Influence of Soil Type, Land Use, and Rootstock Genotype on Root-Associated Arbuscular Mycorrhizal Fungi Communities and Their Impact on Grapevine Growth and Nutrition

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Abstract: Soil characteristics, land management practices, and plant genotypes influence arbuscular mycorrhizal fungi (AMF) communities, leading to the proliferation of AMF taxa with different growth and nutritional outcomes in their hosts. However, the specific patterns driving these relationships are still not well understood. This study aimed to (1) evaluate the influence of soil characteristics, land use, and rootstock on AMF diversity and community structure and (2) assess the effect of those AMF communities on grapevine growth and nutrition. Soil samples were collected from vineyard and non-agricultural areas in Lisbon and Pegões, Portugal, and trap cultures established using Richter 110 and 1103 Paulsen rootstocks. After 3.5 months growth under greenhouse conditions, root-associated AMF communities were assessed by amplicon metagenomic sequencing using AMF-specific primers. Alpha diversity was only influenced by the soil type, while in β-diversity, an interaction was found between the soil type and land use. Both diversity measures were positively correlated with foliar K and negatively with leaf Mn and Mg. Notably, the concentrations of these nutrients were highly correlated with the relative abundance of operational taxonomic units (OTUs) within the genera Glomus, Rhizophagus, and Claroideoglomus. These results are valuable for supporting AMF selection for improved plant nutrition based on varying soil types and land uses.

Keywords: Vineyard; Vitis vinifera; Glomeromycota; diversity; community composition; leaf nutrients

1. Introduction

Arbuscular mycorrhizal fungi (AMF) are key soil components essential for plant development and ecosystem functioning [1,2]. It is estimated that they colonize the roots of almost 80% of vascular plants, establishing one of the most ancient and widespread symbioses on earth: the arbuscular mycorrhiza [3]. Host plants involved in this (usually) mutualistic interaction benefit from enhanced water and nutrient acquisition as well as increased tolerance to biotic and abiotic stresses such as pathogen attacks, soil salinity, metal contamination, and water deficit [4,5]. Soil structure improved by AMF has also been documented, which is related to the synthesis of the glomalin glycoprotein and the formation of an extensive mycelium network in the soil [3,6]. The latter also represents an attractive habitat (mycorrhizosphere or hyphosphere) for several other beneficial microorganisms,
with important soil functions such as nutrient cycling, organic matter decomposition, and pathogen suppression [7–9].

Environmental conditions as well as fungal and plant genotypes are among the main factors determining mycorrhizal function or performance [10]. Although the exact mechanisms underlying plant response to this interaction are not fully understood, some studies indicate that genetic polymorphism among plant varieties/species and differences in root architecture, exudates, and the associated microbiome might have a strong influence on it [10,11].

When it comes to fungi, the high functional diversity among AMF isolates/species [12–16] has also been proven to influence colonization patterns [17,18] as well as P acquisition and transfer capacity to the host plant [13,19]. As a result, different AMF genotypes can lead to heterogenous effects on host performance. In general, diverse mycorrhizal communities tend to promote higher benefits in terms of plant health and productivity [20–22], thus determining plant competitiveness under different environmental conditions [14,23,24]. However, this topic remains controversial since, in some cases, AMF species competition has been observed, with a consequent decrease in plant performance [25–32].

Although AMF are common in nearly every soil [33], in agricultural lands, conventional management practices such as soil tillage, fertilization, and the use of pesticides negatively affect mycorrhizal communities [34–40]. These practices, combined with low plant diversity or monocropping, tend to decrease AMF abundance and diversity by promoting fast-sporulating and generalist taxa with reduced symbiotic efficiency [36,38,39,41–43]. Given the relevance of balanced AMF communities for crop health and productivity [44], it is critical to understand how different AMF communities influence plant growth and nutrition, and if the lower AMF abundance and diversity commonly found in agricultural soils is actually leading to suboptimal crop performance. This knowledge is essential to support healthy soils and to develop sustainable soil management practices that harness all the benefits that AMF communities can provide to crops.

Grapevine (Vitis vinifera L.) is one of the main monocultures cultivated across Europe, with a planted area of 7.3 million ha [45]. In Portugal, grapevine is the second most planted crop, with an area of 175,590 ha [46]. Despite being highly mycotrophic, conventional agricultural practices in vineyard production systems can negatively affect mycorrhizal communities [47]. For instance, the continuous application of Cu-based fungicides and consequent Cu accumulation in vineyard soils decrease AMF species richness and mycorrhizal colonization [48,49], although AMF inoculation can partially mitigate those negative effects [49,50]. However, information on how soil characteristics, land use intensity, and rootstock genotypes influence grapevine AMF diversity is still scarce [48,51].

Therefore, this study aimed (1) to examine the effect of different factors such as rootstock genotype (Richter 110 and 1103 Paulsen), soil type (collected in Lisbon and Pegões, Portugal), and land use (vineyard and non-agricultural) on the diversity and community structure of root associated AMF and (2) to assess the effect of those AMF on grapevine growth and nutrition. The underlying hypotheses were that (1) different rootstocks and soil types under distinct land uses generate different AMF community assembles and that (2) more diverse root-associated AMF communities lead to improved grapevine growth and leaf nutrient concentrations.

2. Materials and Methods

2.1. Study Areas and Soil Sampling

In the present study, two locations in the viticultural regions of mainland Portugal were selected: Tapada da Ajuda in the Lisbon wine region (hereinafter referred to as Lisbon) and PORVID–Central Pole for the Conservation of Autochthonous Grapevine Variability in Pegões, in the Peninsula de Setúbal region (hereinafter referred to as Pegões). According to the Köppen–Geiger climate classification, both locations have a hot-summer Mediterranean climate (Csa). Average temperature and annual precipitation correspond to 11.9–20.9 °C and 680.4 mm in Lisbon [52] and 10.1–22.3 °C and 673.6 mm in Pegões [52]. In the spring
of 2021, at sprouting time, three composite soil samples (~3 kg/sample) were collected in each location (each one 20–30 m away from the others) at a depth of 20 cm (after removing the litter) from two different land uses within each location: (1) vineyard soil (VS) from inter-row spaces and (2) non-agricultural soil (NAS) from the surroundings of the vineyard (at least 200 m far from the vineyard) (Supplementary Figure S1). According to the World Reference Base for Soil Resources [53], soils from the sampled areas in Lisbon and Pegões are classified as Hypereutric Vertic Cambisols and Arenosols, respectively.

Both in Lisbon and in Pegões, the sampled vineyards were planted with red-variety grapevines grafted onto 1103 Paulsen rootstocks, conventionally managed and planted in 2016 and 1996, respectively. In Lisbon, the vineyard inter-row weed control was carried out by regular mowing allowing the growth of a natural vegetation cover, while in Pegões, the vineyard inter-row weed control included tillage and herbicide application (glyphosate). In Lisbon, the vineyard was north–south-oriented on a sloping terrain, while in Pegões, the vineyard was northeast–southwest-oriented, and the terrain was flat. In Lisbon, the vegetation of the non-agricultural areas was characterized by pine and oak trees (Quercus faginea Lam., Pinus pinea L., and Phillyrea latifolia L.) with dense undergrowth vegetation formed by shrubs and herbaceous plants (e.g., Rhamnus alaternus L., Pistacia lentiscus L., and Ruscus aculeatus L.). On the other hand, in Pegões, it was dominated by dispersed oak and pine trees (Quercus suber L. and Pinus pinea L.) and continuous cover of herbaceous plants (e.g., Ornithopus compressus L., Avena barbata Pott ex Link, Bromus sp., and Hordeum sp.).

The three soil samples collected per land use at each location were mixed and homogenized at equal volumes. From each of these soils (Lisbon-NAS, Lisbon-VS, Pegões-NAS, and Pegões-VS), a subsample was dried and sieved (fraction < 2 mm) to conduct soil chemical analyses: pH and electric conductivity in a water suspension (1:2.5 m/V), cation exchange capacity (extraction with ammonium acetate at 1 M); extractable P (Olsen method), total N (Kjeldahl method), organic carbon (OC) by wet combustion (Sauerland method), and macro- and micro-nutrients in available fraction (DTPA method), as in [30] (Table 1). General granulometry analysis was also performed to determine the percentage of coarse and fine fractions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lisbon</th>
<th>Pegões</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coarse soil fraction % (&gt;2 mm)</td>
<td>34.2 NAS</td>
<td>8.7 NAS</td>
</tr>
<tr>
<td>Fine soil fraction % (&lt;2 mm)</td>
<td>65.8 VS</td>
<td>91.3 NAS</td>
</tr>
<tr>
<td>pH</td>
<td>7.9</td>
<td>5.4</td>
</tr>
<tr>
<td>Electric conductivity 1</td>
<td>150.10</td>
<td>28.15</td>
</tr>
<tr>
<td>Cation exchange capacity 2</td>
<td>54.4</td>
<td>3.1</td>
</tr>
<tr>
<td>Total organic carbon 3</td>
<td>27.78</td>
<td>10.98</td>
</tr>
<tr>
<td>Total N 3</td>
<td>2.36</td>
<td>0.46</td>
</tr>
<tr>
<td>Macronutrient concentration in available fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P 4</td>
<td>18.61</td>
<td>4.34</td>
</tr>
<tr>
<td>Ca 3</td>
<td>16.1</td>
<td>9.4</td>
</tr>
<tr>
<td>Na 3</td>
<td>2.9</td>
<td>2.5</td>
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<tr>
<td>K 4</td>
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<td>Mg 4</td>
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<tr>
<td>Micronutrient concentration in available fraction</td>
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<td></td>
</tr>
<tr>
<td>Fe 4</td>
<td>59.12</td>
<td>106.42</td>
</tr>
<tr>
<td>Mn 4</td>
<td>29.41</td>
<td>1.52</td>
</tr>
<tr>
<td>Zn 4</td>
<td>14.39</td>
<td>2.30</td>
</tr>
<tr>
<td>Cu 4</td>
<td>4.00</td>
<td>0.02</td>
</tr>
</tbody>
</table>

1 Measured in µS cm−1; 2 cmol (+) kg−1; 3 g kg−1; 4 mg kg−1.
The number of infective AMF propagules per gram of soil was determined by the most probable number (MPN) method [54,55] using leeks (Alium porrum L.) as host plants. For this, leek seeds were previously surface-disinfected and germinated in sterile sand (1 h at 121 °C 1 atm pressure in the autoclave) under greenhouse conditions. Each fresh soil sample (50 g) was diluted from $10^{-1}$ to $10^{-5}$ by mixing it with sterile sand. The substrate obtained from each dilution was placed in germination trays (five pots per tray), and one three-week-old leek seedling was planted per pot. Six months later, leek root systems were collected and stained with Trypan blue [56,57]. The stained roots were observed under a binocular stereomicroscope at 40× magnification, and the number of AMF-colonized root systems per dilution factor and soil sample were assessed. Mycorrhizal propagule concentration was then calculated according to the mathematical model described by [58].

Table 1 shows the characteristics of the four soils. Soils from Pegões had a smaller coarse fraction compared to those of Lisbon. Furthermore, sand particles were predominant within the fine fraction. On the other hand, the soils of Lisbon presented a larger coarse fraction and higher clay content compared to the soils of Pegões, corresponding with the clay-like and sandy textures previously described for Lisbon [59] and Pegões [30], respectively. Electric conductivity, pH, CEC, OC concentration, and available P, K, N, Mg, and Ca concentrations tended to be higher in Lisbon soils than in the ones of Pegões (Table 1).

In Lisbon’s soils, the concentration of mycorrhizal infective propagules was 170 and 15 propagules g⁻¹ in the NAS and the VS, respectively, and was markedly lower in Pegões’ soils, which had only 3 and 1.1 propagules g⁻¹ in the VS and the NAS, respectively.

### 2.2. Grapevine Trap Culture Establishment

Grapevine trap cultures were established to study the characteristics of AMF communities from the different soils and to evaluate their effect in grapevine performance. Forty non-rooted dormant vines of the red Aragonez variety grafted onto Richter 110 (R 110) and 1103 Paulsen (1103 P) rootstocks were obtained from Viveiros Vitioeste (Bombarral, Portugal). While both rootstocks are very vigorous, 1103 P has a better rooting response than R 110 [60]. Yet, the latter has a resistance of up to 17% to active limestone, is extremely sensitive to salinity and excess humidity, and is well adapted to poor and dry soils [61].

Before the experiment, to induce rooting, plants were washed with tap water and planted into Styrofoam containers (65 × 35 × 16 cm) filled with sterile perlite. Plants remained under greenhouse conditions for one month (temperatures 15–20 °C, natural sunlight) and were watered daily. Every week, they were fertilized with half-strength Hoagland and Arnon solution [62].

Rooted plants from both rootstocks (R 110 and 1103 P) with 4–5 leaves were then transplanted into 1 L forest pot containers filled with the soils collected from the two soil types (Pegões and Lisbon) and land uses (NAS and VS). Thus, eight experimental treatments (2 rootstocks × 2 soil types × 2 land uses) were set up, with five biological repetitions per treatment (Supplementary Figure S2). Plants remained 3.5 months under greenhouse conditions (temperatures 15–25 °C, natural sunlight). They were watered daily and fertilized weekly with a half-strength Hoagland and Arnon solution [62].

### 2.3. Growth, Nutrition, and Mycorrhizal Colonization Assessment in Grapevine Plants

At the end of the growing season, shoot length and root dry biomass were measured. In addition, to determine foliar nutrient concentration, 3 g of leaves was collected per plant, dried at 40 °C, ground, and analyzed as in [30]. To evaluate mycorrhizal colonization, 2 g of fine roots were collected per plant. The root samples were stained with Trypan blue [56,57], and root mycorrhizal colonization was estimated by the gridline intersect method [63].

### 2.4. Analysis of Mycorrhizal Community Characteristics

Root-associated AMF communities were analyzed following a molecular approach. After 3.5 months of grapevine growth in the soils collected from the two locations and land
uses, root samples were collected, washed with tap water, and immediately frozen in liquid nitrogen. Roots of each experimental unit were ground in liquid nitrogen and stored at −20 °C. The DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) was used to extract DNA from the roots following the instructions of the manufacturer. The final DNA concentration in all samples ranged between 20 and 50 ng µL⁻¹, and samples with 200 ng of genomic DNA were sent to Novogene Company (Cambridge, UK) for targeted amplicon sequencing based on the partial ribosomal large subunit (LSU). Novogene performed PCR amplification of the targeted regions using the barcoded-specific AMF primers FLR3 and FLR4 [64] as well as the subsequent purification, equimolar pooling of the PCR products, and their ligation to Illumina adapters. Libraries were sequenced in an Illumina NovaSeq 600 platform to generate 250 bp paired-end raw reads. Appropriate quality-control procedures were carried out at each step. The company also performed raw data cleaning (read merging, filtering, and chimera removal using UCHIME algorithm) and OTU clustering at 97% identity using Uparse software (Uparse v7.0.1090). Species annotation was performed by BLAST with Blastall (version 2.2.25) and Unite version V8.2 database and was further checked using MARJAMM [65]. Singletons were removed from the count table, and data were normalized to 36216 sequences using ‘rarefy’ function from the R package ‘vegan’ (version 2.6-4) [66]. The normalized OTU table was used to perform rarefaction analysis (with ‘rarecurve’ function). Alpha diversity indices were calculated using ‘diversity’ and ‘specnumber’ functions and β-diversity through Bray–Curtis dissimilarity index using ‘vegdist’ function with ‘vegan’ package [66] in RStudio (version 2023.03.0+386).

Afterwards, Venn diagrams were constructed to study the shared and exclusive OTUs present in the experimental treatments using Venny 2.1 tool [67], and the core mycorrhizal community was identified using the following tool: https://bioinformatics.psb.ugent.be/webtools/Venn/ (accessed on 3 July 2023). Linear discriminant analysis of effect size (LEfSe) was performed to identify OTUs whose abundances differed significantly (p < 0.05) between soil types, land uses, or rootstocks using the Microbiome Analyst tool version 2.0 [68] and setting the log LDA score cutoff at 2.

Co-occurrence network analysis was used to identify non-random interactions within grapevine root AMF communities [69]. Six networks were created: two considering each soil type (from Lisbon and Pegões), two considering the land use (VS and NAS), and another two considering the rootstock (R 110 and 1103 P). In the networks, nodes represent the different AMF OTUs and the edges the significant positive or negative associations between OTUs. This connectivity study among mycorrhizal OTUs is indicative of community complexity [70]. Co-occurrence network analyses were performed with the ‘cooccur’ package in R [71]. Visualization of the positive or negative co-occurrence of OTU pairs was performed using the ‘visNetwork’ package (version 2.1.2) [72] in RStudio.

2.5. Statistical Analyses

Root biomass, shoot length, mycorrhizal colonization, and α-diversity indices were compared by a three-way ANOVA, where soil type, rootstock, and land use were considered as the main factors. Mean differences among the individual experimental treatments were evaluated by a Duncan post hoc test. Since root mycorrhizal colonization had a significant interaction between soil type and land use factors, a two-way ANOVA was conducted in each soil type considering land use and rootstock effects.

The Spearman rank correlation test was used to study the relationship between root mycorrhizal diversity indices and soil and plant parameters (soil chemical properties, shoot and root biomass, mycorrhizal colonization, and leaf nutrient concentration). The same test was also used to find significant correlations (p < 0.01) between individual OTUs and leaf nutrient concentration. Data visualization was performed with ‘pheatmap’ package (version 1.0.12) in RStudio.

The effect of soil type (Lisbon or Pegões), land use (VS or NAS), and rootstock (R 110 or 1103 P) on AMF community structure was analyzed with a PERMANOVA [73] based on the Bray–Curtis distances using the ‘adonis’ function of the ‘vegan’ package [66] in R Studio.
The significance of the factors and their interactions was assessed through comparison with 999 randomized data sets. A principal coordinate analysis (PCoA) was conducted based on the Bray–Curtis dissimilarity matrix, also using the ‘vegan’ package. Visualization was carried out using ‘ggplot2’ package version 3.4.2 [74] in RStudio.

On the other hand, a Mantel test was conducted to study the correlation between Bray–Curtis distance matrix in root AMF communities and leaf nutrient content distance matrix (based on Euclidean distances) with the ‘mantel’ function of the ‘vegan’ package [66]. Then, the ‘envfit’ function was used to find significant correlations between the mycorrhizal community and individual leaf nutrients. Result visualization was carried out using the ‘ggplot2’ package [74] in RStudio.

3. Results

3.1. Soil Type and Land Use Effect on the Diversity of Culturable Mycorrhizal Communities

Three and a half months after grapevine trap culture growth in the four soils, the AMF communities associated with grapevine roots were analyzed. Illumina sequencing yielded 1,268,181 reads after sequence filtering and cleaning, which were grouped into 256 OTUs corresponding to the Glomeromycota phylum. The sampling effort was considered sufficient according to the rarefaction curve (Supplementary Figure S3).

Alpha-diversity indices of root-associated AMF (OTU richness and Shannon index) significantly differed among the soils from the two locations (Table 2), being higher in Lisbon’ soils (Figure 1). No significant effect of the land use and the rootstock, or interactions among them, were found (Table 2).

<table>
<thead>
<tr>
<th>Factors/Effects</th>
<th>OTU Richness</th>
<th>Shannon Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rootstock</td>
<td>0.496</td>
<td>0.139</td>
</tr>
<tr>
<td>Soil type</td>
<td>0.007</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Land use</td>
<td>0.192</td>
<td>0.227</td>
</tr>
<tr>
<td>Rootstock × Soil type</td>
<td>0.515</td>
<td>0.685</td>
</tr>
<tr>
<td>Rootstock × Land use</td>
<td>0.347</td>
<td>0.994</td>
</tr>
<tr>
<td>Soil type × Land use</td>
<td>0.882</td>
<td>0.073</td>
</tr>
<tr>
<td>Rootstock × Soil type × Land use</td>
<td>0.267</td>
<td>0.658</td>
</tr>
</tbody>
</table>

Figure 1. Alpha-diversity indices, i.e., OTU richness and Shannon index, in roots of Aragonez vine plants grafted onto Richter 110 (R110) or 1103 P (1103 P) rootstocks grown in vineyard or non-agricultural soils from Lisbon and Pegões. Bars indicate average values (n = 3 ± standard error). The effect of the soil type (collected from Lisbon or Pegões locations) was significant for all the three indices.
The analysis of root mycorrhizal community diversity and soil parameters demonstrated that OTU richness was positively correlated with soil OC and N concentrations and Shannon index with pH, EC, OC, total N, K, Ca, and Zn concentrations (Supplementary Table S1).

A core AMF community of 67 OTUs was identified in grapevine roots. The shared OTUs among all experimental groups belonged mainly to uncultured Glomeromycota and Glomeraceae taxa as well as to *Rhizophagus, Glomus,* and *Funneliformis* genera. According to the Venn diagrams, the soils from the two locations, independently of land use or planted rootstock, shared 191 OTUs (78.6% of the total number of OTUs) (Figure 2A), also belonging to uncultured or undescribed members of Glomeromycota and Glomeraceae taxa as well as to *Glomus, Rhizophagus, Funneliformis,* and *Claroideglomus* genera (Figures 2A and 3A). Most differences between soil types corresponded to low-frequency genera (Figure 3B). Some genera were restricted to one of the soil types, meaning they were only present in Lisbon (36 OTUs) or Pegões (16 OTUs) (Figure 2A). For example, *Racocetra* and *Entrophospora* were exclusively associated with plants grown in Lisbon’s soils.

![Venn diagrams representing shared and exclusive OTUs in grapevine roots according to the (A) soil type (from Lisbon or Pegões), (B) land use, and (C) rootstock.](image)

Regarding the land use, 215 OTUs were shared among VS and NAS (88.5% of the OTUs), which corresponded to uncultured Glomeromycota, as well as Glomeraceae, Claroideoglomeraceae, Gigasporaceae, and Paraglomeraceae families (Figure 2B). However, 5 OTUs were exclusive of VS (including uncultured Glomerales and Glomeromycota, *Entrophosphohora,* and *Racocetra* taxa) and 23 exclusive of the NAS (including uncultured or undescribed Glomeromycota taxa and *Ambispora, Gigaspora, Claroideoglomus, Scutellospora,* and *Dominikia* genera) (Figures 2B and 3A,B).

Richter 110 and 1103 P rootstocks shared 204 OTUs (84%) and had 19 and 20 exclusive OTUs, respectively (Figure 2C). In the first rootstock, the exclusive OTUs belonged to *Claroideoglomus, Entrophospora, Funneliformis,* and *Glomus* genera as well as to uncultured Glomeromycota taxa. In 1103 P, three exclusive OTUs also belonged to *Claroideoglomus, Racocetra, Rhizophagus, Dominikia,* and *Glomus* genera and to uncultured Glomeromycota taxa (Figures 2C and 3A,B).
Linear discriminant analysis of effect size (LEfSe) identified 49 OTUs that were significantly enriched in grapevine roots grown in Lisbon’s soil and 17 that were enriched in Pegões soils (Figure S4). Most of the OTUs whose relative abundance was significantly higher in Lisbon’s soils belonged to undescribed Glomeromycota taxa, but there was one OTU belonging to *Claroideoglomus* genus, one to *Septoglo*minus* genus, two to *Funneliformis* genus, fifteen to *Glomus* genus, and five to *Rhizophagus* genus. In Pegões soils, besides the undescribed Glomeromycota taxa, three OTUs from *Glomus* genus, three from *Rhizophagus* genus, and one OTU from *Paraglomus* genus were identified as biomarkers for that soil (Figure S4, Supplementary Table S2). The LEfSe analysis did not find OTUs that could discriminate between land uses or rootstocks.

Community composition and structure was studied by β-diversity, which was calculated from Bray–Curtis distances. The PERMANOVA test showed a significant interaction between the land use and soil type, while the rootstock factor lacked significant effect ($p = 0.224$) (Table 3). Therefore, this factor was not included in the PCoA analysis. This analysis showed a marked separation between root-associated mycorrhizal communities of plants grown in Lisbon and Pegões soils (Figure 4). Moreover, in Lisbon’s soils, AMF communities were separated according to the land use, but this was not the case for the ones of Pegões soils (Figure 4).

Table 3. Results of PERMANOVA test for β-diversity calculated from Bray–Curtis distances in mycorrhizal communities among rootstocks, land uses, and soil types.

<table>
<thead>
<tr>
<th>Factor/Effect</th>
<th>F</th>
<th>$p$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rootstock</td>
<td>1.3517</td>
<td>0.224</td>
</tr>
<tr>
<td>Land use</td>
<td>5.7826</td>
<td>0.001</td>
</tr>
<tr>
<td>Soil type</td>
<td>7.9101</td>
<td>0.001</td>
</tr>
<tr>
<td>Rootstock × Land use</td>
<td>1.7479</td>
<td>0.121</td>
</tr>
<tr>
<td>Rootstock × Soil type</td>
<td>0.9196</td>
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<tr>
<td>Land use × Soil type</td>
<td>6.4062</td>
<td>0.001</td>
</tr>
<tr>
<td>Rootstock × Land use × Soil type</td>
<td>1.3307</td>
<td>0.246</td>
</tr>
</tbody>
</table>
Racocetra, Rhizophagus, Dominikia, and Glomus genera and to uncultured Glomeromycota taxa (Figures 2C and 3A,B).

Linear discriminant analysis of effect size (LEfSe) identified 49 OTUs that were significantly enriched in grapevine roots grown in Lisbon’s soil and 17 that were enriched in Pegões soils (Figure S4). Most of the OTUs whose relative abundance was significantly higher in Lisbon’s soils belonged to undescribed Glomeromycota taxa, but there was one OTU belonging to Claroideoglomus genus, one to Septoglomus genus, two to Funneliformis genus, fifteen to Glomus genus, and five to Rhizophagus genus. In Pegões soils, besides the undescribed Glomeromycota taxa, three OTUs from Glomus genus, three from Rhizophagus genus, and one OTU from Paraglomus genus were identified as biomarkers for that soil (Figure S4, Supplementary Table S2). The LEfSe analysis did not find OTUs that could discriminate between land uses or rootstocks.

Community composition and structure was studied by β-diversity, which was calculated from Bray–Curtis distances. The PERMANOVA test showed a significant interaction between the land use and soil type, while the rootstock factor lacked significant effect ($p = 0.224$) (Table 3). Therefore, this factor was not included in the PCoA analysis. This analysis showed a marked separation between root-associated mycorrhizal communities of plants grown in Lisbon and Pegões soils (Figure 4). Moreover, in Lisbon’s soils, AMF communities were separated according to the land use, but this was not the case for the ones of Pegões soils (Figure 4).

In the co-occurrence network analysis, we identified the OTU pairs that co-occurred more or less often (positive and negative co-occurrence) than would be expected if randomly distributed. In roots of plants grown in Pegões soils, there were 22% more statistically significant connections among OTUs than in the ones grown in Lisbon soils. Furthermore, in Lisbon soils, we found that 356 (56%) of the significant connections were positive, while in Pegões soils, this number was much higher, with 725 associations (92% of the total number) (Table 4, Supplementary Figure S5).

When AMF associations were studied according to the land use, 22% more significant connections among mycorrhizal OTUs were found in roots grown in the NAS than in the VS (Table 4, Supplementary Figure S5). Positive connections were more prevalent than negative ones in both cases, with positive connections accounting for 92% of total connections in roots grown in VS and 71% in those grown in NAS, respectively (Table 4, Supplementary Figure S5). Concerning rootstocks, 1103 P had 27% more connections among AMF OTUs than R 110, most of them being positive (83% and 91%, respectively) (Table 4, Supplementary Figure S5).
3.2. Effect of Different AMF Communities in the Growth and Nutrition of Grapevine Plants

Three and half months after grapevine growth in the four different soils, their growth, root mycorrhizal colonization rate, and leaf nutrient content were analyzed (Supplementary Table S3). Growth of Aragonez grapevine plants grafted onto R 110 or 1103 P rootstocks was not significantly affected by the different soil types and land uses (Table 5). However, a significant effect of rootstock factor was found in root mycorrhizal colonization percentage ($p = 0.01$), with higher values found in 1103 P rootstock roots ($72 \pm 4.1\%$) than in R110 roots ($61 \pm 1.9\%$) (Table 5, Figure 5). In addition, a significant interaction was also found in root mycorrhizal colonization percentage between soil type and land use factors (Table 5). In Pegões, plants grown in the VS had higher colonization percentage than the ones grown in the NAS, but in Lisbon, the land use did not have a significant effect (Figure 5).

Table 5. $p$-values of the three-way ANOVA test conducted to assess of the effects of soil type, land use, and rootstock on grapevine shoot length, root biomass, and root mycorrhizal colonization.

<table>
<thead>
<tr>
<th>Factors/Effect</th>
<th>Shoot Length</th>
<th>Root Biomass</th>
<th>Mycorrhizal Colonization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil type</td>
<td>0.351</td>
<td>0.708</td>
<td>0.716</td>
</tr>
<tr>
<td>Rootstock</td>
<td>0.635</td>
<td>0.884</td>
<td>0.012</td>
</tr>
<tr>
<td>Land use</td>
<td>0.903</td>
<td>0.675</td>
<td>0.129</td>
</tr>
<tr>
<td>Soil type × Rootstock</td>
<td>0.288</td>
<td>0.351</td>
<td>0.852</td>
</tr>
<tr>
<td>Soil type × Land use</td>
<td>0.052</td>
<td>0.123</td>
<td>0.028</td>
</tr>
<tr>
<td>Rootstock × Land use</td>
<td>0.604</td>
<td>0.382</td>
<td>0.241</td>
</tr>
<tr>
<td>Soil type × Rootstock × Land use</td>
<td>0.279</td>
<td>0.075</td>
<td>0.620</td>
</tr>
</tbody>
</table>

$\alpha$-diversity indices were above each location, the results of the two-way ANOVA for land use and rootstock effects and their interaction are shown. Asterisks indicate significant effect, and "ns" indicates non-significant effect at $p = 0.05$. Different letters indicate significant differences according to Duncan post hoc test conducted to compare experimental groups within each soil type.

Figure 5. Mycorrhizal colonization (%) in Aragonez variety grapevines grafted onto Richter 110 (R110) or 1103 Paulsen (1103 P) rootstocks grown in vineyard or non-agricultural soils from Lisbon and Pegões. Bars indicate average values ± standard error ($n = 5$).
Plant growth parameters and root mycorrhizal colonization percentage were not correlated with α-diversity indices (Figure 6). However, the two α-diversity indices were negatively correlated with leaf Mn nutrient concentration (OTU richness: Spearman rho = −0.432, p = 0.035; Shannon index: Spearman rho = −0.650, p = 0.001). Furthermore, Shannon index was negatively correlated with leaf Mg (Spearman's rho = −0.666) and positively with K concentrations (Spearman’s rho = 0.666), with all p-values below 0.01 (Figure 6).

Accordingly, when the correlations between the relative abundance of each OTU in the roots and leaf nutrient concentrations were studied, foliar Mn, K, and Mg also stood out as having the highest number of significant correlations with AMF OTUs, all at a significance level of $p = 0.01$ (Supplementary Figure S6). In the case of leaf Mn and Mg concentrations, significant correlations were mostly negative (80% and 96%, respectively), while for K concentrations, they were mainly positive (76%). Notably, the relative abundance of 40 OTUs (17% of the total OTUs) displayed simultaneous negative correlations with Mn and Mg leaf concentrations while exhibiting a positive correlation with K concentration. Those taxa corresponded to uncultured Glomeromycota and Glomerales taxa as well as to *Glomus*, *Rhizophagus*, and *Claroideoglomus* genera (Supplementary Figure S6).

In the case of leaf Ca, Na, and P concentrations, the trend was not as clear. Leaf Ca concentration was positively correlated with an uncultured Glomeromyctota OTU and negatively correlated with two OTUs of the genus *Scutellospora* and with uncultured Glomeromycota taxa. Foliar Na concentration was positively correlated with eight OTUs (belonging to uncultured Glomeromyctota, *Rhizophagus*, and *Glomus* genera) but negatively correlated with two OTUs belonging to uncultured Glomeromyctota taxon and to *Claroideoglomus* genus. Foliar P concentration was positively correlated with three OTUs corresponding to uncultured Glomeromyctota and *Rhizophagus* taxa and negatively corre-
lated with OTU 286 (Glomus sp.) (Supplementary Figure S6). Leaf N concentration was not correlated with the abundance of any root AMF OTU. Concerning micronutrients, two OTUs belonging to undescribed Glomeromycota taxa were negatively correlated with leaf Fe concentration, but Zn and Cu concentrations were not correlated with the relative abundance of any OTU.

On the other hand, the Mantel test conducted to study the correlation between Bray–Curtis distance matrix of root AMF communities and leaf nutrient dissimilarity matrix indicated a significant correlation ($p = 0.0017$) between them (Figure 7). Particularly, Bray–Curtis distance matrix showed significant and positive correlations with leaf Ca, Mg, K, and Mn concentrations, with Mantel statistic $r$ of 0.2785, 0.4285, 0.4916, and 0.5277, respectively, and $p$-values of 0.035, 0.006, 0.002, and 0.001, respectively.

![Mantel correlation plot for leaf nutrient concentration distance matrix (based on Euclidean distances) and Bray–Curtis dissimilarity index matrix of root AMF communities. Mantel statistic ($r$) and associated $p$-value are shown in the upper-left corner of the plot.](image)

**Figure 7.** Mantel correlation plot for leaf nutrient concentration distance matrix (based on Euclidean distances) and Bray–Curtis dissimilarity index matrix of root AMF communities. Mantel statistic ($r$) and associated $p$-value are shown in the upper-left corner of the plot.

### 4. Discussion

#### 4.1. Land Use, Soil Type, and Rootstock Effects on Culturable AMF Communities

In this study, where the effects of land use, soil type, and rootstock were studied in grapevine root AMF communities, we found a prevalence of the Glomeraceae family in the roots of all experimental treatments, with prominent representation from genera such as *Rhizopagus*, *Glomus*, and *Funneliformis*. This finding is consistent with the results obtained for most global ecosystems [75], including vineyards [76,77]. In fact, in both VS from Pegões and Lisbon, OTUs of the genera *Rhizopagus* and *Glomus* were dominant. According to their life history strategy, species within these genera are commonly classified as $r$ strategists, i.e., rapid colonizers with abundant sporulation [78] and fast reestablishment of their mycelium following disturbance [79], which gives them a competitive advantage in agricultural environments under conventional practices. Species from those genera are commonly reported in vine-growing areas, in particular *Rhizopagus* spp., *Funneliformis mosseae*, *F. geosporum*, and *Glomus fasciculatum* [37,41,59,77,80,81]. In our study, we also found OTUs from Claroideoglomeraceae and Acaulosporaceae families, which is consistent with the results reported in the aforementioned studies. Additionally, we identified taxa from *Paraglomus* and *Racocetra* genera, which seems to be more controversial, as they have been reported as absent in some vineyards while present in others [37,77,81,82].

Contrastingly, some other taxa seem to be more specific to particular environments. For instance, some OTUs within the genera *Ambispora*, *Gigaspora*, and *Scutellospora* were exclusive of Pegões NAS, suggesting a preference for non-disturbed, sandy, and low-fertility
soils. This was confirmed by [83], which found that Gigasporaceae family taxa tend to be predominant in sandy soils with low levels of humidity, organic carbon (OC), and nutrients. These species are usually K strategists and have high extraradical hyphal production (i.e., they are edaphophilic species) [70,84] providing their hosts with competitive attributes under limiting nutrient conditions [83].

The study of diversity indices revealed that $\alpha$-diversity was solely influenced by the soil type. LEfSe analysis identified 49 OTUs with significantly higher relative abundance in plant roots grown in Lisbon’s soils and 17 showing significantly higher abundance in plant roots from Pegões. In addition, 36 OTUs were exclusive to plant roots grown in Lisbon’s soils and 16 to plant roots grown in Pegões soils. The observed differences between the two soils may be attributed to their distinct physico-chemical characteristics. We disclosed significant positive correlations between Shannon index and several soil parameters, including pH, EC, OC, total N, and K, Ca, and Zn concentrations in the available fraction, with the values of these parameters being markedly higher in Lisbon’s soils. Although high soil nutrient concentrations have been associated with reduced AMF diversity and decreased mycorrhizal activity [19,85–88], the more favorable soil structure of Lisbon’s soils may have contributed to the higher diversity observed in our study. Despite the significant fine fraction (~66% of soil particles) in Lisbon’s soils, primarily comprised of clays, which has been associated with lower abundance of AMF propagules and diversity [83], the presence of a substantial coarse fraction (~34% of soil particles) and a high organic matter concentration may have improved soil structure, porosity, aeration, and water-retention capacity. These factors could explain the higher species richness and diversity observed in those soils when compared to those from Pegões (mostly dominated by sand particles and with low organic matter concentration). However, other factors may have also influenced the $\alpha$-diversity of AMF communities, like the different plant communities originally present in Lisbon and Pegões soils [89–91].

Even though land use did not affect $\alpha$-diversity, the number of mycorrhizal propagules tended to be lower in VS than in NAS. Several management practices such as soil tillage, which impacts the mycelial network within the soil [40,47], the excessive use of P-based fertilizers [92,93] or some pesticides can be detrimental to AMF populations [59,94–97]. The different weed management strategies used in the vineyard soils of Pegões and Lisbon might have also affected AMF propagule formation. While in the latter location, weed control was achieved through mowing, in Pegões, herbicides were applied. In vineyards, herbicide application can lead to a decrease in grapevine root mycorrhizal colonization and in the formation of mycorrhizal propagules in the soil, especially spores and hyphae [98,99].

Concerning AMF community structure ($\beta$-diversity), a significant interaction was found between soil type and land use. In Lisbon, the PCoA analysis showed a clear separation between AMF communities of VS and NAS, but not in Pegões. Jansa et al. [100] also observed that different soil-tillage treatments significantly affected AMF community structure but not AMF $\alpha$-diversity. Similarly, Bouffaud et al. [41] observed that land use type only influenced mycorrhizal $\beta$-diversity, with no significant impact on $\alpha$-diversity. However, it is important to note that in our study, AMF-diversity indexes were not assessed directly in the soil. Instead, we evaluated root-associated AMF from plants grown in pots filled with soil samples collected from the field (known as grapevine trap cultures). Therefore, our results may not fully reflect the effects of the soil type or land use on mycorrhizal communities [81,101].

We found that the rootstock effect was not significant for either root mycorrhizal $\alpha$-diversity or $\beta$-diversity, in contrast with previous studies suggesting that the rootstock influences grapevine microbiome [102] and mycorrhizal community composition in vineyard soils [81,103]. However, we found differences in rootstock preferences at the OTU composition level. For instance, in Pegões soils, *Septoglomus* and *Dominikia* were only found colonizing 1103 P plants, and *Paraglomus* genus was exclusive of R110 rootstock.

Besides AMF community composition and diversity, it has also been demonstrated that the interaction between members of the community network can play an important
role in ecosystem functioning, with potential consequences on host performance and adaptation [70,104]. To evaluate the connectivity of the AMF community, we studied co-occurrence networks according to the different experimental factors (soil type, land use, and rootstock). Although co-occurrence networks may not have clear biological implications [105], they may still be useful for unravelling particular connectivity trends among mycorrhizal OTUs across different experimental treatments. In fact, in our work, we detected a large difference in the degree of connectivity between mycorrhizal OTUs in roots grown in NAS and VS, with the number of OTUs involved in the network being higher in the NAS (indicative of a more complex community). These results are in agreement with previous results showing that agricultural management intensity can affect the connectivity of soil and root-associated microbial communities [97,106]. In line with our findings, Banerjee et al. [97] found higher connectivity between root fungal communities in an organic wheat field compared to a conventional one, representing extensively and intensively managed soils, respectively. They also suggested that in conventionally managed fields, the proliferation of r strategists (fast-growing species adapted to ecological disturbances) may be promoted, leading to microbial assemblies with more random connections (less connectivity), while in less-disturbed soils, the proliferation of K strategists (slow-growing species) is enhanced, favoring the establishment of more stable associations among them (higher connectivity).

In addition, root-associated AMF communities in VS had a higher percentage of positive connections compared to the community found in roots grown in NAS (92% versus 71%, respectively). Positive associations in the co-occurrence analysis do not exclusively reflect mutualistic interactions between AMF OTUs, as they may also indicate a niche overlap among OTUs, i.e., the coexistence of microorganisms with the same niche requirements but without direct interdependence [107–110]. Hence, our results might reflect the presence of a less-connected community formed by particular OTUs, probably r strategists, adapted to the same niche and agricultural soil conditions.

Much less is known about how different soil characteristics (i.e., physico-chemical properties) affect microbial co-occurrence networks. We found a higher connectivity and number of positive associations between AMF OTUs in plants grown in Pegões soils than in the ones from Lisbon’s soils. Yang et al. [111] found that connectivity between microbial species was mostly affected by soil pH. Even though we also detected a difference in pH between both soil types (with soils being slightly alkaline in Lisbon and slightly acidic in Pegões), further studies are necessary to elucidate how different soil parameters affect grapevine root-associated AMF co-occurrence networks.

On the other hand, several works show that host genotypes affect root endophyte co-occurrence networks [70,112–114]. In our study, we observed that the root-associated AMF network was less dense in 1103 P rootstock than in R 110. However, although Guo et al. [70] found that the AMF network with the lowest complexity and highest number of positive interactions was the one leading to greater growth benefits in their hosts, we did not find differences in root biomass or shoot length between the two grapevine rootstocks.

4.2. Effects of Different Root-Associated AMF Communities in Grapevine Growth and Nutrition

In the present study, 1103 P roots had higher mycorrhizal colonization percentage than the ones from R 110 rootstock, as also observed by Karagiannidis et al. [115]. Previous studies have reported variations in mycorrhizal root occupation associated with different grapevine rootstocks [115,116], which is possibly explained by distinct root system structure, such as the density of fine roots and response to different biotic and abiotic conditions. In addition, despite VS in Pegões having lower AMF propagule concentration compared to the NAS, it promoted higher root mycorrhizal colonization rates. Although the application of glyphosate for weed control in the VS of Pegões may be partly responsible for the decrease in propagule concentration in the soil, as suggested by previous studies [117,118], its negative effects on AMF communities are considered species-specific, favoring the selection of herbicide-tolerant mycorrhizal species [94]. Consequently, the
higher mycorrhizal colonization observed in the plants grown in the VS of Pegões could indicate a greater prevalence of glyphosate-tolerant taxa compared to the ones in the NAS, which might have more herbicide-sensitive and edaphophilic species [119].

However, differences in mycorrhizal colonization rates were not translated into differences in shoot or root growth, as previously documented [120]. This may be due to the fact that some AMF species with higher and faster colonization rates, like the ones that tend to proliferate in agricultural soils, are not necessarily the most mutualistic ones and do not promote higher biomass production in the host plant [84].

Arbuscular mycorrhizal fungi usually contribute to better plant nutrition, in both macro- and micronutrients like P, N, K, Ca, Mg, Zn, Cu, S, and Fe, due to several mechanisms like a higher soil exploration capacity of their extraradical mycelium network, and some enzymatic activities that can increase the availability of some nutrients [3,121–123]. Although P is the nutrient that tends to increase its concentration the most in mycorrhizal grapevines [47,121,124], this depends on the AMF species or isolates present in the roots [30,121,125,126]. Moreover, in non-sterile soils, especially in those with high P concentrations, AMF can sometimes produce neutral or even negative effects (although less frequently) on shoot P concentration [30,127–131]. In the present study, which was conducted using non-sterilized soils, we found that the relative abundance of only three OTUs was positively correlated with leaf P concentration, while one OTU corresponding to *Glomus* sp. was negatively correlated, clearly demonstrating a high variability existing in the effect of different AMF in P nutrition.

On the other hand, the relative abundance of 40 OTUs was negatively correlated with foliar Mn and Mg, and positively correlated with K concentrations, which was ultimately translated into significant correlations between α- and β-diversity indices and foliar K, Mn, and Mg concentrations. Compared to P, much less is known about the role of AMF symbiosis in the uptake and accumulation of other nutrients [47,132]. It seems that K shoot concentration is generally increased and Mn decreased in mycorrhizal plants [133–135], as also observed in grapevine leaves [93,125]. Since antagonistic interactions are commonly found between K and Mg uptake in plants [136], it is not unexpected that AMF that enhance K uptake tend to reduce Mg concentration in their host plants.

Results concerning mycorrhizal effect on Ca, Na, Cu, and Fe concentration in grapevines also tend to vary depending on the study [30,93,121,125,133,137–139]. In our experiment, some of the OTU’s relative abundance in roots was positively correlated with foliar Ca, Na, or Fe concentrations, and others were negatively correlated, contrasting with leaf Zn and Cu concentrations, which were not correlated with any OTU. Since AMF species or even isolates may vary in their ability to promote different element uptake in the host plant [19,140], it is important to promote and conserve diverse mycorrhizal communities to guarantee the ecosystem services provided by these fungi.

5. Conclusions

In the present study, we demonstrated that the soil type and therefore its specific characteristics represented the major driver of grapevine root AMF α- and β-diversity. The effect of land use on root-associated AMF community diversity was less obvious, and most differences between VS and NAS were found at OTU composition level. In addition, root AMF communities in the VS soil had less-complex OTU networks than those in the NAS soil. Nevertheless, those differences were not translated into changes in grapevine growth, although the presence of some mycorrhizal OTUs was highly correlated with leaf macro- and micronutrient concentration.

Although grapevine trap cultures may not reflect the real soil AMF community characteristics, their use is important for identifying specific rootstock preferences and for evaluating the effects of different taxa compositions on plant nutrition. Future field studies could further support the results obtained here. Altogether, this knowledge is essential to determine which AMF species/isolates could be worth inoculating for improving plant nutrient concentration and can allow a more profound understanding about the potential
need for introducing specific changes in soil management practices in order to promote the proliferation of key AMF taxa.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13112163/s1, Figure S1: Sampling sites; Figure S2: Experimental design; Figure S3: Rarefaction curve for assessing the sampling effort; Figure S4: Significantly enriched arbuscular mycorrhizal taxa detected by linear discriminant analysis of effect size (LEfSe) in Lisbon and Pegões soils. Significant differences were defined at p < 0.05 and an LDA score (log LDA) > 2.0. Figure S5: Significant co-occurrence networks of grapevine root arbuscular mycorrhizal fungal communities; Figure S6: Correlation analysis between individual operational taxonomic units of arbuscular mycorrhizal fungi and leaf nutrient concentrations; Table S1: Correlation analysis between soil physico-chemical properties and α-diversity indexes; Table S2: Significantly enriched arbuscular mycorrhizal taxa detected by linear discriminant analysis of effect size (LEfSe) in Lisbon and Pegões soils; Table S3: Leaf nutrient concentration.

**Author Contributions:** Conceptualization, A.N., W.V. and P.S.-F.; methodology, E.S.-U. and E.S.S.; formal analysis, E.S.-U., E.S.S., A.N. and P.S.-F.; investigation, A.N., R.O.F. and E.S.S.; data curation, A.N. and R.O.F.; writing—original draft preparation, R.O.F. and A.N.; writing—review and editing, R.O.F., A.N., P.S.-F., E.S.-U. and M.M.A. All authors have read and agreed to the published version of the manuscript.

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