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Correlating the above- and belowground genotype of *Pinus pinaster* trees and rhizosphere bacterial communities under drought conditions

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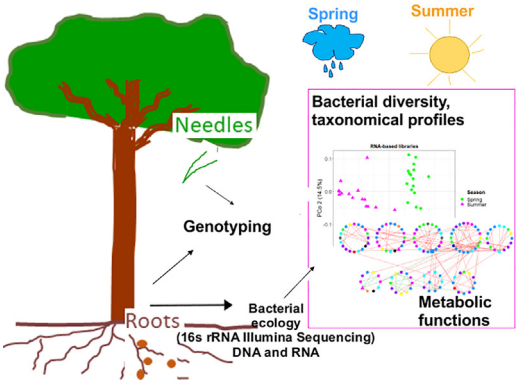
HIGHLIGHTS

- Despite a thorough sampling, root samples are not always taken from the selected tree.
- Genotype variability does not affect the core pine rhizosphere bacteriome.
- Taxonomical composition of bacterial populations of a pine forest changes seasonally.
- Co-occurrence networks are better indicators of seasonal variations than alpha indices.
- Summer affects the assembly of bacterial communities, interacting negative each other.

GRAPHICAL ABSTRACT



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ABSTRACT

Increasing temperatures along with severe droughts are factors that may jeopardize the survival of the forests in the Mediterranean basin. In this region, *Pinus pinaster* is a common conifer species, that has been used as a model species in evolutionary studies due to its adaptive response to changing environments. Although its drought tolerance mechanisms are already known, knowledge about the dynamics of its root microbiota is still scarce. We aimed to decipher the structural (bacterial abundance), compositional, functional and associative changes of the *P. pinaster* rhizosphere bacterial communities in spring and summer, at DNA and RNA level (environmental DNA, live and dead cells, and those synthesizing proteins). A fundamental aspect of root microbiome-based approaches is to guarantee the correct origin of the samples. Thus, we assessed the genotype of host needles and roots from which rhizosphere samples were obtained. For more than 50% of the selected trees, genotype discrepancies were found and in three cases the plant species could not be determined. Rhizosphere bacterial communities were homogeneous with respect to diversity and structural levels regardless of the host genotype in both seasons. Nonetheless, significant changes were seen in the taxonomic profiles depending on the season. Seasonal changes were also evident in the bacterial co-occurrence patterns, both in DNA and RNA libraries. While spring communities switched to more complex networks, summer populations resulted in more compartmentalized networks, suggesting that these communities were facing a disturbance. These results may mirror the future status of bacterial communities in a context of climate change. A keystone hub

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was ascribed to the genus *Phenylobacterium* in the functional network calculated for summer. Overall, it is important to validate the origin and identity of plant samples in any plant-microbiota study so that more reliable ecological analyses are performed.

1. Introduction

The Mediterranean basin ecosystems are often considered as hot spots of global biodiversity, providing a myriad of valuable services for the environment and humans (Palahí et al., 2008). However, the Mediterranean basin is drastically affected by increasing temperatures alongside frequent and severe drought events (IPCC, 2021) and forest fires that threaten the health and survival of forests in this region. Projections for the future include losses of biodiversity in Mediterranean ecosystems, and a substantial expansion of the desert areas is expected in much of the southern Europe (Guiot and Cramer, 2016). Those areas located at the transition between two biomes are usually considered highly sensitive areas (Palahí et al., 2008), so the study of these regions may shed light in advance, on the future effects of climate change at more than the regional scale.

Maritime pine (*Pinus pinaster* Aiton) is a widespread autochthonous conifer species found in different ecosystems of Western Mediterranean Europe. Beyond its limited geographical distribution, this species, that shows versatile adaptation to a wide variety of ecological conditions (Cabezas et al., 2015; Prada et al., 2016; Sánchez-Salguero et al., 2018), has been selected as a model species to study conifer genome structure and evolution (de Miguel et al., 2015; Plomion et al., 2016; Sterck et al., 2021) with the aim to unravel adaptive strategies used to inhabit contrasting ecosystems (Cabezas et al., 2015; de María et al., 2020; López-Hinojosa et al., 2021). Maritime pine response to environmental changes is the result of complex interactions between each tree and its associated microbiota (Jones et al., 2019). In particular, root associated microbes execute essential ecosystem roles in forest soils that can positively affect the tree hosts, namely, through organic matter decomposition and mineralization processes, participating in the biogeochemical cycling, and increasing the bioavailability of soil nutrients (Uroz et al., 2016). PGPB (Plant Growth Promoting Bacteria) and arbuscular mycorrhizal fungi enhance plant growth and development even under a variety of stressful conditions (Kumar and Verma, 2018). For instance, microbes that inhabit the portion of soil under the influence of the roots (rhizosphere) aid in the plant growth and development in several ways. PGPB contribute to the plant nutrient uptake (by solubilizing phosphate or producing siderophores), disease resistance (by synthesizing antibiotics and fungal cell wall degrading enzymes), biotic or abiotic stress tolerance (by means of salicylic acid production), or growth promotion of forest tree species (by producing phytohormones such as Indole-3-acetic *acid*), being key determinants for the plant host fitness (Lucas-García et al., 2004; Gehring et al., 2017; Lladó et al., 2017). As mentioned before, plant microbes can aid in the amelioration of the tolerance of their host to different type of biotic and abiotic stresses. Some PGPB improve the growth of the roots and increase the water absorption ability of their host, and thus, preserve the water use efficiency of the plants under water deficit conditions. Khan et al. (2020) demonstrated that the inoculation of particular PGPB strains and plant growth regulator such as putrescine and salicylic acid in chickpea plants resulted in the enhancement of chlorophyll, protein and sugar levels, and in higher root and shoot weights under water scarcity conditions, improving the adverse effects of the water deficiency stress. Despite the importance of understanding the function of *Pinus pinaster*-microbiota systems (Duhamel et al., 2019), there is scarce information about maritime pine microbiome composition and dynamics (Pérez-Izquierdo et al., 2017, 2019; Proença et al., 2017; Alves et al., 2018).

Knowledge about biotic and abiotic factors that influence soil bacterial communities are essential to predict how bacteria-mediated processes drive ecosystem responses to environmental changes. On the one hand, the structure of soil bacterial populations is not only plant species dependent but also host genotype specific (Fernández-González et al., 2019; Pérez-Izquierdo et al., 2019). Each host plant, based on its genetic composition, organ surface, tissue composition, developmental and physiological stage, associated mycorrhizal activity, or root exudates shape the microbiome assemblage and function (Sánchez-Cañizares et al., 2017; Zhalnina et al., 2018). Indeed, in conifers, the structure and function of rhizosphere microbial communities are strongly influenced by the tree population, as occurs in the case of *P. pinaster* (Pérez-Izquierdo et al., 2019). Furthermore, *Pinus edulis* ectomycorrhizal fungal community composition has been shown to be under strong tree genetic control, and determines drought tolerance among host pine genotypes (Gehring et al., 2017). Nevertheless, compared to grasslands or agroecosystems, very little is known about the effect of tree genetic diversity on forest soil microbial community composition and function.

On the other hand, soil parameters such as temperature, moisture, pH, nutrient content, soil porosity and texture, govern the structure and composition of soil microbial communities intensely (Lauber et al., 2008). Concurrently, changes in environmental factors such as those exposed before cause alterations in terms of soil and forest vegetation, altering rates of photosynthesis, modifying tree carbon allocation and fixation and composition of root exudates (Wang et al., 2014; Williams and de Vries, 2019). The effect that environmental changes have on forest ecosystems depends on the environmental change itself, the specific ecosystem under study and the ecology of the biomes. Some of the most marked environmental changes are those related to seasonality, which entail modifications of tree species-dependent patterns of leaf phenology, litter, root biomass development, root exudation and plant activity (Wang et al., 2018; Dukunde et al., 2019 and references therein). As revealed by Regan et al. (2014), seasonal variation also alters soil edaphic parameters in a straightforward manner (especially soil organic matter (SOM) content, pH, temperature, moisture, belowground carbon (C) allocation, nitrogen (N) availability, among others). As a result, seasonal soil microbial community composition, structure, biomass and enzymatic activity is also affected (Tan et al., 2014), adjusting their compositional and functional features to the new environmental and nutritional conditions (Žifčáková et al., 2016). Under a scenario of climate change, in addition to seasonal dynamics, the microbial communities in the rhizosphere have to cope with changing environmental conditions which alter plant-soil feedbacks (Pugnaire et al., 2019). Water availability is a major constraint on forest ecosystems in the dry Mediterranean basin, due to the high summer temperatures and low levels of rainfall (Sardans and Peñuelas, 2013). Thus, understanding how forest soil microbial communities respond to seasonal fluctuations could aid researchers in gaining more insights into the influence of climate change on microbial populations. Moreover, given the magnitude of the impact of climate change on forest ecosystems, it is essential to decipher whether seasonal microbial dynamics are unique or not in order to make predictions about how microbial populations will respond to future climatic fluctuations. Understanding the seasonal dynamics is of great interest so that devastating processes such as desertification could be mitigated (Lacerda-Júnior et al., 2019). So far, most of the studies coping with seasonal dynamics in forest ecosystems have focused on the consequent structural and compositional changes of soil bacterial communities (Ruiz Palomino et al., 2005; Prevost-Boure et al., 2011). There is a growing body of research that coupled the assessment of the influence of environmental parameters, land use change, elevation or soil depth on soil microbial communities as well (Fernández-González et al., 2020a; Lacerda-Júnior et al., 2019; Siles and Margesin, 2017; Shigyo et al., 2019; Bi et al., 2021). Overall, it has been reported that the composition of forest bacterial populations changes to a great extent with the seasons, because taxonomic groups of soil bacteria differ in their response to changes in edaphic parameters and plant phenology. Season can modify the abundance of bacteria through plant litter and soil properties. Lipson (2007) stated that an increase in the abundance of

different bacterial phyla (which can be offset by a decrease in the abundance of other phyla) could be due to fluctuations in carbon supply from plant litter and exudation. Recently, further progress has been made in the field of seasonal dynamics of microbial communities, unraveling the impact of environmental changes on the activity of those microbes that inhabit forest soils (Žifčáková et al., 2016; Siles and Margesin, 2017; Dukunde et al., 2019; Lacerda-Júnior et al., 2019). Despite the demonstrated marked changes that bacterial communities show among seasons in terms of composition and structure, studies identifying the keystone members in bacterial assembly are not yet available. It should be taken into account the associations established between microbes, which can be either bi- or unidirectional (positive-positive, negative-negative, positive-negative, positive-neutral and neutral-neutral; Song et al., 2019) and will determine the functional competence of the whole community and ecosystem stability (Barberán et al., 2012). Despite the importance of microbial co-occurrence networks in community assembly, the understanding of the potential interactions among bacterial community members inhabiting forests is still fragmentary. Although Siles and Margesin (2017) and Wang et al. (2018) disentangled the most influential taxa in the soil of a mixed coniferous forest and the rhizosphere of *Pinus tabuliformis*, respectively, in different seasons, more studies are needed to address the ability of microbial communities to adapt to seasonal changes in different forest ecosystems. However, the presence of microorganisms in forest soils does not necessarily imply that they are active or influencing plant host's fitness. Most of the studies that analyze soil or plant microbiota focus only on addressing total bacterial communities, however they fail to investigate the active part of the microbiota. DNA-based surveys tend to overestimate bacterial richness since they are not able to distinguish the DNA from living and non-living cells, nor the DNA from bacterial members in quiescent or dormancy state and metabolically active bacteria (Bladodatskaya and Kuzyakov, 2013). The fast turnover of the RNA and its short lifespan make RNA-based approaches to be more suited to investigate metabolically active bacteria (Lasa et al., 2019 and references therein).

In this work we studied rhizosphere bacterial community dynamics of a natural population of *Pinus pinaster* that has exhibited remarkable adaptability to severe droughts (Aranda et al., 2010; Sánchez-Gómez et al., 2010). We also compare communities across two contrasting seasons: spring, when the most vigorous plant growth occurs at mild temperatures and the highest rainfall pattern, and summer, characterized by highly reduced rainfall and high temperatures, aligned with the reduction of plant growth. Considering that the genotype of the host plant is one of the factors that determine the composition of the rhizosphere microbiome (Fernández-González et al., 2019), the powerfulness of these studies depends on the capacity to collect rhizosphere samples from known trees.

Thus, the aims of this research were: i) to track the specific host from which rhizosphere samples were obtained by genotyping the needles and roots of the selected pine trees, ii) to determine whether total and potential bacterial community structure and diversity are host-genotype dependent (both in summer and spring) by metabarcoding techniques, and iii) to unravel changes in total and potentially active bacterial communities at structural, compositional and functional levels while determining whether communities are instable in relation to seasonal fluctuations.

2. Material and methods

2.1. Experimental site and sample collection

The experimental site is located in Sierra de Oria (Almería, SE Spain; 37° 31′ N 2° 21′ W), where a natural population dominated by *Pinus pinaster* and *Pinus halepensis* can be found (Fig. S1). We selected this site since it is representative of dry Mediterranean mountain areas, under the effects of climate change (Eveno et al., 2008; Aranda et al., 2010; Sánchez-Gómez et al., 2010). A total of 18 *Pinus pinaster* trees, separated at least 50 m to avoid inbreeding, were selected and geo-referenced. Samples of needles and roots of the host trees as well as associated rhizosphere soil were collected in two contrasting season: on July 18, 2017 (summer samples) and the sampling of roots and their associated rhizosphere was repeated on April 24, 2018 (spring samples). It should be noted that this mountainous area suffers frequent and severe droughts (López-Hinojosa et al., 2021). The average temperature and precipitation in June and July (2017) are 23.9 °C and 1.6 mm, and means values in March and April (2018) of 12.1 °C and 26.5 mm, respectively. Summer and spring were selected since they are the most contrasting seasons in terms of trees' growth (reduced and intense vegetative growth, respectively), and temperature and rainfall patterns in this mountainous area.

The collection of rhizosphere soil was performed as described previously by Cobo-Díaz et al. (2015). Briefly, at a distance of less than 50 cm from the trunk of each tree, the litter and topsoil were removed by digging, and the main roots of the tree were followed until active non-suberified roots were found (5–25 cm depth). Soil closely adhered to non-suberified roots was collected, and 2 g of each rhizosphere soil sample were mixed with 5 mL of LifeGuard™ Soil Preservation Solution (MoBio Laboratories Inc., CA, USA) in order to preserve bacterial community profiles. Samples were immediately stored on ice, and they were stored at 4 °C until DNA and RNA extraction within 24 h of sampling.

Roots and needles samples were sterilized using 70% ethanol for 5 min and 2% NaClO for 20 min. They were rinsed five times with sterile water, frozen in liquid nitrogen and stored at −80 °C until DNA extraction.

Additional samples of soil near the roots (500 g of soil per tree) were also collected to analyze physicochemical properties, solely on July 2017. The determination of soil physicochemical parameters was performed at the Agri-Food Laboratory of the Andalusian Regional Government (Granada, Spain) under the standardized procedures developed by this service. A scheme of the experimental design followed in this work is represented in Fig. S2.

2.2. Plant DNA extraction and genotyping

Frozen needles and roots from each tree were ground using a MM400 Mixer Mill (Retsch GmbH and Co.) Plant DNAs were independently extracted from needles and roots from each pine tree using DNeasy Plant Mini Kit (Qiagen, Düsseldorf, Germany) and quantified using a spectrophotometer (Thermo Scientific, Nanodrop 1000).

Initially, needle DNAs were amplified using seven nuclear microsatellites developed for *Pinus pinaster*: A5B07, A6F03, A5A11, A6F10, A6A09, A6E05 and A6D12 (Guevara et al., 2005; Arrillaga et al., 2014). Five of them (A6F03, A5A11, A6A09, A6E05 and A6D12) were chosen as being the most informative for amplifying DNAs of summer and spring roots. Additionally, two chloroplast microsatellites (Pt36480 and Pt87268; Vendramin et al., 1996) and one nuclear microsatellite (PtTX4001, González-Martínez et al., 2004) were used to amplify samples from trees that showed different allelic patterns between needles and summer or spring roots. PCRs were performed in 12 μL containing 5 ng of DNA, 1× Qiagen Multiplex PCR master mix, and 0.1 μM of forward and reverse primers in a Veriti 96 Well Thermal Cycler (Applied Biosystem). Reverse primers were labeled on the 5′ end with IRD800. The PCR profile used was 95 °C 10 min, 35 cycles of 94 °C 30 s, 57 °C 90 s, 72 °C 60 s, and final extension at 60 °C 30 min.

Amplified products were denatured by adding an equal volume of formamide-buffer (98% formamide, 10 mM EDTA, pH 8.0, 0.06% bromophenol blue and 0.06% xylene cyanol) and heated for 3 min at 94 °C. Fragments were separated into 25 cm long denaturing polyacrylamide gels (16% Long Ranger™ Gel Solution (Lonza), 7 M urea and 1xTBE) at 1500 V and visualized in a 4300 DNA Analyzer (LI-COR Biosciences, Lincoln, NE, USA).

2.3. Rhizosphere DNA and RNA extraction and Illumina sequencing

RNA and DNA from each individual rhizosphere sample were co-extracted by using the RNA PowerSoil® Total RNA Isolation Kit and the RNA PowerSoil® DNA Elution Accesory kit (MoBio, Laboratories Inc., CA, USA), respectively, following the manufacturer's instructions. By using the extracted RNA as a template, cDNA was synthesized as described previously

by Lasa et al. (2019). Briefly, RNA was treated with DNase I in order to remove all the DNA and avoid contaminations. The absence of DNA in cDNA samples was confirmed by checking the impossibility of amplifying the 16S rRNA gene (Villadas et al., 2017). cDNA was synthesized by means of random primers and SuperScript™ Reverse Transcriptase. RNase H, DNA polymerase I and DNA ligase was used to synthesize the double stranded DNA. Finally, blunt-end DNA was obtained by using T4 DNA polymerase. No RNA spike-in was used as control. DNA and cDNA yields were quantified by means of the fluorometer Qubit 3.0 (Life Technologies, NY, USA).

DNA and cDNA from each individual sample (36 samples per type of nucleic acid) were sequenced through Illumina MiSeq platform at the genomics service of the Institute of Parasitology and Biomedicine López Neyra (CSIC, Granada, Spain). In particular, the hypervariable regions V3-V4 of 16S rRNA gene were sequenced for both DNA and cDNA samples, using the forward and reverse primers Pro341F and Pro805R, respectively, as described by Takahashi et al. (2014). Amplicons were sequenced following a paired-end 2 × 300 bp strategy (PE 300), and three samples of a mock microbial community (ZymoBIOMICS Microbial Community Standard II, in log distribution; ZYMO RESEARCH, CA, USA) were included in the sequencing run in order to stablish de detection limits of the sequencing, as recommended by Bokulich et al. (2013). Samples based on DNA and cDNA will be henceforth named "DNA libraries" and "RNA libraries", respectively.

2.4. Illumina data processing

Raw reads obtained by high-throughput sequencing were processed with *DADA2* package of R (Callahan et al., 2016) following the workflow proposed by the developers (https://benjjneb.github.io/dada2/tutorial.html). At first, a filtering and trimming step was carried out in which all the forward and reverse reads (R1 and R2, respectively) were trimmed, removing the low quality fragments. Reads containing ambiguities and more than two expected errors were excluded from the analysis. Afterwards, the parametric errors were modeled and corrected from the data, after which forward and reverse reads were merged by making use of the default parameters. Subsequently, an initial Amplicon Sequence Variant (ASV) table was constructed, and sequences of a different length than that corresponding to V3-V4 hypervariable regions of 16S rRNA gene were removed (sequences outside the 401–429 nucleotide range). Chimeric sequences were identified and also removed from the dataset and the remaining quality sequences were classified taxonomically. For that purpose, the reference database of the Ribosomal Database Project (RDP-II) training set v.18 was considered. According to the mock community used as quality control, those ASV which accounted for less than 0.0027% of the high quality sequences were eventually removed from the dataset, since they were considered sequencing artifacts.

2.5. Rarefaction curves and alpha diversity

All the ecological and statistical analyses, as well as the plotting were performed by using different functions and packages implemented in R 4.1.0 (R Core Team, 2021).

Firstly, the sampling effort was checked by calculating rarefaction curves for each sample (*rarecurve* function of *vegan* package, Oksanen et al., 2020). The number of observed ASV (richness) as well as Shannon, Simpson, Inverse of Simpson (InvSimpson) and Pielou (evenness) indices were calculated by means of *phyloseq* package (*estimate_richness* function, McMurdie and Holmes, 2013). It should be pointed out that previously, the number of sequences per sample was separately rarefied to the smallest library size of each dataset to minimize potential biases associated with different sample sizes in alpha diversity comparison studies (*rarefy_even_depth* function, *phyloseq* package).

2.6. Beta diversity

Diversity between samples was analyzed taking into account non-rarefied data. All the beta-diversity analyses were conducted based on Weighted UniFrac distantes (*UniFrac* library of *phyloseq* package of R). Firstly, Constrained Analysis of Principal Coordinates (CAP) was performed to determine the effect of soil physicochemical properties on rhizosphere bacterial communities in summer. For that purpose, the function *capscale* was run (*vegan* package; Oksanen et al., 2020) followed by the model building by means of *ordistep* function of the same R package. Based on the results of the modeling, *envfit* function (*vegan* package) was implemented to address the statistical significance of the environmental factors selected by adjusting the p-values with the Bonferroni's method. On the other hand, the dispersion of each group of samples was calculated and compared implementing the permutational analysis of multivariate homogeneity of groups dispersion (PERMDISP2, Anderson, 2006), under *betadisper* and *permutest* functions included in *vegan* package. The *adonis* function of the same R package was run to perform the permutational analysis of variance (PERMANOVA), and check whether bacterial communities were different at structural level (distribution of the taxa and their abundance). To better interpret the results of PERMANOVA, the distribution of each group of samples in the multivariate space was plotted by Principal Coordinate Analysis (PCoA), using *ordinate* function of *phyloseq* package.

2.7. Differential abundance analysis of prokaryotic taxa

Testing for differential abundance of taxa was conducted by implementing Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) methodology developed by Lin and Peddada (2020). Specifically, the *ancombc* function of *ANCOMBC* package developed by the same authors was executed in R. Those taxa which were present in less than 10% of samples were excluded for the analysis. P-values of comparisons were adjusted by implementing Holm's method included in the function.

2.8. Co-occurrence networks

Bacterial networks were separately computed for each season and for each type of library; in short, four co-occurrence networks were constructed, all of them based on 17 replicates. For that purpose, the Molecular Ecological Network Analysis Pipeline (MENAP) website was used (http://ieg4.rccc.ou.edu/mena/main.cgi) as described by Fernández-González et al. (2020b, and references therein). Briefly, ASVs that were present in less than nine out of 17 replicates (prevalence cut-off of 53%) were removed from the analysis, in order to avoid random bacterial correlations. After logarithmic transformation of ASV abundance, a similarity matrix was constructed by using the Spearman's correlation coefficient based on the covariance of the variables. The remaining parameters were kept as default, and fast greedy modularity optimization was chosen for module separation and modularity calculation. Once bacterial networks were obtained, 100 random networks were calculated for each computed network by maintaining the number of nodes and links and changing the topology of each empirical network. Thus, after the randomization, the standard deviation of the global properties was used when empirical networks associated with both seasons were compared with each other (for DNA and RNA libraries) by means of Student's *t*-tests. Finally, Cytoscape v.3.7.0 tool (Shannon et al., 2003) was employed to plot the co-occurrence networks, and R Studio was implemented for ZiPi graphics. Topological roles of each network node were assigned on the basis of within-module (Z_i) and among-module connectivity (P_i), according to Olesen et al., 2007. Thus, network forming-ASV were classified into connectors, module hubs, network hubs and peripherals.

2.9. Prediction of functional profiles of prokaryotic communities

In order to gain insights about the functional traits of rhizosphere bacterial communities, The Functional Annotation of Prokaryotic Taxa v1.2.4 (FAPROTAX) tool was used, according to Louca et al. (2016). In summary, by running the python script *collapse_table.py*, the taxonomic classification of each rhizosphere ASV was compared to the functional assignment for each taxon included in the FAPROTAX database. Both script and functional

database were supplied by the developers of FAPROTAX, and can be found at http://www.loucalab.com/archive/FAPROTAX/lib/php/index.php?section=Home. Since DNA libraries include the DNA of dead or dormant cells, and those based on RNA give a better view of the metabolic activity of bacterial populations, the functional profiles were solely predicted based on RNA libraries.

2.10. Univariate statistical analyses

When alpha indices or the abundance of each ecological function were compared among two groups of samples, univariate statistical approaches were carried out. Firstly, the normal distribution and homoscedasticity of each variable was checked by using *shapiro.test* (base R) and *leveneTest* (*car* package of R, Fox and Weisberg, 2019), respectively. When parametric assumptions were met, Student's *t*-test was applied by running the function *t.test* of base R. Otherwise, the non-parametric Mann-Whitney *U* test was computed to detect differences among the two groups considered (*wilcox.test* of base R). In all cases, confidence levels >95% ($\alpha = 0.05$) were stablished.

2.11. Data availability

The sequencing dataset generated during the current study were deposited and are publicly available at the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) repository under the BioProject accession number PRJNA748008.

3. Results

3.1. Pinus pinaster identification and cross-validation

To validate that roots collected in different seasons belonged to the selected pines, DNA from needles and roots collected in July 2017 (summer roots) and in April 2018 (spring roots) were genotyped using five informative nuclear microsatellite markers, A6F03, A5A11, A6A09, A6E05 and A6D12 (Table 1). Microsatellite analysis of needle DNAs allowed discrimination of the 18 selected trees. Six trees (2, 4, 5, 6, 13 and 18) showed the same genotypes when analyzing needles, summer and spring roots. For other five trees (7, 8, 9, 10 and 15) only one of the root samples, either summer or spring roots, showed the same needle genotype. In five trees (1, 3, 14, 16 and 17), none of the collected roots showed the same needle genotype, while DNA roots from two trees (11 and 12) could not be amplified, which may indicate that roots were collected from a different plant species. Although the selected *P. pinaster* trees were isolated from other plants to reduce the probability of collecting roots from other trees and considering that Sierra de Oria stand includes *P. pinaster* and *P. halepensis*, three additional microsatellites (Pt36480, Pt87268 and PtTX4001) were used to explore if the samples with different summer or spring root genotypes could correspond to neighboring *P. pinaster* or *P. halepensis*. This analysis revealed that 13 summer and spring root samples belonged to different *P. pinaster* trees (1, 3, 7, 8, 9, 14, 15 and 17). Also, summer roots from trees 11 and 12 and spring roots from tree 11 belonged to *P. halepensis* while spring roots from trees 10, 12 and 16 did not belong to pine species (Table 1). That is to say, 30 out of 36 samples corresponded to *P. pinaster*, three samples to *P. halepensis* and the remaining three samples didn't correspond to pine species.

3.2. General characteristics of sequencing datasets

DNA and RNA libraries obtained from the 36 rhizosphere soil samples were analyzed separately considering the already known differences in terms of bacterial community composition and structure between both types of nucleic acids (Lasa et al., 2019). The number of reads derived from 16S rRNA transcripts corresponding to tree 14 (summer) was notably low (<100 reads), thus, both summer and spring rhizosphere samples collected from this tree were removed from the analyses.

From the remaining 17 trees, a total of 2,018,364 and 2,082,146 raw DNA and RNA-derived sequences were obtained by Illumina sequencing, which resulted in 1,317,895 and 1,281,747 quality sequences, respectively, after the trimming step (Table S1). After all the trimming steps, a total of 5241 (DNA library) and 5231 (RNA library) bacterial ASV were considered for further analyses (Table 2).

All the rarefaction curves calculated at ASV level reached the asymptote clearly (Fig. S3), demonstrating that the sampling effort was enough and the diversity of rhizosphere bacterial communities will surely be covered, both in case of DNA and RNA-based libraries, either in summer and spring.

3.3. Are needle and root genotype discrepancies reflected in the diversity and structure of rhizosphere bacterial communities?

In order to determine whether the discrepancies observed in host genotype cross-validation study could also entail differences in bacterial seasonal dynamics, two subsets of samples were considered for further analyses. On the one hand, samples corresponding to *P. pinaster* trees 2, 4, 5, 6, 13 and 18, which showed the same root and needle genotype both in summer and in spring, were retained and will be named "confirmed *P. pinaster*" from now on (Table 1). On the other hand, it was created a group referred to "Pine forest samples" from here on out, including samples related to trees 1, 3, 7, 8, 9, 10, 11, 12, 15, 16 and 17 (showing different root and needle genotype of *P. pinaster* in one or both seasons, or even plant species different to *P. pinaster*). Comparisons among confirmed *P. pinaster* and Pine forest samples were performed separately for each library (DNA and RNA-derived ones), and for each season (summer and spring).

Regarding alpha diversity of total bacterial communities (DNA library), similar numbers of observed ASV were registered (in both seasons, and for both groups of samples). The same trend was observed for samples corresponding to the RNA-derived library (Table 2). When comparing alpha indices of both types of groups of samples (confirmed *P. pinaster* and Pine forest samples) for both total and potentially active bacteria, high values of Shannon's diversity and evenness (Inverse of Simpson and Pielou indices) were observed. Nevertheless, no significant differences were uncovered for any of the seasons (Student's *t*-test and Mann-Whitney *U* test, p-values < 0.09).

The effect of soil physicochemical parameters on rhizosphere bacterial community structure was evaluated solely for the summer season, since there should be no differences in the physicochemical properties of the soil between both seasons. Firstly, differences in the corresponding edaphic properties between confirmed *P. pinaster* and Pine forest samples were calculated, and are summarized in Table S2. The effect on bacterial beta diversity was addressed by means of CAP analysis, which revealed that among all the measured edaphic properties, just the pH was significantly correlated with the axes of the ordination plot (*envfit* test, p-values < 0.002; Table S3). That is to say, the pH was the only measured parameter that explained the distribution of the samples in the CAP plot. Although the environmental model only explained 23.5% of the total variance, the CAP plot (Fig. S4) also revealed that the soil pH is the main environmental driver of bacterial communities associated with the roots of the selected host plant in summer. It should be mentioned that despite the weight of the soil pH in bacterial community structuring, confirmed *P. pinaster* and Pine forest samples were not clearly separated in the CAP ordination plot. Both groups of samples were segregated across the CAP1 and CAP2 axes. Accordingly, no significant differences were recorded in the soil pH between the groups of samples (Table S2).

Concerning beta diversity analyses, it should be noted that in spite of the fact that the group of samples comprised different number of replicates ($N_{\text{Confirmed } P.pinaster} = 6$, $N_{\text{Pine forest}} = 11$; in each season and for each library), PERMDISP2 approach revealed that there were no significant differences in the dispersion of all pairs of groups of samples (Table S4), which turns PERMANOVA into a sound statistical tool for unbalanced experimental designs (Anderson and Walsh, 2013). Thus, according to PERMANOVA results, bacterial communities inhabiting the rhizosphere of confirmed

Table 1
Microsatellite genotyping of the 18 selected trees. Allele sizes of needles (N), summer roots (sm) and spring roots (sp). Each sample was identified as *P. pinaster* (Pp, green cells), *P. halepensis* (Ph, orange cells) or plants whose gender could not be determined (nd, grey cells). ≠, different *P. pinaster* (light green) genotype; na, no amplified.

		SSR								
TREE		A6F03	A5A11	A6A09	A6E05	A6D12	PtTX4001	Pt36480	Pt87268	
1	Needles	250 252	295	265 271	278	356				Pp 1
	Summer roots	248 252	293 295	251 265	278 308	356	207	145	164	Pp ≠
	Spring roots	250	293 295	255 265	278	356 358	207	145	166	Pp ≠
2	Needles	250 252	293	239 241	278 300	354 356				Pp 2
	Summer roots	250 252	293	239 241	278 300	354 356				Pp 2
	Spring roots	250 252	293	239 241	278 300	354 356				Pp 2
3	Needles	248	293	247 269	278 308	354				Pp 3
	Summer roots	248 250	na	269	276 310	354	207	145	164	Pp ≠
	Spring roots	248 250	293	267 269	276 304	356	207	145	164	Pp ≠
4	Needles	248 250	293 295	239 265	278 302	352 356				Pp 4
	Summer roots	248 250	293 295	239 265	278 302	352 356				Pp 4
	Spring roots	248 250	293 295	239 265	278 302	352 356				Pp 4
5	Needles	244 248	293 307	269 271	276 302	354 356				Pp 5
	Summer roots	244 248	293 307	269 271	276 302	354 356				Pp 5
	Spring roots	244 248	293 307	269 271	276 302	354 356				Pp 5
6	Needles	248 262	293 295	269 273	308 318	346 354				Pp 6
	Summer roots	248 262	293 295	269 273	308 318	346 354				Pp 6
	Spring roots	248 262	293 295	269 273	308 318	346 354				Pp 6
7	Needles	252 256	293 299	235 259	278 308	346 354				Pp 7
	Summer roots	252 256	293 299	235 259	278 308	346 354	207	145	166	Pp 7
	Spring roots	248 254	293	271 273	302 318	356	207	145	166	Pp ≠
8	Needles	248	293	253 259	276 278	356				Pp 8
	Summer roots	248	293	239 253	298 308	356	207	145	164	Pp ≠
	Spring roots	248	293	253 259	276 278	356	207	145	166	Pp 8
9	Needles	248 254	293 307	269 273	278	354				Pp 9
	Summer roots	248	293 311	255 271	278 302	356 358	207	145	164	Pp ≠
	Spring roots	248 254	293 307	269 273	278	354	207	145	165	Pp 9
10	Needles	248 256	285 293	265 271	278 308	346 354				Pp 10
	Summer roots	248 256	285 293	265 271	278 308	346 354	207	145	164	Pp 10
	Spring roots	na	na	na	na	na	na	na	na	nd
11	Needles	248	293	259 267	278 302	356				Pp 11
	Summer roots	na	na	na	na	na	201	140	176	Ph
	Spring roots	na	na	na	na	na	201	140	176	Ph
12	Needles	248 252	295 299	265 271	278 298	352 356				Pp 12
	Summer roots	na	na	na	na	na	204	140	176	Ph
	Spring roots	na	na	na	na	na	na	na	na	nd
13	Needles	256 258	285 295	269 271	300 316	344 356				Pp 13
	Summer roots	256 258	285 295	269 271	300 316	344 356				Pp 13
	Spring roots	256 258	285 295	269 271	300 316	344 356				Pp 13
14	Needles	252 256	295 311	261 271	302 332	356				Pp 14
	Summer roots	252 254	295 311	239 271	278	356 360	207	145	164	Pp ≠
	Spring roots	244 256	293 299	269 271	300 314	356 358	207	145	165	Pp ≠
15	Needles	248	299	255	300	350 356				Pp 15
	Summer roots	244 256	293	271	298 312	346 356	207	145	165	Pp ≠
	Spring roots	248	299	255	300	350 356	207	145	164	Pp 15
16	Needles	248 254	293 295	271	280 304	346 356				Pp 16
	Summer roots	248 254	293	271	280 306	346 356	207	145	166	Pp ≠
	Spring roots	na	na	na	na	na	na	na	na	nd
17	Needles	248 254	293	265 275	302 308	356				Pp 17
	Summer roots	248 252	299 311	251 269	300 308	350 356	207	145	166	Pp ≠
	Spring roots	244 248	293 299	259 275	278 308	346 350	207	145	167	Pp ≠
18	Needles	244 248	293 295	227	302 310	354 356				Pp 18
	Summer roots	244 248	293 295	227	302 310	354 356				Pp 18
	Spring roots	244 248	293 295	227	302 310	354 356				Pp 18

Table 2
Alpha indices of bacterial communities inhabiting the rhizosphere of confirmed *P. pinaster* trees and other plants (see Material and methods section). Data show the mean value of all the samples considered in each case ± standard deviation. "Obs. ASV" = Observed ASV; "Comparison" refers to the statistical comparisons computed. Those pair of samples with the same Greek letter were compared among each other. The same Greek letter indicates that there were no significant differences in the considered parameter among the specific group of samples (Student's *t*-test and Mann-Whitney U test for parametric and non-parametric data, respectively). Confidence level of 95% in all cases.

	Season	Genotype	Obs. ASV	Shannon (H′)	InvSimpson (1/D)	Pielou (J′)	Comparison
DNA	Summer[a]	Confirmed *P. pinaster*	758.50 ± 64.86	5.89 ± 0.25	179.68 ± 80.97	0.89 ± 0.04	α
		Pine forest	742.55 ± 160.92	5.84 ± 0.30	159.74 ± 65.29	0.89 ± 0.03	α
	Spring[b]	Confirmed *P. pinaster*	786.00 ± 101.62	5.92 ± 0.21	179.18 ± 55.27	0.89 ± 0.02	β
		Pine forest	717.18 ± 70.39	5.75 ± 0.19	138.61 ± 53.29	0.87 ± 0.03	β
RNA	Summer[c]	Confirmed *P. pinaster*	713.50 ± 142.64	5.77 ± 0.27	140.45 ± 56.81	0.88 ± 0.04	γ
		Pine forest	708.91 ± 148.00	5.86 ± 0.34	174.41 ± 68.41	0.90 ± 0.03	γ
	Spring[d]	Confirmed *P. pinaster*	779.67 ± 150.57	5.94 ± 0.46	215.79 ± 101.05	0.88 ± 0.04	δ
		Pine forest	749.18 ± 79.26	5.94 ± 0.19	201.52 ± 65.14	0.90 ± 0.03	δ
DNA[a]	Summer	(All the trees included in the analysis, N = 17)	748.18 ± 132.52	5.86 ± 0.28	166.78 ± 69.35	0.89 ± 0.03	ε
	Spring		737.29 ± 85.50	5.80 ± 0.21	152.68 ± 57.21	0.88 ± 0.03	ε
RNA[c]	Summer		710.53 ± 141.61	5.83 ± 0.31	162.42 ± 64.91	0.89 ± 0.03	λ
	Spring		754.94 ± 104.25	5.93 ± 0.30	204.35 ± 75.35	0.90 ± 0.03	λ

[a] Rarefaction selection of 20,698 sequences.
[b] Rarefaction selection of 27,612 sequences.
[c] Rarefaction selection of 17,407 sequences.
[d] Rarefaction selection of 25,412 sequences.

P. pinaster trees showed no significant differences with populations characterizing Pine forest samples, nor in spring nor in summer, and in none of the studied libraries (Table S4). This homogeneous structure of rhizosphere bacterial communities was endorsed by the extremely low values of all R^2 values associated with the proposed statistical models ($R^2 < 0.10$). The lack of differences in terms of community structure was visualized in the corresponding PCoA plots, which explained a moderated percentage of variance in all cases (<61.74%). However, samples could not be separated in the multivariate space according to the correspondence of the needle and root genotypes, in none of the considered seasons, nor at DNA nor at RNA-based levels (Fig. 1).

3.4. Deciphering the seasonal dynamics of rhizosphere bacterial communities of a pine forest representative of dry Mediterranean mountains

Taking into account that no significant differences were found in bacterial populations based on the origin of all collected rhizosphere samples, we considered the 17 samples of each library as a whole for each sampling season. That is, seasonal shifts of those bacterial populations dwelling in the rhizosphere of *P. pinaster*, *P. halepensis* or other plants located in the pine forest under study were addressed both in the case of total and potentially active communities (DNA and RNA libraries, respectively).

Comparing alpha diversity indices corresponding to DNA library after a random selection of 20,698 sequences, no significant differences were found between summer and spring, for any of the studied indices. Bacterial communities inhabiting the rhizosphere of the 17 selected trees were as rich in summer as in spring, and there were no dominant ASVs either (Pielou's values close to 1, both in summer and in spring; Table 2). The same homogeneity across seasons was observed when the alpha diversity of potentially active bacteria (RNA) was compared with each other (rarefied subsamples of 17,407 random sequences). Although the number of observed ASV and Inverse of Simpson were greater in spring than in summer, differences were statistically no consistent (Student's *t*- and Mann-Whitney *U* tests, p-values < 0.52).

Structural significant changes associated with the sampling period were found in the case of total bacterial populations (DNA), as revealed by PERMANOVA (p-value < 10^{-4}; Table S4). Yet significant, differences in the structure of bacterial communities characterizing summer and spring samples were moderate, as visualized in the first coordinate of PCoA (Fig. 2a). Seasonal differences were also detected in the case of potentially active bacterial populations (RNA-based library). PERMANOVA uncovered that the season when sampling was carried out is a relevant factor in distinguishing bacterial communities ($R^2 = 0.32$, Table S4). As depicted the PCoA, the segregation of summer and spring samples along the axis 1 supported this hypothesis (Fig. 2b).

No archaeal sequences were detected in the whole dataset, neither for structural (DNA) nor functional levels (RNA). In the DNA library, 19 and 18 bacterial phyla were detected in summer and spring samples respectively, although rhizosphere bacterial communities were dominated mainly by *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia* and *Bacteroidetes* (Summer, 81.35%; Spring, 82.71%; Fig. S5a). It should be noted that phylum *Actinobacteria* was markedly more abundant in summer than in spring (Fig. S5a), with this difference being statistically significant (ANCOM-BC, p-value < $2 \cdot 10^{-5}$). As demonstrated by the results of ANCOM-BC anaysis, the prevalence of *Planctomycetes* and *Chloroflexi* was significantly greater in summer than in spring, although the latter was considered as a minor phylum due to its low relative abundance in both types of samples (<1%). On the contrary, phyla *Proteobacteria* and *Bacteroidetes* were underrepresented in bacterial communities of the summer season, resulting these differences statistically significant according to ANCOM-BC test (p-values < 0.013). Meanwhile, functional bacterial communities were composed of 19 different phyla, both in summer and spring. Just *Proteobacteria*, *Acidobacteria*, *Candidate division WPS-1*, *Actinobacteria* and *Verrucomicrobia* accounted for more than 84% and 82% of the total RNA-derived sequences recorded in summer and in spring, respectively (Fig. S5b). Regarding compositional shifts, *Candidate division WPS-1* showed a noticeable significant difference in its relative abundance when comparing summer and spring samples (ANCOM-BC, p-value = $1.77 \cdot 10^{-11}$). Interestingly, the prevalence of this phylum was more than two-fold greater in samples collected in summer than in those for spring (Fig. S5b). Other phyla such as *Verrucomicrobia*, *Planctomycetes*, *Chloroflexi* and other exiguous (*Firmicutes*, *Gemmatimonadetes* and *Hydrogenedentes*, <1%) were also significantly more abundant in summer than in spring (ANCOM-BC, p-values < $2.38 \cdot 10^{-4}$). On the other hand, *Bacteroidetes*, *Gemmatimonadetes*, *Latescibacteria* and *Acidobacteria* were underrepresented in summer bacterial communities, although the markedly greater prevalence of *Acidobacteria* in spring samples was not statistically significant (Fig. S5b).

Several changes in community dynamics were also registered for lower taxonomic ranks. In the DNA library, more than 62 and 59% of the total sequences recorded in summer and spring, respectively, were classified at genus level. Eventually, the prevalence of 14 bacterial genera was significantly different when both seasons are compared. Specifically, *Gp16*, *Lacipirellula*, and actinobacterial genera *Gaiella*, *Baekduia*, *Nocardiodes* and *Parviterribacter* were more common in summer than in spring (Fig. 3a). The remaining genera showing statistical differences in terms of abundance

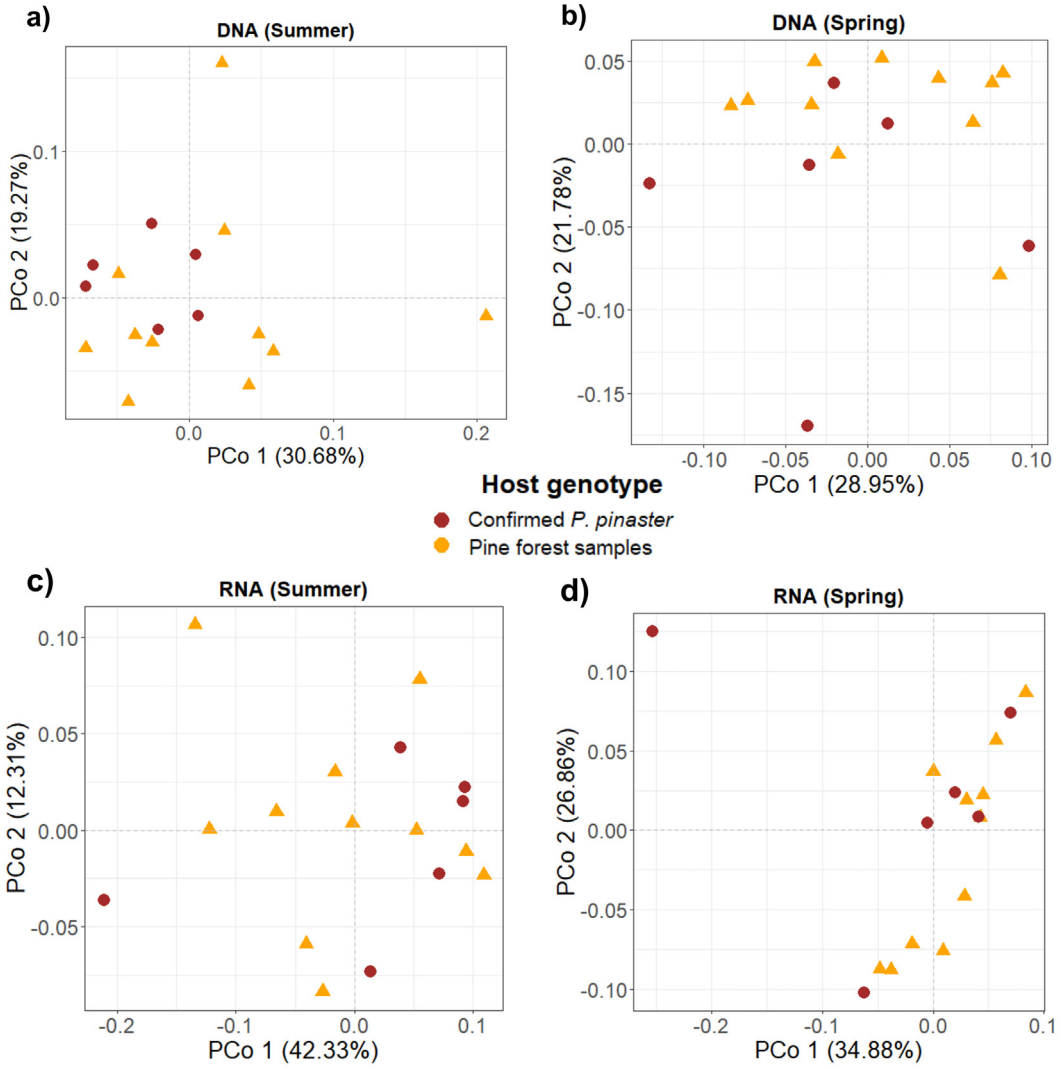

Fig. 1. Principal Coordinate Analysis (PCoA) of rhizosphere bacterial communities dwelling in the pine forest of Sierra de Oria based on Weigthed UniFrac distance for DNA libraries in summer (a), spring (b), and for RNA-based libraries, in summer (c) and spring (d).

accounted for less than 0.15% of the total library sequences in one or either season, including several members that were more represented in spring than in summer (Fig. 3a). It should be noted that acidobacterial subgroup *Gp4* (one of the most predominant in both seasons) was considerably more abundant in summer than in spring samples (7.72 and 3.83%, respectively), even if this differential abundance was not statistically significant (ANCOM-BC, p-value > 0.05). More than 51 and 65% of the RNA-derived sequences retained in summer and spring, respectively, were identified with already known genera (included in taxonomy databases). Moreover, more differentially abundant genera were detected than in the case of the DNA libraries. Comparison between summer and spring samples revealed that 31 potentially active bacterial genera were differentially abundant, being the predominant genus (acidobacterial *Gp3*) significantly more abundant in spring than in summer (Fig. 3b). The abundance of genera *Gp16*, *Tepidisphaera*, *Gemmata*, *Baekduia*, *Desertimonas*, *Gp7*, *Nannocystis*, *Bradyrhizobium*, *Mesorhizobium*, *Devosia* and *Nitrobacter* (the last four belonging to the order *Rhizobiales*) and 11 minor genera (which accounted for less than 0.16% of the sequences) was greater in summer than in spring (Fig. 3b; Table S5). It is worth mentioning that samples collected in spring were significantly enriched in *Rhizobacter*, *Gemmatirosa*, *Gp2* and *Povalibacter* when compared to those obtained in summer, while *Gp6*, *Gp1*, *Mucilaginibacter*, *Reyranella* and *Pseudomonas* were also depleted in summer but no consistent differences were recorded (ANCOM-BC, p-values < 0.26).

Co-occurrence network analysis revealed seasonal differences in rhizosphere bacterial communities. When spring and summer bacterial populations were compared, different network topologies were observed both for DNA and RNA libraries (Fig. S6; Table 3). The overriding change was registered at the level of network complexity level, with the value of the average degree and average cluster coefficients (avgK and avgCC, respectively) being significantly greater in spring than in summer, primarily in the DNA library (Table 3). By contrast, summer networks showed a consistent increase in Geodesic Distance (GD) when compared to those calculated for spring, both at DNA and RNA levels. On the other hand, it should be highlighted that all of the four networks had modular assembly (M > 0.4; Table 3 and Fig. 4), and that summer networks were more modularized than those corresponding to spring, for DNA and RNA-derived cases.

Concerning the topological role of ASV, several keystone members were detected in both types of co-occurrence networks, defined as network hubs, module hubs or connectors (Figs. 4, 5; Tables S5 and S6). Interestingly, when networks of total communities were evaluated (DNA library), more keystone ASVs were recorded in summer than spring (Table S6), although the reverse trend was observed for potentially active populations (RNA library). Notwithstanding, it should be emphasized that one network hub belonging to genus *Phenylobacterium* was registered for the summer network corresponding to potentially active bacteria (RNA library) and, what is more, another ASV belonging to the same genus acted as connector in the same network. As summarized in Tables S5 and S6, different ASVs that

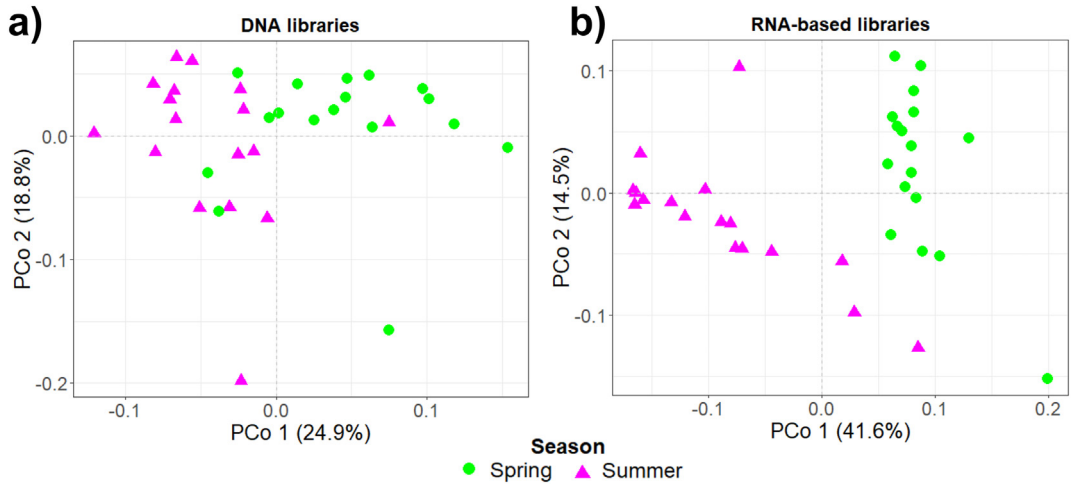

Fig. 2. Principal Coordinate Analysis (PCoA) of rhizosphere bacterial communities dwelling in the pine forest of Sierra de Oria based on Weigthed UniFrac distance, for DNA (a) and RNA-based libraries (b). In both cases, 17 trees were included in each group of samples ($N_{Summer} = 17$, $N_{Spring} = 17$).

belong to the same genus could play relevant topological roles. That was the case of the acidobacterial subgroup *Gp3*, represented by three ASV acting as connectors in the spring network (DNA library). The genera *Pseudonocardia* and *Phenylobacterium* were also represented by two different ASVs that acted as module hub and connector, and as connector and network hub, respectively (network of summer samples, RNA library). Strikingly, the four co-occurrence networks were characterized by a great amount of negative links that connected the nodes (in an intramodular and intermodular way; Table 3, Figs. 4, 5).

3.5. Prediction of functional profiles of potentially active bacteria

Metabolic functions could only be assigned to 14.18% of total ASV registered in the RNA library, and 35 out of 92 functions included in the database were ascribed mostly to one ASV (Table S8). The most prevalent functions in both seasons concerned N and C cycles, (especially (aerobic) chemoheterotrophy and nitrogen fixation, respectively), with the sulphur (S) cycle also represented although to a lesser extent (Table S8). Regarding season-induced changes, 12 metabolic processes were significantly more abundant in summer than in spring, while just two were more represented in the spring. Especially remarkable, was the increase in the rate of nitrogen fixation for the summer sampling, with the main contributing ASV belonging to the genus *Bradyrhizobium* (Table S8). As expected, the prevalence of light depending functions such as phototrophy or photoheterotrophy was significantly greater in summer. On the other hand, it is worth mentioning that no plant pathogens were detected in samples from either of the seasons, although human parasites or pathogens were found to be present in rhizosphere samples.

4. Discussion

The dynamics of rhizosphere microbiome can be approached when samples are collected from the same trees in every season. However, most published studies have been carried out without including the plant-host genotyping step to validate the origin of all collected samples (Žifčáková et al., 2016; Siles and Margesin, 2017; Wang et al., 2018; Lacerda-Júnior et al., 2019; Lasa et al., 2019; Fernández-González et al., 2020b). Our study included an initial step to check if rhizosphere samples were collected or not from the selected *P. pinaster* trees. The microsatellite analysis of needle and root DNAs showed that less than 50% of the samples belonged to selected trees and 11% did not belong to studied species despite the sampling being carried out according to standardized protocols. It is important to highlight that the phenotypically homogeneous stand of trees at Sierra de Oria is of low density, yet with pines showing remarkable genetic variability (Eveno et al., 2008). Moreover, the *P. pinaster* trees were at least 10 m distance from other woody species, shrubs or trees. Nevertheless, it is well known that the root systems of trees are widely disperse and intermixed. Thus, root and rhizosphere samples could have been obtained from trees or plants far away from those from needle samples were taken.

Soil physicochemical parameters (especially pH) are commonly recognized for being strong drivers of microbial community assemblages, although Bonito et al. (2014) proposed that tree species has greater impact on microbial community structure. The composition of root exudation is considered to be plant-species dependent, which results in the recruitment of specific microbial taxa. In addition, it is well stablished that the composition of root microbiota is host genotype dependent (Fernández-González et al., 2019; Terhonen et al., 2019). Plant host intraspecific variation entails shifts at compositional levels of belowground microbial communities (Schweitzer et al., 2018), and thus, affects the ecosystem functioning (Pérez-Izquierdo et al., 2019 and references therein). However, in this study a marked homogeneity was observed among rhizosphere bacterial communities when inter- and even intraspecific variability of host trees was identified in the Mediterranean pine forest, suggesting that there may be biotic or abiotic factors with higher impact on bacterial communities that the rhizosphere effect.

It should be noted that for a given plant species, soil properties are known to have stronger effect over the composition of root microbial populations than the tree host genotype (Cregger et al., 2018; Gallart et al., 2018). Thus, we checked the effect of edaphic parameters on the structure of rhizosphere bacterial communities. In agreement with the abovementioned observations and as revealed by CAP analysis, the soil pH of the representative forest of dry Mediterranean areas was the only factor shaping bacterial communities in summer. However, no significant differences were recorded for this parameter among soil collected from confirmed *P. pinaster* and Pine forest samples. Thus, the homogenized diversity and structure of rhizosphere bacterial populations may be the result of homogeneous pH values among groups of samples in summer. In addition, we cannot rule out that there may be overlooked biotic or abiotic factors (or a combination or both) that shaped the structure and composition of rhizosphere bacterial communities to such an extent that they were homogeneous even when associated with different plant species located in the pine forest of Sierra de Oria. That is to say, the effect of such factors would overcome the host genotype effect.

Considering the homogeneity of the bacterial populations even though the rhizosphere samples did not always match the same tree, seasonal changes were observed for bacterial communities associated with the roots of the 17 trees. Unlike the rest of ecological traits that were assessed for rhizosphere bacterial communities, richness and diversity remained

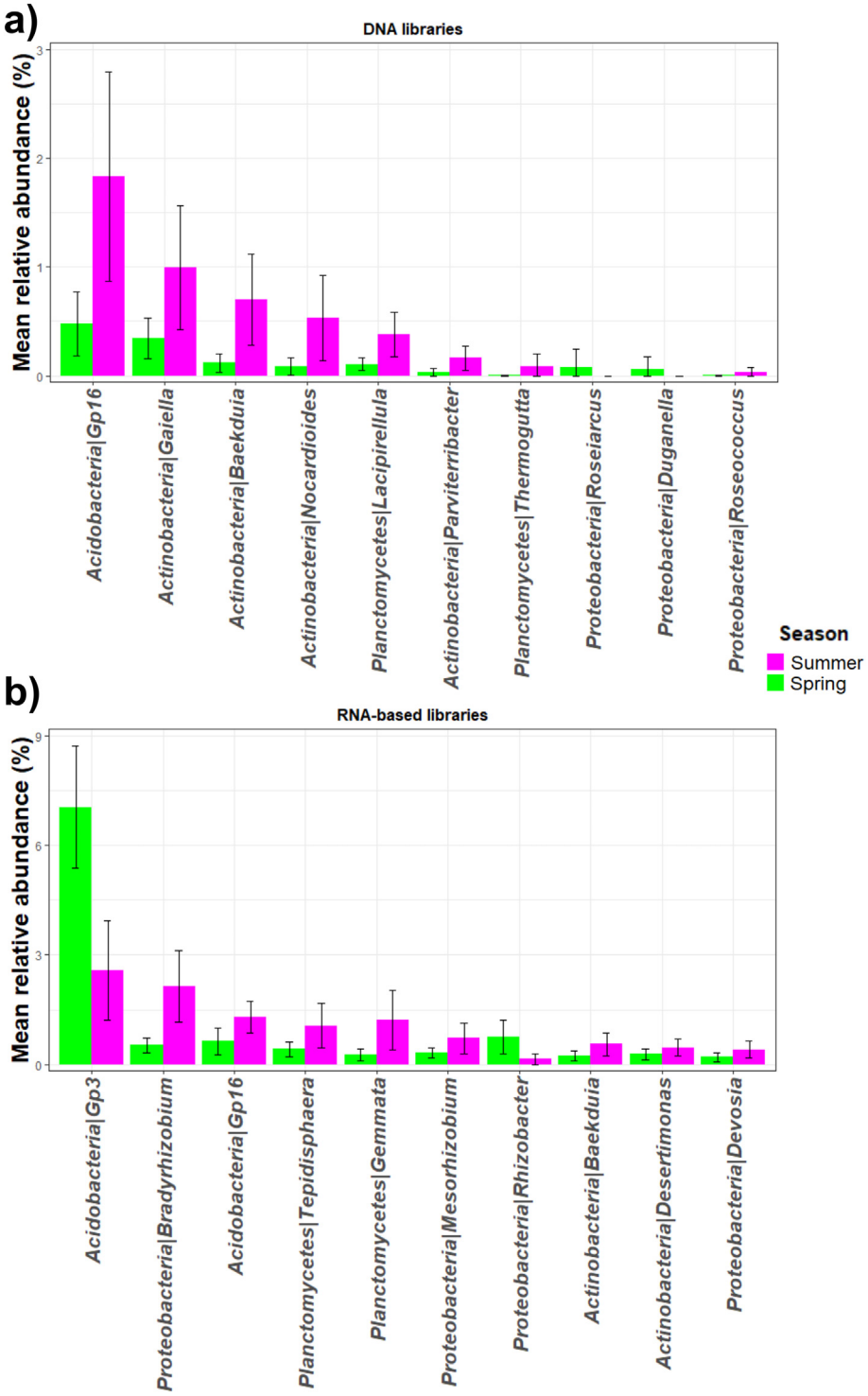

Fig. 3. Mean relative abundance of the dominant and differentially abundant bacterial genera in DNA (a) and RNA-derived (b) libraries. Only the 10 most abundant genera which are show significant differences between seasons (according to ANCOM-BC) are included. Values indicate their mean relative abundance and standard deviation. The name of each genus is preceded by the phylum to each one belongs (and separated by "|" symbol).

stable across seasons. This finding is at odds with that reported by Wang et al. (2018), who demonstrated that alpha diversity indices of rhizosphere bacterial communities for *Pinus tabulaeformis* were sharply different in spring and summer. These differences between studies are likely to be due to different tree host species and forest sites analyzed. Nonetheless, other authors have previously suggested that bacterial diversity is mostly affected by altitude or soil depth rather than by seasonal fluctuations (Shigyo et al., 2019). Likewise, Siles and Margesin (2017) argued about the neutral effect of seasonality on the diversity of microbial communities subjected to the same soil pH, SOM, nutrient availability or temperature in coniferous forests. Although we cannot discuss the effect of seasonal climate on bacterial diversity, based on the observations of Shigyo et al. (2019) we speculate that there may be other uncontrolled factors that govern bacterial diversity to a greater extent than seasonality in the Mediterranean pine forest under study. On the other hand, the stability in terms of bacterial diversity and richness along with structural and compositional

Table 3
Main topological properties of bacterial co-occurrence networks calculated for the 17 host plants located in Sierra de Oria. Numbers in brackets indicate the number of modules obtained. Asterisks indicate significant differences among seasons corresponding to the same library (DNA or RNA; Student's *t*-test). Confidence level of 95% in all cases.

		Number of ASV	Similarity threshold (St)	R^2 of power-law	Total nodes	Total links	Positive edges (PEP)	Average degree (avgK)	Average clustering coefficient (avgCC)	Geodesic distance (GD)	Modularity
DNA	Summer	4106	0.78	0.896	142	150	12%	2.113*	0.008*	5.727*	0.742* (24)
	Spring	4334	0.76	0.841	140	259	24%	3.700*	0.089*	4.262*	0.513* (14)
RNA	Summer	3950	0.74	0.915	157	207	18.36%	2.637*	0.052*	4.861*	0.685* (15)
	Spring	4337	0.75	0.878	159	230	18.26%	2.893*	0.064*	4.263*	0.646* (14)

changes observed between seasons suggest, that a bacterial succession event may have occurred in the rhizosphere, with certain taxa replaced by others without changing neither richness nor bacterial evenness.

It should be highlighted that more pronounced abundance changes were observed in the case of potentially active bacteria (RNA) than in total communities (DNA). While 9 phyla, 52 genera and 266 active ASV were differentially abundant, 5, 22 and 168 phyla, genera and ASV, respectively, with significant abundance changes were recorded in the DNA library. It is not striking that substantial changes occur at RNA level rather than in DNA libraries, since that refers to the active bacterial population. Indeed, Žifčáková et al. (2016) reported that soil bacterial community composition in a coniferous forest remained quite stable whereas remarkable changes were observed in the corresponding metatranscriptome across seasons. It has been also demonstrated for other woody species that the ecology of total and potentially active bacterial communities (DNA and RNA-based libraries, respectively) are different in terms of diversity, structure and taxonomic profiles (Baldrian et al., 2012; Lasa et al., 2019). The same trend was also observed in this work. As described by other authors, only a small part of the bacterial communities is metabolically active in the soil; living bacteria can face adverse conditions by entering into a reversible state of dormancy, or by forming spores (Bladodatskaya and Kuzyakov, 2013). Moreover, extracellular DNA from dead bacterial cells can persist for long periods in soils (Carini et al., 2016), thus, the diversity and richness of soil bacterial populations could be overestimated when just DNA based approaches are performed. To overcome this concern, recent studies have included the analysis of 16S rRNA transcripts in plant-

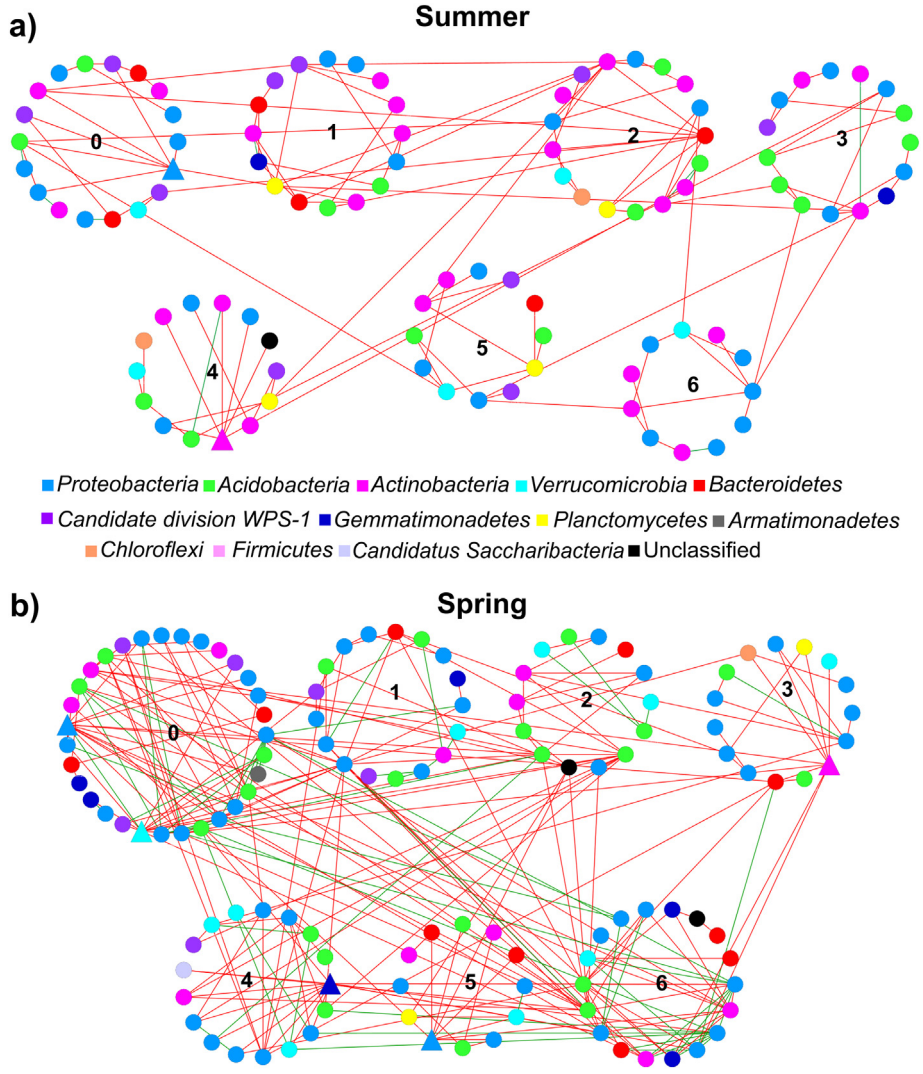

Fig. 4. Co-occurrence networks of total bacterial communities (DNA) in summer (a) and spring (b). Green and red lines (links) connecting nodes indicate positive and negative interactions, respectively. Triangle-shaped nodes represent Module hubs. Only those modules composed by 10 or more nodes are included in the graph. Numbers indicate the number of the module.

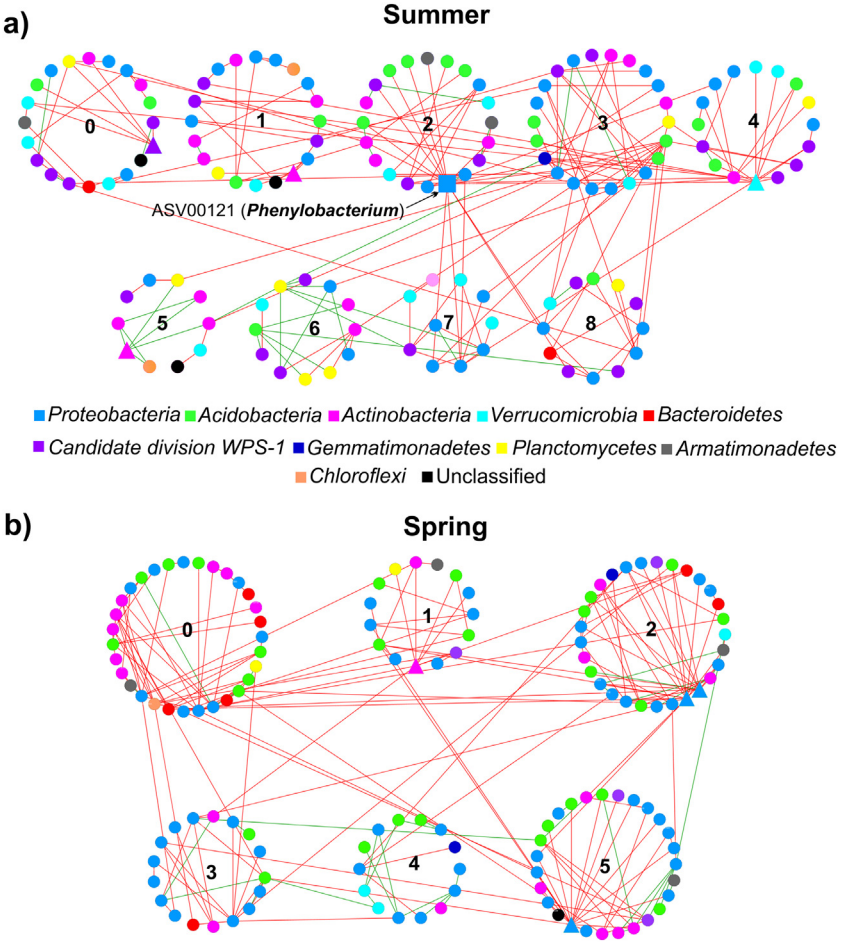

Fig. 5. Co-occurrence networks of active bacterial communities (RNA) in summer (a) and spring (b). Green and red lines (links) connecting nodes indicate positive and negative interactions, respectively. Square-shaped nodes denote Network hubs, while triangle-shaped nodes represent Module hubs. Only those modules composed by 10 or more nodes are included in the graph. Numbers indicate the number of the module.

microbiota analyses (Lasa et al., 2019; Fernández-González et al., 2020b), since it is a very valuable tool to track the metabolically active bacteria. The identification of active (gene translating) bacteria can be hampered by the short lifespan of this nucleic acid and its rapid decomposition during the extraction process from soils (Bladodatskaya and Kuzyakov, 2013). Moreover, there is still a gap of knowledge regarding the influence of the 16S rRNA gene copy number and bacterial ribosome amount per cell in ecological analyses.

Seasonality is widely considered one of the most consistent factors of microbial dynamics in temperate forest soils (Baldrian, 2017). Seasonal climate differences affect plant-related parameters such as leaf litter, dead wood quality, root exudation or root biomass, which result in variations in soil nutrient availability as well as in belowground microbial communities (Dukunde et al., 2019). Consequently, a selective enrichment in certain bacterial taxa occurs in forest soils. In our case, bacterial communities were mainly dominated by phylum Proteobacteria, especially in spring. This result is in agreement with the observations of Pérez-Izquierdo et al. (2019), who suggested that rhizosphere microbiota of *P. pinaster* trees was overrepresented by this copiotrophic phylum due to its ability to feed on varying C sources, which leads it to outcompete other bacterial taxa. Moreover, several genera were enriched in summer samples when compared with spring samples, both for DNA and RNA libraries. For example, acidobacterial *Gp16*, which has been recorded in rhizosphere soils of strawberry soil-borne-pathogen resistant cultivars (Lazcano et al., 2021). A similar trend was also observed for the genera *Gaiella*, *Nocardiodes* and the acidobacterial subgroup *Gp4*. It should be pointed out that one ASV belonging to the acidobacterial subgroup *Gp4* (ASV002849) acted as a connector node in the summer network recorded for the DNA library. As discussed below, connectors could deploy mechanisms to maintain the stability of the community, for instance against pathogens. Thus, in summer, rhizosphere soils of the representative forest of dry Mediterranean areas are enriched in genera potentially involved in the resistance to soil-borne pathogens possibly due to the greater pathogen susceptibility of plant hosts associated with the harsh summer conditions. Notwithstanding, changes in community composition may only facilitate substantial changes in ecosystem processes if microorganisms vary in their functions.

According to Karimi et al. (2017), microbial diversity may not be the best indicator of ecosystem state when perturbations occur. In the pine forest of Sierra de Oria, bacterial diversity remained stable in spite of the low rainfall levels and the rise in temperatures registered in summer, however, co-occurrence networks were more informative, reinforcing the hypothesis that seasonality has a strong influence on bacterial communities, even at associative level. Although at a first glance all the studied networks appear to be of similar complexity (Fig. S6), those calculated for the spring showed greater values of avgK and avgCC and more nodes and links than those in summer, especially in the case of potentially active populations. It has been previously proposed that these kind of topological properties reflect the complexity of the soil microbial networks, which could affect positively host plants by aiding in suppressing plant diseases or improving the ability to deal with environmental changes (Jiemeng et al., 2018; Fernández-González et al., 2020b). Furthermore, other authors have suggested that complex networks are more resistant to microbial invasions (by pathogens

or beneficial microbes). Thus, members of the spring networks could have the capability to improve the fitness of plant hosts, both at structural and functional level. On the other hand, a decrease in complexity and an augmentation in GD and modularity values were observed for summer networks in comparison with those calculated for spring (Table 3). Low complexity is a characteristic of networks inferred for microbial communities subjected to environmental stresses and more sensitive to external changes (Banerjee et al., 2019). This observation is in accordance with the harsh conditions that pine trees regularly face in the Mediterranean region, especially during the summer. Moreover, highly compartmentalized microbial networks are suspected to resist biotic and abiotic stresses (Marasco et al., 2018), maintaining the stability of the microbial population and thus, protecting it from external disturbances (Delmas et al., 2019). Thus, modularity is a network parameter proposed as an indicator of the resistance of the corresponding biological system (Siles and Margesin, 2017). In summer, rhizosphere bacterial networks could have increased the number of intramodular links and diminished the interactions with other modules so that the impact of the harsh summer conditions and the low-growth state of the host trees remained constrained just to certain modules, preventing the negative impact from being disseminated across the whole bacterial networks. Furthermore, negative connections were extremely abundant for summer network of total bacterial communities (DNA library). Although that network had more negative links than the one of RNA library, it should be highlighted that both summer and spring networks (RNA) were characterized by a myriad of potentially negative interactions. Other authors (Faust et al., 2012; Marasco et al., 2018) have already suggested that negative edges represent mutual microbial co-exclusion patterns, which could be the results of a wide variety of mechanisms such as direct competition, toxin excretion or different niche adaptation. Taking both considerations into account, bacterial communities dwelling in the representative forest of the Mediterranean zones may be negatively interacting or excluding each other as a mechanism to cope with the harsh summer conditions and the lower host trees' growth. It is worth mentioning that the Mediterranean climate is distributed throughout the world (Mediterranean basin, southwestern Australia, part of South Africa, California or Central Chile), and the state of rhizosphere bacterial communities inhibiting Mediterranean forests could be a wood indicator of future environmental changes. That is, the potential stress state observed for bacterial populations inhabiting the Sierra de Oria forest, could reflect the status of other bacterial communities dwelling in other regions of the world with a Mediterranean climate. More so, the negative interactions between network members inferred here may reflect the upcoming situation in a context of climate change. Notwithstanding, further experimental approaches are needed to get more insights into the ecological interactions among root-associated bacteria of Sierra de Oria.

Concerning keystone individuals of the networks, it is worth mentioning that a potentially active network hub (RNA) was identified in the summer network, namely the ASV00121 which belonged to genus *Phenylobacterium* (Fig. 5a and Table S7). Moreover, a connector node belonging to that genus was found in the same network. These observations are consistent with the results provided by Longley et al. (2020) and Shang et al. (2021), who detected hubs affiliated to genus *Phenylobacterium* in the rhizosphere and roots of soybean and peanut plants. Network hubs are extensively regarded as crucial to maintain the coherence of the network and the module they comprise, especially in the face of an environmental disturbance (Jiemeng et al., 2018). Thus, in spite of the lack of knowledge regarding the effect of members of genus *Phenylobacterium* on plant health, their detection as network hub and connector in summer hints at their potential role in structuring the rhizosphere microbiome towards the amelioration of the effects of harsh summer conditions. On the other hand, Lasa et al. (2019) disclosed the disproportioned uncoupling between the abundance of *Phenylobacterium* members in RNA and DNA libraries when the rhizosphere of oak trees was studied. Thus, our observations may endorse the hypothesis that *Phenylobacterium* could be an active member playing an important ecological role in maintaining the stability of bacterial communities in forest rhizosphere soils. Understanding the ecological role of keystone individuals could provide novel candidates to be isolated for forestry management strategies aimed at improving the plant resilience to environmental changes.

Seasonality also induced metabolic activity shifts of rhizosphere bacterial communities. One of the constraints of functional prediction tools such as FAPROTAX is that the presence of certain taxa does not necessarily indicate bacterial metabolic activity. Nonetheless, our assignments were based on 16S rRNA transcripts rather than the corresponding gene; whereas, it has been demonstrated that taxonomy-derived functional predictions for soil bacterial communities are in good agreement with that obtained from metagenomes or metatranscriptomes (Dukunde et al., 2019). Even so, the low proportion of ASV with an assigned function (probably due to the large amount of taxonomically unclassified ASV) makes it difficult to shed light on the functional dynamics related to seasonality. It has been proposed that most litter decomposition occurs mainly during the summer (Sohng et al., 2014), however, organic matter degradation processes (ligninolysis, xylanolysis, cellulolysis, aromatic compound degradation, among other) were not detected or at very low levels, being cellulolysis significantly more predominant in spring. One possible explanation is that coniferous litter is rather recalcitrant (high lignin content; Berger and Berger, 2012), which could lead to almost depletion of bacterial decomposition processes. In summer, the relative abundance of metabolic functions related to C and N mobilization was slightly greater than in spring. This finding could be in line with the results of networks analysis: rhizosphere bacterial communities may have been dealing with stressful environmental conditions deploying their adaptive response during the summer (Siles and Margesin, 2017), as suggested by the high percentage of negative linkages in the both DNA and RNA-related networks. This adaptation could have entailed an increase in the metabolic activity of competing bacteria, including metabolic efforts to launch co-exclusion mechanisms. However, future empirical studies (metatranscriptomics or metabolomics) are needed to unravel the functional shifts caused by seasonality and the potential drought adaptive response of rhizosphere bacteria.

5. Conclusions

According to forecasts, the effects of the climate change are going to trigger the expansion of desert areas in the Mediterranean basin in the next century. Here, we propose a novel method for validating the origin of the root samples and uncover how seasonal changes alter rhizosphere soil bacterial communities inhabiting in a Mediterranean pine forest drastically affected by climate change. First of all, our genotyping study reveals that in spite of doing a controlled thorough sampling, root samples did not always correspond to the tree of interest, they can even correspond to plant species other than the ones selected. These novel findings stablish a new paradigm which has global implications and applicability: our results make it advisable to study the plant origin of the samples in further plant-microbiota analyses in order to be aware of the plant species of interest. Nonetheless, bacterial communities dwelling in the rhizosphere soil of dry Mediterranean pine forests with low tree density and diversity are apparently so homogeneous that genotype cross-validation analyses are slightly decisive. On the other hand, the seasonal effect was noteworthy, entailing changes in rhizosphere bacterial communities at compositional, structural, functional, and especially at associative levels. Interestingly, our results demonstrate that bacterial taxa were forming negative interactions among themselves under the stressful conditions of the summer. Eventually, considering the sensitivity of the Mediterranean basin to environmental shifts, the composition and the instability of the bacterial populations observed in the summer serve as a mirror of the climate change effects expected in a near future. Hence, our study has global implications and propose a sample cross-validation procedure to perform more accurate plant-microbiota analyses and shedding light on the marked changes that rhizosphere bacterial populations will have to face in a future of intense drought events, especially with regard to their members' interactions.

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CRediт authorship contribution statement

Ana V. Lasa: Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing – original draft, Visualization; M. Ángeles Guevara: Investigation - plant genotyping, Formal análisis, Writing – original draft, Visualization; Pablo J. Villadas: Formal analysis; Investigation; Resources; María Dolores Vélez: Investigation - plant genotyping, Validation; Antonio J. Fernández-González: Software, Formal analysis; Nuria de María: Investigation - plant genotyping; Miriam López-Hinojosa and Luis Díaz: Resources, Investigation; María Teresa Cervera: Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition; Manuel Fernández-López: Conceptualization, Methodology, Resources Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

All authors contributed to the article and approved the submitted version.

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.

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References

Alves, M., Pereira, A., Vicente, C., Matos, P., Henriques, J., Lopes, H., Nascimento, F., Mota, M., Correia, A., Henriques, I., 2018. The role of bacteria in pine wilt disease: insights from microbiome analysis. FEMS Microbiol. Ecol. 94, 94fiy077. https://doi.org/10.1093/femsec/fiy077.

Anderson, M.J., 2006. Distance-based tests for homogeneity of multivariate dispersion. Biometrics 62, 245–253. https://doi.org/10.1111/j.1541-0420.2005.00440.x.

Anderson, M.J., Walsh, D.C.I., 2013. PERMANOVA, ANOSIM and the mantel test in the face of heterogeneous dispersions: what null hypothesis are you testing? Ecol. Monogr. 83, 557–574. https://doi.org/10.1890/12-2010.1.

Aranda, I., Alía, R., Ortega, U., Dantas, A.K., Majada, J., 2010. Intraspecific variability in biomass partitioning and carbon isotopic discrimination under moderate drought stress in seedlings from four Pinus pinaster populations. Tree Genet. Genomes 6, 169–178. https://doi.org/10.1007/s11295-009-0238-5.

Arrillaga, I., Guevara, M.A., Muñoz-Bertomeu, J., Lázaro-Gimeno, D., Sáez-Laguna, E., Díaz, L.M., Torralba, L., Mendoza-Poudereux, I., Segura, J., Cervera, M.T., 2014. Selection of haploid cell lines from megagametophyte cultures of maritime pine as a DNA source for massive sequencing of the species. Plant Cell Tissue Organ Cult. 118, 147–155. https://doi.org/10.1007/s11240-014-0470-z.

Baldrian, P., 2017. Microbial activity and the dynamics of ecosystem processes in forest soils. Curr. Opin. Microbiol. 37 (128), 134. https://doi.org/10.1016/j.mib.2017.06.008.

Baldrian, P., Kolařík, M., Štursová, M., Kopecký, J., et al., 2012. Active and total microbial communities in forest soil are largely different and highly stratified during decomposition. ISME J. 6, 248–258. https://doi.org/10.1038/ismej.2011.95.

Banerjee, S., Walder, F., Büchi, L., Meyer, M., Held, A.Y., Gattinger, A., Keller, T., Charles, R., van der Heijden, M.G.A., 2019. Agricultural intensification reduces microbial network complexity and the abundance of keystone taxa in roots. ISME J. 13, 1722–1736. https://doi.org/10.1038/s41396-019-0383-2.

Barberán, A., Bater, S.T., Casamayor, E.O., Fierer, N., 2012. Using network analysis to explore co-occurrence patterns in soil microbial community. ISME J. 6, 343–351. https://doi.org/10.1038/ismej.2011.119.

Berger, T., Berger, P., 2012. Greater accumulation of litter in spruce (Picea abies) compared to beech (Fagus sylvatica) stands is not a consequence of the inherent recalcitrance of needles. Plant Soil 358, 349–369. https://doi.org/10.1007/s11104-012-1165-z.

Bi, B., Zhang, H., Yuan, Y., Wu, Z., Wang, Y., Han, F., 2021. Dynamic changes of soil microbial community in Pinus sylvestris var. Mongólica plantations in the Mu Us Sandy Land. J. Environ. Manag. 287, 112306. https://doi.org/10.1016/j.jenvman.2021.112306.

Bladodatskaya, E., Kuzyakov, Y., 2013. Active microorganisms in soil: critical review of estimation criteria and approaches. Soil Biol. Biochem. 67, 192–211. https://doi.org/10.1016/j.soilbio.2013.08.024.

Bokulich, N.A., Subramanian, S., Faith, J.J., Gevers, D., Gordon, J.I., Knight, R., Mills, D.A., Caporaso, J.G., 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat. Methods 10, 54–59. https://doi.org/10.1038/nmeth.2276.

Bonito, G., Reynolds, H., Robeson, M.S., Nelson, J., Hodkinson, B.P., Tuskan, G., Schadt, C.W., Vilgalys, R., 2014. Plant host and soil origin influence fungal and bacterial assemblages in the roots of woody plants. Mol. Ecol. 23, 3356–3370. https://doi.org/10.1111/mec.12821.

Cabezas, J.A., González-Martínez, S.C., Collada, C., Guevara, M.A., Boury, C., de María, N., Eveno, E., Aranda, I., Garnier-Géré, P.H., Brach, J., Alía, R., Plomion, C., Cervera, M.T., 2015. Nucleotide polymorphisms in a pine ortholog of the Arabidopsis degrading enzyme cellulase KORRIGAN are associated with early growth performance in Pinus pinaster. Tree Physiol. 35, 1000–1006. https://doi.org/10.1093/treephys/tpv050.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution simple inference from Illumina amplicon data. Nat. Methods 13, 581–583.. https://doir.org/10.1038/nmeth.3869.

Carini, P., Marsden, P.J., Left, J.W., Morgan, E.E., Strickland, M.S., Fierer, N., 2016. Relic DNA is abundant in soil and obscures estimates of soil microbial diversity. Nat. Microbiol. 2, 16242. https://doi.org/10.1038/nmicorbiol.2016.242.

Cobo-Díaz, J.F., Fernández-González, A.J., Villadas, P.J., Robles, A.B., Toro, N., Fernández-López, M., 2015. Metagenomic assessment of the potential microbial nitrogen pathways in the rhizosphere of a Mediterranean forest after a wildfire. Microb. Ecol. 69, 895–904. https://doi.org/10.1007/s00248-015-0586-7.

Cregger, M.A., Veach, A.M., Yang, Z.K., Crouch, M.J., Vilgalys, R., Tuskan, G.A., Schadt, C.W., 2018. The Populus holobiont: dissecting the effects of plant niches and genotype on the microbiome. Microbiome 6, 31. https://doi.org/10.1186/s40168-018-0413-8.

DADA2, .. https://benjjneb.github.io/dada2/tutorial.html Last accessed: 20 Jun 2021.

de María, N., Guevara, A., Perdiguero, P., Vélez, M.D., Cabezas, J.A., López-Hinojosa, M., Li, Z., Díaz, L.M., Pizarro, A., Mancha, J.A., Sterck, L., Sánchez-Gómez, D., Miguel, C., Collada, C., Díaz-Sala, C., Cervera, M.T., 2020. Molecular study of drought response in the Mediterranean conifer Pinus pinaster Ait.: differential transcriptomic profiling reveals constitutive water-deficit-independent drought tolerance mechanisms. Ecol. Evol. 10, 9788–9807. https://doi.org/10.1002/ece3.6613.

de Miguel, A., Bartholomé, J., Ehrenmann, F., Murat, F., Moriguchi, Y., Uchiyama, K., Ueno, S., Tsumura, Y., Lagraulet, H., de Maria, N., Cabezas, J.A., Cervera, M.T., Gion, J.M., Salse, J., Plomion, C., 2015. Evidence of intense chromosomal shuffling during conifer evolution. Genome Biol. Evol. 7, 2799–2809. https://doi.org/10.1093/gbe/evv185.

Delmas, E., Besson, M., Brice, M.H., Burkle, L.A., Dalla Riva, G.V., Fortin, M.J., Gravel, D., Guimares Jr., P.R., Hembry, D.H., Newman, E.A., Olesen, J.M., Pires, M.M., Yeakel, J.D., Poisot, T., 2019. Analysing ecological networks of species interactions. Biol. Rev. 94, 16–36. https://doi.org/10.1111/brv.12433.

Duhamel, M., Wan, J., Bogar, L.M., Segnitz, R.M., Duncritts, N.C., Peay, K.G., 2019. Plant selection initiates alternative successional trajectories in the soil microbial community after disturbance. Ecol. Monogr. 89, e01367. https://doi.org/10.1002/ecm.1367.

Dukunde, A., Schneider, D., Schmidt, M., Veldkamp, E., Daniel, R., 2019. Tree species shape soil bacteria community structure and function in temperate deciduous forests. Front. Microbiol. 10, 1519. https://doi.org/10.3389/fmicb.2019.01519.

Eveno, E., Collada, C., Guevara, M.A., Léger, V., Soto, A., Díaz, L., Léger, P., González-Martínez, S.C., Cervera, M.T., Plomion, C., Garnier-Géré, P.H., 2008. Contrasting patterns of selection at Pinus pinaster Ait. drought stress candidate genes as revealed by genetic differentiation analyses. Mol. Biol. Evol. 25, 417–437. https://doi.org/10.1093/molbev/msm272.

Faust, K., Sathirapongsasuti, J.F., Izard, J., Segata, N., Gevers, D., Raes, J., Huttenhower, C., 2012. Microbial co-occurrence relationships in the human microbiome. PLoS Comput. Biol. 8, e1002606. https://doi.org/10.1371/journal.pcbi.1002606.

Fernández-González, A., Villadas, P.J., Gómez-Lama Cabanás, C., Valverde-Corredor, A., Belaj, A., Mercado-Blanco, J., Fernández-López, M., 2019. Defining the root endosphere and rhizosphere microbiomes from the World Olive Germplasm Collection. Sci. Rep. 9, 20423. https://doi.org/10.1038/s41598-019-56977-9.

Fernández-González, A.J., Wentzien, N.M., Villadas, P.J., Valverde-Corredor, A., Lasa, A.V., Gómez-Lama Cabanás, C., Mercado-Blanco, J., Fernández-González, M., 2020a. Comparative study of neighboring Holm oak and olive trees belowground microbial communities subjected to different soil management. PLoS ONE 15, e0236796. https://doi.org/10.1371/journal.pone.0236796.

Fernández-González, A.J., Cardoni, M., Gómez-Lama Cabanás, C., Valverde-Corredor, A., Villadas, P.J., Fernández-López, M., Mercado-Blanco, J., 2020b. Linking belowground microbial network changes to different tolerance level towards Verticillium wilt of olive. Microbiome 8, 11. https://doi.org/10.1186/s40168-020-0787-2.

Fox, J., Weisberg, S., 2019. An {R} Companion to Applied Regression. third ed. Thousand Oaks CA.

Gallart, M., Adair, K.L., Love, J., Meason, D.F., Clinton, P.W., Xue, J., Turnbull, M.H., 2018. Host genotype and nitrogen form shape the root microbiome of Pinus radiata. Microb. Ecol. 75, 419–433. https://doi.org/10.1007/s00248-017-1055-2.

Gehring, C.A., Sthultz, C.M., Flores-Rentería, L., Whipple, A.V., Whitham, T.G., 2017. Tree genetics defines fungal partner communities that may confer drought tolerance. Proc. Natl. Acad. Sci. U. S. A. 114, 11169–11174. https://doi.org/10.1073/pnas.1704022114.

González-Martínez, S.C., Robledo-Arnuncio, J.J., Collada, C., Díaz, A., Williams, C.G., Alía, R., Cervera, M.T., 2004. Cross-amplification and sequence variation of microsatellite loci in Eurasian hard pines. Theor. Appl. Genet. 109, 103–111. https://doi.org/10.1007/s00122-004-1596-x.

Guevara, M.A., Chagné, D., Almeida, M.H., Byrne, M., Collada, C., Favre, J.M., Harvengt, L., Jeandroz, S., Orazio, C., Plomion, C., Ramboer, A., Rocheta, M., Sebastiani, F., Soto, A., Vendramin, G.G., Cervera, M.T., 2005. Isolation and characterization of nuclear microsatellite loci in Pinus pinaster Ait. Mol. Ecol. Notes 5, 57–59. https://doi.org/10.1111/j.1471-8286.2004.00830.x.

Guiot, J., Cramer, W., 2016. Climate change: the 2015 Paris agreement thresholds and Mediterranean basin ecosystems. Science 354, 465–468. https://doi.org/10.1126/science.aah5015.

IPCC, 2021. Summary for policymakers. Climate change: the physical science basis. In: Masson-Delmotte, V., Zhai, P., Pirani, A., Connors, S.L., Péan, C., Berger, S., Caud, N., Chen, Y., Goldfarb, L., Gomis, M.I., Huang, M., Leitzell, K., Lonnoy, E., Matthews, J.B.R., Maycock, T.K., Waterfield, T., Yelekçi, O., Yu, R., Zhou, B. (Eds.), Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press In Press.

Jiemeng, T., Meng, D., Qin, C., Liu, X., Liang, Y., Xiao, Y., Liu, Z., Gu, Y., Li, J., Yin, H., 2018. Integrated network analysis reveals the importance of microbial interactions for maize growth. Environ. Biotechnol. 102, 3805–3818. https://doi.org/10.1007/s00253-018-8837-4.

Jones, P., Garcia, B.J., Furches, A., Tuskan, G.A., Jacobson, D., 2019. Plant host-associated mechanisms for microbial selection. Front. Plant Sci. 10, 862. https://doi.org/10.3389/fpls.2019.00862.

Karimi, B., Maron, P.A., Chemidlin-Prevost Boure, N., Bernard, N., Gilbert, D., Ranjard, L., 2017. Microbial diversity and ecological networks as indicator of environmental quality. Environ. Chem. Lett. 15, 265–281. https://doi.org/10.1007/s10311-017-0614-6.

Khan, N., Bano, A.M.D., Babar, A., 2020. Impacts of plant growth promoters and plant growth regulators on rainfed agriculture. PLoS ONE 15, e0231426. https://doi.org/10.1371/journal.pone.0231426.

Kumar, A., Verma, J.P., 2018. Does plant-microbe interaction confer stress tolerance in plants: a review? Microbiol. Res. 207, 41–52. https://doi.org/10.1016/j.micres.2017.11.004.

Lacerda-Júnior, G.V., Noronha, M.F., Cabral, L., Delforno, T.P., de Sousa, S.T.P., Fernandez-Júnior, P.I., Melo, I.S., Oliveira, V.M., 2019. Land use and seasonal effects on the soil microbiome of a Brazilian dry forest. Front. Microbiol. 10, 648. https://doi.org/10.3389/fmicb.2019.00648.

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.

Lauber, C.L., Strickland, M.S., Bradford, M.S., Fierer, N., 2008. The influence of soil properties on the structure of bacterial and fungal communities across land-use types. Soil Biol. Biochem. 10, 2407–2415. https://doi.org/10.1016/j.soilbio.2008.05.021.

Lazcano, C., Boyd, E., Holmes, G., Hewavitharana, S., Pasulka, A., Ivors, K., 2021. The rhizosphere microbiome plays a role in the resistance to soil-borne pathogens and nutrient uptake of strawberry cultivars under field conditions. Sci. Rep. 11, 3188. https://doi.org/10.1038/s41598-021-82768-2.

Lin, H., Peddada, S.D., 2020. Analysis of compositions of microbiomes with bias correction. Nat. Commun. 11, 3514. https://doi.org/10.1038/s41467-020-17041-7.

Lipson, D.A., 2007. Relationships between temperature response and bacterial community structure along season and altitudinal gradients. FEMS Microb. Ecol. 49, 418–427. https://doi.org/10.1111/j.1574-6941.2006.00240.x.

Lladó, S., López-Mondéjar, R., Baldrian, P., 2017. Forest soil bacteria: diversity, involvement in ecosystem processes, and response to global change. Microbiol. Mol. Biol. Rev. 81, e00063-16. https://doi.org/10.1128/MMBR.00063-16.

Longley, R., Noel, Z.A., Benucci, G.M.N., Chilvers, M.I., Trail, F., Bonito, G., 2020. Crop management impacts the soybean (glycine max) microbiome. Front. Microbiol. 11, 1116. https://doi.org/10.3389/fmicb.2020.01116.

López-Hinojosa, M., de María, N., Guevara, M.A., Vélez, M.D., Cabezas, J.A., Díaz, L.M., Mancha, J.A., Pizarro, A., Manjarrez, L.F., Collada, C., Díaz-Sala, C., Cervera Goy, M.T., 2021. Rootstock effects on scion gene expression in maritime pine. Sci. Rep. 11, 11582. https://doi.org/10.1038/s41598-021-90672-y.

Louca, S., Parfrey, L.W., Doebeli, M., 2016. Decoupling function and taxonomy in the global ocean microbiome. Science 353, 1272–1277. https://doi.org/10.1126/science.aaf4507.

Lucas-García, J.A., Domenech, J., Santamaría, C., Camacho, M., Daza, A., Gutierrez Mañero, F.J., 2004. Growth of forest plants (pine and holm-oak) inoculated with rhizobacteria: relationship with microbial community structure and biological activity of its rhizosphere. Environ. Exp. Bot. 52, 239–251. https://doi.org/10.1016/j.envexpbot.2004.02.003.

Marasco, R., Rolli, E., Fusi, M., Michoud, G., Daffonchio, D., 2018. Grapevine rootstock shape underground bacterial microbiome and networking but not potential functionality. Microbiome 6, 3. https://doi.org/10.1186/s40168-017-0391-2.

McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217. https://doi.org/10.1371/journal.pone.0061217.

MENAP, .. .Available online: http://ieg4.rccc.ou.edu/mena/main.cgi Last accessed: 2 Jul 2021.

Oksanen, J., Guillaume Blanchet, F., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2020. vegan: Community Ecology Package. R package version 2.5-7. https://CRAN.R-project.org/package=vegan.

Olesen, J.M., Bascompte, J., Dupont, Y.L., Jordano, P., 2007. The modularity of pollination networks. Proc. Natl. Acad. Sci. U. S. A. 104, 19891–19896. https://doi.org/10.1073/pnas.0706375104.

Palahí, M., Mavsar, R., Gracia, C., Birot, Y., 2008. Mediterranean forests under focus. Int. For. Rev. 10, 676–688. https://doi.org/10.1505/ifor.10.4.676.

Pérez-Izquierdo, L., Zabal-Aguirre, M., Flores-Rentería, D., González-Martínez, S., Buée, M., Rincón, A., 2017. Functional outcomes of fungal community shifts driven by tree genotype and spatial-temporal factors in Mediterranean pine forests. Environ. Microbiol. 19, 1639–1652. https://doi.org/10.1111/1462-2920.13690.

Pérez-Izquierdo, L., Zabal-Aguirre, M., González-Martínez, S.C., Buée, M., Verdú, M., Rincón, A., Goberna, M., 2019. Plant intraspecific variation modulates nutrient cycling through its below-ground rhizospheric microbiome. J. Ecol. 107, 1594–1605. https://doi.org/10.1111/1365-2745.13202.

Plomion, C., Bartholomé, J., Lesur, I., Boury, C., Rodríguez-Quilón, I., Lagraulet, H., Ehrenmann, F., Bouffier, L., Gion, J.M., Grivet, D., de Miguel, M., de María, N., Cervera, M.T., Bagnoli, F., Isik, F., Vendramin, G.G., González-Martínez, S.C., 2016. High-density SNP assay development for genetic analysis in maritime pine (Pinus pinaster). Mol. Ecol. Resour. 16, 574–587. https://doi.org/10.1111/1755-0998.12464.

Prada, E., Climent, J., Alía, R., Díaz, R., 2016. Life-history correlations with seasonal cold hardiness in maritime pine. Am. J. Bot. 103, 2126–2135. https://doi.org/10.3732/ajb.1600286.

Prevost-Boure, N.C., Maron, P.A., Ranjard, L., Nowak, V., Dufrene, E., Damesin, C., Soudani, K., Lata, J.C., 2011. Seasonal dynamics of the bacterial community in forest soils under different quantities of leaf litter. Appl. Soil Ecol. 47, 14–23. https://doi.org/10.1016/j.apsoil.2010.11.006.

Proença, D.N., Francisco, R., Kublik, S., Schöler, A., Vestergaard, G., Schloter, M., Morais, P.V., 2017. The microbiome of endophytic, wood colonizing bacteria from pine trees as affected by pine wilt disease. Sci. Rep. 7, 4205. https://doi.org/10.1038/s41598-017-04141-6.

Pugnaire, F.I., Morillo, J.A., Peñuelas, J., Reich, P.B., Bargett, R.D., Gaxiola, A., Wardle, D.A., van der Putten, W.H., 2019. Climate change effects on plant-soil feedbacks and consequences for biodiversity and functioning of terrestrial ecosystems. ScienceAdvances 27, eaaz1834. https://doi.org/10.1126/sciadv.aaz1834.

R Core Team, 2021. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Regan, K.M., Nunan, N., Boeddinghaus, R.S., Baumgartner, V., Berner, D., Boch, S., Oelmann, Y., Overmann, J., Patri, D., Schloter, M., Schmitt, B., Sorkau, B., Steffens, M., Kandeler, E., Marhan, S., 2014. Seasonal controls on grassland microbial biogeography: are they governed by plants, abiotic properties or both? Soil Biol. Biochem. 71, 21–30. https://doi.org/10.1016/j.soilbio.2013.12.024.

Ruiz Palomino, M., Lucas García, J.A., Gutierrez Mañero, F.J., Probanza, A., 2005. Seasonal diversity changes in Alder (Alnus glutinosa) culturable rhizobacteria communities throught a phenological cycle. Appl. Soil Ecol. 29, 215–224. https://doi.org/10.1016/j.apsoil.2004.12.003.

Sánchez-Cañizares, C., Jorrín, B., Poole, P.S., Tkacz, A., 2017. Understanding the holobiont: the interdependence of plants and their microbiome. Curr. Opin. Microbiol. 38, 188–196. https://doi.org/10.1016/j.mib.2017.07.001.

Sánchez-Gómez, D., Majada, J., Alía, R., Feito, I., Aranda, I., 2010. Intraspecific variation in growth and allocation patterns in seedlings of Pinus pinaster Ait. submitted to contrasting watering regimes: can water availability explain regional variation? Ann. For. Sci. 67, 505. https://doi.org/10.1051/forest/2010007.

Sánchez-Salguero, R., Camarero, J.J., Rozas, V., Génova, M., Olano, J.M., Arzac, A., Gazol, A., Caminero, L., Tejedor, E., de Luis, M., Linares, J.C., 2018. Resist, recover or both? Growth plasticity in response to drought is geographically structured and linked to intraspecific variability in Pinus pinaster. J. Biogeogr. 45, 1126–1139. https://doi.org/10.1111/jbi.13202.

Sardans, J., Peñuelas, J., 2013. Plant-soil interactions in Mediterranean forests and shrublands: impacts of climate change. Plant Soil 365, 1–33. https://doi.org/10.1007/s11104-013-1591-6.

Schweitzer, J.A., Van Nuland, M., Bailey, J.K., 2018. Intraspecific plant-soil feedbacks link ecosystem ecology and evolutionary biology. In: Ohgushi, T., Wurst, S., Johnson, S. (Eds.), Aboveground-Belowground Community Ecology. Ecological Studies (Analysis and Synthesis). Springer, Cham, pp. 69–84.

Shang, J.Y., Wu, Y., Huo, B., Chen, L., Wang, E.T., Sui, Y., Chen, W.F., Tian, C.F., Chen, W.X., Sui, X.H., 2021. Potential of bradyrhizobia inoculation to promote peanut growth and beneficial rhizobacteria abundance. J. Appl. Microbiol. 131, 2500–2515. https://doi.org/10.1111/jam.15128.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., Ideker, T., 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504. https://doi.org/10.1101/gr.1239303.

Shigyo, N., Umeki, K., Hirao, T., 2019. Seasonal dynamics of soil fungal and bacterial communities in cool-temperate montane forests. Front. Microbiol. 10, 1944. https://doi.org/10.3389/fmicb.2019.01944.

Siles, J.A., Margesin, R., 2017. Seasonal soil microbial responses are limited to changes in microbial functionality at two Alpine forest sites differing in altitude and vegetation. Sci. Rep. 7, 2204. https://doi.org/10.1038/s41598-017-02363-2.

Sohng, J., Han, A.R., Jeong, M.A., Park, Y., Park, B.B., Park, P.S., 2014. Seasonal pattern of decomposition and N, P, and C dynamics in leaf litter in a mongolian oak forest and a Korean pine plantation. Forests 4, 2561–2580. https://doi.org/10.3390/f5102561.

Song, H.S., Lee, J.Y., Haruta, S., Nelson, W.C., Lee, D.Y., Lindermann, S.R., Frederickson, J.K., Bernstein, H.C., 2019. Minimal interspecies interaction adjustment (MIIA): inference of neighbor-dependent interactions in microbial communities. Front. Microbiol. 10, 1264. https://doi.org/10.3389/fmicb.2019.01264.

Sterck, L., de María, N., Cañas, R.A., de Miguel, M., Perdiguero, P., Raffin, A., Budde, K.B., López-Hinojosa, M., Cantón, F.R., Rodrigues, A.S., Morcillo, M., Hurel, A., Vélez, M.D., de la Torre, F.N., Modesto, I., Manjarrez, L.F., Pascual, M.B., Alves, A., Mendoza-Poudereux, I., Callejas-Díaz, M., Pizarro, A., El-Azaz, J., Hernández-Escribano, L., Guevara, M.A., Majada, J., Salse, J., Grivet, D., Bouffier, L., Raposo, R., de la Torre, A., Zas, R., Cabezas, J.A., Ávila, C., Trontin, J.F., Sánchez, L., Alía, R., Arrillaga, I., González-Martínez, S.C., Miguel, C., Cánovas, F.M., Plomion, C., Díaz-Sala, C., Cervera, M.T., 2021. Chapter 5: maritime pine genomics in focus. In: de la Torre (Ed.), The Pine Genomes. Springer Nature In press.

Takahashi, S., Tomita, J., Nishioka, K., Hisada, T., Nishijima, M., 2014. Development of a prokaryotic universal primer for simultaneous analysis of bacteria and archaea using next-generation sequencing. PLoS One 9, 8. https://doi.org/10.1371/journal.pone.0105592.

Tan, B., Wu, F., Yang, W., He, X., 2014. Snow removal alters soil microbial biomass and enzyme activity in a Tibetan alpine forest. Appl. Soil Ecol. 76, 34–41. https://doi.org/10.1016/j.apsoil.2013.11.015.

Terhonen, E., Blumenstein, K., Kovalchuk, A., Asiegbu, F.O., 2019. Forest tree microbiomes and associated fugal endophytes: functional roles and impact on forest health. Forests 10, 42. https://doi.org/10.3390/f10010042.

Uroz, S., Buée, M., Deveau, A., Mieszkin, S., Martin, F., 2016. Ecology of the forest microbiome: highlights of temperate and boreal ecosystems. Soil Biol. Biochem. 103, 471–488. https://doi.org/10.1016/j.soilbio.2016.09.006.

Vendramin, G.G., Lelli, L., Rossi, P., Morgante, M., 1996. A set of primers for the amplification of 20 chloroplast microsatellites in Pinaceae. Mol. Ecol. 5, 111–114. https://doi.org/10.1111/j.1365-294x.1996.tb00353.x.

Villadas, P.J., Lasa, A., Martínez-Hidalgo, P., Flores-Félix, J.D., Martínez-Molina, E., Toro, N., Velázquez, E., Fernández-López, M., 2017. Analysis of rhizobial endosymbionts of Vicia, Lathyrus and Trifolium species used to maintain mountain firewalls in Sierra Nevada National Park (South Spain). Syst. Appl. Microbiol. 40, 92–101. https://doi.org/10.1016/j.syapm.2016.11.008.

Wang, M., Shi, S., Lin, F., Jiang, P., 2014. Response of the soil fungal community to multifactor environmental changes in a temperate forest. Appl. Soil Ecol. 81, 45–56. https://doi.org/10.1016/j.apsoil.2014.04.008.

Wang, H.H., Chu, H.L., Dou, Q., Xie, Q.D., Tang, M., Sung, C.K., Wang, C.Y., 2018. Phosphorus and nitrogen drive the seasonal dynamics of bacterial communities in Pinus forest rhizospheric soil of the Qinling Mountains. Front. Microbiol. 9, 1930. https://doi.org/10.3389/fmicb.2018.01930.

Williams, A., de Vries, F.T., 2019. Plant root exudation under drought: implications for ecosystem functioning. New Phytol. 225, 1899–1905. https://doi.org/10.1111/nph.16223.

Zhalnina, K., Louie, K.B., Hao, Z., Mansoori, N., Nunes da Rocha, U., Shi, S., Cho, H., Karaoz, U., Loqué, D., Bowen, B.P., Firestone, M.K., Northen, T.R., Brodie, E.L., 2018. Dynamic root exudate chemistry and microbial substrate preferences drive patterns in rhizosphere microbial community assembly. Nat. Microbiol. 4, 470–480. https://doi.org/10.1038/s41564-018-0129-3.

Žifčáková, L., Větrovský, T., Howe, A., Baldrian, P., 2016. Microbial activity in forest soil reflects the changes in ecosystem properties between summer and winter. Environ. Microbiol. 18, 288–301. https://doi.org/10.1111/1462-2920.13026.