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Above- and below-ground microbiome in the annual developmental cycle of two olive tree varieties

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Abstract

The olive tree is a hallmark crop in the Mediterranean region. Its cultivation is characterized by an enormous variability in existing genotypes and geographical areas. As regards the associated microbial communities of the olive tree, despite progress, we still lack comprehensive knowledge in the description of these key determinants of plant health and productivity. Here, we determined the prokaryotic, fungal and arbuscular mycorrhizal fungal (AMF) microbiome in below- (rhizospheric soil, roots) and above-ground (phyllosphere and carposphere) plant compartments of two olive varieties 'Koroneiki' and 'Chondrolia Chalkidikis' grown in Southern and Northern Greece respectively, in five developmental stages along a full fruit-bearing season. Distinct microbial communities were supported in above- and below-ground plant parts; while the former tended to be similar between the two varieties/locations, the latter were location specific. In both varieties/locations, a seasonally stable root microbiome was observed over time; in contrast the plant microbiome in the other compartments were prone to changes over time, which may be related to seasonal environmental change and/or to plant developmental stage. We noted that olive roots exhibited an AMF-specific filtering effect (not observed for bacteria and general fungi) onto the rhizosphere AMF communities of the two olive varieties/locations/, leading to the assemblage of homogenous intraradical AMF communities. Finally, shared microbiome members between the two olive varieties/locations include bacterial and fungal taxa with putative functional attributes that may contribute to olive tree tolerance to abiotic and biotic stress.

Keywords: olive plant microbiome, bacteria, fungi, arbuscular mycorrhizal fungi, plant developmental stage, plant compartment

Introduction

Olive (*Olea europaea* L.) is a perennial, evergreen diploid species belonging to the *Oleaceae* family. It represents one of the economically most important crop plants of the Mediterranean basin, accounting for 95% of the cultivated olive area worldwide (FAOSTAT 2020). More than 2000 olive varieties are listed in the world, comprising a huge genetic heritage (Lumaret and Quazzani 2001, Díez et al. 2011). Plant—associated microbial communities are key determinants of plant health and productivity, which may contribute to nutrient availability and uptake and enhance tolerance to abiotic and biotic stress (Hardoim et al. 2015, Reinhold-Hurek et al. 2015). We are beginning to understand the mechanisms and factors that shape the plant microbiome. As a primary determinant, the host plant genotype has been identified (Agler et al. 2016, Schlechter et al. 2019), while biogeography (Colleman-Derr et al. 2016) and temporal changes seem to be also important on the natural assemblages of plant-associated microbes (Copeland et al. 2015). Recent studies report on the effect of plant genotype, age, biogeography, and seasonality on the plant microbiom composition and structure (Müller et al. 2015, Gomes et al. 2018, Fernández-González et al. 2019, Materatski et al. 2019, Mina et al. 2020, Castro et al. 2022, Malacrinò et al. 2022).

So far studies on olive tree microbiome have monitored its composition singly in specific plant compartments, like the carposphere (Castro et al. 2022), the phyllosphere (Abdelfattah et al. 2015, Müller et al. 2015, Gomes et al. 2018, Materatski et al. 2019, Mina et al. 2020, Costa et al. 2021, Malacrinò et al. 2022), roots or the plant-associated rhizosphere (Fausto et al. 2018; Fernández-González et al. 2019, Malacrinò et al. 2022), or xylem sap (Anguita-Maeso et al. 2020, Anguita-Maeso et al. 2021, Anguita-Maeso et al. 2022), largely ignoring the well-documented interconnection among microbial communities that colonize different plant-associated compartments (Amend et al. 2019, Tkacz et al. 2020). Studying the microbiome in all plant compartments is needed (Singh and Trivedi 2017) to provide the full context of the complexity of interactions between the host plant and microorganisms. Martins et al. (2016) focused on exploring in parallel the microbiome of above- and below-ground plant parts in olive trees. However, they analysed fungi only, using entirely culture-dependent approaches, which may not provide a high-resolution analysis of the microbial diversity in environmental samples, compared to amplicon sequencing approaches (Hug 2018) and they did not consider the temporal variation of plant microbiome along the growing season. In addition, there is relative scarcity of data

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regarding the diversity of arbuscular mycorrhizal fungi (AMF) colonizing olive tree roots (Mondes-Borrego et al. 2014, Meddad-Hamza et al. 2017, Palla et al. 2020), despite olive plants being mycotrophic and highly dependent on AMF under arid conditions (Mekahlia et al. 2013). Several previous studies have demonstrated that AMF can promote olive plantlet growth and nutrient uptake under both favourable and constraining conditions (Calvente et al. 2004, Porras-Soriano et al. 2009, M'barki et al. 2018, Ouledali et al. 2018).

In our study, we determined the prokaryotic, fungal, and AMF community in different plant-associated compartments (rhizospheric soil, roots, leaves and flowers/fruits) of two widely cultivated olive varieties in Greece, 'Koroneiki' and 'Chondrolia Chalkidikis' (referred here as 'Chondrolia'), over five developmental stages along the annual growth cycle of the olive tree. Own-root trees of each variety, grown in the geographical regions of their traditional cultivation (Southern and Northern Greece, respectively), were selected for the study. We hypothesized for the presence of distinct below- and above- ground microbial communities, and that seasonal and developmental changes would have a larger effect on epiphytic compared to endophytic communities in both varieties and environmental settings. We were particularly interested to investigate for putative similarities in the endophytic microbial communities in the roots, focusing on the arbuscular mycorrhizal fungi and record the effect of the plant genotype in AMF natural assemblages.

Materials and methods

Experiment setup

All samples analysed in the study were collected from the experimental olive groves of (i) the Aristotle University of Thessaloniki in Thermi, Thessaloniki (40°32'5''N 22°59'43''E, 10 m altitude, with mean annual temperature 15.6°C and mean annual rainfall 460 mm), which maintains own—rooted olive trees of the variety Chondrolia and (ii) IELYA experimental station in Chania, Crete (35°29'23.44'' 24°01'37.59'', 28 m altitude, with mean annual temperature 18.1°C and mean annual rainfall 654 mm), which maintains own—rooted olive trees of the variety Koroneiki. Both collections contain mature, young tree plants (over 20 years old). Both studied varieties are certified and included in the National Plant Varieties Catalogue. Soil properties of the two experimental olive groves and monthly meteorological data are presented in Tables S1 and S2. Within each olive grove, six trees were used as biological replicates ($n = 6$) and all plant compartments from each tree (rhizospheric soil, roots, leaves, flowers/fruits) were sampled at five selected developmental stages (stages 60 to 92, flowering to overripe, given with a code letter A–E in the manuscript for simplicity, Fig. 1) (Sanz-Cortés et al. 2002) along the growing season (March to November 2019).

The rhizospheric soil and root samples were collected as follows: the projection of the olive tree canopy was roughly marked out as 100% distance from the trunk and the root was tracked in the soil. Samples of rhizospheric soil and roots were collected with a soil auger from three points around each plant in an area within 50% and 100% distance; samples were collected from the depth of 20–40 cm, which is occupied by the main volume of olive roots. The three soil-root samples per tree were homogenized providing six composite samples per sampling set. Well-developed and healthy leaves, flowers and fruits were collected from different spots in the plant canopy in a sterile 50 ml falcon tube. One falcon tube was obtained per tree. All samples were immediately placed on ice and transferred to the laboratory within the same day. Leaf and flower/fruit samples were stored at −20°C until processed for DNA extraction. Rhizospheric soil samples were sieved (4-mm opening size) while root fragments of up to 3 mm diameter were sieved (1-mm pore size) and thoroughly washed under tap water and surface-sterilized. All samples were stored at −20°C until processed for DNA extraction. Overall, 239 (156 for Chondrolia and 83 for Koroneiki) samples (rhizospheric soil, roots, leaves, flowers, and fruits) were collected of which 83 (46 for Chondrolia and 37 for Koroneiki) represent rhizospheric soil and root samples.

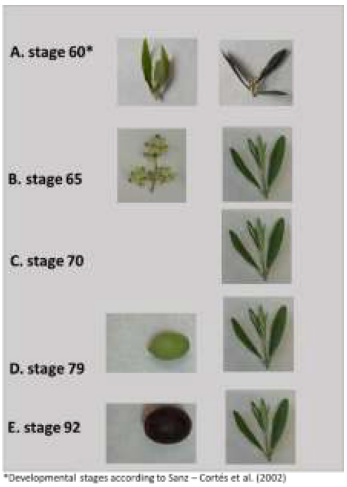
DNA Extraction from rhizospheric soil, roots, phyllosphere, and carposphere

DNA extraction from each rhizospheric soil sample (0.25 g) was performed with the DNeasy Power soil DNA isolation kit following the manufacturers' protocol (Qiagen, Hilden, Germany). DNA from roots was extracted using the CTAB method (Doyle and Doyle 1987). Leaves, flowers and fruits were initially placed in sterile potassium phosphate buffer (8 g/l NaCl; 0.20 g/l KCl; 1.4 g/l Na_2HPO_4; 0.24 g/l KH_2PO_4) in a sonication bath (Transonic 460, frequency 35 kHz) for 10 minutes to detach the microbial biofilms from the plant tissue. This procedure does not strictly exclude the recovery of endophytes (mainly through damaged epidermic cells), however we expect the recovery of strict endophytes to be very low and the captured microbial fingerprint to be highly enriched in epiphytes. Then, a centrifugation step followed (15 min, 9000 rpm, 4°C) and the formed pellet was further processed for DNA extraction with the DNeasy Power soil DNA isolation kit following the manufacturers' protocol (Qiagen, Hilden, Germany). The extracted DNA from all samples was quantified by the Qubit 2.0 Fluorometer (Life Technologies, Paisley, UK).

Amplicon sequencing analysis

The 16S rRNA gene and the fungal ITS region amplification and diversity analysis were performed *via* multiplex amplicon sequencing based on our in-house protocol (Vasileiadis et al. 2015, 2018). Briefly, bacterial and archaeal 16S rRNA genes were amplified with the primer set 515f–806r (Caporaso et al. 2012, Walters et al. 2016), which targets the V4 region of the 16S SSU rRNA, following the protocol of the Earth Microbiome Project (Caporaso et al. 2018). mplification of ITS was performed with the primers ITS7f—ITS4r (Ihrmark et al. 2012, White et al. 1996) following the protocol described by Ihrmark et al. (2012). For the AMF, a semi-nested PCR approach was employed targeting the SSU region of the 18S rRNA gene. The Glomeromycota—specific AML1–AML2 primer pair (Lee et al. 2008) was used initially, followed by WANDA—AML2 (Dumbrell et al. 2011). For all PCR amplifications the Q5® High—Fidelity DNA Polymerase (NEB, Ipswich, Massachusetts, USA) was used. All samples were initially amplified (28 cycles), using the domain-specific primers mentioned above, followed by a PCR (7 cycles), using primers carrying sample—associated indices for performing the multiplex sequencing. The 16S rRNA gene and the ITS region amplicons were sequenced via HiSeq Illumina Rapid Mode generating 2 × 250 bp paired-end reads and the 18S rRNA amplicons of AMF were sequenced via Illumina Miseq generating 2 × 300 bp paired-end reads. The raw, demultiplexed sequences were submitted to NCBI under the BioProjects PRJNA751232, PRJNA751394, PRJNA751395, PRJNA751397, PRJNA751399 and PRJNA751400.

The retrieved sequences were de-multiplexed with Flexbar v3.0 (Dodt et al. 2012). The demultiplexed paired—end sequences were



Development Stages[a]	Code Names of Development Stages	Koroneiki (>50yold)	Chondrolia (>20yold)
1 (stage 60: Flowering - first flowers open)	**A**	April	March - April
2 (stage 65: Flowering - full flowering: at least 50% of flowers open)	**B**	Early May	Late May
3 (stage 68: Flowering - majority of petals fallen or faded)	**C**	Late May	June
4 (stage 79: Fruit development - fruit about 90% of final size: fruit suitable for picking green)	**D**	September	September
5 (stage 92: Senescence – overripe: fruits lose turgidity and start to fall)	**E**	November	November

[a] Developmental stages according to Sanz – Cortés et al. (2002)

Figure 1. Description of the samplings for the two emblematic varieties 'Koroneiki' and 'Chondrolia' based on the developmental stages.

further processed (primers removal, filtering and denoising sequences, removing chimaeras, merging reads) with DADA2 (Callahan et al. 2016) package, and amplicon sequence variants (ASVs) were constructed. Taxonomic affiliation for prokaryotes was performed against the Silva v128 database small ribosomal subunit database (Yilmaz et al. 2014) and for fungal community against the UNITE database (Nilsson et al. 2019). Sequences classified in non-target taxa (e.g. NAs at Kingdom level, Eukaryota, chloroplasts and mitochondria for the 16S rRNA gene and unknown or protists for the ITS) were removed.

For AMF, the demultiplexed paired—end sequences were analyzed with Mothur v1.42.1 (Schloss et al. 2009). Contigs were constructed with 20 bp minimum overlap and those of 500 to 550 bp length were kept for further analysis. No ambiguous bases were allowed. The sequences were aligned against the Silva Eukaryotic v132 (Pruesse et al. 2012; Quast et al. 2012; Yilmaz et al. 2014) with the default parameters. Misaligned sequences, sequences with more than 1% pairwise differences and singletons were removed from the analysis and chimeras were removed using UCHIME v4.2 approach (Edgar et al. 2011). Remaining sequences were clustered (average neighbor clustering, mothur.org/wiki/cluster) at 0.03 sequence distance to operational taxonomic units (OTU). Representative sequences of the OTUs were classified against the MaarjAM database (Öpik et al. 2010) using the RDP classifier (Wang et al. 2007). Prokaryotic and AMF phylogenetic trees were constructed in QIIME2 (Bolyen et al. 2019) with the *fasttree* lgorithm (Price et al. 2010) under a CAT-like rate category approximation (Stamatakis et al. 2005, Stamatakis 2006).

Statistical analysis of microbial diversity data

All analysis, on statistics and diversity, were carried out in R (R Core Team 2022). A total of 6.349.142, 4.006.604, and 1.383.390 sequence paired-end reads were obtained and successfully assembled for the prokaryotic, fungal and AMF community respectively. Further quality control removed low quality reads and chimeras, keeping 3.461.006, 3.219.311, and 439.574 reads, for prokaryotic, fungal and AMF community, which were binned into 18751 prokaryotic ASVs, 6154 fungal ASVs and 233 AM fungal OTUs. Sequencing effort, assessed by rarefaction curve analysis using the R package iNEXT (Hsieh et al. 2016), confirmed that prokaryotic, fungal and AMF diversity was sufficiently captured for all plant compartments, location/variety (a variable used to denote their combined effect for each variety in its respective location) and development stages (Figure S1, Supporting Information). Normalization was performed to standardize the for-sequencing depth among the samples based on proportioning (relative abundance of ASV/OTU). Following, ASVs/OTUs with relative abundance > 0.1% in at least 3 samples were included for downstream analysis, resulting in 4292 prokaryotic ASVs, 1829 fungal ASVs and 122 AM fungal OTUs. The ASVs matrices of bacteria/archaea and fungi, and the OTU matrix of AMF were used to assess the impact of plant compartment and developmental stage on the alpha- and beta- diversity in the two olive cultivars. The alpha—diversity indices for community richness (observed ASVs or OTUs), diversity (Shannon) and phylogenetic diversity (Faith's PD), were determined with the *phyloseq* package (McMurdie and Holmes 2013). The Kruskal-Wallis test ($P < 0.05$) was used to investigate the effects of the plant compartment and the developmental stages on alpha—diversity indices followed by Dunn's test of multiple comparisons.

Differences of the beta–diversity of prokaryotes, fungi and AMF according to plant compartment and developmental stage were visualized with non-metric Multidimensional Scaling (NMDS) based on the Bray-Curtis dissimilarity metric. Permutational multivariate analysis of variance (PERMANOVA; adonis function, in the vegan package with 999 permutations, Wagner 2018) was applied to assess the effect of plant compartment and developmental stage on the beta–diversity in the two olive cultivars separately. Analysis of Similarity (ANOSIM) was applied on the Bray-Curtis dissimilarity matrix to assess the relative similarity of prokaryotic, general fungal and AMF communities, of the different plant compartment between the two varieties/locations.

Identifying the shared microbiome is sensitive to the abundance and prevalence thresholds (Salonen et al. 2012). In the present study, we filtered microbial taxa (for each community and cultivar) and retained those occurring in at least 70% of the samples in each plant—associated compartment at a consistent detection threshold of 0.1% relative abundance to construct shared microbiome estimates. All plots and diagrams were generated in R with the packages *microeco* (Liu et al. 2021), *ggplot2* (Wickham 2016) and *ggpubr* (Kassambara et al. 2020). For the constructed shared pie charts, the mean relative abundance of the shared taxa at the genus level was used to construct Tables S3 and S4.

Results

Effect of plant compartment and developmental stage

Prokaryotic microbiome

Prokaryotic microbial communities at rhizospheric soil (hereafter referred as 'soil') and roots showed higher alpha- diversity metrics compared to above ground plant compartments throughout the study period (Fig. S2) for both varieties in their respective locations. We observed no significant effects of the developmental stage on the alpha-diversity of prokaryotes in soil and plant roots for the two varieties ($P > 0.05$) (Fig. S2). Regarding the phyllosphere, significant differences were evident only in the samples of Chondrolia; Observed, Shannon and Faith PD indices significantly increased at developmental stages C and D compared to the earlier developmental stages (Fig. S2B). Similarly, a statistically significant effect of developmental stage was observed in the carposphere of Chondrolia only, where significantly higher Shannon diversity index values ($P < 0.05$) were noted at earlier developmental stages (C, D) compared to the final harvest stage () (Fig. S2B).

clear separation of samples in an above and belowground pattern was evident for the prokaryotic microbial community in both varieties in their respective locations (Fig. 2A). PERMANOVA based on the Bray–Curtis dissimilarity metric showed that plant compartment had the highest effect among the main factors on the beta-diversity of the prokaryotic communities for all samples ($R^2 = 0.27$, $P < 0.001$; Table S5) and when examined the two varieties separately (Koroneiki: $R^2 = 0.4$, $P < 0.001$ and Chondrolia: $R^2 = 0.41$, $P < 0.001$; Suppl. Table S6). In soil and roots the prokaryotic microbial communities were highly dissimilar (Soil: $R_{ANOSIM} = 0.95$, $P = 0.001$; Roots: $R_{ANOSIM} = 0.87$, $P = 0.001$) between the two varieties/locations, while the different location/variety explained the larger fraction of the community's variation (Table 1). At the aboveground compartments (leaves, fruits and flowers), the prokaryotic microbial communities were similar among the two varieties/locations (Leaves: $R_{ANOSIM} = 0.058$, $P = 0.15$; Flowers: $R_{ANOSIM} = 0.13$, $P = 0.17$; Fruits: $R_{ANOSIM} = 0.053$, $P = 0.24$) and the location/variety explained a relatively minor fraction of the community's variation (Table 1). We further explored the effect of the developmental stage of the olive tree on the prokaryotic microbial communities, separately for each plant compartment and each location/variety, and found a significant effect on beta-diversity in the soil (Koroneiki: $R^2 = 0.33$, $P < 0.01$, Chondrolia: ($R^2 = 0.235$, $P < 0.001$), phyllosphere (Koroneiki: $R^2 = 0.35$, $P < 0.001$, Chondrolia: ($R^2 = 0.34$, $p< 0.001$) and carposphere (Koroneiki: $R^2 = 0.46$, $P < 0.001$, Chondrolia: ($R^2 = 0.3$, $P < 0.001$) of the two studied varieties (Suppl. Fig. S3). On the contrary, no significant effect was observed in the root samples for both varieties (Koroneiki: $R^2 = 0.20$, $P > 0.05$, Chondrolia: ($R^2 = 0.17$, $P > 0.05$; Suppl. Fig. S3), indicating stable prokaryotic communities over time.

Fungal microbiome

Alpha-diversity metrics for the fungal communities were higher for soil and roots compared to above ground plant compartments throughout the study period (Fig. S4) for the samples of Koroneiki. The developmental stage differentiated ($P < 0.05$ increased) the number of observed ASVs only for Koroneiki's soil and did not affect the alpha-diversity of the fungal community in Chondrolia soil and the roots in both varieties in their respective locations ($P > 0.05$). In the phyllosphere, except from the Shannon diversity index for Koroneiki, the Observed ASVs for Koroneiki and both indexes of Chondrolia showed a significant increase along plant development ($P < 0.001$) (Fig. S4A and B). Fungal communities of the carposphere were not differentiated ($P > 0.05$) along plant development in both varieties/locations (Fig. S4A and B).

NMDS analysis of the fungal community showed a clear separation of samples according to the plant compartment, similar to that of the prokaryotic communities, with the below ground samples (soil and root) clustering separate from the above ground (phyllosphere and carposphere) samples but also separate between the different varieties/locations (Fig. 2B). The significant differences in the fungal communities between above and belowground plant parts was verified by PERMANOVA analysis based on the Bray–Curtis dissimilarity metric, for all samples ($R^2 = 0.22$, $P < 0.001$; Table S5) and for both varieties separately (Koroneiki: $R^2 = 0.46$, $P < 0.001$ and Chondrolia: $R^2 = 0.36$, $P < 0.001$; Table S6). PERMANOVA and ANOSIM analysis, based on the Bray–Curtis dissimilarity metric, further confirmed the separate clustering between the two varieties/locations for all plant compartments, where the fungal communities were highly dissimilar (Soil: $R_{ANOSIM} = 0.99$, $P = 0.001$; Roots: $R_{ANOSIM} = 0.88$, $P = 0.001$; Leaves: $R_{ANOSIM} = 0.37$, $P = 0.001$; Flowers: $R_{ANOSIM} = 0.77$, $P = 0.003$; Fruits: $R_{ANOSIM} = 0.20$, $P = 0.01$) and the different location/variety explained the larger fraction of the community's variation (Table 1). Finally, the plant developmental stage had a significant effect on the beta-diversity of the fungal community in the rhizospheric soil (Koroneiki: $R^2 = 0.338$, $P < 0.001$, Chondrolia: $R^2 = 0.245$, $P < 0.001$), phyllosphere (Koroneiki: $R^2 = 0.35$, $P < 0.001$, Chondrolia: $R^2 = 0.34$, $P < 0.001$) and carposphere (Koroneiki: $R^2 = 0.43$, $P < 0.001$, Chondrolia: $R^2 = 0.43$, $P < 0.001$) of the two studied varieties (Fig. S5). On the contrary, the root tissue samples of both varieties hosted a rather stable fungal community throughout the sampling period (Koroneiki: $R^2 = 0.21$, $P > 0.05$, Chondrolia: $R^2 = 0.14$, $P > 0.05$; Fig. S5).

AMF community

Rhizospheric AMF communities were richer and more diverse compared to the intraradical communities, throughout the study period for both varieties in their respective locations (Fig. S6). The plant developmental stage had no effect on the alpha-diversity of AMF in the soil and plant roots of both varieties, with the sole exception of the Observed species richness, which showed a significant increase ($P < 0.001$) along the summer period in the rhizosphere samples of Koroneiki (Fig. S6).

NMDS visualization clearly separated the rhizospheric soil and intraradical AMF communities of samples and revealed that while the rhizospheric communities where distinct between the two varieties/locations, the intraradical highly overlapped (Fig. 2C). Plant compartment (soil and roots) was the major determinant for the AMF communities' variations for all samples ($R^2 = 0.20$, $P < 0.001$; Table S5) and for each location/variety (Koroneiki: $R^2 = 0.18$, $P < 0.001$ and Chondrolia: $R^2 = 0.29$, $P < 0.001$; Table S6). When examined within each plant compartment, PERMANOVA and ANOSIM analysis based on the Bray-Curtis dissimilarity metric, confirmed that AMF communities in the rhizosperic soil were highly dissimilar between the varieties/locations (Soil: $R_{ANOSIM} = 0.55$, $P = 0.001$), whereas in the roots were highly similar (Roots: $R_{ANOSIM} = 0.05$, $P = 0.15$). The location/variety variable explained the larger fraction of the community's variation in the rhizospheric soil compared to roots (Table 1). The plant developmental stage had a significant effect only for the rhizospheric AMF community of the Koroneiki variety (Soil Koroneiki: $R^2 = 0.30$, $P < 0.01$), while no significant effect was recorder for the root community variation or for the Chondrolia samples (Soil Chondrolia: $R^2 = 0.17$, $P > 0.05$, Root Koroneiki: $R^2 = 0.47$, $P > 0.05$, Root Chondrolia: $R^2 = 0.17$, $P > 0.05$; Fig. S7).

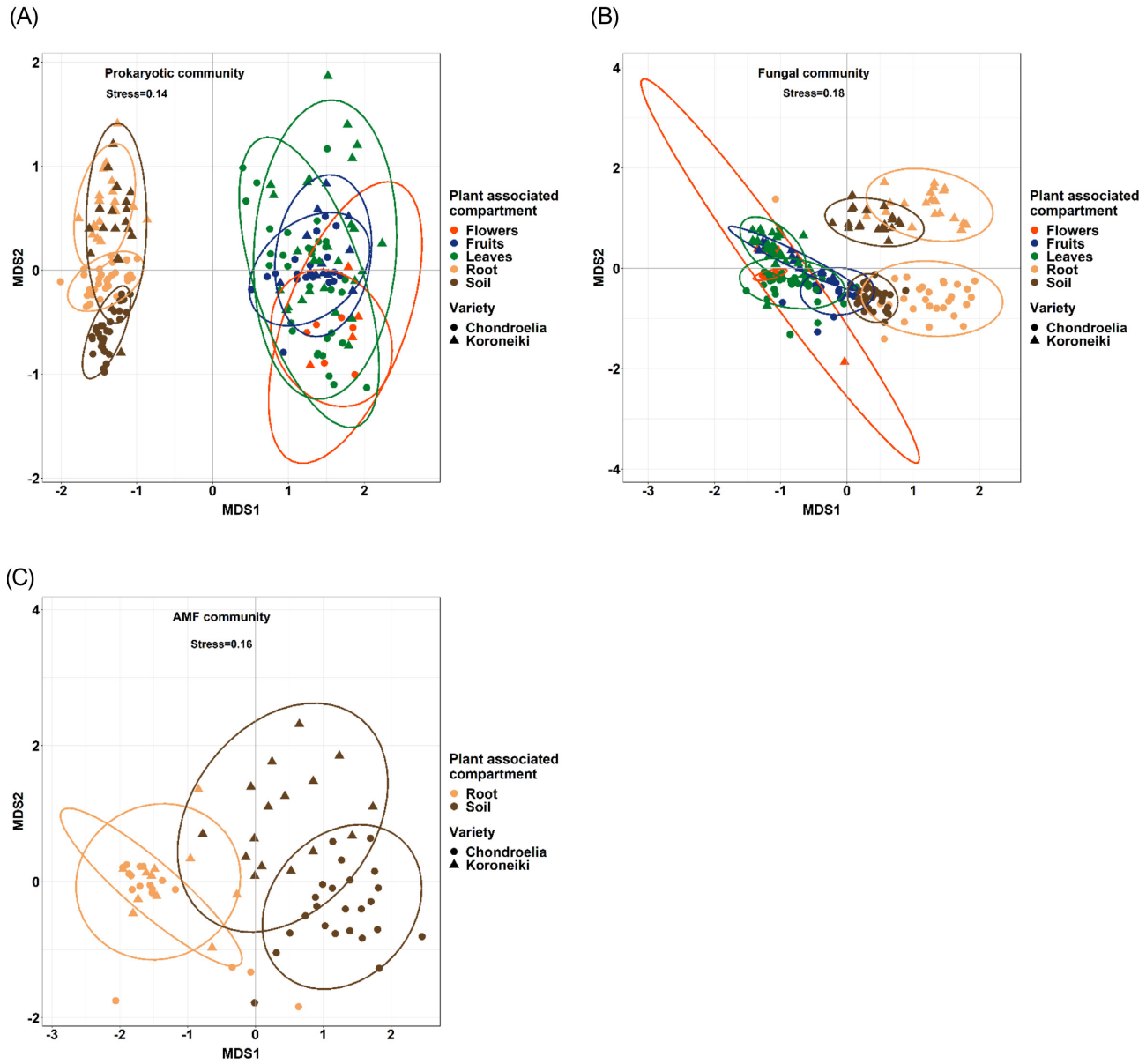


Figure 2. Nonmetric multidimensional scaling (NMDS) ordination plots based on the Bray–Curtis dissimilarly metric for the prokaryotic community **(A)**, the fungal community **(B)** and the arbuscular mycorrhizal fungi (AMF) **(C)** in the olive varieties Koroneiki and Chondrolia across the different plant compartments. Ellipses represent the 95% confidence interval around the group's centroid.

Microbial community composition and shared microbiome

Prokaryotic microbiome

The composition of the prokaryotic microbiome in the different plant compartments at all developmental stages in the two studied varieties at their respective locations was dominated by *gamma-Proteobacteria*, *Actinobacteria*, *Bacilli*, *alpha-Proteobacteria*, *Bacteroidia*, and *Thermoleophilia* (Fig. 4A). Specifically, the epiphytic bacterial communities were dominated by *αlpha-* and *gamma-Proteobacteria* (adding up to a RA> 50%), while the root communities were dominated by *Actinobacteria*. When looking at microbial composition at different developmental stages, we noted dominance of *gamma-Proteobacteria* throughout the season in the phyllosphere and carposphere of both varieties, with the exception of the young plant leaves (stage A) of the Koroneiki variety where *alpha-Proteobacteria* dominated. Within the prokaryotic microbiome, archaea contributed with a low number of reads in all aboveground plant- associated compartments (0.1% phyllosphere, 0.16% carposhere), but showed higher RA for both cultivars in roots and especially in the rhizospheric soil (1.41% roots, 4.37% rhizospheric soil). *Nitrososphaeria* were the most dominant members of the archaeal community, showing increasing abundance in the rhizospheric soil and roots of Chondrolia (Fig. 4A).

We then investigated the community members that were shared in all plant-associated compartments within each variety in its respective location independently. In total and for the Koroneiki variety, 114 out of the 3130 ASVs were shared in all plant-associated compartments (Table S7A) while 16 ASVs had relative abundance >1% at least in one plant-associated compartment and belonged to classes *alpha*—and *gamma*—*Proteobacteria, Actinobacteria,* and *Bacilli* (Table S7B). For the Chondrolia variety, 310 out of the 3550 ASVs were shared in all plant-associated compartments (Suppl. Table S8A) and 16 ASVs had relative abun-

Table 1. PERMANOVA results based on Bray–Curtis dissimilarities for prokaryotic, fungal, and AMF communities for each plant compartment (soil, root, leaves, fruits, and flowers) to investigate the effect of different location/variety and development stage.

Soil						
Factor	Prokaryotic community R^2	Sig	Fungal community R^2	Sig	AMF community R^2	Sig
Location/Variety	0.30	***	0.33	***	0.12	***
Development stage	0.11	**	0.098	***	0.10	*
Interaction	0.084	ns	0.093	***	0.11	**
Residual	0.51		0.48		0.67	
Root						
Location/Variety	0.25	***	0.21	***	0.06	ns
Development stage	0.076	ns	0.068	ns	0.072	ns
Interaction	0.064	ns	0.064	ns	0.22	ns
Residual	0.61		0.65		0.65	
Leaves						
Location/Variety	0.053	***	0.21	***		
Development stage	0.21	***	0.15	***		
Interaction	0.11	***	0.14	***		
Residual	0.62		0.49			
Fruits						
Location/Variety	0.087	***	0.31	***		
Development stage	0.12	**	0.07	ns		
Interaction	0.15	***	0.075	ns		
Residual	0.65		0.55			
Flowers *(exist in only one stage)*						
Location/Variety	0.14	ns	0.47	**		
Residual	0.86		0.53			

Sig: *P*-values based on 999 permutations, ns $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

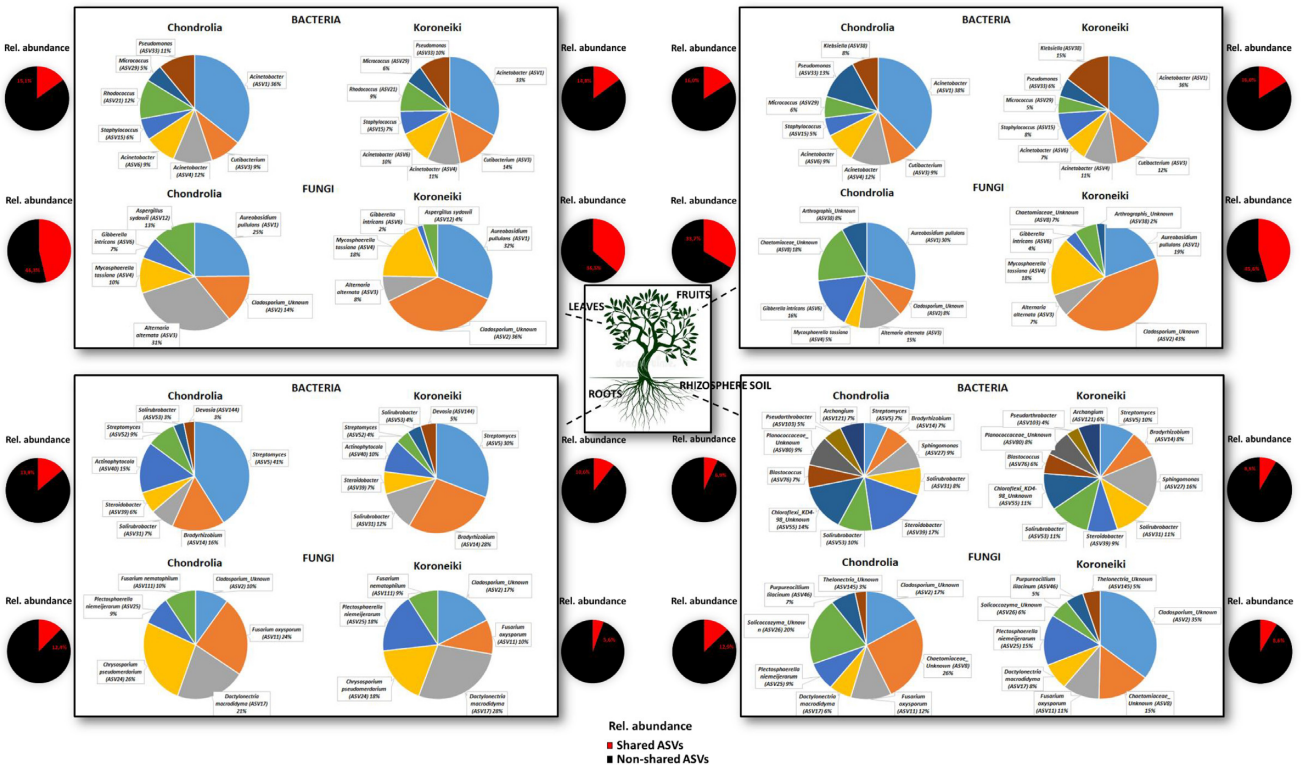


Figure 3. Shared microbiome pie charts illustrating the relative abundance of bacterial and fungal shared ASVs and their taxonomy (taxa represented in at least 70% of all samples) at each plant-associated compartment (soil, root, leaves, fruits) of olive varieties, Koroneiki, and Chondrolia. The phylogenetic assignment of the ASVs presented is given in Tables S3 (prokaryotic ASVs) and S4 (fungal ASVs).

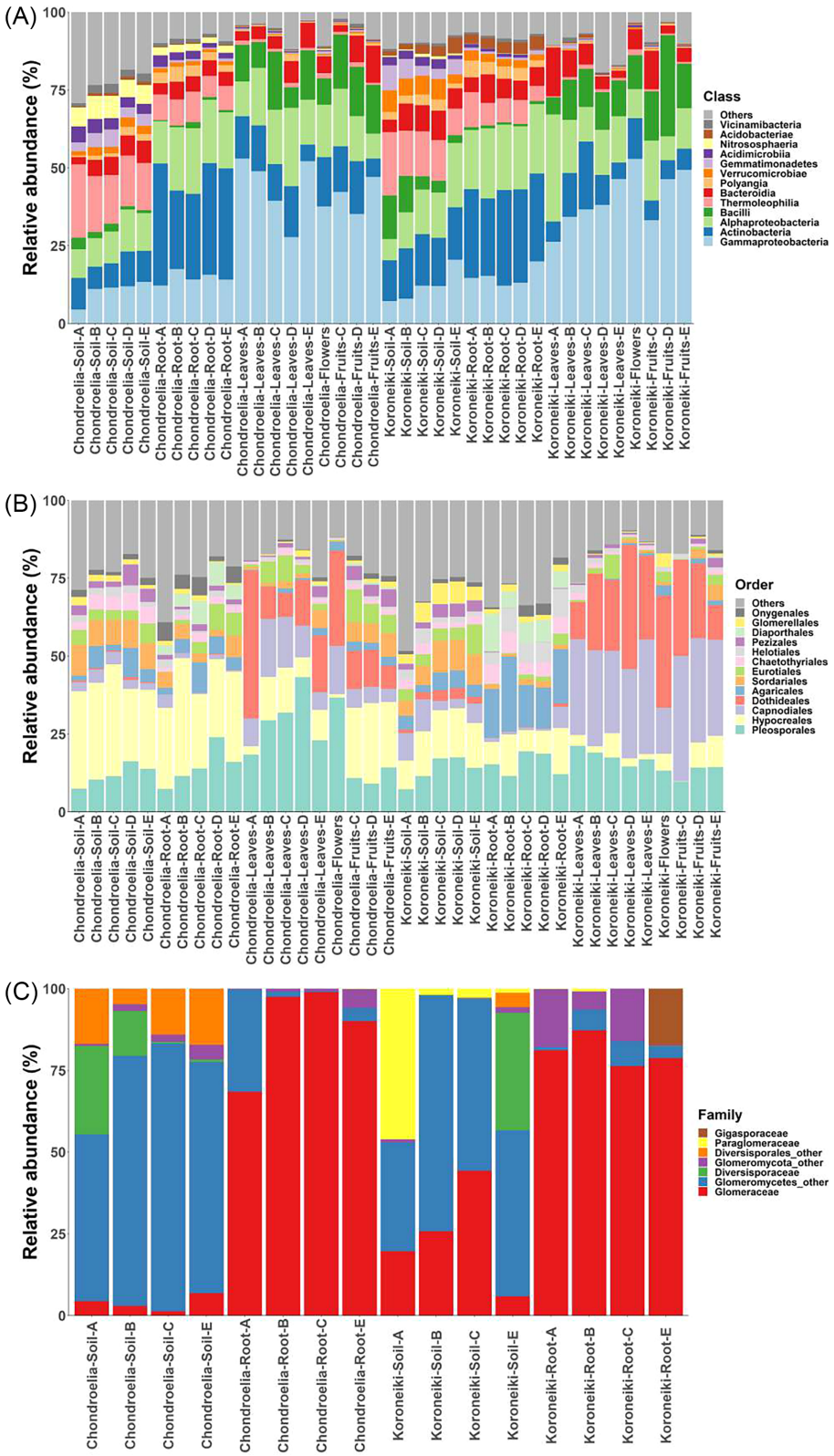


Figure 4. Stacked bar plots presenting the average relative abundance (%) of **(A)** the top thirteen prokaryotic classes, **(B)** the top thirteen fungal orders and **(C)** all arbuscular mycorrhiza fungal (AMF) families detected in the different plant-associated compartments of the olive varieties Koroneiki and Chondrolia. For each variety, the plots show all the habitats in the following order, first the roots, and then the soil samples. For each plant-associated compartment the samples are in order according to the development stage of the olive tree as they are described in Fig. 1.

dance >1% at least in one plant-associated compartment and belonged to classes *alpha*—and *gamma*—*Proteobacteria, Actinobacteria, Thermoleophilia* and the archaeon class *Nitrososphaeria* (Table S8B). These abundant shared ASVs account for the 7.72%, 11.65%, 19.1%, and 20.22% of the total prokaryotic community in the rhizosphere soil, roots, phyllosphere and carposphere of the Koroneiki variety and 8.76%, 17.46%, 15.72%, and 12.81% of the total community in the soil, roots, phyllosphere, and carposphere of the Chondrolia variety (Tables S7B and S8B).

We aimed to identify prokaryotes that could potentially be part of a shared prokaryotic microbiome of the olive trees (shared by the two varieties in their respective locations). The most dominant ASVs in the rhizospheric soil and root compartments belonged to (i) *Actinobacteria*, more specifically to the classes of *Actinobacteria* and *Thermoleophilia* and (ii) *Proteobacteria* and specifically to *alpha*—and *gamma*—*Proteobacteria* (Fig. 4). These ASVs were present in more than 80% of rhizospheric soil and roots samples of the two varieties (Fig. 3; Table S3). Five bacterial ASVs in the shared root microbiome belonged to *Streptomyces, Bradyrhizobium, Solirubrobacter* and *Steroidobacter* and these were also the ASVs in the shared rhizospheric soil microbiome (Table S3). Likewise, ASVs assigned to *gamma*—*Proteobacteria* (genus *Acinetobacter* and *Pseudomonas*) and *Actinobacteria* (genus *Cutibacterium* and *Micrococcus*) constituted the main members of the shared microbiome of the phyllosphere and carposphere, being presented in > 70% of the leaves and fruits samples of both varieties and shared between the two above-ground plant parts (Fig. 3; Table S3).

Fungal microbiome

The epiphytic and below-ground community of fungi in both varieties in their respective locations was dominated by *Ascomycota* (mainly of the orders *Dothideales*, *Hypocreales*, *Pleosporales*, *Capnodiales*), while *Basiodiomycota* (mainly of the order *Agaricales*) were detected at lower abundance (Fig. 4B). When looking at the above-ground plant parts, the phyllosphere and carposphere of Koroneiki was dominated throughout the sampling period by *Dothideales* and *Capnodiales*, while, in Chondrolia, the *Dothideales* prevalence was accompanied by *Pleosporales* and *Hypocreales*. In the root and rhizospheric soil the fungal community in both varieties was dominated primarily by *Hypocreales* and *Pleosporales*.

Shared fungal members in all plant-associated compartments for the Koroneiki variety (Table S9A) were 110 out of the 1387 ASVs and 292 out of the 1217 ASVs for the Chondrolia variety (Table S10A). From them, 24 ASVs had relative abundance >1% at least in one plant-associated compartment for Koroneiki variety, and belonged mainly to *Dothideales*, *Pleosporales, Hypocreales*, and *Diaporthales* orders (Table S10B) and 41 ASVs, for Chondrolia variety, had relative abundance >1% at least in one plant-associated compartment and belonged mainly to orders *Pleosporales, Hypocreales* and *Diaporthales* (Suppl. Table S8B). These abundant shared ASVs account for the 17.17%, 16.28%, 62.23%, and 69.71% of the total fungal community in the rhizosphere, roots, phyllosphere and carposphere of the Koroneiki variety and 45.85%, 51.66%, 61.23%, and 51.77% of the total community in the rhizosphere, roots, phyllosphere and carposphere of the Chondrolia variety (Table S7B and S8B).

We further determined the main members of the fungal microbiome shared by the two olive varieties in their respective locations. Four ASVs, all belonging to different genera of Ascomycota (*Cladosporium, Fusarium, Dactylonectria*, and *Plectosphaerella*) were present commonly in the rhizospheric soil and root samples from the two varieties (Fig. 3; Table S4). When looking at the above ground plant parts, the six shared ASVs belong again to Ascomycota, albeit to different genera: *Aureobasidium, Cladosporium, Alternaria, Mycosphaerella, Gibberella*, and *Arthrographis* (Fig. 3; Table S4).

Arbuscular mycorrhizal fungal community

The rhizospheric AMF communities in both studied varieties in their respective locations were dominated by members of the family *Glomeraceae* and unclassified *Glomeromycetes* (Fig. 4C). Members of *Diversisporaceae* and unclassified *Diversisporales* were common inhabitants of the rhizospheric soil of the variety Chondrolia, while *Paraglomeraceae* were specifically observed in the rhizospheric soil samples of the variety Koroneiki. In contrast, the intraradical AMF community in both cultivars encompassed members of *Glomeraceae* and unclassified *Glomeromycota* (mostly in Koroneiki), while members of the family *Gigasporaceae* were detected in the root samples of the variety Koroneiki at the later developmental stage (Fig. 4C).

Rhizospheric soil and roots shared 19 out of the 80 AMF OTUs for the Koroneiki variety (Table S12A) and 7 out of the 94 AMF OTUs for the Chondrolia variety (Table S12A). Ten OTUs of the shared ones for the Koroneiki variety and 4 OTUs for the Chondrolia variety had relative abundance >1% at least in one plant-associated compartment, and belonged to the order *Glomerales*, *Diversiporales* and to unclassified *Glomeromycetes* (Table S11B), or to the order *Glomerales* and to unclassified *Glomeromycetes* (Table S12B), respectively. These abundant shared OTUs account for the 22.1% and 71.5% of the total AMF community in the rhizosphere and the roots of the Koroneiki variety and 3.56% and 94.79% of the total AMF community in the rhizosphere and the roots of the Chondrolia variety (Tables S7B and S8B). Furthermore, the two varieties shared 40 of the OTUs among their rhizospheric soil samples while this was reduced to 14 for the intraradical AMF community.

Discussion

In the present study, the prokaryotic, fungal and AMF diversity associated with the rhizospheric soil, root, leaf and fruit tissues of olive trees were investigated in two varieties (Koroneiki and Chondrolia) grown at the geographical regions of their traditional cultivation, at specific developmental stages, coinciding with the seasonal progression from early spring to early winter. This experimental setup allowed us to follow changes in the plant microbiome in its different compartments that may exhibit variable seasonal/developmental patterns, and to identify shared taxons that may contribute to a plant microbiome shared by the two olive varieties.

Effects of plant compartment and developmental stage on the diversity of prokaryotes, fungi and AMF on olive trees

It is hard to disentangle plant developmental stage effects from environment-derived factors and from their combined effects (plant phenology). We, therefore, discuss seasonal patterns that correspond to a sequence of plant developmental stages but do not necessarily derive *per se* from them. Alpha-diversity in the phyllosphere and carposphere generally increased along the growing season, in contrast to rhizospheric soil and roots, where prokaryotic and fungal alpha-diversity was stable throughout the sampling period. In line with our findings, Xiong et al. (2021) showed that the maize phylloplane microbiome diversity exhibited strong seasonal patterns along the growing season compared

to the seasonally stable soil microbiome. This might reflect the increasing immigration of airborne microbial inocula on the expanding interface surfaces of above-ground plant parts. This hypothesis is supported by previous studies in similar semi-arid Mediterranean ecosystems, which reported a significantly higher abundance and alpha-diversity of bacteria (Vokou et al. 2019) and higher abundance of classic airborne fungi like *Cladosporium* and *Alternaria* (Katsoula et al. 2021) on the phyllosphere of native plants during the summer period. The stronger seasonal patterns of the alpha-diversity of prokaryotes and fungi in the above-ground plant parts could also be associated with the higher exposure of the phyllosphere and carposphere microbiome to seasonal fluctuations (UV radiation, temperature, drought/humidity) that are characteristic of such plant niches (Vorholt 2012).

We further explored the composition of prokaryotes and fungi in the different plant compartments. We noted that in both olive varieties, studied in their respective locations, below- and above-ground compartments supported distinct prokaryotic and fungal microbiomes. This has been reported before for other crop plants (e.g. sugarcane, Hammonts et al. 2018). Accordingly, Martins et al. (2016) showed that plant organs were strong determinants of the composition of the fungal community in three olive cultivars in Portugal. The physiological, chemical and nutritional changes in the relevant organs of olive plants (leaves, flowers and fruits) (Sahin et al. 2012, Stateras and Moustakas 2018, Wang et al. 2019), and the changes in environmental conditions that occur during the growing season in Mediterranean semi-arid ecosystems, where olive groves are established, are expected to affect the capacity of microorganisms to colonize those plant microhabitats (Copeland et al. 2015, Katsoula et al. 2021). Similarly, seasonal variation on abiotic characteristics of the soil environment have also been related to microbial composition changes (Fierer 2017, Anguita-Maeso et al. 2020). Moreover, the observed temporal changes in the composition of the prokaryotic and fungal communities in the rhizospheric soil of olive trees could reflect changes in the composition of the root exudates in the different developmental stages of olive tree plants, as suggested for several other crops (Chapparo et al. 2014; Micallef et al. 2009). The rhizospheric soil is indeed a more substrate-driven environment (Zhalnina et al. 2018) but specific studies on the role of root exudation of olive plants are still lacking. In contrast to rhizospheric soil and above-ground plant compartments, the root microbiome for both olive cultivars was stable throughout the sampling period. We speculate that the root system of such long-lived plants, like the mature olive trees examined in this study, may accommodate established microbiomes, with members often linked mutualistically to their hosts. Moreover, as endophytes are less exposed to external perturbations (i.e. climatic fluctuations during the season, management practices) compared to the epiphytic plant compartments (Fernandez-Gonzalez et al. 2019), they are expected to be less responsive to seasonal changes. In support of this hypothesis, Gomes et al. (2018) showed that the endophytic fungal microbiome of olive leaves was stable and less responsive to seasonal fluctuations compared to the corresponding epiphytic microbiome. However, comparisons including more soil environments and olive tree varieties and extreme climatic conditions are needed to investigate factors that may limit this stability.

We expanded our analysis to AM fungi specifically and noted that intraradical AMF communities were rather stable across the different developmental stage within both studied cultivars. Montes-Borrego et al. (2014) proposed that the AMF assemblage structure and composition in olive trees is influenced primarily by soil type and climate, including rainfall and temperature. However, the AMF community varied through the season in the rhizopspheric soil, mainly in the variety Koroneiki. The soil is a dynamic environment where AMF may be more directly affected by seasonality compared to the more stable host root environment which also presents minimal antagonism by other microbes for AMF.

The composition of the plant microbiome

The epiphytic prokaryotic community on the phyllosphere and carposphere of the two olive cultivars in their respective locations was dominated by *Proteobacteria* and *Actinobacteria*. This is in accordance with earlier studies on olive phyllosphere (Müller et al. 2015, Fausto et al. 2018, Mina et al. 2020); however, carposphere microbial dynamics remain as yet largely unknown. The rhizospheric soil and root bacteriome was dominated by *Actinobacteria* followed by *gamma*—and *alpha*—*Proteobacteria* and *Bacilli*, (Fig. 4A) in line with previous studies (Fausto et al. 2018, Fernandez-Gonzalez et al. 2019). Archaea were under-represented in all studied compartments, although a clear trend of higher abundance in soil and lower abundance in above-ground plant parts was noted. This seems to be a common observation in previous studies that investigate the composition of the prokaryotic microbiome of olive trees (Fausto et al. 2019) and, in general, of other plants as well (Tafner et al. 2019, Borrel et al. 2020). An interesting observation was the significantly higher abundance of *Nitrososphaeria* in the rhizospheric soil and roots of Chondrolia compared to Koroneiki. *Nitrososphaeria* are common soil ammonia-oxidizing archaea (Alves et al. 2018), being hallmark organisms in soil N cycling (Kuypers et al. 2018). Their presence in the soil of other olive cultivars has been reported before (Cáliz et al. 2015), while Muller et al. (2015) identified a thaumarchaeal candidate genus *Nitrososphaera* as member of the core microbiome of the endosphere of 10 olive varieties. Their specific presence in Chondrolia could be associated with the lower soil pH, but this warrants further research.

We further identified prokaryotic microbes shared by the two studied olive varieties. These encompassed bacteria with known plant beneficial functional traits like (i) *Streptomyces* spp., which carry a versatile biosynthetic machinery of antimicrobials that could protect plants from pathogenic organisms (Viaene et al. 2016) and known for their potential use as plant growth promoters (Palaniyandi et al. 2014, Qin et al. 2017), (ii) *Bradyrhizobium* and *Devosia,* known as N_2-fixing bacteria and root endophytes of various plants (Rouws et al. 2014, Dong et al. 2018), although their functional role in olive roots remains unknown, (iii) *Steroidobacter*, a common rhizospheric soil bacterium and root endophyte that has been associated with beneficial plant traits like promotion of stem, and root elongation or stress protection (Zarraonaindia et al. 2015), (iv) *Actinophytocola*, a root endophyte of olive plants (Fernández-González et al. 2019), exhibiting antimicrobial activities against several bacteria and fungi including human pathogens (Malek et al. 2015, Chaouch et al. 2021) and (v) *Solirubrobacter*, an Actinobacterium which often prevail in the rhizosphere and roots of plants grown in organically managed and undisturbed ecosystems (Calleja-Cervantes et al. 2017). Fernández-González et al. (2019) analysed the microbiome of 36 olive varieties and identified *Actinophytocola*, *Streptomyces*, *Bradyrhizobium*, and *Steroidobacter* as major members of the root endophytic core microbiome reinforcing their potential role in the fitness of olive plants. Our data derived from rhizospheric soil and root microbiome are in accordance with their findings. When looking at the shared bacterial taxa of the above-ground plant parts, carposphere and phyllosphere shared most of the domi-

nant members like: (i) *Acinetobacter* and *Pseudomonas*, common encounters in the core microbiome of several other crops (Dong et al. 2019), (ii) *Cutibacterium* and *Staphylococcus* both encompassing important human pathogens, still commonly observed in the phyllosphere of urban and natural landscapes (Imperato et al. 2019, Katsoula et al. 2020), (iii) *Micrococcus*, plant growth promoters which suppress plant pathogens and increase plant tolerance to abiotic stress (Bringel and Couée 2015).

Olive trees supported a fungal community dominated by *Ascomycota* and *Basidiomycota*, in line with previous studies focusing on above- (Abdelfattah et al. 2015, Gomes et al. 2018, Materatski et al. 2019) and below-ground plant parts (Fernandez-Gonzalez et al. 2019). Above-and below-ground plant parts were dominated by *Dothideales, Capnodiales, Hypocreales* and *Pleosporales*, *Hypocreales, Agaricales,* in accord with previous studies (Abdelfattah et al. 2015, Gomes et al. 2018, Fernández-González et al. 2019).

The shared root fungi of the two varieties belong to *Cladosporium, Fusarium, Dactylonectria* and *Plectosphaerella* that seem to be ubiquitous in the olive trees (Abdelfattah et al. 2015, Gomes et al. 2018, Materatski et al. 2019, Costa et al. 2021). *Fusarium* strains could act as weak root pathogens in olive trees (Trabelsi et al. 2017) or biological control agents against *Verticillium* infecting olive trees (Mulero-Aparicio et al. 2019). *Dactylonectria* was identified as the causal agent of root rots in olive trees (Úrbez-Torres et al. 2012), while *Plectosphaerella* are common root endophytes of various plants (Caruso et al. 2020). Similarily to bacteria, the carposphere and the phyllosphere fungal communities of the two olive varieties shared several of the most abundant fungal taxa like (i) *Aureobasidium* and *Gibberella,* previously identified as members of the olive tree microbiome known to suppress anthracnose in olive trees (Preto et al. 2017, Nigro et al. 2018) and (ii) putative pathogens like *Alternaria* (Abdelfattah et al. 2015). Unlike bacteria, roots, leaves and fruits shared two of the most abundant ASVs belonging to *Cladosporium* encountered as endophytes or epiphytes in previous studies of the olive plant microbiome (Abdelfattah et al. 2015, Preto et al. 2017, Gomes et al. 2019).

We finally determined the composition of the AMF community both in the rhizospheric soil and the plant roots. The former compartment represents the main reservoir of AMF propagules available in the vicinity of plant roots, which exhibit a strong filtering effect on the AMF community. This is mirrored in the AMF diversity in the two compartments: soil supported a more diverse AMF community compared to the plant root, with the two compartments sharing only a few OTUs for Chondrolia and Koroneiki in their respective locations (7 and 19, respectively) (Table S11 and S12). While prokaryotic and general fungal communities were highly dissimilar in soil and roots (Fig. 2A and B), AMF communities were highly dissimilar in the soil only, suggesting a homogenizing filtering effect for AMF colonizers by the roots of the olive trees (Fig. 2C).

Our data showed that the prokaryotic microbial communities of the two different varieties/locations moved from distinct assemblages in belowground compartments to more homogenized ones in aboveground compartments (Fig. 2A). This may suggest a homogenizing filtering effect by above-ground plant biological surfaces/substrates; this should be viewed with caution since we did not directly assess dissimilarity in the source microbial communities, which may be airborne but are also expected to be affected by soil surface communities. Fungal communities also tended to be less similar in the above ground plant parts but again it is hard to conclude whether this is an effect of dissimilar source microbial communities (Fig. 2B). The two varieties, despite being grown in distant geographical areas, shared AMF OTUs belonging to *Glomeraceae*, in line with the generalist and r-strategy lifestyle of this taxon (Sýkorová et al. 2007, van der Heyde et al. 2017). Interestingly, members of the families *Paraglomeraceae* and the order *Diversisporales* were specifically associated with the rhizospheric soil of Koroneiki and Chondrolia respectively, but they did not appear to efficiently colonize plant roots, in line with the k-strategy lifestyle of these AMF taxa (Lumini et al. 2010; Gosling et al. 2016). Similar studies by Montes-Borrego et al. (2014) detected the same AMF families being present in olive rhizospheric soil and plant roots.

Conclusions

We provide a comprehensive analysis of the structure and dynamics of the microbiome of two landmark olive tree cultivars in Greece grown in the geographical regions of their traditional cultivation. To achieve that we determined the diversity of all microbial domains known to interact and affect the productivity of olive trees (prokaryotes, fungi, and AMF) in the different plant-associated compartments of olive trees at different developmental stages along a full fruit-bearing period. Prokaryotic and fungal communities shared common response patterns to the studied variables (plant compartment and developmental stage), a result consistent in both varieties. Above and below-plant parts supported distinct prokaryotic and fungal communities and showed seasonal patterns along the plant developmental stages studied, with plant roots being the sole compartment that hosted a seasonally stable microbial community. Although the two studied varieties were established in geographically distant regions, they shared members that have been reported to be part of an olive tree core microbiome. AMF soil communities showed a similar seasonal pattern, while a strong plant root filtering on the AMF soil communities, apparently operating at plant species rather than variety level, resulted in the assemblage of similar intraradical AMF communities in both varieties. Our findings support the notion that plant microbiome studies should consider the dynamics of all microbial players in different plant-associated compartments to disentangle complex plant—microbe interactions that determine microbiome composition and plasticity. Further studies will aim to unravel the functional potential of the olive tree microbiome through shotgun metagenomics and microbiome-driven isolation of members of persistent shared taxa.

Author contribution

CE, DGK, KK, NK, and KKP conceived and designed the study; MF, KL, KK, and NK collected all samples; MK, MF, NK, KL performed research; MK, MT, SV, KL, KK, CE, DGK, and KKP analyzed data; MK, MT, CE, DGK, and KKP wrote the paper with the contribution of all authors.

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Supplementary data

Supplementary data is available at *FEMSMC* online.

Conflict of interest statement. The authors declare that they have no conflict of interest.

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