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Plant genotype and seasonality drive fine changes in olive root microbiota☆

Matteo Chialva *, Silvia De Rose, Mara Novero, Luisa Lanfranco, Paola Bonfante

Department of Life Sciences and Systems Biology, University of Torino, Viale P.A. Mattioli 25, I-10125 Torino, Italy

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ABSTRACT

Due to global warming, the cultivation range of many crops is extending at higher altitudes and latitudes exposing plants to new climatic and environmental conditions, as early spring frosts. To face these issues in a sustainable agriculture context, new innovative technologies, as the use of biostimulants and the manipulation of plant microbiota, are emerging. Here, we focused on anarea of Northern Italy in which olive tree cultivation, a traditionally and economically-relevant item of Mediterranean agriculture, is rapidly extending to inland cold-temperate areas. We conducted an assessment of the prokariotic and fungal microbiota present in the root endosphere of a cold-hardy and a cold-susceptible Italian olive cultivar (Leccino and Frantoio, respectively) along spring and winter seasons. Microbiota assembly and diversity analysis revealed that the root microbiotas of more than 20 years-old plants were highly stable with few variations occurring across seasons and genotypes. Notably, we detected fine seasonal-dependent community adjustments in the cold-susceptible genotype, which involved beneficial microbes and pathogens. Moreover, different patterns of abundance were found for arbuscular mycorrhizal fungi and their endobacteria revealing the presence of intimate tripartite interactions. Overall, the results suggest that a healthy and highly stable root microbiota could provide a useful tool to help olive trees to face new environmental issues as those related to climate change.

1. Introduction

Olive tree (*Olea europaea* L.) is one of the major oil-producing crops being also a symbol of identity, environment, food, and culture of the populations bordering the Mediterranean sea since the Homeric times [1]. Even if still nowadays the European Union is the world leading producer, olive tree cultivation is spreading in many other areas of the world especially in the southern hemisphere [2], because of the global warming and the global increase in olive oil consumption.

In addition to the threat represented by dangerous pests as *Xylella fastidiosa* [3], olive cultivation in Mediterranean area is expected to be highly influenced by climate changes [4]. Cultivable areas are expected to extend Northward and Eastwards and at higher altitudes with an increase by 25 % in next 50 years [4,5]; in many areas this phenomenon has already been documented [6].

Such an expansion of cultivation range is expected to increase climatic risks related to drought events, heat waves or frosts for many Mediterranean crops [7,8]. In particular, olive trees cultivated at the boundary of their areal are exposed to increased frost damage risks in early-spring due to the anticipation of the plant phenology [7]. On the other hand, the cultivation of olive tree under cold climates may have unexpected benefits, such as the increase in olive oil quality. Indeed, it has been shown that cold improves olive fruit quality by slowing down the post-maturation process [9]. For these reasons new management practices, are urgently required to promptly face these issues [8].

Microbial communities, that live associated to plant niches (*i.e.*, the plant microbiota), can effectively protect plants against biotic and abiotic stresses [10] leading to innovative potential solutions to afford issues linked to climate change in a sustainable perspective [11]. Indeed, it has been demonstrated that microbiota manipulation can be effective in alleviating drought [12], osmotic stress [13] and cold [14,15]. Particularly, at the root level, microbiota plays a crucial role in modulating plant physiology and metabolism under different environmental conditions, with consequences on plant immunity [16]. The root microbiota may also influence plant responses at systemic levels [17], shaping agronomically relevant traits. The olive root microbiota has been recently characterized [18] in a panel of European cultivars showing that plant genotype is the main factor shaping roots microbial

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* Corresponding author.
E-mail addresses: matteo.chialva@unito.it, matteo.chialva@hotmail.it (M. Chialva), silvia.derose@unito.it (S. De Rose), mara.novero@unito.it (M. Novero), luisa.lanfranco@unito.it (L. Lanfranco), paola.bonfante@unito.it (P. Bonfante).

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assemblages with a stronger effect in the rhizosphere compared to the endosphere. Moreover, olive root-associated microbiota hosted a reservoir of beneficial microbes including plant-growth promoting species that can be potentially used as biocontrol agents. Intriguingly, microbiota has already been suggested as a tolerance modulator towards pathogens as *Verticillium dahliae* [19] and *Xylella fastidiosa* [20]. Moreover, being a mycotrophic species [21] olive tree is also a very good model to study arbuscular mycorrhizal (AM) symbiosis. This pervasive symbiosis has multiple effects on plant responses [22]: in response to AM colonization an increase in olive plant development and production, photosynthesis and transpiration rate [23], tolerance to salt stress [24] and drought [25] were observed.

While many researches have investigated cold-acclimatation in olive tree [26–28] or the olive microbiota in a number of accessions [18,29], the impact of season and temperature on the olive root microbiota remains unknown.

Here, we took advantage of a study area located in the Piedmont region (Northern Italy), where the establishment of olive cultivation has been well-documented in the past 30 years with the aim to recover marginal lands [6]. Our aim was to investigate the potential microbiota changes of the olive root endosphere across winter and spring seasons in one cold-hardy and one non-hardy cultivar by considering both fungal and bacterial communities. We hypothesize that i) both season and genotype have an impact on the microbial community assembly and ii) the cold-resistant genotype recruits different microbes during winter under harsher conditions.

2. Materials and methods

2.1. Field site, root sampling and endosphere isolation

The study was conducted in an olive grove located in Piedmont region at Azienda Agricola Mimosa (Pinerolo, Torino, Northern Italy, 350 m a.s.l.). The site is a productive field located on south-facing hills characterized by a mean annual temperature of 12.2 °C and rainfall of 787 mm (source, climate.data.org). The bedrock is Carboniferous graphitic micaschist (Northern Dora-Maira massif) [30] with a fully grassed Endoleptic Cambisol (pH 6.5) soil type. Olive plants (*Olea europaea* L., 1753) were twenty three-years old and have been growing under constant conventional farming practices. The lawn is mechanically mowed once a month from April to October; a green manure composed of branch cuttings is applied yearly and trees are occasionally watered using an automatic drip irrigation system during the summer (July-August). Plants were sprayed with copper oxychloride (4 kg × ha^{-1} per year) and dimethoate (0.6 kg × ha^{-1} per year) against *Bactrocera oleae* parasite and, occasionally, fertilized by spraying using boron and amino acids-based biostimulants.

Roots for metagenomic analysis were sampled from 'Leccino' and 'Frantoio' cultivars which are two well-established genotypes in Northern Italy and already characterized to be cold-hardy and cold-susceptible, respectively [28,31,32]. Sampling was performed in 2017 at the end of the winter (start of March) and at the end of spring season (early June). Climatic parameters detected in March and June at the Metereological station of the Regional Agency for Environmental Protection (https://www.arpa.piemonte.it/) located 2 km from the field site, are reported in Table S1. Weather conditions at the two time points mainly differed by temperature features, while minor or no variation at all were detected for rainfall and humidity (Table S1). Plants were under vegetative rest (BBCH00) in March and at the fruit-set (BBCH69) in June according to the phenological growth stages of olive trees [33].

Root samples were extracted from soil blocks excavated using a shovel at 70 cm from the trunk at a depth ranging from 5 to 20 cm. For each genotype and season, roots from six plants were sampled and pooled into 3 replicates. For each plant two soil blocks were sampled accounting for local variability of microbial populations. Root samples were kept at 4 °C and processed the same day in the laboratory.

To isolate the root endosphere, a modification of the protocol described by Bulgarelli et al. [34] was used. Briefly, 10 g of roots for each sample were washed twice under stirring (15 min, 120 rpm) in a 50 ml falcon tube containing 10 mM sterile phosphate-buffer saline (10 mM, pH 7.4) supplemented with 0.02 % Tween 80 (PBS-T) to remove rhizosphere. Rhizoplane was further depleted by 30 sonication cycles (30 s pulses, 30 s rest) in PBS-T. Root samples were finally washed with sterile distilled water, freeze-dried ground to powder using sterile mortar and pestle and stored at −20 °C until DNA extraction.

2.2. DNA extraction, 16S/ITS rDNA amplification and sequencing

DNA was isolated from 20 mg of dry root material (corresponding to 100 mg fresh weight, approximately) using the NucleoSpin Plant II Mini kit (Macherey-Nagel) following manufacturer's protocol and samples concentration spectrofotometrically measured using a Nanodrop-1000 instrument (Thermo Scientific, Wilmington, Germany).

To analyze prokaryotic community assembly the V3-V4 hyper-variable region of the 16S rDNA was used. A 464 bp-long amplicon was obtained using 341F-805R primers pair [35] containing 8 base-long barcodes in a double-multiplexing system [36]. A 4 Ns cap was added upstream to barcodes to allow ligation of sequencing adapters. PCR amplification reactions were carried out in a final volume of 30 μl using 1U of Phusion High Fidelity DNA polymerase kit (Thermo Fisher Scientific, Courtaboeuf, France) 0.5 μM of each primer and 40 ng of DNA template following manufacturer's instructions. Cycling conditions were as follows: an initial denaturation step (98 °C, 5 min), followed by 30 cycles (98 °C for 40 s, 53 °C for 40 s and 72 °C for 1 min) and a final extension step of 7 min at 72 °C.

Fungal communities were analyzed using the ITS2 region of 18S rDNA as marker. Since in some samples it was not possible to directly amplify the ITS2 region, as also described in other studies and to avoid the amplification of plant sequences, a nested PCR approach was undertook [37,38]. In the primary PCR reaction the whole ITS region was amplified using ITS1f-ITS4 primers [39] as previously described but the following cycling conditions: 5 min at 98 °C, 35 cycles of 30 s at 98 °C, 45 s at 54 °C and 60 s at 72 °C and a final extension of 10 min at 72 °C. The secondary reaction was carried out using fITS9-ITS4 primers [40] and using 2 μl of the previous reaction as template. Primers contained a 4 Ns cap and a 8-nucleotide barcode as previously described. Cycling conditions were: 5 min at 98 °C, 30 cycles of 30 s at 98 °C, 40 s at 54 °C and 30 s at 72 °C and a final extension of 10 min at 72 °C.

For both 16S and ITS2 amplicon each DNA sample was amplified in triplicate on a TProfessional thermocycler (Biometra GmbH, Germany), checked for the expected size range on 1% agarose gel (Fig. S1) and pooled. Amplicons were then purified using the Wizard® SV Gel and PCR Clean-Up System Protocol (Promega, Madison, Winsconsin, USA) and quantified using Qubit™ 2.0 fluorometer with the dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA). Samples were assembled with equimolar concentration into 1 Fungal and 1 Prokaryotic library (12 samples each) to a total of 2 μg/100 μl. Libraries were prepared and sequenced at IGA Technology Services (Udine, Italy; http://igatechnology.com/) using Illumina MiSeq (2 × 300 bp).

2.3. Bioinformatics

Raw reads were quality checked using FastQC v0.11.9 software [41]. Since reads were in mixed orientation (reverse and forward reads in both R1 and R2) due to undirectional sequencing, libraries were corrected using a custom R script which separates forward from reverse reads by recognizing primers using fqgrep v0.4.4 [42] and trims a variable amount of Ns upstream to the barcode. Libraries were then demultiplexed using 'demultiplex' command in Je v2.0.RC suite [43] using double- and single-multiplex settings for 16S and ITS2 libraries, respectively allowing 1 mismatch per barcode and a minimum quality of 2.

Libraries were imported into QIIME 2 (Quantitative Insights Into Microbial Ecology) v2020.08 [44] for denoising and Amplicon Sequence Variants (ASVs) detection. Demultiplexed reads were first denoised using DADA2 plugin [45]. For 16S rDNA libraries 13 nucleotide at 5' were trimmed ('–p-trim-left-f/r' parameters), while for ITS2 libraries no trimming was applied as recommended in QIIME2 manual. Due to a decrease in quality, reads were truncated at 3' after 280 nt (forward) and 220 nt (reverse) for 16S. To account for ITS2 rDNA length variation the ITSxpress pipeline was performed [46] before DADA2 analysis and no truncation was applied (–p-trunc-len 0). Due to uneven quality in forward and reverse reads only forward reads were used. ASVs were taxonomically annotated using the pre-formatted SILVA v138 database for QIIME [47,48] at 99 % level identity for Prokaryotes and the QIIME release of the UNITE + INSDC v8 database without singletons in developer and dynamic mode [49] for Fungi. Taxonomy was assigned using the q2-feature-classifier command [50] which implements a sklearn naïve Bayes classifier [51]. For 16S ASVs taxonomical classification the 341–805 region was extracted using the 'extract-reads' command and used to train the classifier.

Downstream analyses were conducted under R v3.6.3 programming environment [52]. Diversity analyses were performed using 'phyloseq' v1.30.0 [53] and 'vegan' 2.5-6 R package [54] while differential abundance analysis was performed using DESeq2 [55]. Graphical elaboration were performed using ggplot2 v3.3.2 [56] and UpSetR v1.4.0 [57] packages.

All the analyses were performed on a Dell XPS system (8 cores, 16 GB RAM) running Ubuntu 18.04. Raw sequencing data were submitted to Sequence Read Archive (NCBI-SRA) under BioProject accession number PRJNA679714.

2.4. Identification of fungal endobacteria

Since many AM fungi may host endobacteria in their cytoplasm [58], endobacteria ASVs were identified by clustering and validated by phylogenetic placement as described in Bodenhausen et al. [59] as well as using the reference dataset provided in the same study with minor modifications. Bacterial ASVs (bASV) were clustered with the reference dataset containing *Burkholderia*-related endobacteria (BRE) and *Mycoplasma*-related endobacteria (MRE) retrieved from Glomeromycotina (*Candidatus* Moeniiplasma glomeromycotorum), Endogonaceae (Mucoromycotina), and Mortierellomycotina using vserach v2.7.0 [60] allowing up to 10 % sequence divergence (–usearch_global, –id 0.90, –strand both). The four candidate bASVs identified were further validated by fine mapping on a reference phylogeny. Candidate bASVs were aligned to the reference dataset using MAFFT v7.402 [61] with default parameters and the multiple sequence alignment was trimmed using Gblocks v0.91b [62] using '-b2 50 %+1 -b3 20 -b4 10 -b5 h' parameters. Phylogeny was inferred combining both Bayesian and Maximum likelihood analysis using MrBayes v.3.2.7 [63] and RAxML-HPC2 v.8.2.12 [64], respectively. The best-fitting nucleotide substitution model was predicted prior to phylogenetic reconstructions using ModelTest-NG v0.1.6 [65]. Bayesian analysis was performed running Markov chain of Monte Carlo for 10 million generation until convergence under GTR + G model using default parameters, sampling trees every 1000 generations and discarding the first 2500 trees. RaxML analysis was performed under the 'GTRCAT' model using '-f a -N autoMR' parameters. All the pylogenetic analysis were performed using the the CIPRES Science Gateway V. 3.3 public service [66].

2.5. Statistical analyses

The appropriate statistical test was chosen to compare means across treatments by checking data normality (Shapiro-Wilkey test, $P > 0.05$) and homoschedasticity (Levene's test, $P < 0.05$). The pairwise Kruskal-Wallis non-parametric test ($P < 0.05$) was applied for non-normal homoschedastic data while analysis of variance (ANOVA) followed by Tukey's post-hoc test ($P < 0.05$) was applied for normal and homoschedastic data. Analyses were performed using the 'agricolae' v1.3-3 package in R [67].

2.6. Root staining and microscopical analysis

A portion of the roots sampled for the molecular analysis were thoroughly washed under tap water, blotted on filter paper and clarified at room temperature in 10 % KOH w/v in distilled water for 1 h. Roots were then carefully washed in distilled water, stained for 12 h in 0.1 % water blue (Fluka, Buchs, Switzerland) in lactic acid and further clarified in pure lactic acid (3 wash, 2 h each). Samples were than mounted on glass slides with glycerol and observed under an optical microscope.

3. Results and discussion

3.1. Alpha- but not beta-diversity is driven by sampling season and plant genotype

The olive root endosphere bacterial and fungal communities were analyzed in one cold-hardy (Leccino) and one cold-sensitive (Frantoio) cultivar across a winter and a spring season. MiSeq amplicon sequences yielded a total of 150,058 (Bacteria) and 200,812 (Fungi) raw reads resulting in 482 (Bacteria) and 480 (Fungi) Amplicon Sequence Variants (ASVs), respectively. Despite the relatively low number of raw reads obtained, samples diversity was fully covered for both target regions (16S rDNA and ITS2) in all the samples, as evidenced by rarefaction curve analysis (Fig. S2).

The analysis of bacterial alpha diversity, measured through the observed number of ASVs and Shannon index (Fig. 1A) consistently showed a significant effect for season (ANOVA, $p < 0.01$, Table S2) and a weak interaction effect between season and genotype ($p < 0.05$) but not for genotype only ($p > 0.05$). Considering single condition, the cold-hardy cultivar Leccino had constant diversity across seasons while the cold-susceptible Frantoio, showed significantly higher diversity in winter than in spring.

Mycobiota alpha diversity clearly responded to season, in term of raw numbers of observed ASVs ($p < 0.01$), but not in Shannon diversity ($p > 0.05$, Fig. 1B, Table S2). Indeed, the Shannon diversity index was constant across all the conditions and only a significantly higher amount of fungal ASVs (fASVs) was detected during spring in 'Leccino' genotype (Fig. 1B).

The 16S rDNA amplicon library showed that no prokaryotic ASVs (bASVs) were classified as Archaea as also emerged from a previous study [18]. The analysis of the bacteriota assembly (Fig. 1C) showed that Proteobacteria were the most abundant component (50 % of relative abundance in all conditions), followed by Actinobacteriota (formerly Actinobacteria), Bacteroidota (formerly Bacteroidetes), Myxococcota, Acidobacteriota (formerly Acidobacteria) and Verrucomicrobiota (formerly Verrucomicrobia).

The mycobiota was dominated by a few taxonomic groups (Fig. 1D; Fig. S3): Basidiomycota (42 % relative abundance, including cl. Agaricomycetes), Glomeromycotina [Glomeromycota in UNITE + INSD database] (34 %, cl. Glomeromycetes) and Ascomycota (21 %, cl. Sordariomycetes and Eurotiomycetes).

All these evidences partly agree with previous data obtained on olive root endosphere microbiota studies using the same marker regions [18,68]. Our results showed a similar number of observed ASVs and a rather small root endosphere core community of 39 and 18 genera for bacteriota and mycobiota, respectively (Fig. S4). This result is in line with a proposed scenario where woody species root-associated niches would not be as rich as those of graminoid plants [69]. Notably, in contrast with these previous studies, we here detected a dominance of Basidiomycota fungal phyla, while Ascomycota were poorly represented. These latter have been usually found as dominant within the plant root mycobiota in both annual crops such as rice [70,71], or tomato [72,73]

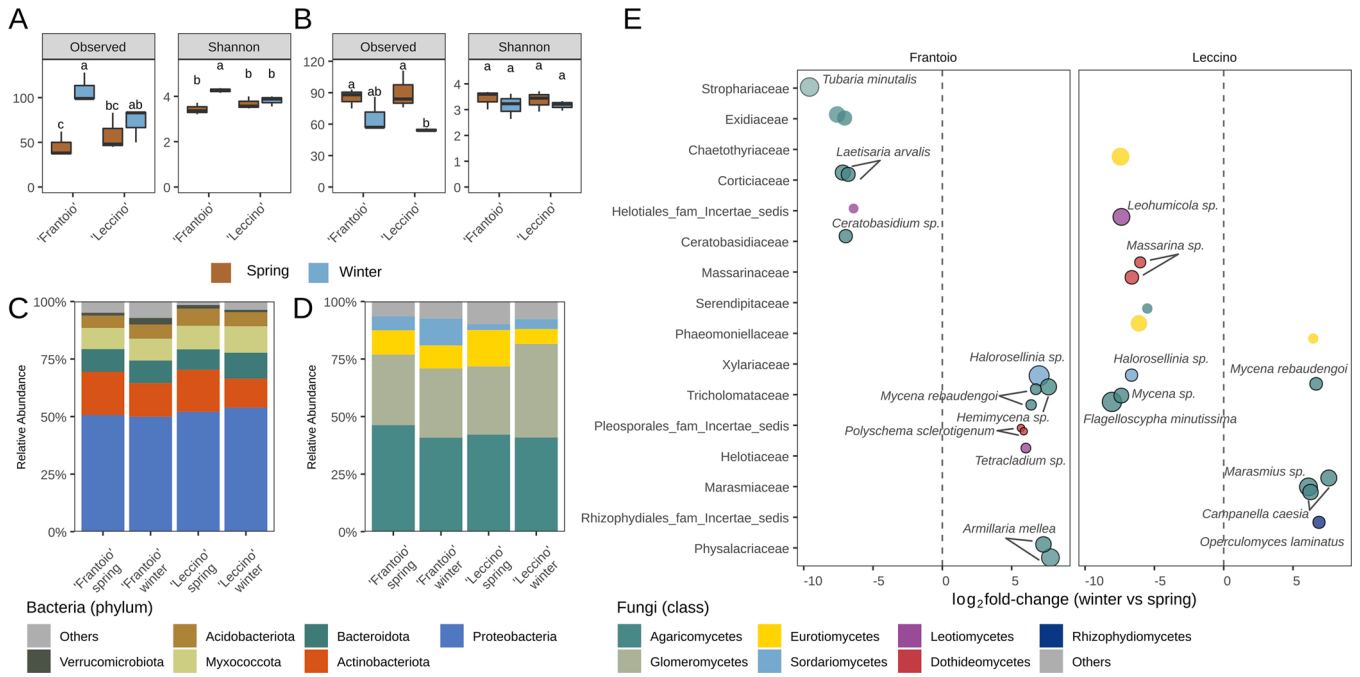

Fig. 1. Root endosphere bacteriota and mycobiota diversity and assembly of the cold-hardy Leccino and cold-susceptible Frantoio olive (*Olea europaea* L.) cultivars in spring and winter season reconstructed by 16S and ITS2 rDNA meta-barcoding. (**A,B**) alpha-diversity of bacterial (A) and fungal (B) communities; number of amplicon sequence variants (ASV) by condition (observed) and Shannon diversity index are shown. Boxplots display median value (horizontal line), quartiles (boxes) and 1.5 x interquartile ranges (whiskers) while different letters indicate differences among means according to the non-parametric pairwise Kruskal-Wallis test ($P < 0.05$). (**C,D**) Relative abundances of bacteria (C) and fungi (D) at phylum and class level, respectively. (**E**) Differentially-abundant ($P_{adj}<0.1$) non-AMF fungal ASVs in winter *versus* spring comparison in both the analyzed cultivars by family (x axis) and class (color); dots radius is log-proportional to the mean ASV abundance in the dataset; only ASV annotated down to the genus level are contoured and annotated with text labels.

and other tree crops as citrus [74] or grape [75].

Unexpectedly, nor genotype or season had strong impact on overall community assembly in both 16S and ITS2 rDNA datasets (PERMANOVA, $P > 0.05$; Table S3) suggesting that the olive root endophytic communites under our field-conditions were highly stable. This result, together with changes emerged studying the alpha-diversity, allows us to speculate that the overall community assembly does not vary significantly in response to genotypes and seasons but minor fine modulations in microbiota composition occurs. Similar results were obtained for two different olive cultivars investigated under *Verticillium dahliae* infection [19] and other tree species. Differently from annual and non-woody species, citrus and poplar plants showed a stable below-ground microbiota [76]. These observations suggest that perennial plants establish more durable and permanent associations with the soil biota, possibly due to specific traits, such as an extensive root system able to regrow after unfavorable conditions such as drought and freezing winters. These anatomical and physiological features can therefore offer a more stable niche to the microbiota.

3.2. Differentially-abundant ASVs in winter versus spring season

To better understand the fine microbiota variations emerged from the previous diversity analysis, differential abundance analysis was performed on both bacteriota and mycobiota using DESeq2 algorithm trying to dissect fine seasonal shifts (winter *versus* spring) in communities assembly, if any., No differentially abundant bASVs were found in both cultivars (data not shown). Indeed, despite the significantly higher bacteriota diversity displayed by the cold-susceptible genotype 'Frantoio', no ASVs were significantly enriched in winter *versus* spring season due to high variability among biological replicates. Moreover, unique taxa in Frantoio genotype during winter season (150 ASVs encompassing 37 genera), *i.e.* those contributing to the higher alpha diversity, had low relative abundance in the dataset justifying their negligible contribution to explain season- and genotype-dependent changes in the overall community assembly.

By contrast, 46 and 39 fASVs emerged as differentially abundant ($P_{adj}<0.1$) in winter *versus* spring in Frantoio and Leccino cultivars, respectively. In Frantoio, 25 were enriched and 21 depleted in winter, while in Leccino 19 were enriched and 20 depleted (Fig. 1E). Notably, there was no overlap between these differentially abundant ASVs highlighting a strong cultivar-dependent response. However, few fungal genera were similarly detected as differentially abundant in both the cultivars and had contrasting differential abundance in winter *vs* spring season comparison; these included a member of *Halorosellinia* sp. (Xylariaceae), a few *Mycena* sp. ASVs (Tricholomataceae), plus a number of Glomeromycotina ASVs (Fig. 2A, see next paragraph). *Halorosellinia* sp. was found enriched in Frantoio during winter but depleted in Leccino. The genus *Mycena* was mostly found as enriched during winter in both cultivars: interestingly, it has been described as a highly abundant endophyte in *Taxus chinensis* tree [77], associated to *Aesculus hippocastanum* (horse-chestnut) trunk [78] and as a saprotroph in arctic tundra soil [79] indicating a potential correlation with cold-adapted woody species and cold biomes. Moreover, a recent report showed that *Mycena* sp. associated to birch tree may switch from saprotrophy to symbiotic associations [80].

Among ASVs genera detected as enriched during winter only in Frantoio, two ASVs belong to the *Armillaria mellea,* which is the agent of the root rot disease, well-known to also occur in olive trees [81]. This pathogen was not found in the cold-hardy Leccino genotype. This evidence was in line with the in field observation of *A. mellea* fruiting bodies during winter and early-spring, especially in proximity or near the trunk of Frantoio trees, but rarely on Leccino plants (data not shown). Similarly, few other ASVs including *Hemimycena* (Tricholomataceae), *Polyschema sclerotigenum* and *Tetracladium* sp. have been found as enriched in Frantoio during winter. Interestingly, members of *Tetracladium* genus has been frequently identified as plant endophyte under

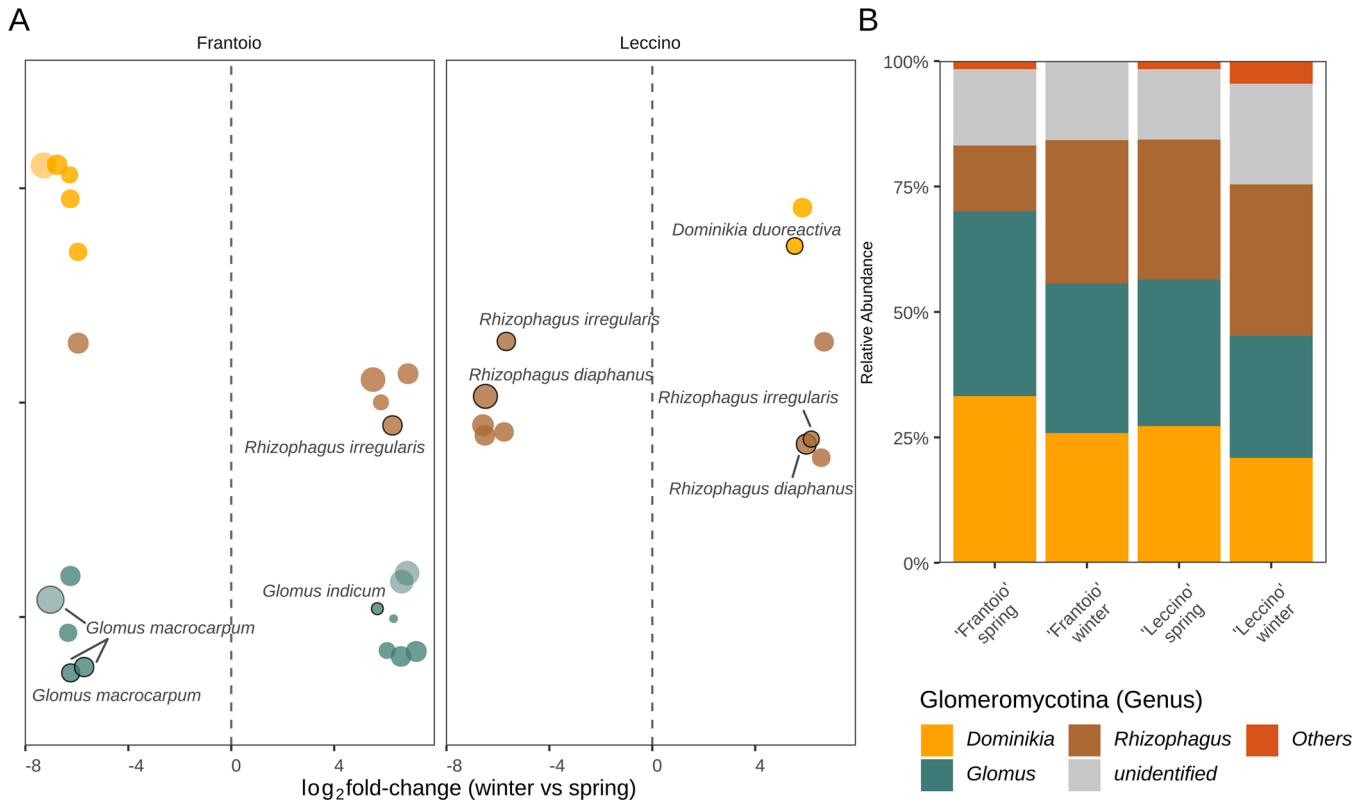

Fig. 2. Arbuscular Mycorrhizal fungi (AMF) assembly in olive root endopshere in winter and spring season. (**A**) Differentially abundance of AMF fASVs by genera (P_{adj}<0.1); dots radius is log-proportional to the mean ASV abundance in the dataset; only ASV annotated down to the species level are contoured and annotated with text labels. (**B**) Relative abundance of AMF genera across seasons and genotypes.

cold and extreme environments [82]. Similarly, *P. sclerotigenum* has been found in association to Norway spruce roots [83]. Winter-depleted taxa included *Tubaria minutalis* (Strophariaceae) and *Laetisaria arvalis* (Corticiaceae family). This last species has been proposed as a biocontrol agent of *Rhizoctonia solani* plant pathogen [84].

Overall our data revealed that while the bacteriota of both cultivars was highly stable across seasons, mycobiota displayed fine seasonal and cultivar-dependent variations involving different fungal ecological groups such as pathogens, beneficial microbes (AMF) and saprotrophs.

In particular, *Mycena* sp., that was often described in cold biomes, was found as enriched during winter in both cultivars. In the cold-susceptible genotype, Frantoio, we found the occurrence of the fungal pathogen *A. mellea*, which suggests a more compromised plant health status. Moreover, comparing the mycobiota in winter *vs* spring, we also detected a differential modulation of saprotrophs and endophytes (such as *Mycena* sp., *Hemimycena* sp., *Tetracladium* sp.) which highlights different interactions with soil fungi in the two cultivar.

3.3. Arbuscular Mycorrizal fungi and occurrence of their endobacteria

Fungal community assembly analysis showed that arbuscular mycorrhizal fungi were the second most abundant taxa within olive endosphere (>30 % of relative abundance in all conditions; Fig. S4). Out of the 480 fASVs identified 166 (34.5 %) belonged to the Glomeromycotina subphylum and in particular to Glomeraceae and Claroideoglomeraceae families. Out of these, 106 AMF fASV were taxonomically assigned to the genus level and 30 ASVs to the species level. This probably reflects the use of the universal ITS2 marker region which allows a reliable analysis of the overall assembly and diversity patterns but it is not fully suitable for fine-scale AMF taxonomic resolution [85]. In analogy to what was found in other studies on olive roots [19,86,87] and on several tree species [88,89], Glomeraceae was the most abundant family but its almost exclusive dominance, as we documented here, was never reported. The most represented genera in our dataset were *Glomus* followed by *Dominikia* and *Rhizophagus* (Fig. 2).

Interestingly, 29 and 17 AMF fASVs were differentially abundant in winter *versus* spring in Frantoio and Leccino cultivar, respectively (Fig. 2A). While all fASVs assigned to genus *Dominikia* were depleted during winter in Frantoio, two fASVs were enriched in Leccino. The *Glomus* genus was also responsive to season in Frantoio while no differential abundance was found in Leccino. *G. macrocarpum* was depleted during winter (2 fASVs) while *G. indicum* was enriched (1 fASV). By contrast, the *Rhizophagus* genus was highly responsive to the season in both genotypes being some ASV depleted and other enriched during winter in Leccino while only enriched in Frantoio. Notably, different fASVs were affiliated to the species *R. irregularis* in both olive cultivars.

Results highlighted that some species/strains showed seasonal preferences probably due to their better tolerance to changing temperature maintaining olive root colonized also under less favorable environmental conditions in winter. This was further confirmed by qualitative microscopical observation on stained root samples (Fig. S5) which confirmed high levels of AM colonization in both cultivars and both seasons.

The high abundance of Glomeromycotina taxa among fASVs and the simultaneous sequencing of both bacterial and fungal rDNA markers prompted us to search fungal endobacteria among bASVs. The distribution of AMF endobacteria have been investigated in many AMF grown in culture collections [90,91], but their detection in natural condition is still very limited [59,92]. This task has been performed by integrating clustering and phylogenetic placement approach on a reference phylogeny as detailed in Bodenhausen et al. [59]. Interestingly, the analysis revealed two candidate endobacterial ASVs (bASV 172 and 397) which mapped to the Mollicutes/Mycoplasma-related endobacteria (MRE) group of AMF (Fig. 3A). Both showed the highest similarity with

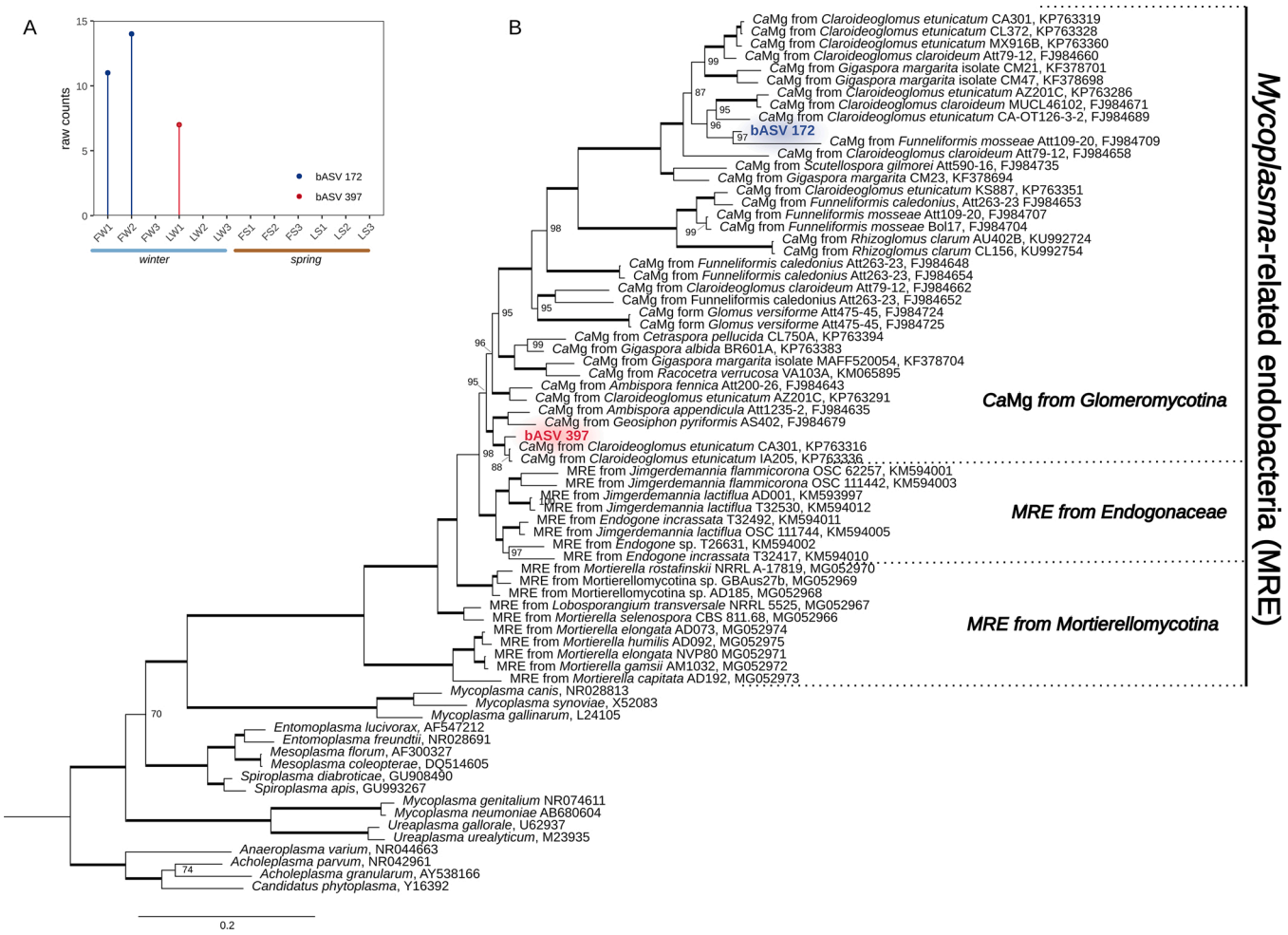

Fig. 3. Abundance and phylogenetic placement of the identified Mollicutes/Mycoplasma-related endobacteria (MRE) ASVs. (**A**) Lollipop chart indicating raw counts of endobacterial ASVs occurring across each single sample. (**B**) Phylogenetic placement of the two candidate ASVs (highlighted in red color) within reference phylogeny of MRE. The tree shows Bayesian inference; branches with Bayesian Posterior Probability (BPP) ≥95 and Maximum Likelihood (ML) bootstrap support values ≥70 are thickened; numbers indicate BPP values for branches with ML bootstrap support values <70.

Candidatus Moeniiplasma glomeromycotorum (*Ca*Mg) and, notably, both occurred only in winter season samples (Fig. 3B). The first, bASV 172, had a closest match to *Ca*Mg from *Funneliformis mosseae* (family Glomeraceae) while the second, bASV 397 had the closest match to *Ca*Mg from *Claroideoglomus etunicatum* (family Glomeraceae). However, *F. mosseae* was not detected in our dataset and, even if 4 fASV were assigned to the *Claroideoglomus* genus, none of these co-occurred with candidate endobacterial bASV 397. It is worth noting that, due to the relatively high number of unidentified sequences, we cannot exclude that both these AMF species occurred in our samples. In an alternative scenario we can envisage that the candidate endobacteria associate to other genera.

The detection of candidate AMF endobacteria exclusively in association with a given environmental conditions, the winter season, paves the way to investigate the relevance of these tripartite interactions in conferring some relevant traits to the fungal isolates, for example a resilience to abiotic stress as cold. However, this hypothesis needs to be supported by a larger number of environmental samples and/or by further targeted investigations.

4. Conclusions

While seasonal changes in bulk soil microbiota have been extensively investigated [93,94] variations in root-associated microbiota are poorly documented. A few studies have demonstrated that root bacterial communities diversity and assembly are highly influenced by season in both tree crops, as mulberry [95], and in grasses, as *Agave* [96].

In this study we investigated the composition of microbiota in olive roots across two seasons, spring and winter, and in two cultivars, with different sensitivity to low temperatures, grown in a site located at the Northern boundary of the olive tree range in a cold-temperate climate.

Results revealed a stable structure in the olive root microbiota which, however, revealed changes in its diversity across cold and warm seasons also depending on genotype tolerance to cold. The results suggest that the root microbiota may be a relevant determinant of climate adaptation of olive trees to higher latitudes, where winter frosts often threaten productivity. The data showed that the microbiota, and especially the prokaryotic component, thriving in the olive root endosphere was highly stable in its composition across seasons and genotypes, confirming previous data on tree species. However, we could notice distinct features in the mycobiota. At first, the analysis revealed a dominance of Basidiomycota, which is unusual for plant roots and can be potentially linked to the age of the sampled olive trees or to the specific environmental conditions of the experimental field. The high abundance and the seasonal vicariance of AMF strains/species points to a functional significance.

In addition, we were able for the first time to identify sequences unambiguously related to AMF endobacteria, and, notably, they were present only in winter samples. Our morphological and molecular results reveal that the AMF are maintained also during the harsh winter

season, and suggest that, possibly together with endobacteria, they can help their perennial hosts to better face environmental constraints.

Author contributions

Matteo Chialva: Conceptualization, Investigation, Formal analysis, Writing - Original Draft **Silvia De Rose:** Investigation, Writing - review & editing **Mara Novero:** Investigation, Writing - review & editing **Luisa Lanfranco:** Writing - review & editing **Paola Bonfante:** Supervision, Writing - review & editing.

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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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cpb.2021.100219.

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