and pure stands in the Pieolu's eveness vector ($H = 4.82$; $p < 0.05$).

The bacterial community resulted in more homogeneity among stands with higher index values than fungal data (Table 3). In this case, 151 OTUs (45.07 %) were common in the 10 plots. Positive correlations were found between Shannon's diversity index and the percentage of trees evaluated with cankers ($r = 0.84$; $p < 0.01$) and percentage of trees evaluated with disease ($r = 0.73$; $p < 0.01$). In the same way that in the fungal community, a negative correlation was found for the percentage of healthy trees ($r = -0.74$; $p < 0.01$). Pielou's evenness index shows the same result between the bacterial evenness and a positive correlation for the health parameters of the percentage of trees evaluated with cankers ($r = 0.87$; $p < 0.001$), percentage of trees evaluated with disease ($r = 0.68$; $p < 0.05$) and negative correlation with the percentage of healthy trees ($r = -0.70$; $p < 0.05$). As with the fungal community analysis, significant differences were found between mixed and pure stands in Pielou's evenness vector ($H = 6.81$; $p < 0.01$).

3.2.3. *Microbial guilds*

Of the OTUs confidently classified in an ecological guild (Fig. 5), the ectomycorrhizal and Soil-Plant Saprotroph guild were the most abundant, except in A38001 (Nogarejas), where plant pathogen-saprotroph was the most-abundant guild (over 50 %).

The Spearman's correlation matrix between functional guilds shows a significant positive correlation between Soil-Plant Saprotroph guilds

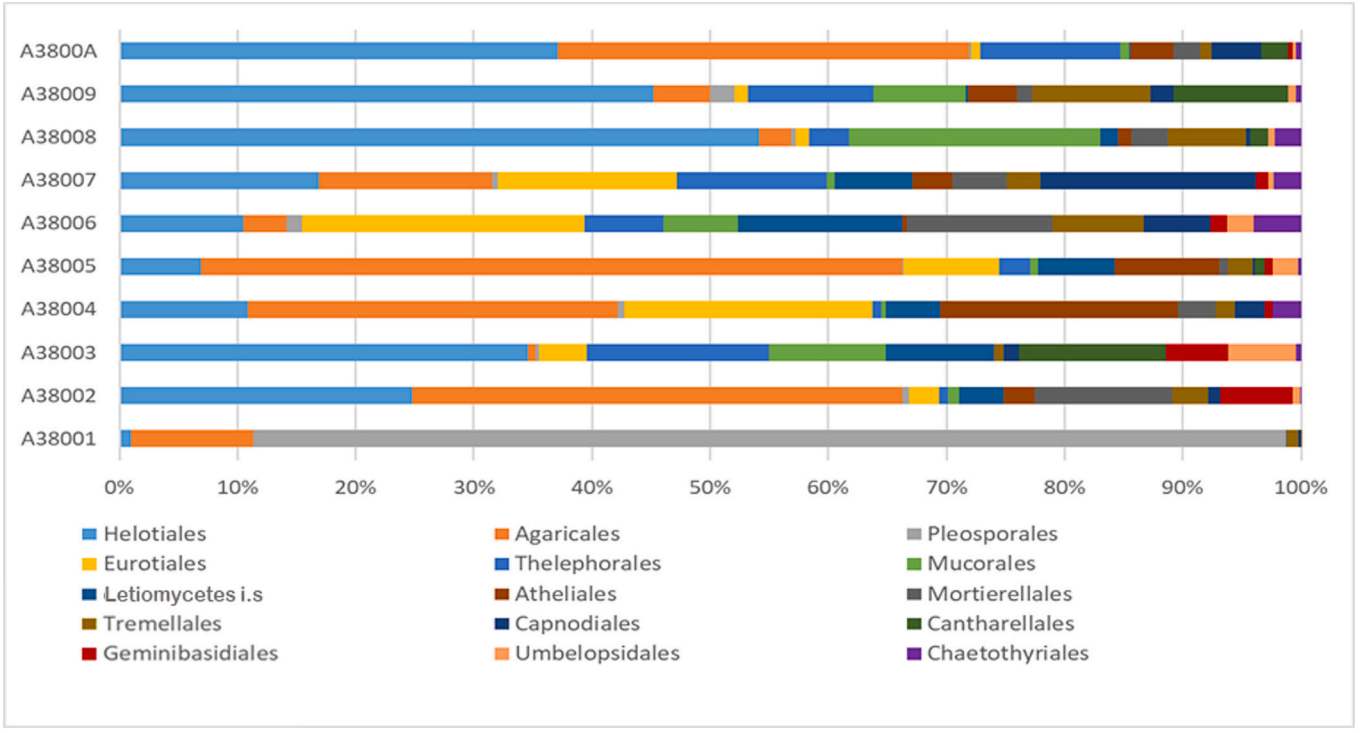


Fig. 3. Relative abundances of the most abundant 15 fungal orders from the ten natural stands of *P. pinaster* ssp. *mesogeensis* studied.

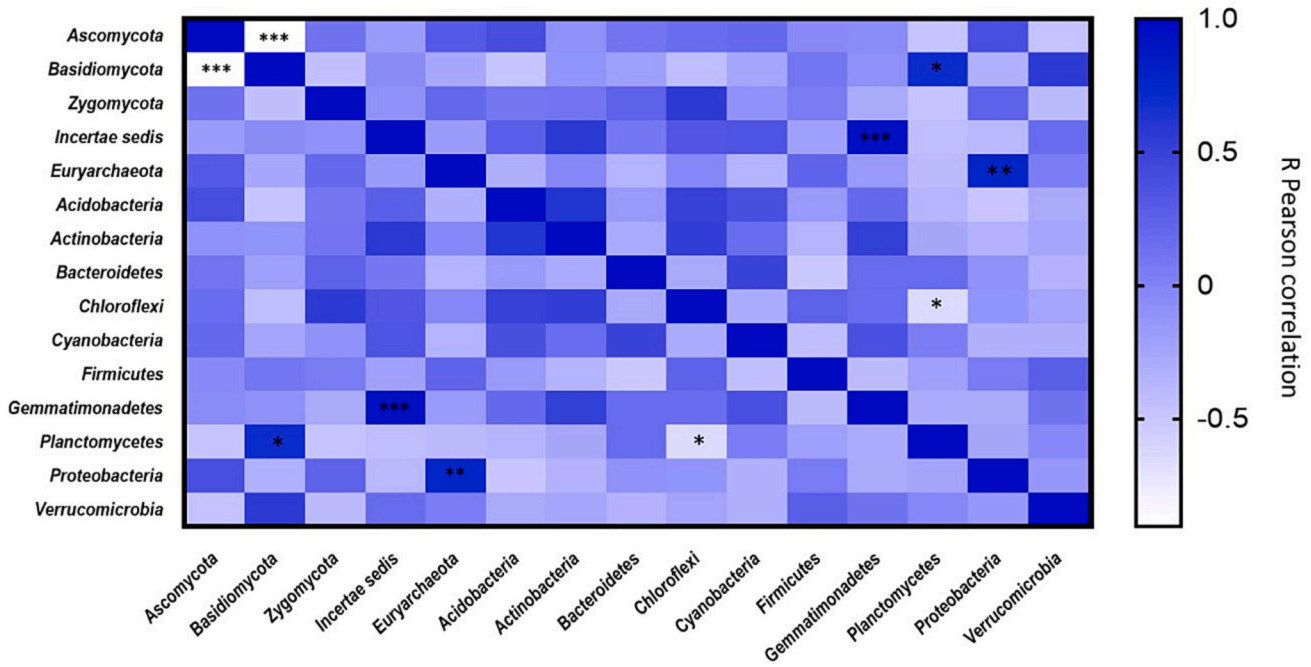


Fig. 4. Relationships between fungal and bacteria phyla on the studied ten natural stands of *P. pinaster* ssp. *mesogeensis*. Correlation matrix between phyla relative abundance sample. *Significant correlation at $P < 0.05$; **significant correlation at $P < 0.01$, ***significant correlation at $P < 0.001$. *Incertae sedis* correspond to the Mortierellales order.

Table 3
Alpha diversity of the ten soils sampled for fungal and bacterial populations.

Location	ID sample	Fungal			Bacterial		
		Shannon	Observed otus	Pielou evenness	Shannon	Observed otus	Pielou evenness
Nogarejas	A38001	2.02	38	0.38	6.3	261	0.79
Pinilla de la Valdería	A38002	4.36	171	0.59	6.8	291	0.83
Aguas Cánidas	A38003	4.66	164	0.63	6.8	294	0.83
Iscar	A38004	5.13	185	0.68	6.6	261	0.82
El Arenal	A38005	4.44	190	0.59	6.3	269	0.78
La Horra	A38006	5.83	238	0.74	6.7	259	0.84
Quintana del Pidio	A38007	5.72	221	0.73	6.8	284	0.84
Huerta del Rey	A38008	4.21	202	0.55	6.3	209	0.81
Gete	A38009	4.47	173	0.6	6.4	268	0.8
Bayubas de Arriba	A3800A	4.55	191	0.6	6.5	272	0.8

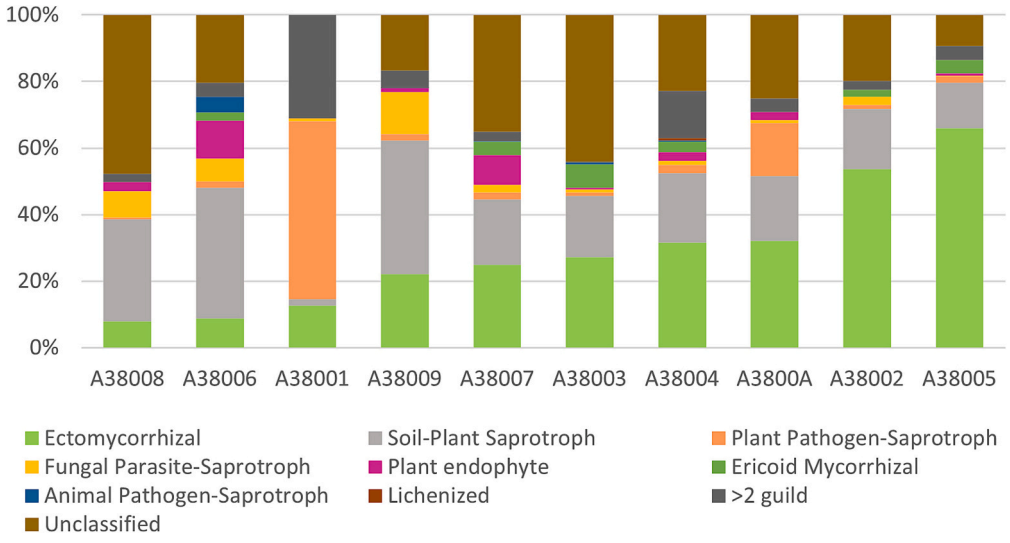


Fig. 5. Relative abundances of fungal functional guilds from the ten natural stands of *P. pinaster* ssp. *mesogeensis* studied.

and Endophyte-Saprotroph ($r = 0.72$; $p < 0.05$), Fungal parasite ($r = 0.79$; $p < 0.05$) and Fungal parasite-Saprotroph ($r = 0.77$; $p < 0.05$). Negative correlation was found between Ectomycorrhizal and Fungal parasite-Saprotroph ($r = -0.74$; $p < 0.05$).

The Spearman's correlation matrix between functional guilds and forest health parameters showed a significant, positive correlation for animal pathogen and percentage of death trees ($r = 0.65$; $p < 0.05$) and negative with the crow status ($r = -0.77$; $p < 0.05$). Ectomycorrhizal-Fungal parasite guild showed a positive correlation with the age of the tree ($r = 0.63$; $p < 0.05$). Endophyte-Saprotroph guild presents a positive correlation with percentage of the trees with fungal presence on the needles ($r = 0.71$; $p < 0.05$), average health status ($r = 0.72$; $p < 0.05$), percentage of medium discoloration ($r = 0.67$; $p < 0.05$), percentage of trees evaluated dead ($r = 0.68$; $p < 0.05$), percentage of trees evaluated with disease symptoms ($r = 0.69$; $p < 0.05$) and conversely negative correlation with percentage of evaluable crown ($r = -0.74$; $p < 0.05$) and percentage of trees evaluated healthy ($r = -0.73$; $p < 0.05$). Fungal parasite-saprotroph and Soil-Plant Saprotroph showed a positive correlation with the average of health status ($r = 0.66$ and $r = 0.64$; $p < 0.05$). The rest of fungal functional guild didn't show any correlation with the parameters studied.

On the other hand, five out of the 11 bacterial phyla showed correlations with the health parameters studied; being Firmicutes these who showed a higher number of correlations. The Spearman's correlation matrix between bacteria phyla and forest health parameters showed positive correlation between Euryarchaeota and Proteobacteria and percentage of the tress with fungal presence on the trunk ($r = 0.78$; $p < 0.01$ and $r = 0.68$; $p < 0.05$); and Bacteroidetes and average health status ($r = 0.74$; $p < 0.05$). Firmicutes showed a positive correlations with the age tree ($r = 0.68$; $p < 0.05$), percentage of evaluable crow ($r = 0.73$; $p < 0.05$) and percentage of trees evaluated healthy($r = 0.65$; $p < 0.05$) and negative with percentage of trees with fungal presence on the needles ($r = -0.67$; $p < 0.05$), percentage of medium discoloration ($r = -0.76$; $p < 0.01$) and percentage of trees evaluated dead ($r = -0.64$; $p < 0.05$).

3.3. Relationship of plot characteristics and microbial biodiversity

When biodiversity indices, health status and site characteristics were analyzed together with the multivariable discriminant methodology, the results confirmed the high level of influence of the environmental variables and the fungal alpha biodiversity indices in the separation of plots (Fig. 1C and D), being bacterial biodiversity indices poorly related with the PCA factors in all the cases, and thus, eliminated from the multivariable analysis. The eight variables accounting for the maximum variance in the optimal PCA solution for environmental characterization also appeared among the 10 selected variables for the mixed dataset, together with Shannon and Pielou's indices of the fungal community. In this case, distances in the bi-dimensional plot were lower for most of the locations, except for A38001 (Nogarejas) and A38008 (Huerta del Rey) plots, which appeared clearly separated from the rest, although A38008 was classified inside Cluster 2. The HCA result coincided with the analysis of environmental characteristics despite slight differences in the distance between clusters.

The NMDS analysis showed the aggregation pattern among some locations in the case of the fungal dataset (Fig. 6), which resulted in significance for the geographic zones (ANOSIM; $r = 0.86$, $p < 0.05$). Huerta del Rey (A38008) was grouped with Gete (A38009) (both zone 7) because of similar taxonomic composition, which might be the reason for the differences in the classification of both plots regarding multivariable analysis between environmental characteristics and biodiversity indices. No differences were found for the bacterial community regarding all the analyzed variables (ANOSIM, $r \approx 0$), confirming the low degree of variability of this community also considering beta diversity.

Among the core biome of the fungal community, OTUs 4 and 24, both *Umbelopsis* sp., were the most relevant OTUs influencing the separation between plots. The most abundant fungal OTU (OTU 1, identified as *Epicoccum nigrum*) presented the lower influence in the plot classification, probably due to the high homogeneity and low abundance of their frequency among plots. Between the bacterial OTUs, although no relationships were found with any dependent variable, four OTUs showed great influence in the separation between plots, with high differences from the rest of the bacterial OTUs. Those OTUs were OTU 6 (*Herbaspirillum* sp.), OTU 13 (*Pseudomonas* sp.), OTU 32 (*Mucilaginibacter ximonensis*) and OTU 10 (unidentified species of the Phyla Acidobacteria).

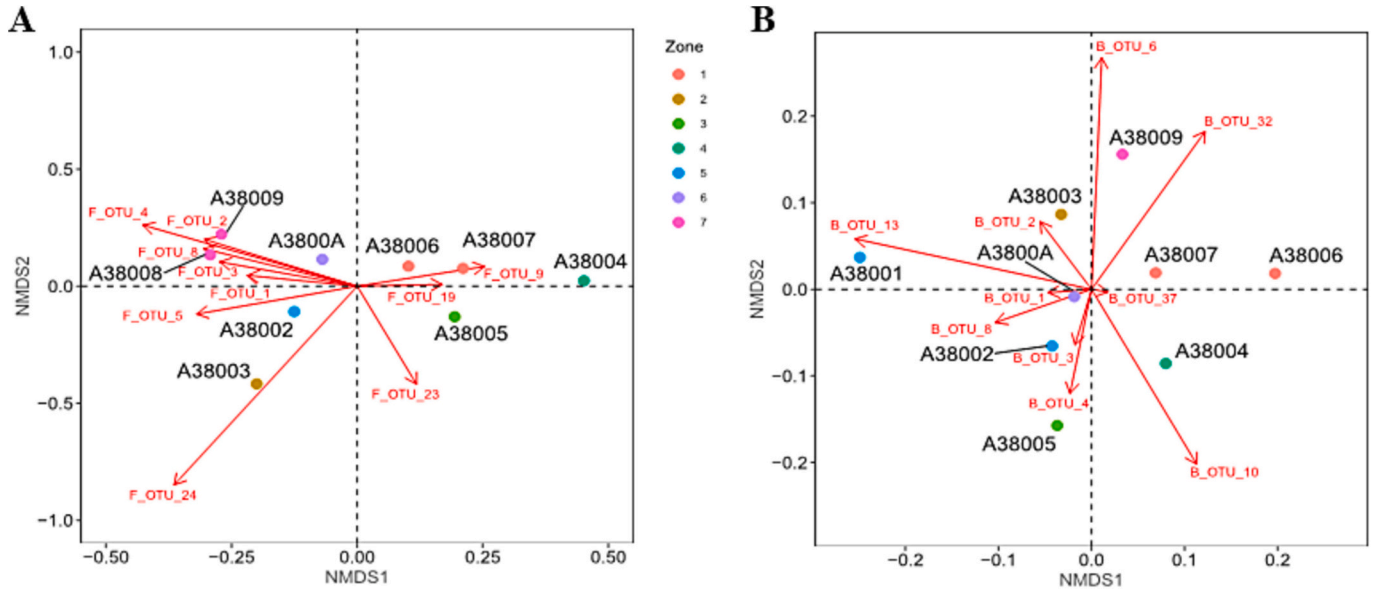


Fig. 6. NMDS analysis for fungal (A) and bacterial (B) communities. Arrows showed partial importance vectors for the 10 most abundant species present in the core biome. F_OTU_2 Hyaloscyphaceae; F_OTU_3 *Alternaria alternata*; F_OTU_4 *Umbelopsis* sp.; F_OTU_5 Hyaloscyphaceae; F_OTU_8 *Solicoccozyma terricola*,; F_OTU_9 Capnodiales sp.; F_OTU_19 *Mortierella elongata*; F_OTU_*23 Oidiodendron chlamydosporicum*; F_OTU_24 *Umbelopsis* sp. B_OTU_1 *Bradyrhizobium lupini*; B_OTU_2 *Burkholderia* sp.; B_OTU_3 Chthoniobacteraceae; B_OTU_4 unknown.; B_OTU_6 *Herbaspirillum* sp.; B_OTU_8 *Paenibacillus* sp.; B_OTU_10 Acidobacteria; B_OTU_13 *Pseudomonas* sp.; B_OTU_32 *Mucilaginibacter ximonensis*; B_OTU_37 Chthoniobacteraceae.

4. Discussion

To the best of our knowledge, this work is the first study of soil biodiversity in *Pinus pinaster* ssp. *mesogeensis* ecosystems, exploring the interaction between fungal and bacterial communities and their influence on forest health parameters, focusing on the soil microbiome.

In comparison with recent analyses of the mycobiota in soils of different ecosystems (Jimu et al., 2017; Veach et al., 2018; Chen et al., 2018; Ruiz-Gómez et al., 2019), the *P. pinaster* stands had a high Shannon H′ index (>4 in all the studied zones, except A38001) and a high value of evenness (exceeding 0.6 in most of the case). This shows a high diversity and good equilibrium of the microbiota (Ruiz-Gómez et al., 2019). The low biodiversity found in the A38001 samples would be explained by the overrepresentation of OTU 1 corresponding to *Epicocum nigrum* (Table S2), this may be due to the presence of aggregated sporodochia in the soil sample. The large representation of this organism has obscured the presence of others due to a large amount of DNA resulting in low diversity indices and a low number of OTUS shared among the 10 samples.

The animal pathogen-saprotroph guild was positively associated with the abiotic damage of the trees and the fungal presence. This guild group saprotrophs genera *Pseudogymnoascus* (OTU 57 and OTU 210) or *Exophiala moniliae* (OTU 425). The increase of organic material due to the abiotic or biotic damage could be the reason for the positive correlation with this guild. A comprehensive screening carried out by Smith et al. (2017) showed that a relatively high proportion of wood-decay fungi are occasionally able to associate in vitro with otherwise intact roots of *Pinus sylvestris* or *Picea abies* and suggested that the association of wood-decay fungi with roots of coniferous trees is not an exception and might indicate the optional endophytic lifestyle of some fungi. Endophytism can be seen as a newly acquired lifestyle option for these typical saprotrophs, which might be an intermediary between saprotrophic and mycorrhizal lifestyles (Selosse et al., 2009). The beneficial effect of this new guild could explain the positive correlation between the average health status of the trees and the fungal parasite-saprotroph, soil-plant saprotroph and endophyte-saprotroph guilds. A positive correlation between ectomycorrizal-fungal parasite guild and the age of the trees was found. Ectomycorrizal-fungal parasite guild groups the Agaricales *Tricholoma portentosum* (OTU 12) and *T. terreum* (OTU 331). Ectomycorrhizal species present in any ecosystem depend on the host species and also on plant age (Dighton and Mason, 1985). Some studies on the succession of ectomycorrhizal fungi reveal fungi characteristic of early-seral stages and others of late-seral stages of stands (Arnolds, 1995). For example, in *Pinus sylvestris* stands in the northeast zone of the Iberian Peninsula, *Boletus edulis* sporocarp production is highly influenced by the *P. sylvestris* stand class age with higher production in the fourth stand age class, between 51 and 70-years-old, which could be considered mature stands (Martínez-Peña et al., 2012).

In accordance with the previous studies, bacterial community composition was relatively stable across the ten studied sites; being Proteobacteria, Acidobacteria and Actinobacteria phylum most abundant (Fierer et al., 2007; Lasa et al., 2019). The dominance of Proteobacteria and Acidobacteria as generally observed in most of soils indicated low impact by land-use type (Rampelotto et al., 2013). Proteobacteria constitute the largest and phenotypically most diverse division among prokaryotes (Gupta, 2000). Historically, members of phylum Acidobacteria (one of the most abundant phyla in soils habitats) have been considered as K-strategists or oligotrophs in terms of lifestyle (Lasa et al., 2019), being well adapted to low substrate availabilities and exhibiting slow growth rates (Fierer et al., 2007).

In the present study, Firmicutes phylum was positively associated with a high percentage of healthy trees, a good crown, and an older tree age. On the contrary, it showed a negative correlation with disease parameters as percentage of fungal needles, discoloration and percentage of dead trees. The activity and beneficial effects on plant health from Firmicutes are well documented (Dias et al., 2015; Grady et al., 2016). Specifically, the most abundant genera of the Firmicutes phyla founded was *Paenibacillus* (OTU 8) the genus *Paenibacillus* comprises bacterial species relevant to plants and to the environment (Grady et al., 2016). Many *Paenibacillus* species can promote crop growth directly via biological nitrogen fixation, phosphate solubilization, production of the phytohormone indole-3-acetic acid (IAA), and release of siderophores that enable iron acquisition. They can also offer protection against insect herbivores and phytopathogens, including bacteria, fungi, nematodes, and viruses (Grady et al., 2016). Bacteroidetes were positively associated with the health status of the trees. It is a common root-associated phylum (Angel et al., 2016) within these Sphingobacteriales (the most abundant order found in our study) are understood as copiotrophic bacteria, referring to their ability to metabolize a wide array of carbon sources and being present at great abundances in soils with high carbon availability (Fierer et al., 2007) and have been reported to be particularly sensitive to O_2 availability (Hester et al., 2018). The Proteobacteria group also plays a key role in organic matter decomposition (Lladó et al., 2016) by producing many kinds of glycosyl hydrolases, such as cellulases, chitinases, xylanases and amylases (Cristóbal et al., 2015), and then generating a large number of oligosaccharides and aromatic alcohols, which can be used as carbon resource by microorganism (Wei et al., 2017). Overall, Proteobacteria may play an important ecological role by collaborating with other microorganisms (the members of Acidobacteria) in the process of degrading polysaccharides of plant and fungal origin (Lladó et al., 2016). These functions could explain the positive correlation between proteobacteria and the presence of fungi in the rhizosphere.

The multivariate classification of plots by environmental conditions and health status gives a clear grouping of stands, which did not vary when the dataset included the biodiversity indices extracted from the microbial community analysis. This second classification included only 2 biodiversity variables and retained the same 8 variables identified for the first dataset. Also, when only microbial diversity was used, stand classification differed. These results revealed that environmental conditions had in our case larger influence on the stand status than the presence of pathogens or other microbial-associated species, agreeing with other studies (Sánchez-Cuesta et al., 2021; Terhonen et al., 2023). On the other hand, it would be interesting to assess at which point environmental variables different from the host species genetics influenced microbial community. Differences in stand classification between environmental and soil biodiversity datasets might be explained by other parameters not considered in this work, as for example tree genetics or stand management factors (e.g.: cattle, wildlife, recreative use) (Bastida et al., 2017; Guo et al., 2023). There is scarce literature about the influence of these factors on soil microbial diversity, and our results would help to focus future research on these changes, coming to understand whether the microbiome is influencing forest health or it is forest health that is influencing the microbiome.

5. Conclusions

This work is a novel contribution to the study of soil biodiversity in *P. pinaster* ecosystems, analyzing for the first time the fungal and bacterial communities together with environmental and health status characteristics of studied stands. Our results could reveal high levels of diversity in the fungal community and medium to low diversity of bacteria, which appeared related with the stand status, although explaining lesser variability than environmental variables. However, changes in soil microbial community composition were more relevant in extreme cases, such as the plot A38001. Although it was impossible to know whether it was a consequence of the environmental variables or another unknown factors, drastic changes in community composition and soil functioning appeared related with clear differences in the stand status. Moreover, it is very important to note that these data represent a specific momentary state of the communities based on a sample, which should be studied in greater depth in the future.

Many soil bacterial and fungal taxa, even those that are dominant, have not been extensively studied and their ecological role remain largely unknown so further research is needed in this sense. This study will help as first step to unravel the complexity of fungal and bacterial communities and the presence of key taxa influencing *P. pinaster* health in the analyzed plots.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2023.105142.

CRediT authorship contribution statement

Conceptualization and supervision, JD; laboratory experiment, CM and JM; data curation, CM, FR and JP; writing—original draft preparation, CM and FR; writing—review and editing, JD, JM and JP. All authors have read and agreed to the published version of the manuscript.

Funding

Research was funded by the Spanish Ministry of Science and Innovation and by the European Union through the Next Generation funds (project numbers: PID2019-110459RB and PLEC2021-008076) and project VA208P20 funded by Junta Castilla y León (JCYL, Spain) and EU funds. FR was supported by a post-doctoral fellowship of the Junta de Andalucía (Spain) and the European Social Fund 2014–2020 Program (Ref. DOC_0055).

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.

Data availability

No data was used for the research described in the article.

Acknowledgements

The authors want to thank the technicians Raul Arcadio and Mariano Rodríguez for helping in the field work.

References

Amin, M., Flowers, T.H., 2004. Evaluation of Kjeldahl digestion method. J. Res. Sci. 15, 159–179.

Angel, R., Conrad, R., Dvorsky, M., Kopecky, M., Kotilínek, M., Hiiesalu, I., et al., 2016. The root-associated microbial community of the world's highest growing vascular plants. Microb. Ecol. 72, 394–406. https://doi.org/10.1007/s00248-016-0779-8.

Aprile, F., Lorandi, R., 2012. Evaluation of cation exchange capacity (CEC) in tropical soils using four different analytical methods. J. Agric. Sci. 4, 278. https://doi.org/10.5539/jas.v4n6p278.

Arnolds, E., 1995. Conservation and management of natural populations of edible fungi. Can. J. Bot. 73, 987–998. https://doi.org/10.1139/b95-349.

Ashworth, J., Keyes, D., Kirk, R., Lessard, R., 2001. Standard procedure in the hydrometer method for particle size analysis. Commun. Soil Sci. Plant Anal. 32, 633–642. https://doi.org/10.1081/CSS-100103897.

Bálint, M., Schmidt, P.-A., Sharma, R., Thines, M., Schmitt, I., 2014. An Illumina metabarcoding pipeline for fungi. Ecol. Evol. 4, 2642–2653. https://doi.org/10.1002/ece3.1107.

Bastida, F., Torres, I.F., Andrés-Abellán, M., Baldrian, P., López-Modejar, R., Větrovský, T., Richnow, H.H., Starke, R., Ondoño, S., García, C., López-Serrano, F.R., Jehmlich, N., 2017. Differential sensitivity of total and active soil microbial communities to drought and forest management. Glob. Chang. Biol. 23, 4185–4203. https://doi.org/10.1111/gcb.13790.

Bokulich, N.A., Mills, D.A., 2013. Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. Appl. Environ. Microbiol. 79, 2519–2526. https://doi.org/10.1128/AEM.03870-12.

Cailleret, M., Heurich, M., Bugmann, H., 2014. Reduction in browsing intensity may not compensate climate change effects on tree species composition in the Bavarian Forest National Park. For. Ecol. Manage. 328, 179–192. https://doi.org/10.1016/j.foreco.2014.05.030.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., et al., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335. https://doi.org/10.1038/nmeth.f.303.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., et al., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. 108, 4516–4522. https://doi.org/10.1073/pnas.1000080107.

Chen, W., Xu, R., Wu, Y., Chen, J., Zhang, Y., Hu, T., et al., 2018. Plant diversity is coupled with beta not alpha diversity of soil fungal communities following N enrichment in a semi-arid grassland. Soil Biol. Biochem. 116, 388–398. https://doi.org/10.1016/j.soilbio.2017.10.039.

Connor, S.E., Araújo, J., Boski, T., Gomes, A., Gomes, S.D., Leira, M., et al., 2021. Drought, fire and grazing precursors to large-scale pine forest decline. Divers. Distrib. 27, 1138–1151. https://doi.org/10.1111/ddi.13261.

Cristóbal, H.A., Benito, J., Lovrich, G.A., Abate, C.M., 2015. Phylogenentic and enzymatic characterization of psychrophilic and psychrotolerant marine bacteria belong to γ-Proteobacteria group isolated from the sub-Antarctic Beagle Channel, Argentina. Fol. Microbiol. 60, 183–198. https://doi.org/10.1007/s12223-014-0351-1.

Dias, T., Dukes, A., Antunes, P.M., 2015. Accounting for soil biotic effects on soil health and crop productivity in the design of crop rotations. J. Sci. Food Agric. 95, 447–454. https://doi.org/10.1002/jsfa.6565.

Díaz-Urbano, M., Goicoechea, N., Velasco, P., Poveda, J., 2023. Development of agricultural bio-inoculants based on mycorrhizal fungi and endophytic filamentous fungi: co-inoculants for improve plant-physiological responses in sustainable agriculture. Biol. Control 182, 105223. https://doi.org/10.1016/j.biocontrol.2023.105223.

Dighton, J., Mason, P.A., 1985. Mycorrhizal dynamics during forest tree development. In: Developmental Biology of Higher Fungi, pp. 117–139.

Dobbertin, M., Brang, P., 2001. Crown defoliation improves tree mortality models. For. Ecol. Manage. 141, 271–284. https://doi.org/10.1016/S0378-1127(00)00335-2.

Duque-Lazo, J., Van Gils, H., Groen, T.A., Navarro-Cerrillo, R.M., 2016. Transferability of species distribution models: the case of *Phytophthora cinnamomi* in Southwest Spain and Southwest Australia. Ecol. Model. 320, 62–70. https://doi.org/10.1016/j.ecolmodel.2015.09.019.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10, 996–998. https://doi.org/10.1038/nmeth.2604.

Eichhorn, J., Roskams, P., Ferretti, M., Mues, V., Szepesi, A., Durrant IV, D., 2010. Manual part IV: visual assessment of crown condition and damaging agents. In: Manual on Methods and Criteria for Harmonized Sampling, Assessment, Monitoring and Analysis of the Effects of Air Pollution on Forests, 49. UNECE ICP Forests Programme Co-ordinating Centre.

El Khoury, Y., Noujeim, E., Bubici, G., Tarasco, E., Al Khoury, C., Nemer, N., 2021. Potential factors behind the decline of *Pinus pinea* nut production in Mediterranean pine forests. Forests 12, 1167. https://doi.org/10.3390/f12091167.

EU Council Directive, 1992. 92/43/EEC of May 21, 1992 on the Conservation of Natural Habitats and of Wild Fauna and Flora. European Union.

Faria, M., Bertocco, T., Barroso, A., Carvalho, M., Fonseca, F., Delerue Matos, C., et al., 2023. A comparison of analytical methods for the determination of soil pH: case study on burned soils in Northern Portugal. Fire 6, 227. https://doi.org/10.3390/fire6060227.

Ferreti, M., Peña, G.S., 1994. Especies forestales mediterráneas: guía para la evalución de las copas. Comisión Económica de las Naciones Unidas para Europa.

Fierer, N., Bradford, M.A., Jackson, R.B., 2007. Toward an ecological classification of soil bacteria. Ecology 88, 1354–1364. https://doi.org/10.1890/05-1839.

Gerenfes, D., Giorgis, A.G., Negasa, G., 2022. Comparison of organic matter determination methods in soil by loss on ignition and potassium dichromate method. Int. J. Hortic. Food Sci. 4, 49–53.

Gomes, T., Pereira, J.A., Moya-Laraño, J., Poveda, J., Lino-Neto, T., Baptista, P., 2023. Deciphering plant health status: the link between secondary metabolites, fungal community and disease incidence in olive tree. Front. Plant Sci. 14, 1048762. https://doi.org/10.3389/fpls.2023.1048762.

Goyal, S.S., 2002. Use of high performance liquid chromatography for soil and plant analysis. Commun. Soil Sci. Plant Anal. 33, 2617–2641. https://doi.org/10.1081/CSS-120014468.

Grady, E.N., MacDonald, J., Liu, L., Richman, A., Yuan, Z.C., 2016. Current knowledge and perspectives of *Paenibacillus*: a review. Microb. Cell Fact. 15, 1–18. https://doi.org/10.1186/s12934-016-0603-7.

Guo, J., Gong, X., Yu, S., Wei, B., Chu, L., Liu, J., He, X., Yu, M., 2023. Responses of soil microbial diversity to forest management practices after pine wilt disease infection. Forests 14, 862. https://doi.org/10.3390/f14050862.

Gupta, R.S., 2000. The phylogeny of proteobacteria: relationships to other eubacterial phyla and eukaryotes. FEMS Microbiol. Rev. 24, 367–402. https://doi.org/10.1111/j.1574-6976.2000.tb00547.x.

Hennon, P.E., McWilliams, M.G., 1999. Decline symptoms do not develop with grafting from dying yellow-cedar. Can. J. For. Res. 29, 1985–1988. https://doi.org/10.1139/x99-161.

Hennon, P.E., D'Amore, D.V., Schaberg, P.G., Wittwer, D.T., Shanley, C.S., 2012. Shifting climate, altered niche, and a dynamic conservation strategy for yellow-cedar in the North Pacific coastal rainforest. BioScience 62, 147–158. https://doi.org/10.1525/bio.2012.62.2.8.

Hester, E.R., Harpenslager, S.F., van Diggelen, J.M., Lamers, L.L., Jetten, M.S., Lüke, C., et al., 2018. Linking nitrogen load to the structure and function of wetland soil and

rhizosphere microbial communities. Msystems 3, e00214–e00217. https://doi.org/10.1128/mSystems.00214-17.
Hurel, A., de Miguel, M., Dutech, C., Desprez-Loustau, M.L., Plomion, C., Rodríguez-Quilón, I., et al., 2021. Genetic basis of growth, spring phenology, and susceptibility to biotic stressors in maritime pine. Evol. Appl. 14, 2750–2772. https://doi.org/10.1111/eva.13309.
Hurtado, M.D., Carmona, S., Delgado, A., 2008. Automated modification of the molybdenum blue colorimetric method for phosphorus determination in soil extracts. Commun. Soil Sci. Plant Anal. 39, 2250–2257. https://doi.org/10.1080/00103620802289125.
Huson, D.H., Mitra, S., Ruscheweyh, H.J., Weber, N., Schuster, S.C., 2011. Integrative analysis of environmental sequences using MEGAN4. Genome Res. 21, 1552–1560. https://doi.org/10.1101/gr.120618.111.
Jimu, L., Kemler, M., Mujuru, L., Mwenje, E., 2017. Illumina DNA metabarcoding of Eucalyptus plantation soil reveals the presence of mycorrhizal and pathogenic fungi. Forestry 91, 238–245. https://doi.org/10.1093/forestry/cpx046.
Kavanagh, K., 2017. Fungi: Biology and Applications. John Wiley & Sons.
Kumawat, K.C., Razdan, N., Saharan, K., 2022. Rhizospheric microbiome: bio-based emerging strategies for sustainable agriculture development and future perspectives. Microbiol. Res. 254, 126901. https://doi.org/10.1016/j.micres.2021.126901.
Lasa, A.V., Fernández-González, A.J., Villadas, P.J., Toro, N., Fernández-López, M., 2019. Metabarcoding reveals that rhizospheric microbiota of *Quercus pyrenaica* is composed by a relatively small number of bacterial taxa highly abundant. Sci. Rep. 9, 1695. https://doi.org/10.1038/s41598-018-38123-z.
Lindahl, B.D., Nilsson, R.H., Tedersoo, L., Abarenkov, K., Carlsen, T., Kjøller, R., et al., 2013. Fungal community analysis by high-throughput sequencing of amplified markers—a user's guide. New Phytol. 199, 288–299. https://doi.org/10.1111/nph.12243.
Lladó, S., Žifčáková, L., Větrovský, T., Eichlerová, I., Baldrian, P., 2016. Functional screening of abundant bacteria from acidic forest soil indicates the metabolic potential of Acidobacteria subdivision 1 for polysaccharide decomposition. Biol. Fertil. Soils 52, 251–260. https://doi.org/10.1007/s00374-015-1072-6.
Lloyd, E.A., Wade, M.J., 2019. Criteria for holobionts from community genetics. Biol. Theory 14, 151–170. https://doi.org/10.1007/s13752-019-00322-w.
Manion, P.D., 1981. Tree Disease Concepts. Prentice-Hall, Inc.
Martínez-Peña, F., Ágreda, T., Águeda, B., Ortega-Martínez, P., Fernández-Toirán, L.M., 2012. Edible sporocarp production by age class in a Scots pine stand in Northern Spain. Mycorrhiza 22, 167–174. https://doi.org/10.1007/s00572-011-0389-8.
McDonald, D., Clemente, J.C., Kuczynski, J., Rideout, J.R., Stombaugh, J., Wendel, D., et al., 2012. The Biological Observation Matrix (BIOM) format or: how I learned to stop worrying and love the ome-ome. Gigascience 1, 1–6. https://doi.org/10.1186/2047-217X-1-7.
Morales-Rodríguez, C., Sferrazza, I., Aleandri, M.P., Dalla Valle, M., Speranza, S., Contarini, M., Vannini, A., 2021. The fungal community associated with the ambrosia beetle Xylosandrus compactus invading the mediterranean maquis in central Italy reveals high biodiversity and suggests environmental acquisitions. Fungal Biol. 125, 12–24. https://doi.org/10.1016/j.funbio.2020.09.008.
Navarro-Cerrillo, R.M., Rodriguez-Vallejo, C., Silveiro, E., Hortal, A., Palacios-Rodríguez, G., Duque-Lazo, J., Camarero, J.J., 2018. Cumulative drought stress leads to a loss of growth resilience and explains higher mortality in planted than in naturally regenerated *Pinus pinaster* stands. Forests 9, 358. https://doi.org/10.3390/f9060358.
Nguyen, N.H., Song, Z., Bates, S.T., Branco, S., Tedersoo, L., Menke, J., et al., 2016. FUNGuild: an open annotation tool for parsing fungal community datasets by ecological guild. Fungal Ecol. 20, 241–248. https://doi.org/10.1016/j.funeco.2015.06.006.
Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al., 2018. vegan: Community Ecology Package. R Package Version 2.5-2. 2018.
Pantigoso, H.A., Newberger, D., Vivanco, J.M., 2022. The rhizosphere microbiome: plant–microbial interactions for resource acquisition. J. Appl. Microbiol. 133, 2864–2876. https://doi.org/10.1111/jam.15686.
Parks, D.H., Mankowski, T., Zangooei, S., Porter, M.S., Armanini, D.G., Baird, D.J., et al., 2013. GenGIS 2: geospatial analysis of traditional and genetic biodiversity, with new gradient algorithms and an extensible plugin framework. PloS One 8, e69885. https://doi.org/10.1371/journal.pone.0069885.
Poveda, J., Abril-Urias, P., Escobar, C., 2020. Biological control of plant-parasitic nematodes by filamentous fungi inducers of resistance: *Trichoderma*, mycorrhizal and endophytic fungi. Front. Microbiol. 11, 992. https://doi.org/10.3389/fmicb.2020.00992.
Poveda, J., Díaz-González, S., Díaz-Urbano, M., Velasco, P., Sacristán, S., 2022. Fungal endophytes of Brassicaceae: molecular interactions and crop benefits. Front. Plant Sci. 13, 932288. https://doi.org/10.3389/fpls.2022.932288.
Prieto-Recio, C., Martín-García, J., Bravo, F., Diez, J.J., 2015. Unravelling the associations between climate, soil properties and forest management in *Pinus pinaster* decline in the Iberian Peninsula. For. Ecol. Manage. 356, 74–83. https://doi.org/10.1016/j.foreco.2015.07.033.
Rampelotto, P.H., de Siqueira Ferreira, A., Barboza, A.D.M., Roesch, L.F.W., 2013. Changes in diversity, abundance, and structure of soil bacterial communities in Brazilian Savanna under different land use systems. Microb. Ecol. 66, 593–607. https://doi.org/10.1007/s00248-013-0235-y.
Ribeiro, S., Cerveira, A., Soares, P., Fonseca, T., 2022. Natural regeneration of maritime pine: a review of the influencing factors and proposals for management. Forests 13, 386. https://doi.org/10.3390/f13030386.
Rubio-Cuadrado, Á., López, R., Rodríguez-Calcerrada, J., Gil, L., 2021. Stress and tree mortality in Mediterranean pine forests: anthropogenic influences. In: Pines and Their Mixed Forest Ecosystems in the Mediterranean Basin, pp. 141–181.
Ruiz-Gómez, F.J., Navarro-Cerrillo, R.M., Pérez-de-Luque, A., Oβwald, W., Vannini, A., Morales-Rodríguez, C., 2019. Assessment of functional and structural changes of soil fungal and oomycete communities in holm oak declined dehesas through metabarcoding analysis. Sci. Rep. 9, 1–16. https://doi.org/10.1038/s41598-019-41804-y.
Sánchez-Cuesta, R., Ruiz-Gómez, F.J., Duque-Lazo, J., González-Moreno, P., Navarro-Cerrillo, R.M., 2021. The environmental drivers influencing spatio-temporal dynamics of oak defoliation and mortality in dehesas of Southern Spain. For. Ecol. Manage. 485, 118946. https://doi.org/10.1016/j.foreco.2021.118946.
Sarker, A., Ansary, M.W.R., Hossain, M.N., Islam, T., 2021. Prospect and challenges for sustainable management of climate change-associated stresses to soil and plant health by beneficial rhizobacteria. Stresses 1, 200–222. https://doi.org/10.3390/stresses1040015.
Schlatter, D., Kinkel, L., Thomashow, L., Weller, D., Paulitz, T., 2017. Disease suppressive soils: new insights from the soil microbiome. Phytopathology 107, 1284–1297. https://doi.org/10.1094/PHYTO-03-17-0111-RVW.
Selosse, M.A., Dubois, M.P., Alvarez, N., 2009. Do Sebacinales commonly associate with plant roots as endophytes? Mycol. Res. 113, 1062–1069. https://doi.org/10.1016/j.mycres.2009.07.004.
Sleighter, R.L., Liu, Z., Xue, J., Hatcher, P.G., 2010. Multivariate statistical approaches for the characterization of dissolved organic matter analyzed by ultrahigh resolution mass spectrometry. Environ. Sci. Technol. 44, 7576–7582. https://doi.org/10.1021/es1002204.
Smith, G.R., Finlay, R.D., Stenlid, J., Vasaitis, R., Menkis, A., 2017. Growing evidence for facultative biotrophy in saprotrophic fungi: data from microcosm tests with 201 species of wood-decay basidiomycetes. New Phytol. 215, 747–755. https://doi.org/10.1111/nph.14551.
Tahat, M.M., Alananbeh, K.M., Othman, Y.A., Leskovar, D.I., 2020. Soil health and sustainable agriculture. Sustainability 12, 4859. https://doi.org/10.3390/su12124859.
Terhonen, E., Blumenstein, K., Kovlachuck, A., Asiegbu, F.O., 2023. Forest tree microbiomes and associated fungal endophytes: functional roles and impact on forest health. Forests 10 (1), 42. https://doi.org/10.3390/f10010042.
Thomas, F.M., Blank, R., Hartmann, G., 2002. Abiotic and biotic factors and their interactions as causes of oak decline in Central Europe. For. Pathol. 32, 277–307. https://doi.org/10.1046/j.1439-0329.2002.00291.x.
Veach, A.M., Stokes, C.E., Knoepp, J., Jumpponen, A., Baird, R., 2018. Fungal communities and functional guilds shift along an elevational gradient in the southern Appalachian Mountains. Microb. Ecol. 76, 156–168. https://doi.org/10.1007/s00248-017-1116-6.
Wei, Z., Hu, X., Li, X., Zhang, Y., Jiang, L., Li, J., et al., 2017. The rhizospheric microbial community structure and diversity of deciduous and evergreen forests in Taihu Lake area, China. PloS One 12, e0174411. https://doi.org/10.1371/journal.pone.0174411.