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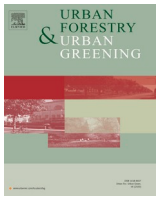

Effects of pavements on diversity and activity of mycorrhizal symbionts associated with urban trees

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ABSTRACT

This study investigated the impact of different types of soil sealing on the communities of a group of beneficial plant symbionts, arbuscular mycorrhizal fungi (AMF), colonizing the roots of two shade trees, *Celtis australis* and *Fraxinus ornus*, frequently grown in urbanized sites. Such plants were grown in an experimental site, in northern Italy, established in November 2011 and subjected to four different pavement treatments: impermeable monolithic asphalt, permeable pavers, permeable concrete and unpaved soil. The diversity and composition of root AMF communities were assessed by PCR denaturating gradient gel electrophoresis of partial 18S rRNA gene, AMF taxa were identified by amplicon sequencing and mycorrhizal colonization was evaluated after root clearing and staining. For the first time, our molecular work revealed that impermeable pavements induced shifts in the composition of AMF communities associated to the roots of *C. australis* and *F. ornus* and impacted on the percentage of mycorrhizal root length. When the root-zone was covered with permeable pavements, a similar AMF community as that observed in the unpaved soil was detected, providing novel information to be utilised for reducing the disturbance caused by specific types of soil sealing on AMF symbionts, which play a key role in plant nutrition and health. A total of 45 AMF sequence types were detected, with *Sclerocystis* and *Septoglomus* as the most abundant phylotypes, accounting for 84% of the sequences. The predominance of *Sclerocystis* species in the roots of both tree species under impermeable pavements indicated their high and unforeseen tolerance towards harsh environmental conditions. Such species could be utilized as AMF inocula specifically selected for their proven resilience in paved sites, in order to exploit their ability to boost biogeochemical processes fundamental for energy fluxes and plant nutrition and health.

1. Introduction

Soil sealing resulting from the covering of soil by manmade constructions currently represents a predominant form of soil degradation in urbanised countries. It influences soil water cycle, soil-gas migration, increases sensible heat producing the Urban Heat Island effect, and causes a depression of soil organic carbon (Asaeda and Ca, 2000; Milly et al., 2002; Scalenghe and Marsan, 2009). Moreover, soil sealing significantly decreases potential carbon mineralization rate, nitrification, soil respiration, and the activity, diversity and composition of beneficial soil microbial communities, impairing their resulting ecosystem services (Zaho et al., 2012; Wei et al., 2014; Piotrowska-Długosz and Charzyński, 2015; Hu et al., 2018, 2021; Pereira et al., 2021).

Yet, human activities cannot be carried out without implementing and maintaining hard surfaces and pavements, which can reach 67% of urban surface areas (Matthews et al., 2015). Recent investigations envisaged solutions inspired by and utilising nature (nature-based solutions), in order to pursue the overall objective of the highest use efficiency of natural resources in urban areas (Maes and Jacobs, 2015). Such an approach has been recently recognised also by the European Commission (2015), claiming that the re-establishment of natural water

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and biogeochemical cycles may represent a smart solution to enhance sustainability in degraded urban sites. One of the solutions proposed to heal the negative impact of pavements is the use of permeable pavements, consisting of either impermeable modular elements with voids which allow water infiltration and permeable concrete made of an even-graded inert bound by a permeable binder (Scholz and Grabowiecki, 2007). Such permeable pavements have been assessed for their technical characteristics, while few works investigated their impact on the underlying soil and the plants growing in sealed areas. Indeed some findings, indicating that tree decline was to be ascribed to drought or to a negative impact of impermeable pavements on root development, were not consistent with other results (Volder et al., 2014; Morgenroth and Buchan, 2009, 2011; Weltecke and Gaertig, 2012; Savi et al., 2015). A 4-year study comparing permeable pavements, permeable concrete and impermeable pavements showed that permeable concrete caused less intense alterations of soil water cycle, allowing higher infiltration and evaporation of water, higher CO_2 efflux rates and lower soil temperatures, compared with other soil cover types (Fini et al., 2017). No sign of stress induced by pavements was found on the tree species tested; slight declines in CO_2 assimilation and transpiration were found in *Fraxinus ornus* growing in impermeable pavements, compared with those growing in the unpaved soil, although soil water availability was lower in unpaved soil than under impermeable pavements (Fini et al., 2017). Successive studies carried out in the same experimental site showed altered soil moisture dynamics below impermeable pavements, which delayed both soil dehydration during dry periods and its rehydration after rainy events compared to other treatments (Fini et al., 2022). Root morphology was also affected by soil sealing, which caused root thickening and shortening in both *Celtis australis* and *F. ornus*. Nonetheless, no sign of stress to the photosynthetic apparatus or reduced growth were found over a 9-year monitoring period (Fini et al., 2022).

A major factor for plant performance and health is represented by root systems with well-developed arbuscular mycorrhizal (AM) symbioses, that are established by a group of soil beneficial fungi (AMF, Glomeromycota) with the roots of about 80% of land plants, including most food crops and shade trees (Smith and Read, 2008). AMF extraradical hyphae function as an auxiliary absorbing system, as they spread from colonized roots into the soil, uptake and transfer water and mineral nutrients, such as P, N, S, K, Ca, Fe, Cu, and Zn, from the soil to host plants, promoting plant growth and nutrition and enhancing soil natural resources and water use efficiency (Smith and Read, 2008). Moreover, AMF improve plant tolerance to biotic and abiotic stresses, and induce changes in plant secondary metabolism leading to higher production of antioxidant compounds able to protect plants against oxidative damages caused by drought, salinity and adverse growing conditions (Rouphael et al., 2015). Given the key role played by AMF in several soil processes, they have been proposed as a potential indicator of soil health (Abbott and Lumley, 2014).

The occurrence, activity and diversity of AM symbionts are modulated by diverse environmental factors, including edaphic variables and agricultural practices, that affect spore germination, root colonization and the abundance, diversity and composition of AMF communities (Helgason et al., 1998; Giovannetti et al., 2010; Davison et al., 2021).

Previous works investigated the changes in soil AMF communities across gradients of urbanization, showing lower proportions of AMF and reduced AMF richness and diversity in the most urbanized sites (Stabler et al., 2001; Cousins et al., 2003; Bainard et al., 2011; Chen et al., 2021). Timonen and Kauppinen (2008) highlighted that poor health of street *Tilia* trees growing in sealed soils was correlated to low mycorrhiza diversity. To the best of our knowledge, no information is available about the effects of pavements suitable for water sensitive urban designs, such as permeable and porous pavements, on AMF communities actually colonizing the roots of shade trees. To fill this knowledge gap, we investigated the impact of the different types of soil sealing on AMF colonizing the roots of the shade trees *C. australis* and *F. ornus*. To this aim, we i) assessed the mycorrhizal status and colonization of the root systems of the two tree species, ii) investigated the diversity and composition of AMF communities colonizing the roots by using PCR-denaturating gradient gel electrophoresis (PCR-DGGE) of partial 18S rRNA gene, iii) identified native AMF by amplicon sequencing. The results of this study can increase our understanding of how different pavements can affect root colonization and dynamics of AMF communities, compared with unpaved control, providing also data on the fungal symbionts differentially boosted or depressed in the different environments. Such data could be useful for the identification of the most resilient AMF species, to be isolated and inoculated in the root systems of plants growing in such harsh environments.

2. Materials and methods

2.1. Site description, experimental design and plant material

The study was carried out at the experimental site described in Fini et al. (2017, 2022), located at Fondazione Minoprio (Vertemate con Minoprio, CO, Italy). Average annual rainfall and temperature in the site (measured over the 1990–2020 period) were 1138 mm and 13.8 °C, respectively. The soil, a slightly alkaline sandy silt topsoil, showed the following chemical traits: pH 7.6, organic matter 2.1%, cation exchange capacity 13.2 meq 100 g^{-1}, total nitrogen 1.4 g/Kg, assimilable phosphorus (Olsen) 19 mg/Kg, carbon to nitrogen ratio 8.9. Briefly, 24 plots of 50 m^2 each were established in November 2011 and subjected to four different pavement treatments, using a randomized block design with six blocks. The four treatments were: impermeable monolithic asphalt laid on a 13 cm thick concrete sub-base (IM); permeable modular inter-locking pavers laid on a crushed rock sub-base (PP); permeable concrete, even graded inert bound by epoxy resin (PC); unpaved soil, compacted to the same density as the other treatments (C) (see Fini et al., 2017, 2022). Plots were physically separated using PVC barriers buried in the soil down to 70 cm.

In March 2012, *C. australis* L. and *F. ornus* L., two tree species widely-used in temperate and Mediterranean urban areas, were planted in each of the 24 plots, into 1 m^2 planting holes which were left unpaved. Seedlings of the two species were produced and cultivated by a single local nursery (Vivai Ghilotti, Canneto sull'Oglio, MN, Italy) until they were 12–14 in circumference (measured at 1.3 m). The trees were transplanted to the experimental site as balled and burlapped. No fertilization or organic amendments were performed; irrigation was only performed twice during the first 6 months after transplant, using a drip system.

In October 2020, pavements were removed using an excavator. For IM treatment, the excavator was also used to remove the monolithic concrete subgrade. Conversely, for PP and PC treatments, the crushed rock subgrade was removed manually with a shovel, having care not to damage roots grown at the soil-subgrade interface. Within 2 days since pavement removal, root sampling was carried out as previously described (Corsini et al., 2022).

Three root+soil sub-samples (approx. 400 g each) per species, treatment, and replicate (72 sub-samples in total) were harvested at about 120° from each other by manual excavation conducted using a shovel and a hand-made soil-corer (Amoroso et al., 2010). Sub-samples were collected at a horizontal distance of 1.5–2 m from the root flare (1–1.5 m horizontal distance from the planting pit edge) at a depth of 20–35 cm below grade (5–20 cm below the pavement subgrade), where preliminary Ground Penetrating Radar assessments revealed higher root density (Fini et al., 2022).

The sub-samples were then pooled together to obtain a compound sample from each replicate plant per each of the four soil sealing treatments, per each plant species. Overall, a total of 24 samples were analysed. The roots were cleaned from the soil on a sieve using tap water, and processed for AMF colonization and molecular analyses.

2.2. Assessment of mycorrhizal status and colonization

The root system of each plant was thoroughly washed in running tap water to remove adhered soil and then analysed for AMF colonization. Percentage of mycorrhizal root length was determined on 5 g samples of fine roots (≤ 2 mm in diameter) after clearing and staining, as described in Turrini et al. (2017). Briefly, 10 g of thoroughly washed root samples were cleared in 10% KOH in an 80 °C water bath for 15 min, neutralized in 2% aqueous HCl, and stained with 0.05% Trypan blue in lactic acid. The percentage of AMF colonization was calculated using a dissecting microscope at × 25 or × 40 magnification and the gridline intersect method (Giovannetti and Mosse, 1980).

Samples of mycorrhizal roots were selected under the dissecting microscope, mounted on slides and observed at magnification of × 125 and × 500 under a Polyvar light microscope (Reichert-Jung, Vienna, Austria) for assessing the occurrence of appressoria, entry points and intracellular structures. Root colonization for each tree species was analysed by one-way ANOVA after checking the homogeneity of variances and significant differences among pavement treatments were established by Tukey's test. The statistical analyses were carried out in IBM SPSS statistics version 23 software (IBM Corporation, Armonk, NY, USA).

2.3. Diversity and composition of mycorrhizal symbionts by PCR-DGGE

2.3.1. DNA extraction from roots and PCR amplification

Genomic DNA was isolated from 250 mg of fine roots (≤ 2 mm in diameter) by grinding with mortar and pestle in liquid nitrogen and then using the DNeasy® PowerSoil Kit® (QIAGEN Group, Germantown, MD), according to the manufacturer's protocol. The isolated DNA was stored at − 20 °C for subsequent analyses. The AMF community composition was studied by PCR-DGGE, using a semi-nested PCR approach. A 550 bp fragment of the 18S rRNA gene was amplified by using the primer NS31 (Simon et al., 1992) in combination with the primer AM1 (Helgason et al., 1998). Amplification reactions were performed as previously described in Turrini et al. (2017), using an iCycler-iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Milan, Italy). The presence of amplicons was confirmed by electrophoresis in 1.5% (w/v) agarose gels in TBE 1 × buffer (Tris-borate-EDTA, pH 8.0) (Sigma-Aldrich, Milan, Italy), stained with 0.5 μg mL^{-1} REALSAFE Nucleic Acid Staining Solution 20,000 × (Durviz S.L., Valencia, Spain) at 80 V for 1 h. A 100 bp DNA ladder (BioLabs, New England) was used as a molecular weight marker.

Amplification products from the first PCR reaction were then diluted 1:100 and 1 μL was used as template in a second PCR using the NS31 and the Glo1 (Cornejo et al., 2004) primers. A GC clamp (5′-CGCCCGGGGCGCGCC CCGGGCGGGGCGGGGGCACGGGGG −3') was added to the 5′ end of the forward primer NS31 (Kowalchuk et al., 2002). PCR amplifications were performed as described above, except for the addition of BSA and the annealing temperature of 56 °C. All gels were visualized using UV light and captured as TIFF format files using the UVI 1D v. 16.11a program for the FIRE READER V4 gel documentation system (Uvitec Cambridge, Eppendorf, Milan, Italy).

2.3.2. DGGE and profile analyses

For the DGGE analysis, 20 μL of the PCR products plus 20 μL of buffer 2 × made as described in Palla et al. (2020) were separated in a 8% polyacrylamide-bisacrilamide (37.5:1) gel with a 20–55% urea-formamide gradient, using the DCode™ Universal Mutation Detection System (Bio-Rad, Milan, Italy). A sample of unpaved control from *C. australis* was added on each side and in the centre of DGGE gels as DGGE Marker (M). Gels were run at 90 V and 60 °C for 16 h, stained for 30 min in 500 mL of TAE 1X buffer (Tris-acetate-EDTA, pH 8) containing 50 μL of Sybr Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific, Italia) and visualized as previously described (Section 2.3.1).

DGGE profiles were digitally processed by BioNumerics software version 7.6 (Applied Maths, St-Martens-Latem, Belgium), and AMF community composition was assessed by cluster analysis of DGGE profiles, as reported in Turrini et al. (2017). Similarities between DGGE patterns were calculated by determining Dice's similarity coefficients for the total number of lane patterns from the DGGE gel using the band matching tool with a position tolerance and optimization of 0.5% and 0.5%, respectively. The similarity coefficients were then used to generate the dendrogram utilizing the clustering method UPGMA (Unweighted Pair Group Method Using Arithmetic Average).

DGGE banding data were used to estimate six different indices treating each band as an individual operational taxonomic unit (OTU). Richness (S) indicates the number of OTUs present in a sample and was determined from the number of fragments. Shannon (Hs) and the dominance index of Simpson (D) were calculated using the equations $Hs= -\Sigma(P_i \times \ln P_i)$ and $D= \Sigma P_i^2$, respectively, where the relative importance of each OTU is $P_i = n_i N^{-1}$, and n_i is the peak intensity of a band and N is the sum of all peak intensities in a lane. Evenness index (E), which allows the identification of dominant OTUs, was calculated as $E = H (\ln S)^{-1}$ and diversity indices $Hill_1$ (H_1) and $Hill_2$ (H_2) were calculated using the equations $H_1 = D^{-1}$ and $H_2 = \exp Hs$. Two-ways ANOVA was applied to diversity indices with tree species and pavement treatments as variability factors, after checking the homogeneity of variances. The means were compared by the Tukey's test ($P < 0.05$). Analyses were carried out with the SPSS version 23 software (IBM Corp., Armonk, NY, USA).

2.3.3. DGGE band sequencing

The main bands of DGGE profiles were cut out from the gels for sequencing. DNA was extracted by eluting overnight in 50 μL UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen) at room temperature. One μL of the supernatant diluted 1:100 was used to re-amplify a fragment of the 18S rRNA gene according to the PCR protocol described above, using the primers NS31 and Glo1 without the GC clamp (Cornejo et al., 2004). PCR products were than purified by Ultra-Clean PCR CleanUp Kit (MO-BIO Laboratories, San Diego, CA) according to the manufacturer's protocol, quantified and 5′ sequenced at the Eurofins Genomics MWG Operon (Ebersberg, Germany). Sequences were analyzed using BLAST on the web NCBI–GeneBank (https://www.ncbi.nlm.nih.gov/genbank) and MaarjAM (https://maarjam.ut.ee/) databases accessed on September 2022. The related sequences were collected and aligned using MUSCLE (Edgar, 2004a, 2004b), and phylogenetic trees were constructed using the Maximum Likelihood method in Mega 11 (Tamura et al., 2021) software (http://www.megasoftware.net/) with 1000 bootstrap replicates. The sequences were submitted to GenBank (https://submit.ncbi.nlm.nih.gov) (Benson et al., 2013) under the accession numbers from OP555071 to OP555090 (*F. ornus*) and from OP559534 to OP559556 (*C. australis*).

3. Results

3.1. Mycorrhizal colonization as affected by the diverse pavements

Roots of *C. australis* and *F. ornus* showed different levels of AMF colonization, depending on the treatment. In *C. australis* mycorrhizal colonization ranged from 11% to 27%, with significant differences among the treatments ($p = 0.017$). In particular, 58% and 50% decreases in root colonization were detected in plants grown in impermeable pavement and permeable pavers, respectively, compared with unpaved control. In *F. ornus* the percentage of mycorrhizal root length ranged from 27% to 35% and was not significantly affected by the pavement treatments ($p = 0.54$) (Fig. 1).

Both *C. australis* and *F. ornus* showed *Arum*-type colonization pattern, with AM hyphae spreading intercellularly among root cortical cells and many arbuscules formed terminally on intracellular hyphal branches (Fig. 2). Vesicles, which are spore-like storage structures containing lipids, occurred in both plant species, although in *F. ornus* they could

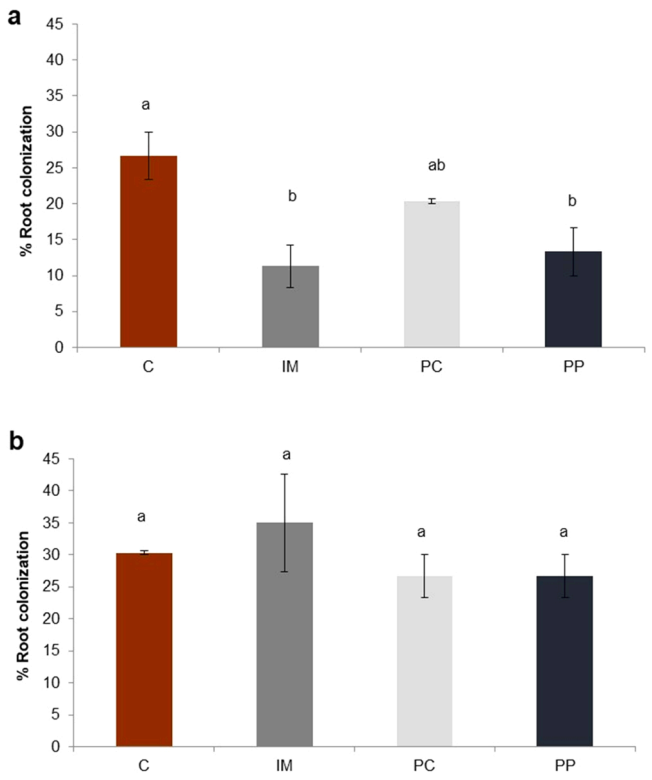

Fig. 1. Histograms showing the percentage of root mycorrhizal colonization of *Celtis australis* (a) and *Fraxinus ornus* (b) growing in soil covered by impermeable pavements (IM), permeable pavers (PP), permeable concrete (PC) or left unpaved (C). Data are averages ± standard errors of three replicates per treatment. Different letters indicate significant differences by Tukey's test ($P < 0.05$). ANOVA: *C. australis* ($p = 0.017$), *F. ornus* ($p = 0.54$).

represent the early stage of intraradical spores, produced by *Rhizoglomus/Rhizophagus* species, whose sequences have been found in its roots. It is worth noting that the roots of *C. australis* and *F. ornus* were lignified and highly pigmented, with distorted, inflated appressoria, empty germination pegs and many coils, characteristics which have been previously described in other woody plants (Fig. 2) (Turrini et al., 2017).

3.2. AMF community diversity in C. australis and F. ornus roots

The DNA extracted from *C. australis* and *F. ornus* roots was analysed by PCR- DGGE to assess the diversity of AMF communities as affected by the diverse pavements, compared with unpaved soil. A DNA fragment of approximately 230 bp, corresponding to the V3-V4 region of the 18S rDNA, was successfully amplified from all samples by the semi-nested PCR approach.

DGGE analysis of PCR products shows in each lane a pattern representing the AMF communities occurring in the relevant root sample, where each fragment represents a species or an operational taxonomic unit (OTU) with variable intensities depending on their abundance. The obtained DGGE patterns were characterized by a high number of intense and clearly defined fragments in all the samples obtained from the roots of the two tree plants (Figs. 3 and 4).

AMF community profiles of *C. australis* and *F. ornus* were assessed by cluster analysis (Fig. 5). The two dendrograms were characterized by two clusters, differentiating AMF communities of root samples of impermeable pavement from all the others. In addition, root samples of both plant species from permeable pavers and unpaved control showed similar AMF community profiles (Fig. 5). The dendrogram referring to *C. australis* detected two main clusters, characterized by a similarity of 34.6%. The main cluster grouped all the samples originated from permeable concrete, permeable pavers and unpaved control treatments, the second one was represented by samples of the impermeable pavement (Fig. 5a).

The dendrogram referring to *F. ornus* detected two clusters, characterized by a similarity of 25.3%, discriminating two samples of the impermeable pavement from all the other samples. The other cluster included two sub-clusters with a similarity of 41.4%. The first one grouped samples obtained from permeable concrete, while the second one grouped samples from unpaved control and permeable pavers treatments (Fig. 5b).

DGGE profiles were also analysed to assess Richness (S), Shannon

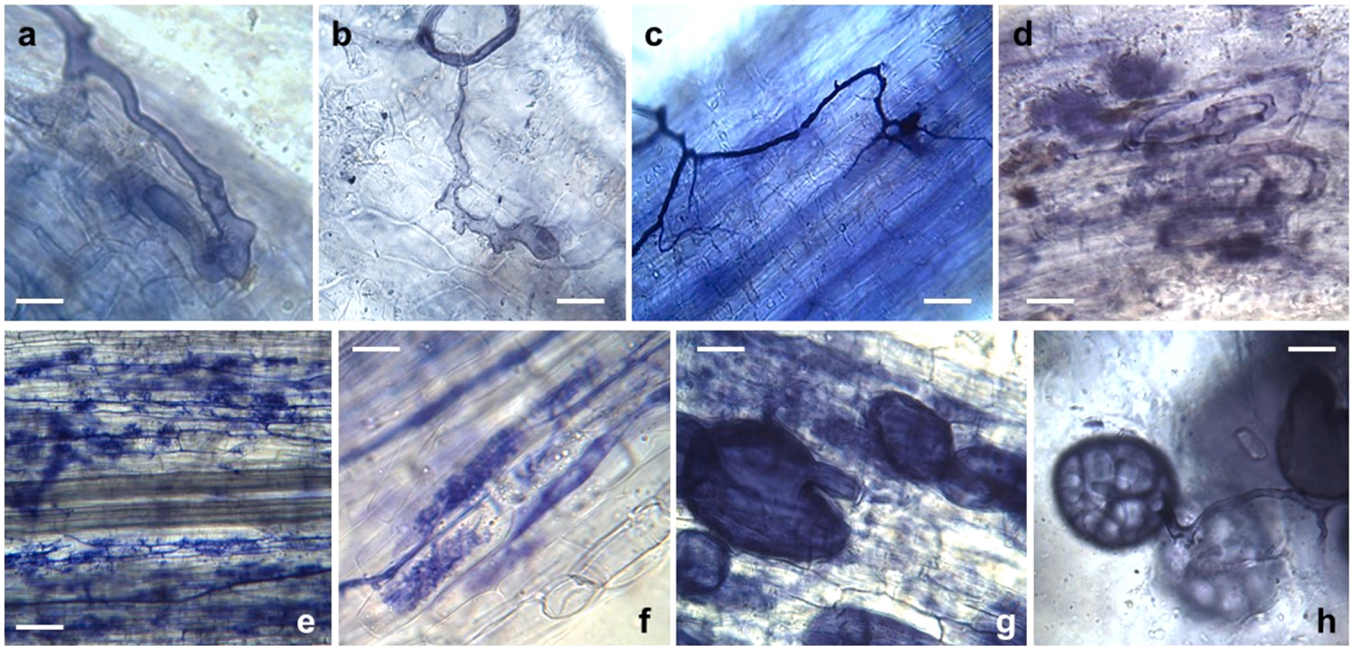

Fig. 2. Micrographs showing fungal structures characterizing the arbuscular mycorrhizal colonization of *Celtis australis* (a-d) and *Fraxinus ornus* (e-h) roots: (a), (c) entry points with appressoria, bar= 30 μm; bar= 65 μm, respectively; (b) empty appressorium, bar= 30 μm; (d) hyphal coils, bar= 30 μm; (e) root cortex colonized by intraradical hyphae and arbuscules, bar= 300 μm; (f) arbuscules, bar= 45 μm; (g) vescicles, bar= 45 μm; (h) spores, bar= 45 μm.

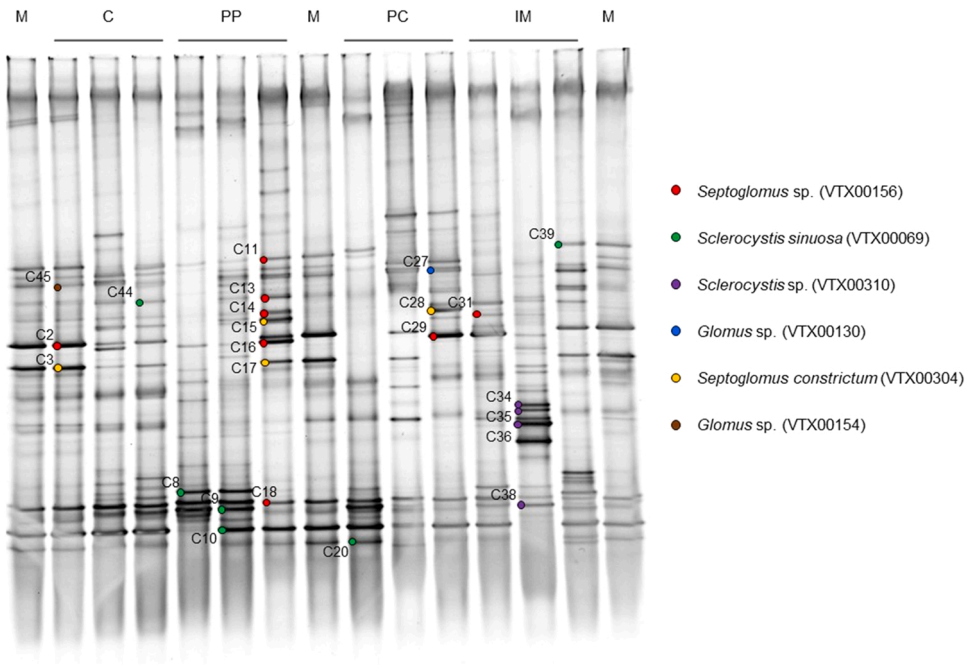

Fig. 3. DGGE analysis of AMF communities characterizing the roots of *Celtis australis* . The numbers indicate sequenced DNA fragments and the colored circles the relevant AMF species (virtual taxa) affiliation. Marker (M).

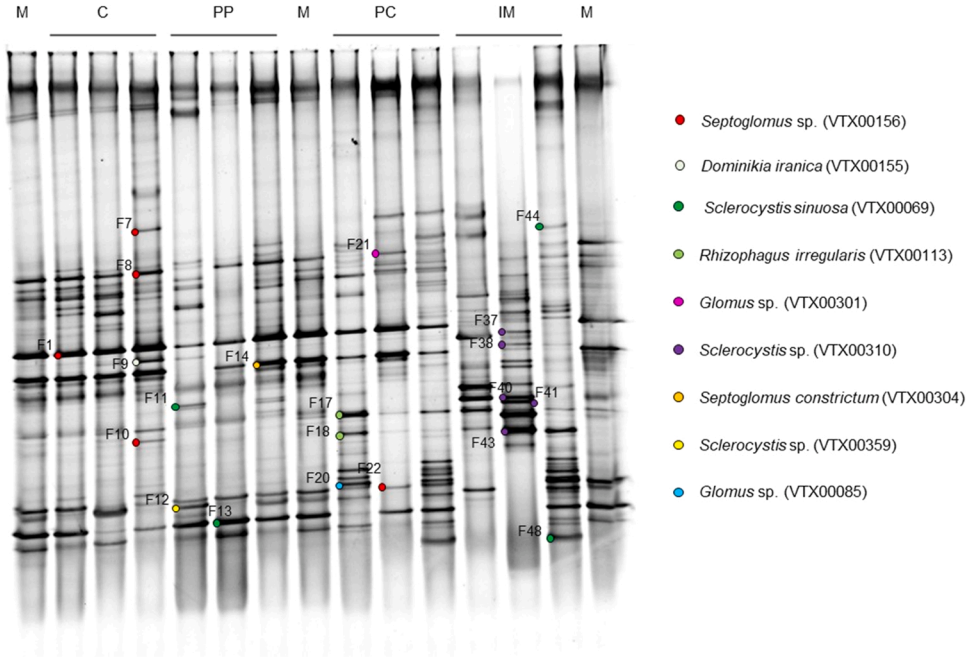

Fig. 4. DGGE analysis of AMF communities characterizing the roots of *Fraxinus ornus*. The numbers indicate sequenced DNA fragments and the colored circles the relevant AMF species (virtual taxa) affiliation. Marker (M).

(Hs), Simpson (D), Evenness (E), Hill$_1$ (H_1) and Hill$_2$ (H_2) indices. The lack of differences among the four pavement treatments in relation to all biodiversity indices may be due to the low number of replicates. Significant differences between the two plant species, *C. australis* and *F. ornus* were found in H_1 and H_2 indices (Table 1) with an overall mean of 11.76 and 13.86 respectively for *C. australis* and 13.82 and 16.39 respectively for *F. ornus*. Such data are consistent with the DGGE profiles (Figs. 3, 4), where a higher number of predominant bands were detected in *F. ornus*, compared with *C. australis*.

3.3. Identification of AMF colonizing C. australis and F. ornus roots

In order to identify AMF genera and species colonizing the roots of the tree plants, the main DGGE bands were excised, sequenced and affiliated to fungal species by using BLAST and phylogenetic trees analyses. Figs. 3 and 4 show the sequenced fragments marked with a progressive number, as well as with their correspondent affiliation to the virtual taxa, while Figs. 6 and 7 show the related phylogenetic trees with the affiliation of sequences to each fungal taxa.

It is important to point out that the taxa affiliation reported in

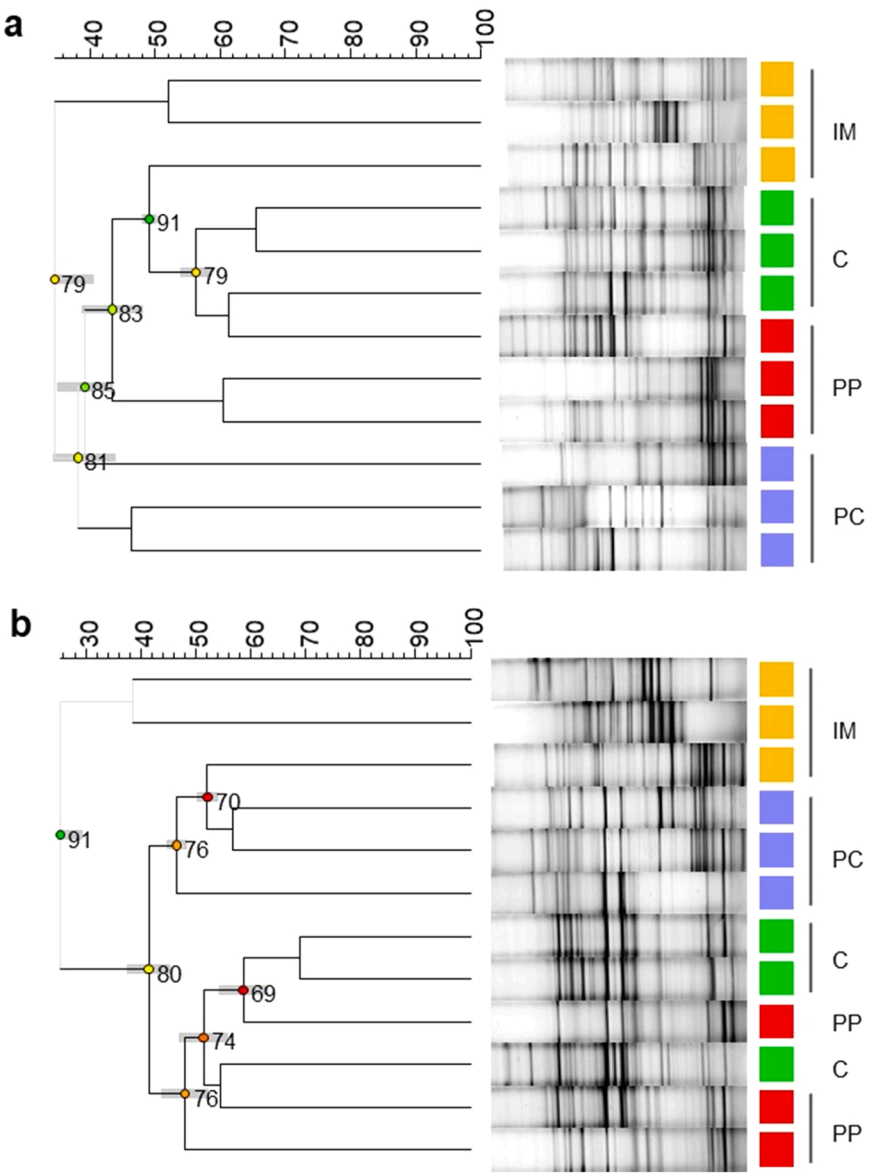

Fig. 5. Cluster analysis of AMF DGGE profiles indicating the relationships among samples, based on similarity, as shown by the numeric scale above each dendrogram, obtained by UPGMA (Unweighted Pair Group Method Using Arithmetic Average) analysis, using Dice's similarity coefficient. Dendrograms are based on AMF DGGE profiles obtained from the roots of *Celtis australis* (a) and *Fraxinus ornus* (b) growing in four different types of soil sealings: impermeable pavements (IM, yellow squares); permeable pavers (PP, red squares); permeable concrete (PC, violet squares) and unpaved soil (C, green squares). Cophenetic correlation, expressing the consistency of clusters, is shown at each node by numbers and colored dots, ranging between green-yellow-orange-red, according to decreasing values. Standard deviation is shown at each node by a grey bar.

Table 1
Richness (S), Simpson (D), Shannon (Hs), Evenness (E), $Hill_1$ (H_1) and $Hill_2$ (H_2) indices calculated from AMF DGGE profiles associated with *Celtis australis* and *Fraxinus ornus* roots growing in different pavements. Data are averages ± standard errors of three replicates per treatment.

Tree species	*Pavements*	S	D	Hs	E	H_1	H_2
Celtis australis	Unpaved control (C)	17.67 ± 1.33	0.08 ± 0.01	2.66 ± 0.10	0.93 ± 0.02	12.36 ± 1.42	14.36 ± 1.33
	Impermeable pavement (IM)	19.67 ± 3.84	0.08 ± 0.01	2.74 ± 0.04	0.92 ± 0.02	13.49 ± 1.16	15.52 ± 0.61
	Permeable concrete (PC)	15.00 ± 4.10	0.10 ± 0.01	2.48 ± 0.09	0.92 ± 0.02	10.33 ± 1.03	12.00 ± 1.01
	Permeable pavers (PP)	21.00 ± 3.71	0.10 ± 0.01	2.59 ± 0.12	0.85 ± 0.02	10.85 ± 1.42	13.56 ± 1.49
Fraxinus ornus	Unpaved control (C)	21.33 ± 1.20	0.07 ± 0.00	2.76 ± 0.05	0.90 ± 0.00	13.42 ± 0.47	15.86 ± 0.74
	Impermeable pavement (IM)	22.33 ± 0.88	0.07 ± 0.01	2.82 ± 0.13	0.92 ± 0.02	14.51 ± 1.79	17.04 ± 2.37
	Permeable concrete (PC)	24.67 ± 1.15	0.07 ± 0.01	2.87 ± 0.19	0.90 ± 0.01	15.33 ± 3.07	18.25 ± 3.44
	Permeable pavers (PP)	19.67 ± 1.73	0.09 ± 0.01	2.65 ± 0.15	0.90 ± 0.01	12.03 ± 1.48	14.42 ± 2.22
	ANOVA						
p-values	Tree species	0.137	0.165	0.074	0.966	**0.024**	**0.022**
	Pavements	0.435	0.295	0.315	0.126	0.346	0.386
	Pavements x tree species	0.656	0.879	0.954	0.155	0.944	0.944

Significantly different values are reported in bold.

Tables 2 and 3 are those originally deposited, as retrieved from the NCBI–GeneBank database. The predominant DGGE fragments originated sequences affiliated with the genera *Sclerocystis*, *Septoglomus* and uncultured *Glomus* in *C. australis*, and to *Sclerocystis*, *Septoglomus*, *Rhizoglomus*, *Dominikia* and uncultured *Glomus* in *F. ornus* (Tables 2, 3).

Overall, 38 sequences (84%) belonged to the two genera *Sclerocystis* and *Septoglomus*. Actually, 20 different sequences (44%), corresponding to the VTX00310–00069–00359, clustered in the *Sclerocystis* group,

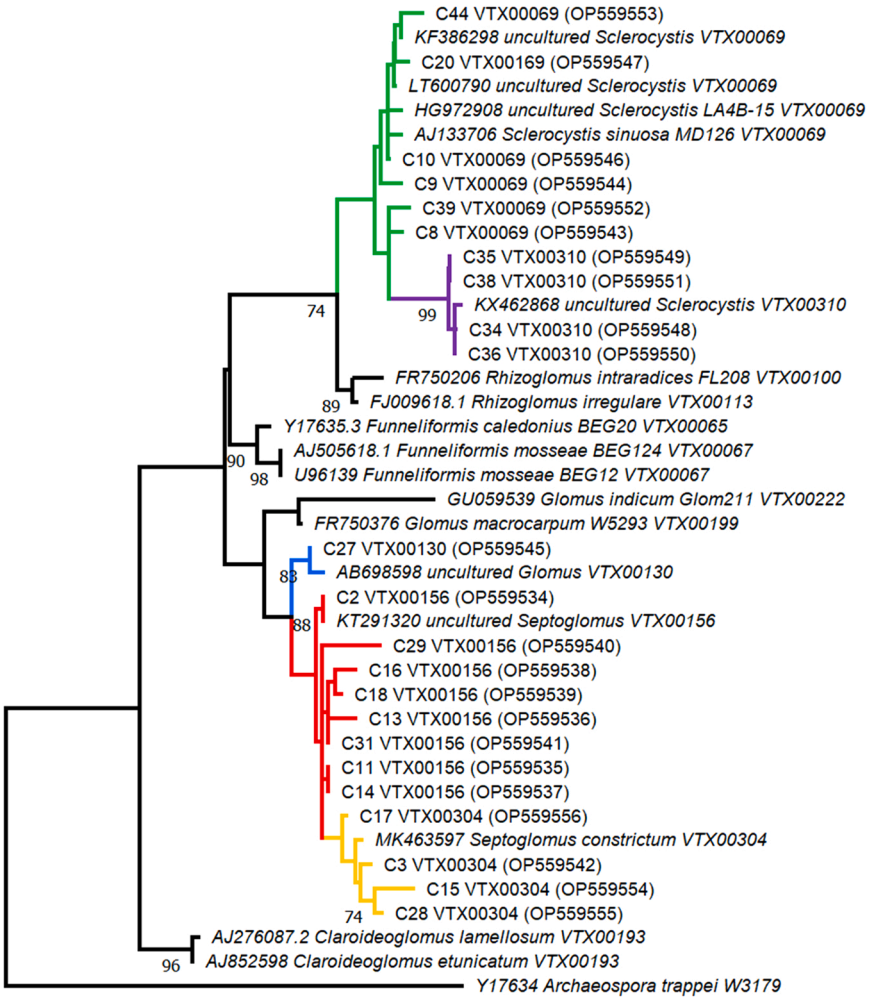

Fig. 6. Affiliation of the sequences retrieved from DGGE gel bands obtained from the roots of *Celtis australis* (marked in Fig. 3) with the sequences of the NS31-Glo1 18S rRNA gene retrieved in gene banks. Phylogenetic analysis was inferred by using the Maximum Likelihood method. The evolutionary distances were computed using the Kimura 2-parameter model. Bootstrap (1000 replicates) values below 70 are not shown. Evolutionary analyses were conducted in MEGA 11. The sequences from the database are indicated by their accession numbers. The DNA sequences retrieved in this work are indicated by their corresponding band number and their accession number. The MaarjAM database Virtual Taxa of each sequence are also shown. Black branches refer to taxonomic reference species sequences retrieved from databases only. Colors are used for sequences obtained in this work and for their related sequences from GenBank. Branches of different colors correspond to different AMF species (virtual taxa).

where VTX00069 corresponded to the described species *Sclerocystis sinuosa* (Figs. 6, 7). Further 18 sequences (40%), corresponding to the VTX00304 and 00156, clustered in the *Septoglomus* group, where VTX00304 corresponded to the described species *S. constrictum* (Figs. 6, 7).

Interestingly, in both plant species a specific virtual taxon (00069), fragments N° 34, 35, 36, 38 in *C. australis* and N° 37, 38, 40, 41, 43 in *F. ornus*, affiliated to the genus *Sclerocystis*, and grouping together with the VTX00310, was predominant in the impermeable pavement. Moreover, in *F. ornus*, the genus *Septoglomus* disappeared in the impermeable pavement treatment (Fig. 4).

It is interesting to note that only 10 sequences were affiliated to AMF isolated in pot-culture, *i.e.* 7 sequences corresponding to VTX00156 affiliated to *Septoglomus* sp. MH1, one sequence corresponding to VTX00301 affiliated to *Glomus* sp. SH3, one sequence corresponding to VTX00113 affiliated to *Rhizophagus irregularis* (*Rhizoglomus irregulare*), and one sequence corresponding to VTX00155 affiliated to *Dominikia iranica* DP11_P1. The other 35 sequences were affiliated to uncultured AMF.

4. Discussion

This is the first molecular study investigating the identity of AMF colonizing the roots of two shade trees species, *C. australis* and *F. ornus,* and characterizing their diversity and community composition under different degrees of soil sealing. Our data showed that diverse pavement types shaped AMF species community diversity in the roots of the two plant species, affecting also mycorrhizal colonization. Such data can be crucial for a better understanding of the effects of soil sealing on plant-soil-microbiota interaction, as well as to evaluate the potential of permeable pavements to mitigate such disturbance.

4.1. Mycorrhizal colonization of C. australis and F. ornus as affected by the diverse pavements

In this study *C. australis* and *F. ornus* showed good levels of AMF root colonization. Little was previously known about the mycorrhizal status of these two species. Only one work reported that *C. australis* was colonized by the AM fungus *Glomus fasciculatum*, showing a percentage of mycorrhizal root length of about 50% after 60 days, that increased seedlings' dry weight by 108% (Chamola et al., 2014). However, the type of AM symbiosis was not described. Here, we found that *C. australis* showed the *Arum*-type colonization pattern, which has been widely studied, as it is distributed in most food crops (Smith and Smith, 1997). To the best of our knowledge, there is only a mention on the occurrence of AM symbiosis in *F. ornus* root system (Maremmani et al., 2003). In this work we confirmed the AM status, showing also, for the first time, the *Arum*-type colonization pattern of *F. ornus*. Earlier evidence suggested that AMF colonization pattern was under the genetic control of host plants, while later data revealed that also the genome of AMF may affect structural differences of the symbiosis (Dickson et al., 2007). However, in this work mycorrhizal symbionts were represented by the native AMF occurring in the experimental soil, none of which appeared to produce *Paris*-type pattern. Knowledge of the type of AM colonization pattern, either *Arum* or *Paris*, is important as they may play different functional and ecological roles, *i.e.* in plant nutrition and P transfer to the host plant

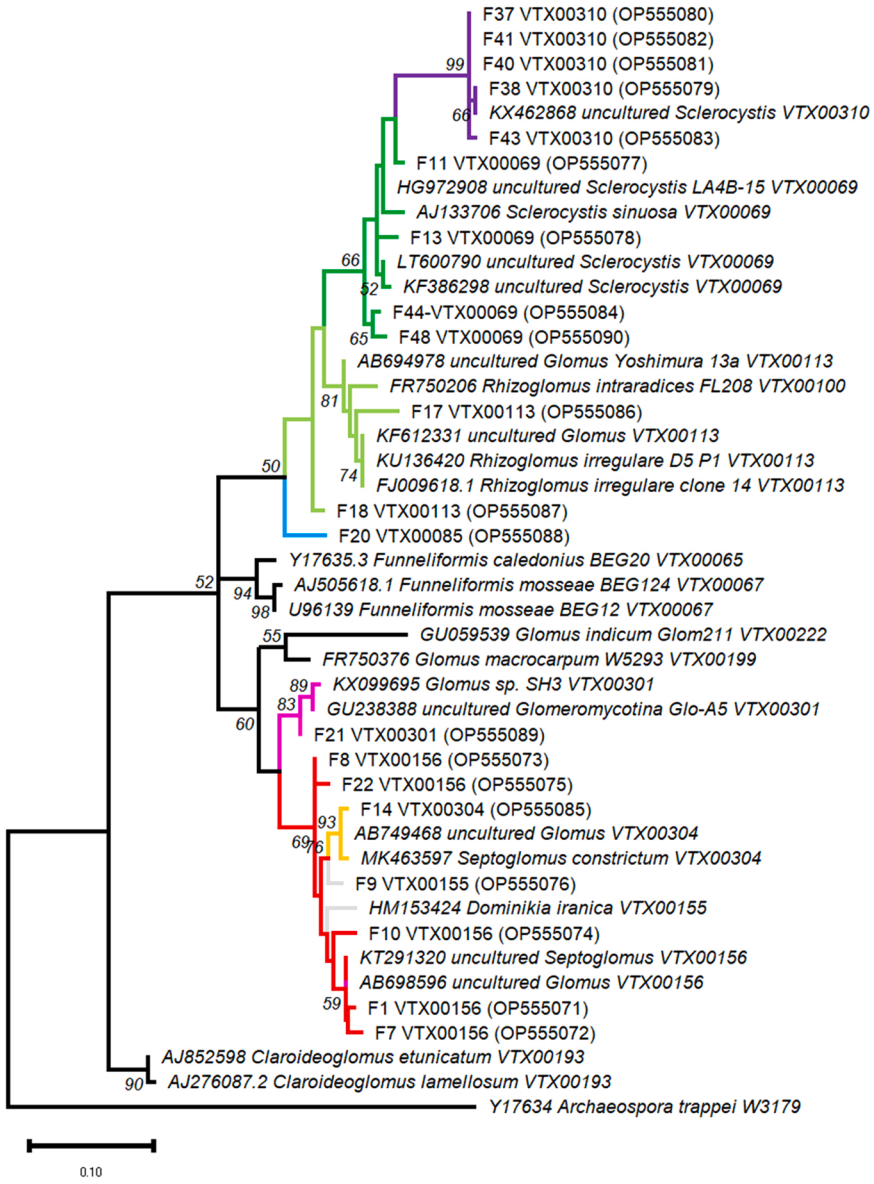

Fig. 7. Affiliation of the sequences retrieved from DGGE gel bands obtained from the roots of *Fraxinus ornus* (marked in Fig. 4) with the sequences of the NS31-Glo1 18S rRNA gene retrieved in gene banks. Phylogenetic analysis was inferred by using the Maximum Likelihood method. The evolutionary distances were computed using the Tamura 3-parameter model. Bootstrap (1000 replicates) values below 70 are not shown. Evolutionary analyses were conducted in MEGA 11. The sequences from the database are indicated by their accession numbers. The DNA sequences retrieved in this work are indicated by their corresponding band number and their accession number. The MaarjAM database Virtual Taxa of each sequence are also shown. Black branches refer to taxonomic reference species sequences retrieved from databases only. Colors are used for sequences obtained in this work and for their related sequences from GenBank. Branches of different colors correspond to different AMF species (virtual taxa).

(Dickson et al., 2007).

Data on the percentage of mycorrhizal root length provided evidence that native AMF were present in the experimental soil and in such abundance as to establish a good mycorrhizal colonization regardless of the soil sealing treatments. The responses of *C. australis* and *F. ornus* to the diverse types of pavements were divergent: in *C. australis* mycorrhizal root length showed statistically significant changes across the treatments, while in *F. ornus* it was not affected, may be due to the low number of replicates. Indeed, permeable concrete had less impact on mycorrhizal symbiosis, compared with impermeable pavement and permeable pavers, which caused decreases of 58% and 50% in root colonization, respectively, compared with unpaved control. Many factors affect AMF life cycle and colonization, from soil management practices (Oehl et al., 2010; Avio et al., 2013; Meyer et al., 2021) to soil climatic and chemical parameters, including temperature and pH (Coughlan et al., 2000; Fitter et al., 2004; Giovannetti et al., 2010; Davison et al., 2021). In our experimental site, soil temperature beneath impermeable pavements (IM) and permeable pavers (PP) was significantly higher than in the other treatments (Fini et al., 2022), possibly negatively influencing AMF colonization in *C. australis*. Moreover, changes in soil physical, rather than chemical, traits may have indirectly affected AMF colonization by altering root system morphology. The occurrence of shorter, thicker roots, with a lower occurrence of non-woody roots (the ratio between fine root dry biomass and woody root dry biomass of individual roots decreased 3-fold) in both plant species growing in impermeable pavement and, to a lesser extent, in permeable pavers, compared to control (Fini et al., 2022), may have affected the establishment of AMF symbiosis, hindering fungal root penetration by germinating appressoria (Turrini et al., 2017).

Only a few studies assessed root colonization by AMF as affected by different urban environments, which, however, were hardly comparable with our experimental site. The effects of urban expansion on AMF were studied in native trees at remnant Sonoran Desert sites and in landscape trees at nearby residential metropolitan sites in Phoenix, Arizona, USA. Native desert trees showed higher mycorrhizal colonization than residential landscape trees (Stabler et al., 2001). Similar data were reported in red maples from native forests, showing greater AMF colonization than those from recently developed landscapes (Wiseman and Wells, 2005) and in sycamores from forests located in areas with a low degree of urbanization, displaying higher AMF colonization than those from highly urbanized forests (Rusterholz et al., 2020). A work evaluating the mycorrhizal status of 26 tree species in cities in southern Ontario,

Table 2
Identification (identity %) of *Celtis australis* root AMF sequences retrieved from the DGGE gel bands (marked in Figs. 3 and 6) with the existing 18S rRNA gene sequences (reported with their origin/isolation source) in NCBI–GeneBank and MaarjAM database, as obtained using BLASTN analysis.

DGGE fragments	Identity (%)	Taxon name	Gene Bank.N°	Isolation source	Maarjam Virtual Taxon
C2	99.15%	Uncultured *Septoglomus* clone P5–20–23	KT291328.1	Soil	VTX00156
C3	95.36%	Uncultured *Glomus* clone C04_node322	MN597021.1	*Lycium chinense* roots	VTX00304
C8	98.31%	Uncultured *Glomus*	JN788350.1	Soil	VTX00069
C9	98.42%	Uncultured *Glomus* isolate Borracho cay clone BO2B-11	HG972874.1	*Coccoloba uvifera* rhizosphere	VTX00069
C10	98.72%	Uncultured *Sclerocystis* clone LaOH24–5	LT600790.1	*Malus domestica* colonised roots	VTX00069
C11	98.30%	*Septoglomus* sp. isolate MH1	KX099693.1	Hyphae	VTX00156
C13	97.06%	*Septoglomus* sp. isolate MH1	KX099693.1	Hyphae	VTX00156
C14	98.82%	*Septoglomus* sp. isolate MH1	KX099693.1	Hyphae	VTX00156
C15	97.00%	uncultured *Glomeraceae*	LR792912.1	Soil and root	VTX00304
C16	95.57%	*Septoglomus* sp. isolate MH1	KX099693.1	Hyphae	VTX00156
C17	98.29%	Uncultured *Glomeromycotina* clone KRSZ-145	MH021761.1	*Plantago lanceolata* root	VTX00304
C18	98.25%	*Septoglomus* sp. isolate MH1	KX099693.1	Hyphae	VTX00156
C20	99.15%	Uncultured *Sclerocystis* clone TeOL11–4.	LT600787.1	*Malus domestica* colonised roots	VTX00069
C27	99.15%	uncultured *Glomus* clone 3AaB_if	AM946872.1	Roots of *Acinos alpinus*	VTX00130
C28	98.36%	uncultured *Glomeraceae*	LR792912.1	Soil and root	VTX00304
C29	94.98%	*Septoglomus* sp. isolate MH1	KX099693.1	Hyphae	VTX00156
C31	99.13%	*Septoglomus* sp. isolate MH1	KX099693.1	Hyphae	VTX00156
C34	99.15%	Uncultured *Sclerocystis* clone 1	KX462868.1	Fruit tree	VTX00310
C35	99.15%	Uncultured *Sclerocystis* clone 1	KX462868.1	Fruit tree	VTX00310
C36	99.15%	Uncultured *Sclerocystis* clone 1	KX462868.1	Fruit tree	VTX00310
C38	99.15%	Uncultured *Sclerocystis* clone 1	KX462868.1	Fruit tree	VTX00310
C39	97.88%	Uncultured *Sclerocystis* clone LaOH24–5.	LT600790.1	*Malus domestica* colonised roots	VTX00069
C44	96.81%	Uncultured *Sclerocystis* clone RAL36	KF386298.1	*Pilea pumila* roots	VTX00069
C45F	95.15%	Uncultured *Glomus* isolate MK5R81VTX00371	LN900749.1	Mixed roots	VTX00154

Table 3
Identification (identity %) of *Fraxinus ornus* root AMF sequences retrieved from the DGGE gel bands (marked in Figs. 4 and 7) with the existing 18S rRNA gene sequences (reported with their origin/isolation source) in NCBI–GeneBank and MaarjAM database, as obtained using BLASTN analysis.

DGGE fragment	Identity (%)	Taxon name	Gene Bank N°	Isolation source	Maarjam Virtual Taxon
F1	96.97%	Uncultured *Septoglomus* clone P5–20–23	KT291328.1	Soil	VTX00156
F7	97.92%	*Septoglomus* clone P5–1–20–4	KT291320.1	Soil	VTX00156
F8	98.70%	Uncultured *Septoglomus* clone P5–20–23	KT291328.1	Soil	VTX00156
F9	97.83%	*Dominikia iranica* isolate D11_P1	KU136399.1	Almond rhizosphere	VTX00155
F10	96.34%	Uncultured *Septoglomus* clone P5–20–23	KT291328.1	Soil	VTX00156
F11	99.15%	Uncultured *Sclerocystis* clone LA4B-15	HG972908.1	*Coccoloba uvifera* rhizosphere	VTX00069
F12	97.81%	Uncultured Sclerocystis clone c4–31	LT158745.1	Biocrust	VTX00359
F13	96.64%	Uncultured *Sclerocystis* clone LaOH24–5	LT600790.1	*Malus domestica* roots	VTX00069
F14	97.41%	Uncultured *Glomeraceae* clone OTU61	KX110007.1	*Vitis vinifera* cv. Pinot Noir	VTX00304
F17	97.51%	*Rhizophagus irregularis* isolate D5_P1	KU136420.1	-	VTX00113
F18	96.05%	Uncultured *Rhizoglomus*	LS997526.1	Root	VTX00113
F20	97.44%	Uncultured *Glomus* clone OTU125	KP988449.1	Flax	VTX00085
F21	97.98%	*Glomus* sp. isolate SH3	KX099695.1	Soybean hyphosphere	VTX00301
F22	98.72%	Uncultured *Septoglomus* clone P5–1–20–4	KT291320.1	Soil	VTX00156
F38	100%	Uncultured *Sclerocystis* clone 1	KX462868.1	Fruit tree	VTX00310
F37	98.99%	Uncultured *Sclerocystis* clone 1	KX462868.1	Fruit tree	VTX00310
F40	99.56%	Uncultured *Sclerocystis* clone 1	KX462868.1	Fruit tree	VTX00310
F41	99.12%	Uncultured *Sclerocystis* clone 1	KX462868.1	Fruit tree	VTX00310
F43	99.12%	Uncultured *Sclerocystis* clone 1	KX462868.1	Fruit tree	VTX00310
F44	97.37%	Uncultured *Sclerocystis* clone LaOH24–5	LT600790.1	*Malus domestica* roots	VTX00069
F48	97.01%	Uncultured *Glomus* clone EJD4	JN788364.1	Soil	VTX00069

Canada, revealed that AM colonization of trees growing in urban environments was significantly lower than in rural sites (Bainard et al., 2011). Other authors found that soil infectivity decreased across a gradient of soil disturbance in four urban sites surrounded by paved roads, in the city of Córdoba, in central Argentina, and that the highest soil infectivity was related with the lowest soil compaction (Buil et al., 2021). The idea that chronic stressors such as root hypoxia and exposure to elevated soil CO_2 concentration frequently occurring in sealed soils may also indirectly affect AMF colonization by acting on tree vitality cannot be not supported here, because no change in net CO_2 assimilation, maximum quantum yield of PSII, leaf chlorophyll content, water relations, as well as canopy size and Leaf Area Index among pavement treatments was found over a 9-year period after transplanting (Fini et al., 2022). Conversely, it was surprising to observe similar above ground growth rate and leaf gas exchange in trees growing in impermeable pavements and in unpaved soil, although the former was supported by much lower root density, compared to the latter. The different composition and activity of AMF communities associated to roots growing in sealed soil may explain such findings.

4.2. AMF community diversity in C. australis and F. ornus roots as affected by the diverse pavements

To the best of our knowledge, this is the first molecular work showing that root AMF community diversity of *C. australis* and *F. ornus* growing under identical soil conditions varied significantly because of soil sealing. As shown by cluster analyses, in both plant species roots grown under impermeable pavements were characterized by an AMF community composition different from those of the other three treatments. In particular, in the impermeable pavements one species of the

genus *Sclerocystis* (VTX00310) predominated in both plant species and the genus *Septoglomus* disappeared in *F. ornus*. The higher soil temperature, restricted gas exchange, lower soil respiration and reduced soil moisture, previously detected in the soil covered with impermeable pavements (Fini et al., 2017, 2022) may have exerted a selective pressure on AMF communities, differentially affecting the diverse AMF species colonizing the roots of *C. australis* and *F. ornus*. Several works reported that soil physicochemical properties caused by soil sealing with impervious surfaces negatively affected nitrogen mineralization, soil microbial biomass, microbial and enzymatic activity (Zhao et al., 2012; Wei et al., 2013; Piotrowska-Długosz and Charzyński, 2015; Pereira et al., 2021) and altered the diversity and composition of soil bacterial communities (Hu et al. 2018; 2021; Yu et al., 2019). In contrast, only few studies have been carried out on the impact of urbanization on AMF community diversity, despite the important role played by these beneficial symbionts in the modulation of a number of soil processes, including nutrient cycling, carbon sequestration and soil aggregation (Tedersoo and Bahram, 2019). Using morphological identification of spores, variations in AMF species composition and diversity were assessed in 20 different sites representing four predominant land-use types found in Phoenix metropolitan area: urban-residential, urban non-residential, agriculture and desert. The results showed that AMF spore density in desert sites was significantly higher than that of agriculture or urban-residential sites, suggesting also that shifts in AMF community composition could be associated with urbanization (Cousins et al., 2003). In an urban ecosystem in Argentina, other authors found that the community composition of AMF morphospecies differed between urban forests and the most disturbed parklands (Buil et al., 2021).

Molecular analyses carried out using Illumina sequencing found significant changes in soil AMF communities across an urbanization gradient, with lower proportion of AMF in highly urbanized locations (Chen et al., 2021). Overall, the results reported in the cited studies are consistent with our findings, which were, however, obtained in a completely controlled experimental site.

The $Hill_1$ (H_1) and $Hill_2$ (H_2) biodiversity indices were significantly different between the two plant species, consistently with the DGGE profiles (Figs. 3, 4), where a higher number of predominant bands were detected in *F. ornus*, compared with *C. australis*. Such data may be ascribed to host preference, which may encompass a differential recruitment of specific AMF species by the two host plants (Gollotte et al., 2004; Sýkorová et al., 2007; Turrini et al., 2016).

4.3. Identification of native AMF colonizing C. australis and F. ornus roots

The dominant DNA bands of the DGGE profiles, excised and sequenced, identified genera belonging exclusively to the Glomeraceae family: *Sclerocystis*, *Septoglomus*, *Rhizoglomus*, *Glomus* and *Dominikia*. These data confirm and complement previous findings reporting the common occurrence of members of Glomeraceae in agricultural soils, which has been attributed to their ruderal lifestyle and high tolerance to disturbance (Jansa et al., 2002; Oehl et al., 2003, 2005; Turrini et al., 2017). The absence of sequences belonging to other families, including the genera *Acaulospora*, *Gigaspora* and *Scutellospora*, is consistent with previous studies reporting a low AMF diversity and the prevalence of Glomeraceae species in managed soils, compared with natural ecosystems, and may be ascribed to diverse factors, including soil type and previous agronomic practices, which strongly affect the occurrence of such AMF genera (Helgason et al., 1998; Menéndez et al., 2001; Njeru et al., 2015). Actually, recent molecular works reported that soil environment may play a key role in shaping AMF communities (Hazard et al., 2013; De Beenhouwer et al., 2015; Van Geel et al., 2015).

In this work the AMF communities colonizing the roots of the two tree plants were characterized by sequences affiliated to the genera *Sclerocystis* and *Septoglomus*, that represented 92% and 76% in *C. australis* and *F. ornus*, respectively. The occurrence of additional taxa in *F. ornus* may be ascribed to a differential host preference. Indeed, host plant identity play an important role in the selection of AMF able to establish the mycorrhizal symbiosis, with a strong host preference (Gollotte et al., 2004; Sýkorová et al., 2007) which has been recently confirmed in maize plants hosting AMF communities entirely different from those of the previous cover crops (Turrini et al., 2016). The detection of *Sclerocystis* species in *C. australis* and *F. ornus* root systems supports previous findings suggesting a host preference for woody plants, a subject worth of in depth investigations, given the importance of trees not only in forestry, but also in agriculture, *i.e.* for the production of most economically important foods and beverages, form fruits like apples and citrus to wine and olive oil.

Here, the abundance of sequences affiliated to uncultured AMF taxa (78%) - that is those known only on the basis of their DNA sequences and not cultivated in pure culture and morphologically described - is consistent with data obtained by massive NGS sequencing (Ohsowski et al., 2014). This fact may represent a strong weakness in order to reach a complete understanding of these beneficial symbionts, hindering knowledge not only of their taxonomic diversity and magnitude, but also of their biology and functional diversity.

Although no comparison with previous data from similar soil sealing treatments is possible, however, our data match well with works reporting shifts in AMF community composition associated with urbanization, as assessed by culture-dependent/morphological approaches (Cousins et al., 2003; Buil et al., 2021) or culture-independent Illumina MiSeq sequencing (Chen et al., 2021). Consistently with our data, the quoted works found that the predominant genera and species detected in urbanized sites belonged to the family Glomeraceae, whose most studied members are considered generalists and resilient to harsh environmental conditions.

In our work we found that 42% of sequences matched those retrieved in woody plants, such as *Coccoloba uvifera* (HG972908.1 and HG972874.1), *Lycium chinense* (MN597021.1), *Malus domestica* (LT600790.1 and LT600787.1), *Prunus amygdalus* (KU136399.1) and *Vitis vinifera* (KX110007.1) (Tables 2, 3), suggesting a possible preference for woody species by particular AMF taxa in relation to their functional significance.

5. Concluding remarks and future perspectives

This study was aimed at evaluating the impact of different types of pavements on AMF colonizing the roots of the *C. australis* and *F. ornus*, two shade trees frequently grown in urbanized sites. The results obtained revealed that impermeable pavements induced shifts in the composition of AMF communities associated with the roots of the two plant species and impacted on the percentage of mycorrhizal root length. Such effects were mitigated when the root-zone was covered with permeable pavements, which promoted a similar AMF community, as that observed in the unpaved soil. Thus, this research provides novel information to be utilised for reducing the disturbance caused by specific types of soil sealing on AMF symbionts, which play a key role in plant nutrition and health. A second goal of the research was to assess the mycorrhizal status of *C. australis* and *F. ornus*, together with the identity of the AMF symbionts. The predominance of *Sclerocystis* species in the roots of both trees under impermeable pavements indicated their high and unforeseen tolerance towards harsh environmental conditions. Such species could be utilized as AMF inocula specifically selected for their proven resilience in paved sites, in order to exploit their ability to boost biogeochemical processes fundamental for energy fluxes and plant nutrition and health.

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CRediT authorship contribution statement

Caterina Cristani: Investigation, Methodology. **Arianna Grassi:** Investigation, Methodology. **Irene Pagliarani:** Investigation, Methodology. **Michela Palla:** Investigation, Methodology, Formal analysis. **Alessandra Turrini:** Investigation, Formal analysis. **Manuela Giovannetti:** Conceptualization, Supervision, Writing – original draft, Funding acquisition. **Monica Agnolucci:** Conceptualization, Supervision, Writing – original draft, Funding acquisition, Writing – review & editing. **Alessio Fini:** Conceptualization, Investigation, Funding acquisition, Writing – review & editing. **Sebastien Comin:** Investigation. **Piero Frangi:** Conceptualization, Funding acquisition.

Declaration of Competing Interest

None.

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