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BRIEF REPORT

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Bacterial inoculation of *Quercus pyrenaica* trees alters co-occurrence patterns but not the composition of the rhizosphere bacteriome in wild conditions

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Abstract

Quercus pyrenaica is a woody species of high landscape value, however, its forests show an advanced state of degradation in the Iberian Peninsula. Afforestation typically has low success, thus, it is necessary to improve the fitness of oaks plantlets to be transplanted, for instance, by inoculating beneficial microorganisms. In adding microorganisms to ecosystems, there must be balanced efficacy with potential effects on native microbial communities. We addressed changes in diversity, richness, composition and co-occurrence networks of prokaryotic communities in the rhizosphere of inoculated and control trees outplanted to three different sites located in the Sierra Nevada National and Natural Park (Spain). After 18 months in wild conditions, we did not detect changes due to the inoculation in the richness, diversity and structure in none of the sites. However, we observed an increase in the complexity of the co-occurrence networks in two experimental areas. Modularization of the networks changed as a result of the inoculation, although the sense of the change depended on the site. Although it was impossible to unravel the effect of bacterial inoculation, our results highlighted that inoculation alters the association of rhizosphere bacteria without entailing other changes, so networks should be analysed prior to inoculating the plantlets.

INTRODUCTION

Quercus pyrenaica Willd., commonly known as melojo oak, is a key tree species of Mediterranean forests, such as the Sierra Nevada National and Natural Park (Southeast Spain), the most southern mountainous area in Europe where this species can be found (Nieto Quintano et al., 2016). The ecosystem services derived from *Q. pyrenaica* are numerous and valuable: among others, melojo oaks are superb soil builders and protectors (Pérez-Luque et al., 2021) and its forests act as excellent biodiversity reservoirs (Lasa, Mašinová, et al., 2019; Nieto Quintano et al., 2016; Pérez-Luque et al., 2021). Despite its high landscape value, melojo oak forests have faced a dramatic reduction in their distribution area and show a worrying state of deterioration nowadays (Camacho-Olmedo et al., 2002; Pérez-Luque et al., 2021). Two of the most commonly proposed strategies to boost the expansion and vigour of *Q. pyrenaica* formations are the afforestation of mountainous areas and the naturalization of stands that formerly were melojo oak forests (Aspizua et al., 2012; Bonet et al., 2015). To overcome the frequent high mortality rate of the introduced plantlets (Gómez-Aparicio et al., 2004), afforestation tasks should be performed with vigorous plantlets resilient to harsh forest

conditions (Karličic et al., 2016). Some studies have already demonstrated the beneficial effects of Plant Growth Promoting Microorganisms (PGPM) inoculation on woody plants' vigour (Anand et al., 2013; Karličić et al., 2015; Lucas-García et al., 2004; Mafia et al., 2009; Puente et al., 2010). In particular, bacterial inoculation of plantlets at the nursery stage followed by outplanting in the field is a promising strategy to improve the efficiency of afforestation tasks (Chanway, 1997; Gómez-Lama Cabanás et al., 2018).

Notwithstanding the above, very little is known about the ecological impact of bacterial inoculations in wild conditions. Formulations based on microorganisms (bioformulations) set aside for agricultural or forestry purposes still have major bottlenecks that should be overcome (Kaminsky et al., 2019). On the one hand, bacteria included in the bioformulations might be excluded by the plant host microbiota through competitive mechanisms (Maghnia et al., 2019). On the other hand, invasion phenomena mediated by the introduced bacteria may occur, which could trigger the displacement of some native taxa (Mallon et al., 2015). On the contrary, other authors have demonstrated that plant inoculation can entail an increase in the diversity of the resident communities (Ciccillo et al., 2002). These inoculation-associated changes are the result of the complex networks of interactions (e.g., positive, negative, or neutral) that take place between microorganisms (Faust & Raes, 2012; Karimi et al., 2017). As a result, they can recruit or exclude other microorganisms (for instance, PGPM or plant pathogens), having a great regulatory effect on the community assembly (Toju et al., 2018). If a plant acts on a microbe that interacts with many network members, it can transmit the information to the whole microbial network (Agler et al., 2016). Thus, these interactions can have direct consequences on host health but also on soil fertility and the surrounding environment (van der Heijden & Hartmann, 2016).

Beyond the functional screenings for PGPM and classical microbial diversity analyses, it is also important to unravel whether the PGPM included in the bioformulations establish interactions with other microorganisms of the studied ecosystem, employing co-occurrence network analyses (Barberán et al., 2012; Faust & Raes, 2012). Although this approach is still in its infancy and suggested hypotheses should be validated experimentally, it offers the opportunity to gain more insights into the taxa that are most likely to mediate interactions within microbial populations and to predict the efficiency of the microbiome-based afforestation tasks (Toju et al., 2018 and references therein).

The main aim of this work was to assess the long-term downstream impact of the inoculation of melojo oak plantlets with a bacterial consortium composed of two strains previously isolated from the National Park of Sierra Nevada on native rhizosphere bacterial communities. For that purpose, we followed a holistic strategy based on deep sequencing which comprised the study of the diversity, structure, taxonomic profiles and co-occurrence networks of inoculated plantlets in comparison with controls (not inoculated), after 18 months in wild conditions. We hypothesize that there are no long-term differences between the inoculated and control plants in terms of diversity, taxonomical profiles and association of microbial communities, as the equilibrium of the ecosystem under study is re-established over time.

EXPERIMENTAL PROCEDURES

Bacterial inoculation, field studies and sample collection

In November 2012, approximately 4000 *Q. pyrenaica* acorns were collected manually in melojo oak forests located on the slopes of the municipal district of Cáñar, National Park of Sierra Nevada (Granada, Spain). Within 48 h, acorns were sowed in pots containing non-sterile peat (one acorn per pot) and grown in the commercial nursery Paisajes del Sur (Colomera, Granada, Spain) under environmental conditions for 6 months. Thus, in May 2013, when plantlets were actively growing, they were inoculated with a bacterial consortium composed of two strains previously isolated from the Sierra Nevada National and Natural Park. On the one hand, *Bradyrhizobium canariense* GV101, which was one of the most abundant genera in the oak rhizosphere and in the soil of one of the sites studied in this work (located in the municipality of Cáñar, Cobo-Díaz et al., 2014; Cobo-Díaz et al., 2017; see Table S1). Furthermore, previous studies have suggested the genus *Bradyrhizobium* is one of the most metabolically active in the rhizosphere of melojo oaks (Lasa, Fernández-González, et al., 2019). On the other hand, we were interested in the genus *Arthrobacter* because of its high abundance in the rhizosphere of holm-oak trees after a wildfire that took place in 2005 in the National Park of Sierra Nevada. In particular, the strain *Arthrobacter globiformis* AFG20 was selected due to its plant growth-promoting potential (Fernández-González et al., 2017). This strain can solubilize inorganic phosphate, and produce organic matter degrading enzymes, cellulose and the phytohormone indole acetic acid (IAA), all of them related to plant growth promotion.

These two strains had been never employed in any previous inoculation experiments on plants belonging to the genus *Quercus*.

Bradyrhizobium and *Arthrobacter* strains were grown on Yeast Extract Mannitol (YEM) and HH' (Fernández-González et al., 2017) liquid media, respectively, at 28°C on a rotatory shaker (170 rpm)

during 24–72 h. 100 μL of a bacterial suspension of each strain (10^9 cfu/mL) in sterile NaCl solution (0.9% v/v) were inoculated on the root collar of each plantlet. Plantlets not treated with the consortium (negative controls) were inoculated with 100 μL of sterile NaCl solution (0.9%, v/v) in the same way. Inoculation was done without calculation of the root surface since it is quite risky for the seedling survival because the seedlings were 6 months old at the time of the first inoculation. However, the amount of bacterial inoculum was significant since 10^8 cfu were applied to each tree directly below the stem crown over the beginning of the radicular system; thus bacteria can spread on tree roots. The experimental design included 800 plantlets in each condition. All the plantlets (1600) were kept in the commercial nursery for six additional months watering them regularly with tap water. In November 2013, plants were reinoculated as described above to minimize the possibility of the inoculum being lost. That is to say, a first inoculum in springtime with plantlet active growth and a second one in autumntime before the period of plant dormancy. One month later (just when plants began to lose the leaves) they were outplanted to three areas located in the Sierra Nevada National and Natural Park. Two places were selected in the municipality of Cáñar: AZC (Afforested Zone in Cáñar), characterized by a densely padded brushwood of the legume *Genista versicolor*; and NPF (Naturalized Pine Forest), which was covered by a Scot pine forest (*Pinus sylvestris*) developed in the context of an afforestation program in the 1950s, and thinned out during the spring of 2013 (Lasa, Mašinová, et al., 2019). On the other hand, a third mountainous site was chosen in the nearby valley of Lanjarón River (AZL, Afforested Zone in Lanjarón), which was affected by a wildfire in September 2005 and where plenty of leguminous plants and some resprouted holm-oak trees (*Q. ilex* subsp. *ballota*) were found. The main characteristics, altitude and coordinates of each study area are detailed in Table S1.

Within each site, three sampling plots (15 × 15 m) were delimited along a 1 km length and outplanting practices were performed there. Ten rows were marked in each plot: control trees and 10 melojo oaks (one-year-old) per row were outplanted, alternating bacterially inoculated (1–1.5 m apart). A total of 900 trees (450 corresponding to each treatment) were outplanted altogether. The experimental design is summarized in Figure S1. It should be pointed out that the authorities of the Sierra Nevada National and Natural Park were actively involved in the afforestation works.

To assess the long-term effect of bacterial inoculation, 18 months after the outplanting practices (May 2015) eight randomly selected control and inoculated trees were uprooted at each study site to obtain rhizosphere soil (48 trees in total, see Figure S1). Oak roots were manually rubbed until 2 g of rhizosphere soil were obtained, which were mixed with 5 mL of LifeGuard™ Soil Preservation Solution (MoBio Laboratories Inc.) to maintain microbial community profiles and immediately stored at 4°C until DNA extraction.

A total of 200 g of the soil near the roots (per tree) was collected to determine the edaphic properties. Soil physicochemical analyses were performed at the Agrifood laboratory of the Andalusian Regional Government under the standardized protocols developed by this service.

DNA extraction and Illumina sequencing

Soil DNA was extracted from each sample within 24 h of sample collection using the PowerSoil™ DNA Isolation Kit (MoBio, USA), according to the manufacturer's recommendations. DNA quality and yield were checked using the fluorometer Qubit® 3.0 and the Qubit® dsDNA High Sensitivity Assay Kit (Life Technologies, USA). The hypervariable regions V4-V5 of the prokaryotic 16S rRNA gene were sequenced using U519F and U926R primers described by Suzuki and Giovannoni (1996) and Baker et al. (2003), respectively. Library preparation and sequencing were carried out by Macrogen Incorporated (Korea). For that purpose, a 2 × 300 bp strategy was followed and the Illumina MiSeq platform was used.

Deep-sequencing data processing

The quality of sequencing reads was checked with the bioinformatic tool FastQC v.0.11.5 (Andrews, 2010) and they were end-trimmed by using the software FASTX-Toolkit v.0.0.14 (FASTX-Tookit, 2009), specifically, running the script *fastx_trimmer*. All low-quality sequences were removed, guaranteeing values of Q score (Q) higher than 19. The script *fastq-join* (Aronesty, 2011) was then employed to overlap paired-end reads. For that purpose, an overlapping size of 40 bp was selected, allowing a maximum of 15% of mismatches in the overlapping region. An additional step of trimming was performed using the software SEED2 (Větrovský et al., 2018) in which overlapped sequences with an average quality value lower than Q30 were discarded. The trimming process also included the removal of specific primers. In addition, those sequences shorter than 362 bp, with ambiguities, or with at least one nucleotide with a quality lower than Q10 were eliminated. Next, the software Mothur v.1.40.5 (Schloss et al., 2009) was employed to remove chimeric sequences using SILVA gold fasta as a reference (Quast et al., 2013). Quality sequences were subsequently clustered into Operational Taxonomic Units (OTUs) at 3% of genetic distance utilizing the algorithm UPARSE included in the USEARCH V. 8.1.161 tool

(Edgar, 2013). As recommended by Bokulich et al. (2013), those OTUs that accounted for less than 0.005% of the total sequences were removed from the analysis. Finally, OTUs were taxonomically classified using the Ribosomal Database Project (RDP) 16S rRNA reference database, specifically the training set v.16 previously formatted with Mothur (Cole et al., 2014). All the sequences that were classified as chloroplasts, mitochondria or that could not be identified at the kingdom level were discarded from the dataset.

Analysis of the diversity and structure of prokaryotic communities

All the ecological analyses were performed using different packages and functions developed in the statistical tool R v.4.1.2 (R Development Team, 2016). Rarefaction curves were obtained employing the function *rarecurve* included in the package vegan (Oksanen et al., 2016). A rarefaction step was carried out to the minimum library size with the function *rarefy_even_depth* of package phyloseq (McMurdie & Holmes, 2013) to avoid biases associated with different sample sizes. Chao-1, Shannon (H′), Simpson's inverse (1/D) and Pielou (J') alpha diversity indices were then computed (function *estimate_richness*, package phyloseq). Good's coverage was also calculated. All these estimations were calculated at the OTU level.

All the sequences of the dataset (non-rarefied) were taken into account for all the analyses made hereafter according to McMurdie and Holmes (2014). Sequences were aligned by using the multiple alignment tool MAFFT V.7040 (Katoh & Standley, 2013) and a phylogenetic tree was constructed using the software FastTree v.2.1.3 (Price et al., 2010). In terms of beta diversity, a Constrained Analysis of Principal Coordinates (CAP) was performed to elucidate the edaphic parameters governing the structure of prokaryotic communities. The functions *capscale* and *ordistep* (both from the package vegan) were used for model building. Then, the statistical significance of the edaphic parameters included in the model was checked through the function *envfit* (vegan package), correcting the *p*-values with the adjustment of Bonferroni. A non-parametric permutational multivariate analysis of variance (PERMANOVA) was conducted with 9999 permutations using the function *adonis2*, included in the package vegan. PERMANOVA was performed based on Weighted UniFrac distances, which in turn were calculated from the previously computed phylogenetic tree. The interaction between the main factors [Treatment (two levels, Inoculated and Control) and Site (three levels, NPF, AZC, AZL); model: *response* ~ *Treatment*Site*] was tested. Where applicable, differences between pairs of groups of samples were calculated using the function *pairwise. Adonis* implemented in the package pairwiseAdonis (Martinez-Arbizu, 2017). In this case, *p*-values were corrected by the Benjamini-Hochberg method for multiple comparisons.

Differential abundance analysis of rhizosphere communities

To determine which bacterial taxa were differentially abundant between treatments or experimental areas, the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) algorithm was implemented (Lin & Peddada, 2020). For that purpose, the function *ancombc* of the package ANCOMBC was used. The *p*-values associated with each comparison were adjusted by using Holm's correction. The main factors (Treatment and Site) were analysed independently in the model.

In addition, potential differences in the abundance of genera *Bradyrhizobium* and *Arthrobacter* between control and inoculated trees were specifically examined.

Co-occurrence network construction and analysis

Co-occurrence networks were constructed as described by Fernández-González et al. (2020). For that purpose, networks were built independently for each study site (NPF, AZL and AZC) and each condition (inoculated and control trees), all of them at the OTU level. The six networks were constructed by using the Molecular Ecological Network Analysis Pipeline (MENAP) online server (http://ieg4.rccc.ou.edu/mena/main.cgi) following the developer's suggestions (Deng et al., 2012; Tao et al., 2018; Zhou et al., 2010; Zhou et al., 2011). In brief, settings were adjusted to a prevalence cut-off of 50%, the Pearson coefficient was selected for correlation calculation (as recommended by the developers for these kinds of data), and modules were separated by the greedy modularity optimization method. Indirect relationships were removed from the networks employing the iDIRECT framework (Inference of Direct and Indirect Relationships with Effective Copula-based Transitivity) (Xiao et al., 2022). In addition, 100 random networks were constructed for each empirical network, with the same characteristics (number of nodes and links). After the randomization, the standard deviations of the network's global properties were used in Student's *t*-test to compare the average cluster coefficient (avgCC), the Geodesic Distance (GD) and the Modularity (M) values of empirical networks between treatments. Finally, the six co-occurrence networks were plotted using the software

Cytoscape v.3.7.1 (Shannon et al., 2003) and Z_i-P_i plots were obtained as well. Topological roles of each node OTU were assigned based on within-module (Z_i) and among-module connectivity (P_i): OTUs with an associated $Z_i \leq 2.5$ and $P_i > 0.62$ were considered *connector* OTUs, *module hubs* were defined as those with a high value of Z_i (>2.5) and low P_i (≤0.62), while *network hubs* have high values of both parameters ($Z_i > 2.5$ and $P_i > 0.62$) and peripherals values of $Z_i \leq 2.5$ and $P_i \leq 0.62$ (Olesen et al., 2007).

Univariant statistical analyses

All the statistical tests needed to compare the physicochemical parameters of the soils and alpha diversity indices among groups of samples were assessed in R software. The normal distribution and homoscedasticity of each variable were checked with Shapiro–Wilk's and Levene's tests, by using *shapiro.test* (base R) and *leveneTest* functions (included in package car; Fox & Weisberg, 2011), respectively. When data met the assumptions of normality and equality of variances, parametric approaches were applied to compare groups of samples. Thus, a two-factor design was followed in both cases to test the effect of the two main factors [experimental site (three levels: AZC, AZL, NPF) and treatment (two levels: inoculated and control trees)] and their interaction on each alpha diversity index or edaphic property. For that purpose, a two-way ANOVA was computed employing the function *anova_test* from the package rstatix (Kassambara, 2021). Thus, the statistical model tested was: *response* ~ *Site*Treatment*. *pairwise_t_test* (package rstatix) was applied as a post-hoc test when applicable. The experimental design was defined as balanced in all cases and confidence levels >95% ($\alpha = 0.05$) were taken into account for all the statistical tests.

RESULTS

Soil physicochemical parameters

All analysed soils were sandy-loam soils with acidic pH (Table S2). There was a significant effect of the interaction of the experimental area and the inoculation of the trees solely on the content of available water (two-way ANOVA, *p*-value <0.002), having the soil of control trees higher percentage of available water than that of the inoculated, in AZL (Table S2). In the case of slime, pH, the content of soil organic matter, nitrogen, phosphorus and potassium, C:N ratio, and salinity, no significant effect of the interaction of experimental area and inoculation was found, but just of the experimental area under study (Table S2).

General properties of sequencing data and alpha diversity indices

A total of 4,926,626 raw reads were obtained from the Illumina sequencing platform, which resulted in 1,209,559 high-quality sequences after the trimming and filtering steps. A total of 2474 different OTUs were compiled as a result of the bioinformatics processing of the sequences. As shown in Figure S2, most of the rarefaction curves tended to the asymptote when considering all the sequences of the dataset (non-rarefied) and individual Good's coverage values ranged from 97.6% to 99.5% (Table 1). Thus, the samples collected were considered fairly representative of the original ecosystems under study.

Data were rarefied to diminish the effect of different sample sizes over alpha diversity indices, selecting a total of 13,427 sequences randomly per sample. The site, but neither the inoculation nor the interaction of the two factors showed a significant effect on the number of observed OTUs, and the indices Chao-1, Shannon, and Inverse of Simpson (two-way ANOVA, *p*-values <0.40; Table 1 and Table S3). Thus, the rhizosphere prokaryotic communities inhabiting control trees were as rich and diverse as those inoculated with the consortium. It should be marked that high values of Shannon's index were recorded in both inoculated and non-inoculated trees, and the proximity of Pielou's index to 1.0 revealed the high evenness of all populations (Table 1). Although prokaryotic communities differed in terms of Shannon's diversity among sites, those differences were practically negligible (Table 1). With respect to the richness, NPF was the site with the statistically lowest number of observed and estimated OTUs (Table 1, Table S3).

Influence of soil physicochemical parameters on rhizosphere prokaryotic communities

For the analysis of the influence of edaphic properties on the structure of rhizosphere prokaryotic communities, just those parameters that resulted independently of each other were included in the model of CAP. They were the C:N ratio, pH, soil salinity and the content of assimilable K (Table S4). The statistical analysis of CAP distribution revealed that all four variables had a significant influence on the structure of prokaryotic communities, but the former two had a stronger impact (*envfit*, $R^2 > 0.66$). As depicted in Figure S3, rhizosphere communities of the trees located at NPF (corresponding to those soil samples with the significantly highest content of assimilable K and ratio C:N and the most acidic soils) clustered together in the CAP axis 1 and slightly separated from AZL and AZR (higher pH and with lower contents of assimilable K and ratio CN).

TABLE 1 Quality parameters and alpha diversity indices of prokaryotic communities of the rhizosphere of *Quercus pyrenaica* trees.

Site	Treatment	Raw sequences	Quality sequences	Good's coverage (%)	Observed OTUs	Chao-1	Shannon (H')	Inv. Simpson (1/D)	Pielou (J')
NPF	I	631,803	165,881 (20375.13)	98.49 ± 0.62	1283.88 ± 118.29	1430.59 ± 126.31	6.24 ± 0.11	205.25 ± 27.22	0.87 ± 0.01
	NI	716,373	181,793 (22724.13)	98.67 ± 0.66	1242.63 ± 61.44	1393.46 ± 89.43	6.22 ± 0.08	216.15 ± 34.41	0.87 ± 0.01
AZC	I	841,352	203,465 (25433.13)	98.64 ± 0.67	1569.50 ± 110.02	1788.98 ± 106.28	6.46 ± 0.20	256.45 ± 97.64	0.88 ± 0.02
	NI	879,918	223,169 (27896.13)	98.91 ± 0.46	1558 ± 186.40	1756.9 ± 183.52	6.50 ± 0.23	287.43 ± 82.31	0.89 ± 0.02
AZL	I	1,000,620	236,465 (29558.13)	99.18 ± 0.24	1521.75 ± 240.79	1718.87 ± 267.10	6.43 ± 0.25	255.76 ± 88.33	0.88 ± 0.02
	NI	856,560	198,786 (24848.25)	99.60 ± 0.61	1370.88 ± 146.61	1557.04 ± 171.68	6.26 ± 0.19	197.01 ± 42.87	0.87 ± 0.02

Note: In the case of the raw and quality sequences, the sum of the total sequences recorded in each condition is shown. Data in brackets represent the mean number of quality sequences obtained. In the remaining cases, average values of all replicates ± standard deviation are indicated. All the alpha diversity indices were calculated at 3% of genetic distance (OTU level) based on 13,427 high-quality sequences (rarefied data). For results of two-way ANOVA, see Table S3.
Abbreviations: I, Inoculated trees; Inv., Inverse; NI; non-inoculated trees.

Analysing the effect of bacterial inoculation on the structure and taxonomic profiles of rhizosphere prokaryotic communities

At this point of the analyses, all high-quality sequences (not rarefied) were taken into account. As depicted in Figure 1 and Figure S4, samples corresponding to inoculated and control trees of each study site clustered together along the first two coordinates of the PCoA plot. Although the first two dimensions of the PCoA explained 46.2% of the total variance, the distribution pattern of the samples observed in the PCoA was supported by the PERMANOVA test. No significant effect of the interaction of the two studied factors (site and inoculation) on the structure of the prokaryotic communities was found; that is, no significant differences between prokaryotic communities of treated and control trees were observed, regardless of the site (Table S5). Just the site as an individual factor resulted in statistically significant PERMANOVA, p-value $= 10^{-4}$. Thus, the prokaryotic communities inhabiting the rhizosphere of the oaks located in NPF, AZC, and AZL were different from each other, and these differences were captured subtly along a gradient on the first PCoA axis (Figure 1). The differences in the distribution of the samples in the ordination plot was mainly due to the experimental site from which they were collected (PERMANOVA, $R^2_{\text{Site}} = 0.30$).

Taxonomic profiles of prokaryotic communities inhabiting the rhizosphere of the trees outplanted in the field were represented by 23 taxonomically classified *phyla*, among which *Thaumarchaeota*, *Euryarchaeota* and *Pacearchaeota* corresponded to the kingdom Archaea (less than 0.41% of the sequences for all studies areas and conditions). All prokaryotic communities were dominated by bacterial *phyla Proteobacteria*, *Bacteroidetes*, *Acidobacteria, Verrucomicrobia*, *Planctomycetes* and *Actinobacteria* (from 82.6% to 86.4% of the sequences, Figure 2).

No significant differences were observed in the abundance of any of the *phyla* between treatments, in none of the studied areas (ANCOM-BC, p-values >0.35). By contrast, the studied sites were significant albeit subtly different from each other in terms of taxonomical profiles. Among the main *phyla* (>1% sequences), *Gemmatimonadetes* were significantly more abundant in AZL than in NPF, both in the case of inoculated and control trees. NPF was slightly enriched in *Proteobacteria* and *Actinobacteria* compared to non-inoculated and treated samples of AZL, respectively (Figure 2; Table S6).

Going into lower taxonomic ranks, we detected a total of 162 known prokaryotic genera. It should be noted that a great proportion of the sequences could not be identified with any classified genus included in the database used for the analysis (Table S7).

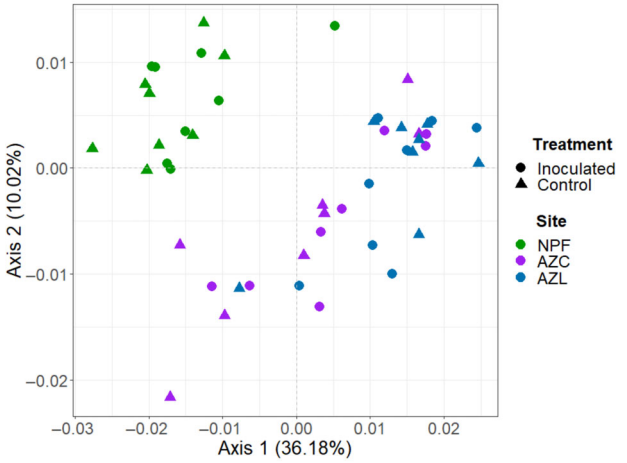

FIGURE 1 Principal coordinate analysis (PCoA) based on Weighted UniFrac distances of prokaryotic communities inhabiting the rhizosphere of *Quercus pyrenaica* inoculated and non-inoculated trees.

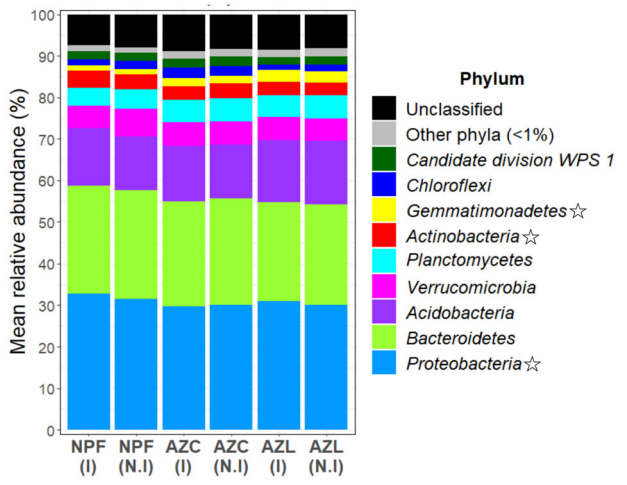

FIGURE 2 Mean relative abundance of the main bacterial phyla dwelling in the rhizosphere of *Q. pyrenaica* trees. I, Inoculated trees; N.I, non-inoculated (control) trees. The artificial group 'Other phyla (<1%)' represents the sum of all phyla accounting for less than 1% of the total quality sequences. Stars indicate the existence of significant differences in the abundance of the corresponding phyla among the sites under study, according to the results of the ANCOM-BC test.

Notwithstanding, acidobacterial *Gp6* was the predominant classified genus in every study area independently of the considered treatment, accounting for more than 4% of the total sequences in all cases. As shown in Table S7, this genus was followed by *Bradyrhizobium*, *Terrimonas*, *Gemmatimonas*, the acidobacterial *Gp4*, *Opitutus, Ferruginibacter* and *Ohtaekwangia*, which represented on average more than 1% of total sequences in all of the considered experimental sites. Among these genera, just *Bradyrhizobium* and *Gemmatimonas* showed significant differences among sites (Table S7). Not a single classified genus showed differences between control and inoculated trees of AZC,

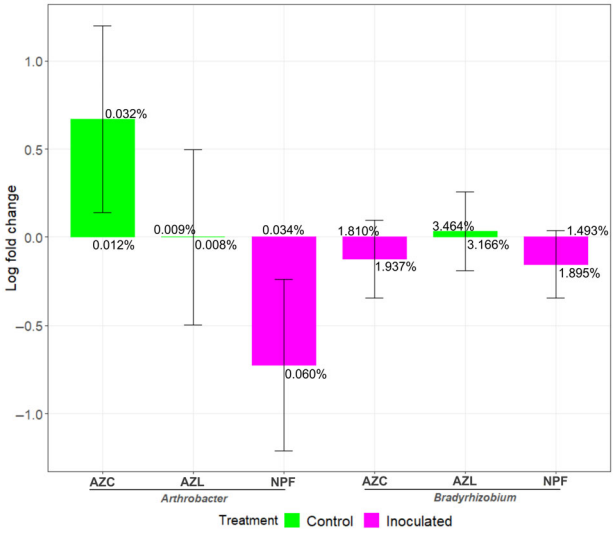

FIGURE 3 Distribution of the genera *Arthrobacter* and *Bradyrhizobium* in the rhizosphere of *Q. pyrenaica* inoculated and non-inoculated (control) trees. The log-fold change obtained by the ANCOM-BC test is represented. Bars in green indicate the cases in which the corresponding genus was more abundant in non-inoculated (control) than inoculated trees, whereas bars in pink show those genera that were more abundant in inoculated trees than in controls. Values above and below the bars represent the relative abundance of each genus in each site in control and inoculated trees, respectively.

and just three and five classified genera were differentially abundant in the rhizosphere of inoculated and control trees in AZL and NPF, respectively. Inoculation affected the distribution of bacterial genera to a very small extent, triggering an increase in the abundance of *Arenimonas* and *Solirubrobacter* in NPF (Table S7). Indeed, non-inoculated rhizosphere soils of AZL and NPF were depleted in two and three genera, respectively, which were present in the corresponding inoculated samples, although at low levels (Table S7).

We assessed whether the consortium had established at the plant roots after the inoculation. Regarding the presence of those genera to which inoculated strains belonged, *Bradyrhizobium* accounted for more than 1.4% of the sequences in every studied area. Although its relative abundance depended on the experimental site, this genus was homogeneously distributed in the rhizosphere of inoculated and control trees (ANCOM-BC, p-values = 1; Table S7; Figure 3). On the other hand, sequences associated with genus *Arthrobacter* were almost unrecoverable: no more than 0.06% of the total sequences on average corresponded to this genus in any of the mountainous areas. Although *Arthrobacter* was more abundant in inoculated than in control trees in NPF (Figure 3), these differences did not result statistically significant (ANCOM-BC, p-values = 1). Indeed, the dispersion of the abundance of genus *Arthrobacter* was noticeable.

However, the number of genera that showed significant differences in their abundance between

experimental sites, depended on the considered area and treatment, and it was pretty broad (from 4 to 56 genera differentially abundant were found when compared control trees of AZL with NPF, and AZL with AZC, respectively). It should be mentioned that discrete differences were observed in most of the cases. For example, the highest and lowest ANCOM-BC log-fold change values were recorded for the genus *Candidatus Hydrogenedens* when compared to non-inoculated NPF and AZC, and control trees in AZL and NPF, respectively, and the relative abundance in these samples was lower than 0.35% (Table S7).

Deciphering the changes of co-occurrence patterns between prokaryotic communities of inoculated and non-inoculated trees

Tree inoculation triggered changes in rhizosphere prokaryotic communities, as demonstrated by the association network analyses. In the case of the AZC site, networks related to inoculated trees were comprised of more modules less connected to each other than those corresponding to control trees (Figures S5 and S6). The opposite trend was appreciated for AZL: the network of bacterially treated trees was composed of several modules with more intramodular links than that of the control trees (Figures S7 and S8). Indeed, the value of Centralization of stress centrality (Cs) was significantly higher for the inoculated trees than for controls. No significant differences were measured in terms of intramodular connections in NPF (Figures S9 and S10). Significant changes were also found in other topological properties between the networks of inoculated and non-inoculated trees in all the experimental areas. The network of prokaryotic communities inhabiting the rhizosphere of the oaks in AZC switched to a less complex network after the inoculation, as revealed by the decrease in the average degree (avgK) and average clustering (avgCC) coefficients and the statistically significant increase in modularity (M) and geodesic distance (GD) values (Table 2). It should be pointed out that although both networks had a modular structure (M > 0.4), the network corresponding to inoculated trees was comprised of a higher number of modules and the modularity index was significantly higher than in the case of control trees. As depicted in Figure 4, the network corresponding to non-inoculated trees was characterized by modules composed of a higher number of nodes when compared with treated trees.

On the contrary, prokaryotic communities dwelling in the rhizosphere of the inoculated trees located in AZL (Figure S8) and NPF (Figure S10) experimental areas were more complex and less modularized than that of control trees (Figures S7 and S9), although for some topological properties such as avgCC (AZL) subtle changes were registered (Table 2). Moreover, in NPF the geodesic distance of the networks corresponding to inoculated and non-inoculated trees was not significantly different, and both networks comprised 145 modules. It is worth mentioning that the percentage of positive interactions among nodes was markedly higher in the case of inoculated than control trees in AZL (Table 2; Figure 5). Almost no differences between treatments were recorded in the case of NPF (66.92% and 65.08% in control and inoculated trees, respectively; Figure 6). Figure S11 summarizes in a schematic view the main differences observed for all the co-occurrence networks.

Bacterial inoculation also triggered changes regarding the taxonomic affiliation of the networks' keystone members. It should be mentioned that

TABLE 2 Main global properties of association networks of prokaryotic communities inhabiting the rhizosphere of inoculated and control oaks.

	AZC		AZL		NPF	
	Control	Inoculated	Control	Inoculated	Control	Inoculated
RMT cut-off	0.97	0.96	0.96	0.96	0.94	0.94
Total nodes	566	880	774	763	832	852
Total links	942	1449	824	964	925	1071
R^2 of power-law	0.924	0.950	0.949	0.858	0.92	0.882
PEP (%)	90.87	82.33	65.41	80.61	66.92	65.08
Average degree (avgK)	3.329	3.293	2.129	2.527	2.224	2.514
Average clustering coefficient (avgCC)	0.088*	0.067*	0.074*	0.077*	0.076*	0.113*
Geodesic distance (GD)	4.064*	6.662*	10.941*	8.183*	8.312	8.320
Centralization of stress centrality (Cs)	0.461*	0.257*	0.747*	1.419*	0.308	0.298
Total modules	110	154	146	135	145	145
Modularity (M)	0.660*	0.788*	0.937*	0.834*	0.937*	0.896*

Abbreviation: PEP, percentage of positive links.
*Significant differences among treatments in the corresponding topological properties (Student's *t*-test).

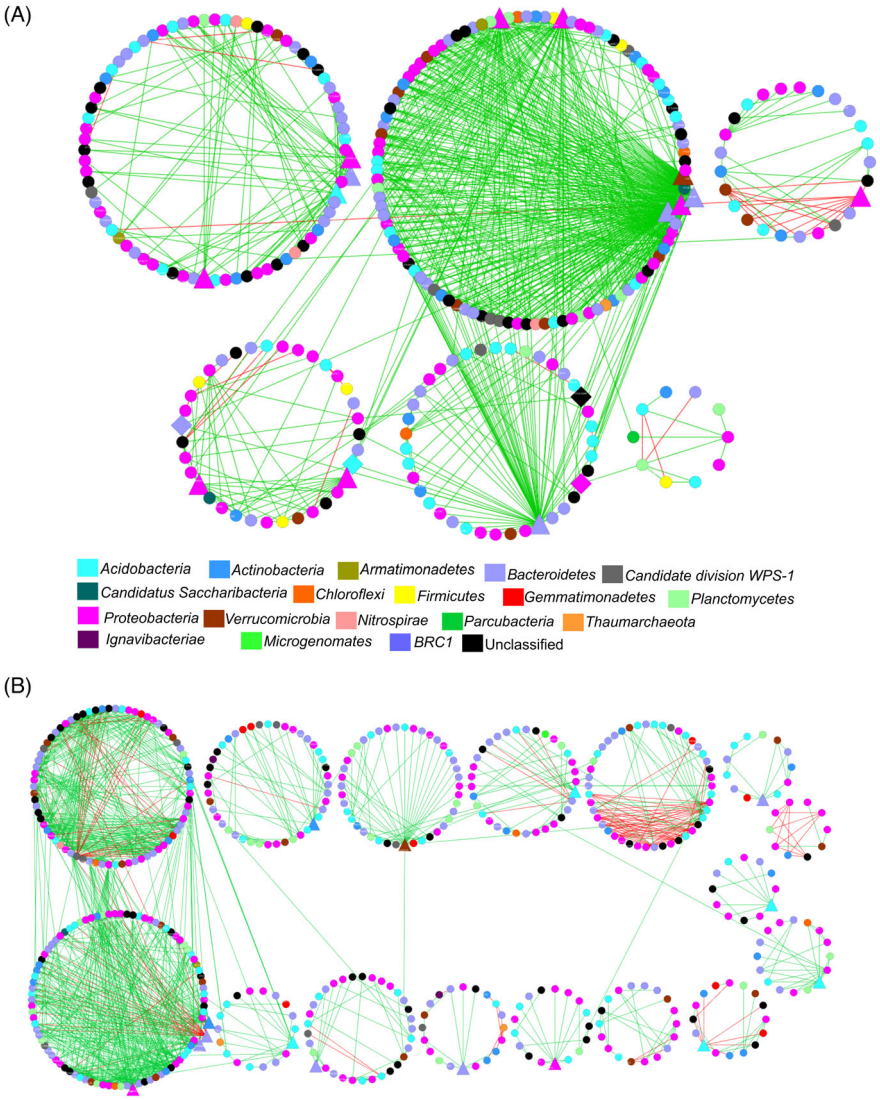

FIGURE 4 Associative networks of prokaryotic communities inhabiting the rhizosphere of control (A) and inoculated (B) trees located at AZC experimental site. Green and red lines represent positive and negative links among nodes, respectively. Triangle-shaped nodes indicate module hubs, while diamond-shaped represent connector nodes. Modules with less than 10 nodes were removed from the plot.

one OTU belonging to the genus *Bradyrhizobium* acted as a module hub in the network corresponding to inoculated trees in AZL, and no other OTUs ascribed to this genus were found in the control trees' network (Figure 5; Table S8). On the other hand, most of the keystone hubs were not shared between the networks corresponding to inoculated and non-inoculated oaks in every studied area, just the OTU000227 (belonging to genus *Reynarella*) was classified as a module hub in both networks of the AZL site. Meanwhile, networks calculated for inoculated and control trees were comprised of some different OTUs ascribed to the same genera, for instance to the acidobacterial group *Gp6* (AZC), *Ohtaekwangia*, *Spartobacteria* (AZL), and acidobacterial groups *Gp4* and *GP6* in NPF (Table S8).

DISCUSSION

Within the field of restoration biotechnology, microbiome-based approaches are of great expectation due to the relevant outcomes that have been obtained in nursery conditions in terms of plant fitness (Barriuso et al., 2008). To elucidate the ecological effect of afforestation practices with bacterially-inoculated plantlets, we performed a reductionist strategy by adapting a process (inoculation followed by the outplanting) in a pre-existing ecosystem, as described by Maghnia et al. (2019). We hypothesized that the bacterial treatment will not entail changes in the rhizosphere bacterial communities in the long term.

Firstly, we conclude that the working hypothesis was partly true since the bacterial treatment did not affect in a significant way the diversity, structure and composition of rhizosphere prokaryotic communities

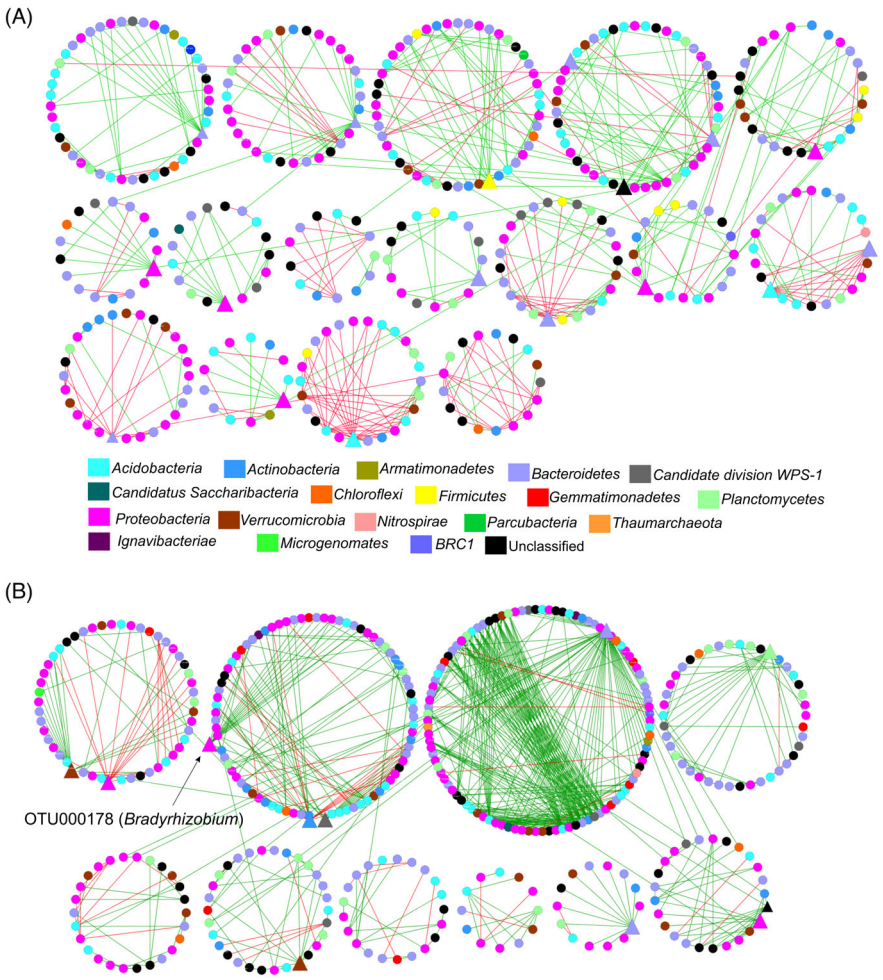

FIGURE 5 Associative networks of prokaryotic communities inhabiting the rhizosphere of control (A) and inoculated (B) trees located at AZL experimental site. Green and red lines represent positive and negative links among nodes, respectively. Triangle-shaped nodes represent module hubs. Modules with less than 10 nodes were removed from the plot.

18 months after the outplanting. We observed significant differences in alpha or beta diversity between sites (NPF, AZC and AZL) regardless of the treatments. Thus, inherent environmental or edaphic properties of each studied site, their different location (valleys of Río Chico and Lanjarón rivers), specific vegetation of each area and other uncontrolled factors may have much more influence on rhizosphere prokaryotic communities than bacterial inoculation did, as reviewed Philippot et al. (2013).

Taking into account the scarcity of data on the subject, it is very difficult to come to a single consensus about the persistence and effect of inoculation in wild conditions. While some authors have demonstrated by different strategies that resident bacterial communities changed due to the application of a bioformulation in field conditions (Schwieger & Tebbe, 2000; Wang et al., 2018), others have not detected any alteration of the autochthonous populations (Chowdhury et al., 2013). In addition to the lack of long-term effect over diversity and taxonomic composition of rhizosphere bacterial populations, we observed that these populations were rather similar to those addressed by other authors in two mountainous areas close to AZL and AZC (Fernández González, 2014; Lasa, Fernández-González, et al., 2019). Moreover, other works have already reported a similar abundance of the genera *Bradyrhizobium* and *Arthrobacter* in the bulk and rhizosphere soil of holm-oak trees located in a mountainous region in Lanjarón close to AZL (Fernández González, 2014; Fernández-González et al., 2017). Taking into account the homogeneity between control and untreated trees, and the distribution of both genera in different positions in the Sierra Nevada National and Natural Park, it is tempting to speculate about an initial loss of the inoculated consortium, followed (or accompanied) by the horizontal acquisition of members of both genera from the surrounding soil biome from where both inoculants were isolated; alternatively, an initial loss till reaching their natural abundance in these soils. It should be mentioned that there are conflicting research findings regarding the lifespan of the bioformulations once inoculated (Araújo et al., 2018; Frey-Klett

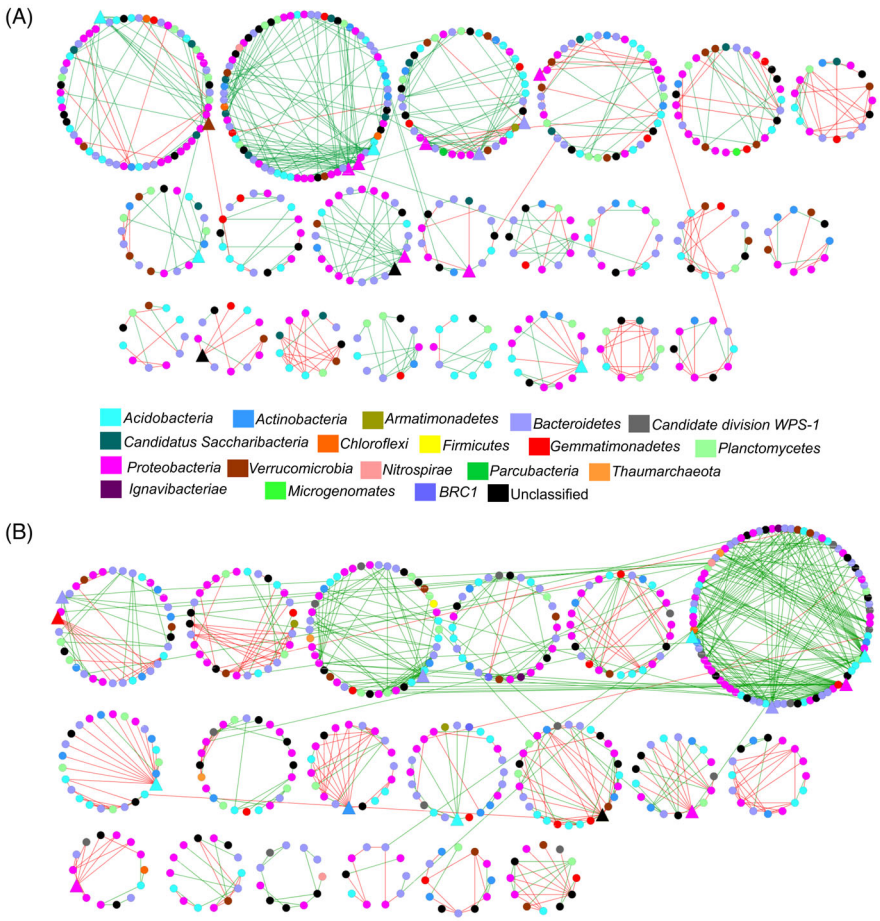

FIGURE 6 Associative networks of prokaryotic communities inhabiting the rhizosphere of control (A) and inoculated (B) trees located at NPF experimental site. Green and red lines represent positive and negative links among nodes, respectively. Triangle-shaped nodes represent module hubs. Modules with less than 10 nodes were removed from the plot.

et al., 1997; Galiana et al., 1994; Narożna et al., 2015 and references therein), which hinders the validation of the idea of an initial loss and subsequent horizontal acquisition of the consortium. On the other hand, it is worth mentioning that most of the previous works that addressed the lifespan of the bioinoculants in field conditions implemented low-sensitivity techniques such as fingerprinting or culture-dependent methods. Here we used a next-generation sequencing approach known for its high resolution and sensitivity, accounting for most of the prokaryotic diversity. Other more sensitive and specific techniques such as quantitative PCR (qPCR) would be needed to estimate the persistence of the consortium in field conditions.

Although no changes were recorded in bacterial diversity and taxonomic profiles, inoculation triggered associative changes in the long term. Bacterial treatment induced an increase in the number of links and the value of avgK of the networks corresponding to melojo oaks located in AZL and NPF, which in addition to a high number of nodes, has been considered as indicators of complex microbial networks. In turn, complex networks are linked to the promotion of the plant host's health and production, and a better adaptation to biotic and abiotic stresses (Fernández-González et al., 2020; Tao et al., 2018; Yang et al., 2017). Thus, the inoculation of melojo oaks in AZL and NPF sites could have entailed an increase in the hosts' fitness.

On the other hand, we observed that bacterial inoculation of melojo oak trees arouse different effects on rhizosphere prokaryotic communities: while inoculation supposed a more compartmentalized network in the AZC site, non-inoculated trees were more prone to higher modularized networks in AZL and NPF. The phenomenon of network modularization has been suggested as a strategy to maintain the stability of microbial communities, protecting them from disturbances (Delmas et al., 2019; Marasco et al., 2018). In addition to that, the inoculation induced an increase in the proportion of negative interactions solely in the case of AZC. Other authors have already proposed that negative interactions also have protective effects against external disturbances (Hernandez et al., 2021 and references therein). The increase in the modularity and the percentage of negative interactions points out to an enhancement in the stability of the bacterial community

of inoculated AZC trees, which could prevent a possible perturbation from spreading throughout the rest of the network by confining it to one module (Rybakova et al., 2017). Bacterial inoculation would have triggered just the opposite effect in the AZL site.

It is important to stress that network keystones are taxa that underlie the network structure. In all cases, the taxonomic affiliation and the number of keystones varied between inoculated and control trees (Table S8). Several OTUs belonging to the acidobacterial subgroup 6 (Gp6) were identified as connectors or module hubs in all networks except that calculated for the inoculated oaks in AZL, highlighting their potential essential role in the maintenance of the networks' stability. Other authors have already suggested that this acidobacterial subdivision may represent the keystone taxa in plant-associated microbial communities (Jiang et al., 2017). It is important to point out that one of the module hubs of the network corresponding to the inoculated trees of AZL belonged to the genus *Bradyrhizobium*. Although we cannot guarantee that this OTU is the one we inoculated, its role in structuring the prokaryotic community of inoculated trees in AZL is thought to be important. Previous works in other plant hosts suggested that members of the genus *Bradyrhizobium* could play an essential role in excluding plant pathogens, enhancing plant host's health, or having a generalist role within the rhizosphere (Floc'h et al., 2021; Lewin et al., 2021). Notwithstanding, networks should be interpreted cautiously since significant correlations among nodes do not necessarily entail microbial interactions, but they reflect co-existing members that could have similar or complementary functions or that share ecological preferences (Deng et al., 2012; Freilich et al., 2010; Preto et al., 2017; Tao et al., 2018).

Bacterial inoculation changed associations among prokaryotic members without changing the diversity or taxonomical profiles of overall communities, in none of the studied areas. The outplanting of treated oaks could have entailed the rearrangement of taxa associations (by the enhancement or exclusion of specific members of resident populations), altering their topological role, the structure of the networks, and therefore the complexity of prokaryotic populations, as other authors have already reported (Kong et al., 2019). Thus, it is very difficult to decipher the effect of the consortium inoculation over native prokaryotic communities but also over the trees.

The sign of the changes in associative patterns and keystone taxa resulted dependent on the experimental area under study. This variability could be a consequence of the history, environment and characteristics inherent to each experimental site. While AZL and AZC were two afforested zones, NPF was a naturalized pine forest where pine trees could have acted as nurse plants of the young melojo oak plantlets. On the other hand, AZC and AZL were more exposed to solar radiation than NPF due to the absence of tree canopy, nevertheless, the historical characteristics of the AZL site included a wildfire in 2005. On the other hand, *Q. pyrenaica* is a tree that commonly establishes symbiotic ectomycorrhizal (ECM) interactions, which may vary depending on the type of forest stand (Martín-Pinto et al., 2021). Indeed, plant-fungal communities harbour their own microbiota, commonly composed of bacteria inhibiting on/inside fungal tissues (Bonfante et al., 2019). ECM interactions are important drivers of bacterial variation in plant microbiomes, so the inoculation could have affected the ECM fungal community, having such changes affect the bacterial networks. Thus, the absence of analyses considering ECM and other fungi in this work makes it limited. Local differences not measured in this work could have triggered different associative responses to bacterial inoculation, so further research is needed to address the factors involved in the rearrangement of the bacterial communities, such as the putative role of ECM.

Regardless of the effects of bacterial inoculation over melojo oak trees and their rhizosphere bacterial communities, here we reported associative changes which persisted at least 18 months after the outplanting, not detected by diversity and taxonomic-based analyses. By evaluating microbial networks, it could be possible to address alterations in prokaryotic communities due to different treatments or conditions that were imperceptible by performing traditional analyses. Despite the uncertainty of the effect on the plants, our results point out the necessity to study changes in the networks prior to certifying the environmental safety of bioformulations, especially when these products are intended to be applied in protected natural areas.

AUTHOR CONTRIBUTIONS

Ana V. Lasa: Conceptualization (equal); data curation (equal); methodology (equal); writing – original draft (equal). **Antonio J. Fernandez-Gonzalez:** Conceptualization (equal); methodology (equal); writing – review and editing (equal). **Pablo J. Villadas:** Conceptualization (equal); methodology (equal); writing – review and editing (equal). **José F. Cobo Díaz:** Conceptualization (equal); writing – review and editing (equal). **M. Fernández-López:** Conceptualization (equal); funding acquisition (lead); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors have no relevant financial or non-financial interests to disclose.

DATA AVAILABILITY STATEMENT

The sequencing dataset generated during this 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 PRJNA807884.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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