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Diversity of arbuscular mycorrhizal fungi in a long-term no-tillage onion production system

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ABSTRACT: Onion is predominantly grown under conventional management. Alternatively, the no-tillage vegetable system uses cover crops to form a residue layer, which improves soil physical, chemical, and biological quality. Aiming to understand the effect of mycorrhizal and non-mycorrhizal cover crops on arbuscular mycorrhizal fungus diversity, we used morphological characterization of spores and high-throughput sequencing in soil from a long-term experiment with no-tillage onion. Treatments were black oats (*Avena strigosa* Schreb.); rye (*Secale cereale* L.); oilseed radish (*Raphanus sativus* L.); rye + oilseed radish; black oats + oilseed radish before the onion crop, and the control was a fallow area. In spring, all plots had onions, followed by velvet-bean in summer. Additionally, a conventional tillage system area and a forest, both adjacent to the experiment, were evaluated. Morphological identification of spores of arbuscular mycorrhizal fungi showed dominance of the Glomeraceae and Acaulosporaceae families. The DNA sequencing of rhizospheric soil confirmed those data and estimated 75 operational taxonomic units, with a predominance of the genus *Glomus*. Presence of oilseed radish, a non-mycorrhizal cover crop, did not reduce the occurrence of fungal species in relation to mycorrhizal cover crops. The use of different cover crop species in a long-term succession system maintains the natural mycorrhizal community.

Keywords: *Allium cepa*, AMF, conventional tillage, cover crops, high-throughput sequencing, taxonomy.

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INTRODUCTION

Onion (*Allium cepa* L.) is the third most-produced vegetable worldwide (Hanci, 2018). Its cultivation is predominantly managed in a conventional tillage system (CVS), which includes plowing and harrowing, as well as high rates of soluble fertilizers and agrochemicals, which may lead to the degradation of chemical, physical, and biological soil properties (Sheoran et al., 2018; De-Mastro et al., 2019), and a decrease in soil microbial activity (Lori et al., 2017). The no-tillage vegetable system (NTVS) uses minimal soil disturbance, fractionated fertilizer applications, and crop rotation, including cover crops (CC). This method aims to reduce or even eliminate the use of agrochemicals, thereby aiding in the recovery and improvement of soil properties (Fayad et al., 2019). In onions, those practices increase soil organic carbon and nitrogen (Comin et al., 2018; Bortolini et al., 2021), pH, exchangeable calcium, magnesium, potassium (Santos et al., 2018), and available phosphorus (Oliveira et al., 2017; Ventura et al., 2021), improve soil structure (Loss et al., 2017), and can modify microbial-related traits, such as glomalin contents, which are related to arbuscular mycorrhizal fungi (AMF) (Bortolini et al., 2021).

Arbuscular mycorrhizal fungi (AMF), the soil fungi associated with over 80 % of plant species (Hijri and Bâ, 2023), are obligate symbionts that enhance nutrient and water uptake and bolster plant defenses against biotic factors (Heijden et al., 2015). The diversity of AMF in agricultural systems needs to be better studied (Powell and Rillig, 2018), particularly in tropical and subtropical soils (Jemo et al., 2018). A review of 54 studies conducted under various environmental conditions revealed an 11 % increase in the diversity of these microorganisms in non-conventional systems (Bowles et al., 2016). Similarly, a 7-year experiment showed that minimum tillage increased spore numbers by 39 % compared with conventional practices (Sale et al., 2015). A 13-year study associated no-tillage with 8 and 66 % increase in spore numbers and AMF species diversity, respectively (Wetzel et al., 2014). In Brazilian tropical conditions, increases of 30 and 12 % in spore numbers and AMF species diversity were observed in no-tillage compared to conventional systems (Pontes et al., 2017).

Management and the selection of plant species affect AMF diversity (Silva et al., 2015; Stürmer et al., 2018), especially when non-mycorrhizal plant species are used, i.e., those that do not form associations with symbiotic fungi (Conceição et al., 2022). Continued use of canola (*Brassica napus* L.), a non-mycorrhizal plant, for ten years in monoculture, led to reduced AMF diversity, with the occurrence of only two genera and predominance of *Claroideglomus*, while crop rotation with wheat had five AMF genera, and barley-wheat-canola sequence had six genera (Floch et al., 2022). In the Cerrado (Brazilian Savanna), morphological analysis of spores revealed 64 AMF species, with Glomeraceae and Acaulosporaceae predominating in systems with mycorrhizal plants (Pontes et al., 2017). Use of high-throughput sequencing techniques in the analysis of ryegrass (*Lolium multiflorum* Lam.) and hairy vetch (*Vicia sativa* L.), which are mycorrhizal plants, followed by mustard (*Sinapis alba* L.), a non-mycorrhizal species, before corn (*Zea mays* L.) and soybean (*Glycine max* (L.) Merr.) showed that the soil AMF community is affected by the mycorrhizal status of the previous crops (Higo et al., 2019). However, we did not find studies on no-tillage onion production that characterize AMF communities in mycorrhizal and non-mycorrhizal plants.

Black oats (*Avena strigosa* Schreb.) and rye (*Secale cereale* L.), cover crops that are associated with AMF, and oilseed radish (*Raphanus sativus* L.), which do not form such associations (Miranda et al., 2001), are widely used as cover crops in southern Brazil (Kurtz et al., 2013). We hypothesize that the characteristic of forming or not forming mycorrhizal associations modifies the soil AMF community; mycorrhizal species increase the diversity of these microorganisms, while non-mycorrhizal species reduce it. Furthermore, conducting studies of this nature in long-term experiments under the same system can facilitate the identification of biological temporal dynamics and evaluate the system stability (Reinke et al., 2019).

This study aimed to evaluate the effects of cover crops (mycorrhizal and non-mycorrhizal) on the diversity of arbuscular mycorrhizal fungi, using morphological approaches and high-throughput sequencing in soil from a long-term no-tillage onion production system.

MATERIALS AND METHODS

Location, history, and experimental design

The study was conducted in Ituporanga, southern Brazil (27° 24' 52" S, 49° 36' 9" W, 475 m). According to the Köppen classification system, the climate is humid subtropical mesothermal (Cfa), with an average annual temperature of 17.6 °C and precipitation of 1,400 mm. The soil in the area is a *Cambissolo Distrófico Alumínico típico* or Humic Cambisol (Santos et al., 2013; Soil Survey Staff, 2014) with a loam-clay texture (380, 200, and 420 g kg^{-1} of clay, silt, and sand, respectively). The onion production with a conventional tillage system (CTS) lasted for approximately 20 years until 1996, when lime was applied to raise the soil pH to 6.0, and a minimum-tillage onion production was implemented with crop rotation and cover crops [black-oats, velvet-bean (*Mucuna aterrima* (Piper & Tracy) Holland), millet (*Pennisetum glaucum* (L.) R. Br.), sunn-hemp (*Crotalaria juncea* L.), and hairy-vetch] from 1996 to 2007. Sweet potato (*Ipomoea batatas* (L.) Lam.) was grown in 2008, and subsequently, the no-tillage onion experiment was established. In the first year (2009), weeds were desiccated with glyphosate®, and no agrochemicals were applied thereafter. The history of the area is summarized in figure 1.

In the no-tillage vegetable system (NTVS), winter species were sown each year in autumn (April) by broadcasting, and the plants were rolled down in mid-winter (July) at full bloom using a knife roller (model RF240, MBO Ltda). Until 2011, 96 kg of P_2O_5, 125 kg of K_2O, and 100 kg of N per hectare were applied annually in the form of poultry bedding, half at seedling transplanting and the remainder 45 days later. The poultry bedding had 193.2 g kg^{-1} total organic carbon and 20 g kg^{-1} total N. From 2011 onward, the application of natural phosphate ceased because phosphorus levels were considered very high, according to regional criteria (SBCS, 2016). Subsequently, furrows were made with a machine adapted for direct onion planting, and onion seedlings (cv. Empasc 352 - Bola Precoce) were manually transplanted, with a spacing of 0.40 m between rows and 0.10 m between plants, with 10 onion rows per plot. Weeding was carried out at 60 and 90 days after transplanting.

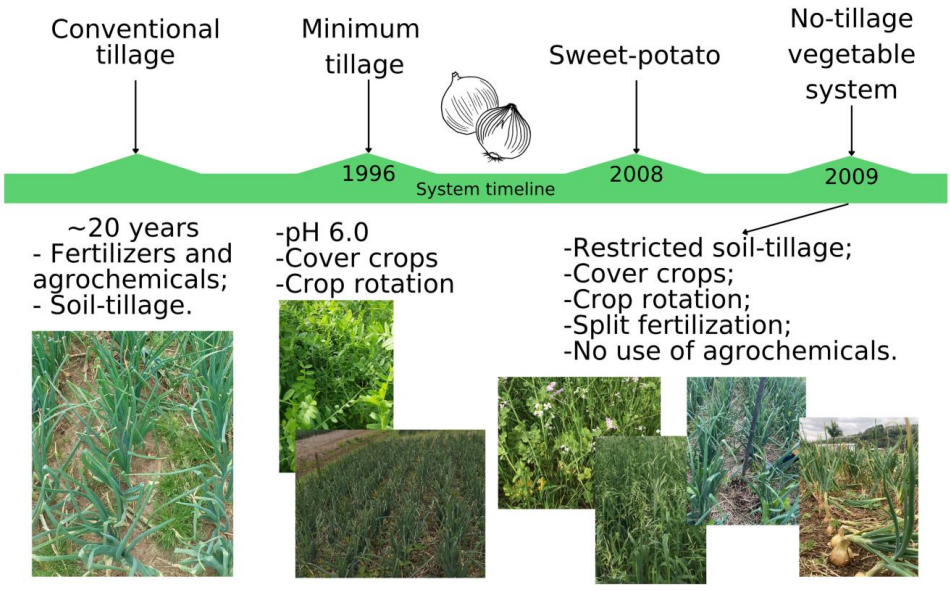

Figure 1. Timeline of the experimental area with no-tillage onion production system.

Treatments consisted of single and mixed cover crops during winter: black oats (*Avena strigosa* Schreb.) (BO); rye (*Secale cereale* L.) (RY) 120 kg seeds ha^{-1}, oilseed radish (*Raphanus sativus* L.) (OR) (20 kg ha^{-1}); BO + OR (60 and 10 kg ha^{-1}) and OR + RY (60 and 10 kg ha^{-1}), and fallow (weeds). The plots (5.0 × 5.0 m) were in a randomized block design with six replicates. All plots had onion in the spring and velvet bean (*Mucuna pruriens* (L.) DC.) in summer.

Two studies were carried out: the first characterized soil AMF diversity in 2017 using spore morphology, while the second used high-throughput sequencing in 2020 and 2021. In the first study, the treatments included: single rye (RY), single oilseed radish (OR); a consortium of RY + OR, and fallow. Additionally, we evaluated an area with 41 years of onion with a conventional tillage system, where millet (*Pennisetum glaucum* (L.) R. Br.) has been grown since 2007 in summer, and a secondary forest area with over 35 years of regeneration, both adjacent to the experimental site. In the second study, rye was replaced by black oats due to seed availability and to lower pathogenic propagule numbers. Treatments were single BO, single OR, a consortium of BO + OR, and fallow. Soil chemical properties from both studies are provided in Supplementary Material 1.

Total root mycorrhizal colonization of cover crops in 2017 was 20 and 12 % in rye roots at the treatments containing rye single and intercropped (rye + oilseed radish). In 2020, black oats roots showed 59 and 53 % total colonization in single and intercropped (black oats + oilseed radish) treatments, respectively. In both years, oilseed radish did not demonstrate root colonization.

Spore extraction and identification (Study 1)

In 2017, 300 g of rhizospheric soil (0.00-0.10 m) per plot were collected 85 days after winter cover crop sowing and 92 days after the onion transplanting (spring). In the conventional system and the forest, samples were collected during the same period, following the methodology proposed by TSBF (Tropical Soil Biology and Fertility) (Hart et al., 2015). The samples were transported and kept at 4 °C until processing.

A 100-gram sample was used for direct spore extraction to perform taxonomic identification of AMF using morphological methods, and the remainder was used to establish trap cultures (Morton et al., 1993) to induce sporulation of species that were not previously captured. Extraction of AMF spores from 50-cm^3 soil subsamples was performed by wet-sieving, followed by separation using a sucrose gradient (Gerdemann and Nicolson, 1963). Spores were mounted on permanent slides with polyvinyl lactoglycerol (PVLG) and PVLG mixed with Melzer reagent (1:1, v/v), and classified into morphotypes based on shape, size, color, and hyphal accessories (Stürmer and Siqueira, 2011).

For the morphological identification, slides of each morphotype were analyzed using information from the International Culture Collection of Glomeromycota (CICG) at the Regional University of Blumenau (FURB). Comparisons were made with the original species descriptions (Schenck and Perez, 1990) and online species description references from the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) at West Virginia University, USA (https://invam.wvu.edu/) and the Department of Phytopathology, University of Agriculture in Szczecin, Poland (http://www.agro.ar.szczecin.pl).

High-throughput sequencing of soil arbuscular mycorrhizal fungi (Study 2)

At the end of the cover crop cycles (full bloom) in 2020 and 2021, as well as at the end of the onion cycle in 2020 and the end of the velvet bean cycle in 2021, 20 g of rhizospheric soil attached to the plant root systems were collected. This material was placed in plastic bags, transported to the laboratory in a thermal box, and stored at -80 °C for subsequent characterization of the soil AMF community. The DNA was extracted from 0.25 g of soil using the DNeasy PowerSoil® Kit (QIAGEN, Hilden, Germany), eluted in 50 µL, in triplicate, according to the manufacturer's instructions. The quantity of extracted DNA was verified with the NanoDrop Lite® spectrophotometer (Thermo Scientific).

The 18S region was amplified using the nested-PCR technique. In the first PCR cycle, the primers NS31 (5' GAA CCC AAA CAC TTT GGT TTC C 3') (Simon et al., 1992) and AML2 (5' GAA CCC AAA CAC TTT GGT TTC C 3') (Lee et al., 2008) were used. The reaction mix contained 1 µL of DNA, 0.2 µmol L^{-1} of each primer, 10.0 µL of 2x PCRBio Ultra Mix® (PCRBiosystems), and ultrapure water to complete a final volume of 25 µL. The reaction included a denaturation step at 94 °C for 2 min, followed by 33 cycles of 30 s at 94 °C for denaturation, 30 s at 65 °C for annealing, and 40 s at 72 °C for extension. The reaction was completed with a final extension step of 10 min at 72 °C. Subsequently, the PCR product was purified using the AMPure® system (Beckman Coulter, California, USA). The second PCR reaction used the primers AMV4.5NF (5' AAG CTC GTA GTT GAA TTT CG 3') and AMDGR (5' CCC AAC TAT CCC TAT TAA TCA T 3') (Geel et al., 2014). The reaction included 1 µL of the product from the first PCR (NS31/AML2), 0.2 µmol L^{-1} of each primer, 10.0 µL of 2x PCRBio Ultra Mix (PCRBiosystems), and ultrapure water to achieve a final volume of 25 µL. The thermocycler was programmed for an initial step of 2 min at 95 °C, followed by 28 cycles of 30 s at 95 °C for denaturation, 30 s at 58 °C for annealing, 40 s at 72 °C for extension, and a final extension step of 10 min at 72 °C (Sato et al., 2005). After the cycles were completed, the PCR product was purified, and Nextera XT Index Primer 1® (N7xx) and Nextera XT Index Primer 2® (S5xx) adapters were attached to the amplified DNA.

The AMF diversity was analyzed using NGS on the Illumina MiSeq® platform, configured for 2x250 bp reads with an average coverage of 10,000 reads per sample (Vasar et al., 2017). Bioinformatic processing was conducted as follows: data were processed using QIIME 2® software version 2020.8 (Pauvert et al., 2019). Primers were removed using Cutadapt, while reads were merged with PEAR, and chimeras were filtered using the denoise-paired algorithm from DADA2. Operational taxonomic units (OTU) were classified using the MaarjAM reference database (http://maarjam.botany.ut.ee/) (Hart et al., 2015) version 2019. Phylogenetic trees were constructed from alignments with similarities above 97 %.

Statistical analyses

In the first study, a heatmap was constructed to visualize the frequency of AMF species across different systems, presented in percentages (0, 25, 50, 75, and 100 %). Principal Coordinates Analysis (PCoA) and distance-based Redundancy Analysis (dbRDA) were conducted using Jaccard dissimilarity (Clarke, 1993). These analyses aimed to assess the similarity among AMF communities in areas with different soil coverages and in winter and spring. Additionally, a permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2006) was applied to determine differences in the AMF community composition between the different soil coverages and the mentioned seasons. This analysis was also used to compare mycorrhizal community data with soil chemical properties, after verifying multivariate homogeneity.

In the second study, the data obtained were analyzed using PERMIDISP and again by PCoA. A phylogenetic tree was constructed from OTU generated based on alignments with >97 % sequence similarity, using the MAFFT software for sequence alignment, the maximum likelihood method for phylogenetic inference, and 999 bootstrap replicates to assess node support. The trees were visualized using the FigTree software.

The AMF diversity was assessed based on the Shannon-Weiner and Margalef indices, and evenness was calculated using the Pielou index. All tests and analyses were conducted using the R software version 4.1.2 (R Core Team, 2023).

RESULTS

First study: Spore morphology of the AMF

During the 2017 winter cover crop cycle, we identified 37 arbuscular mycorrhizal fungus morphotypes, distributed across 11 genera and five families within the phylum Glomeromycota (Figure 2). The Glomeraceae family had the highest richness, with 19 species. There were nine Acaulosporaceae species, five Gigasporaceae, three Claroideoglomeraceae, and Paraglomeraceae had one species.

A total of 11 AMF species were identified in the treatments with single rye (RY) and oilseed radish (OR), 21 species in the rye + oilseed radish consortium (RY+OR), and 19 species occurred in both the conventional tillage system (CTS) and the forest soil (Figure 2). Six species were predominant: *Acaulospora mellea* was dominant in the OR, *Claroideoglomus etunicatum* was predominant in the RY+OR or OR, *Rhizophagus* sp4 in Forest, *Glomus* sp1 in RY+OR. *Funneliformis* genus was present in all treatments, and *Funneliformis geosporum* was dominant.

During the onion cycle (spring), 35 morphotypes were identified, distributed across 12 genera and six families. The family Glomeraceae was the most frequent, with 17 species, followed by Acaulosporaceae with 9, Gigasporaceae with 4, Claroideoglomeraceae with 3, and Archaerosporaceae and Paraglomeraceae with 1 species each (Figure 2). Fifteen AMF species were found in the RY, 16 in the OR, 13 in RY+OR, 11 in OR, 14 in CTS, and 17 species in the forest. The six dominant species were: *Glomus* sp9 in the RY treatment; *Glomus* sp1 in Forest; *Funneliformis mosseae* in the OR and RY+OR; and CTS, *Claroideoglomus etunicatum* was found in all treatments except RY+OR, and *Funneliformis geosporum* was dominant in all areas. For photographs of the dominant species (Supplementary Material 2).

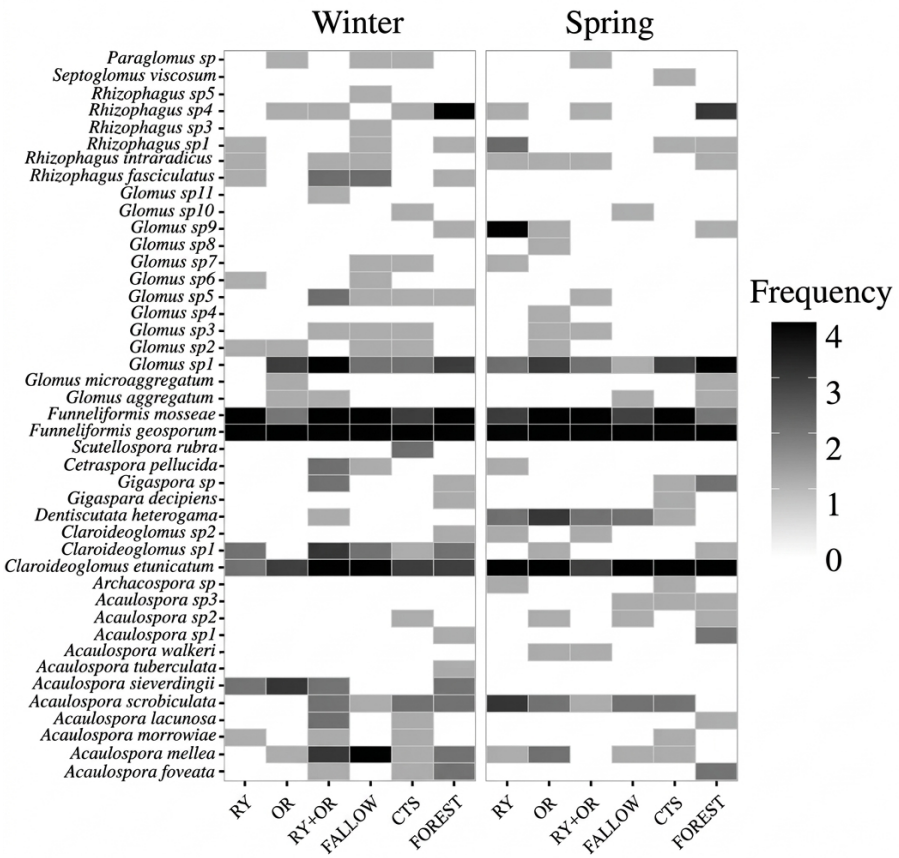

Figure 2. Relative frequency of AMF species assessed by morphological techniques, during the cover crop cycle (winter) and onion cycle (spring) in soil covered with rye (RY), oilseed radish (OR), rye + oilseed radish (RY+OR), fallow in the No-Tillage Vegetable System (NTVS) for onions, in a Conventional Tillage System (CTS), and a Forest.

In the families with the highest number of species, Glomeraceae had 13 species in OR, 10 in RY+OR, nine in CTS, eight in Forest, seven in RY, and seven in OR, while Acaulosporaceae had six species in the RY+OR, CTS, and Forest, and two species in RY, OR, and OR (Figure 3a).

During the onion cycle, the families with the highest number of species, Glomeraceae and Acaulosporaceae, showed varied distributions among the treatments. We found nine Glomeraceae species in the OR and Forest, eight in RY, seven in RY+OR, and five in CTS and OR. Acaulosporaceae had five species in Forest, four in OR, OR, CTS, and two in RY and RY+OR (Figure 3b). Some species occurred exclusively in certain seasons: during winter, i.e., the cover crop cycle, exclusive species included *A. sieverdingii*, *A. tuberculata*, *S. rubra*, *G.* sp6, *G.* sp11, *R. fasciculatus*, *R.* sp3, and *R.* sp5. In the onion cycle (spring), the exclusive species were *A. walkeri*, *A.* sp3, *Archaeospora* sp., *G.* sp4, *G.* sp8, and *Septoglomus viscosum*.

The Forest showed differences in species diversity and family composition with the rye and fallow (Figures 2 and 3). In the Forest, species not found in the rye cultivation included six from the family Acaulosporaceae, three from Glomeraceae, and two from Gigasporaceae. Compared to fallow, the Forest had five species from Acaulosporaceae, three from Glomeraceae, two from Gigasporaceae, and one from Claroideoglomeraceae.

PERMANOVA indicated an effect of both cover crops and seasons on AMF diversity (Figure 4), with Pseudo $F_{5,36}$ = 1.64; p = 0.008 for cover crops and Pseudo $F_{1,36}$ = 4.03; p = 0.001 for seasons, with no interaction between those factors (Pseudo $F_{5,36}$ = 1.41; p = 0.053). The AMF community structure in the Forest differed from the RY treatment (p = 0.03) and fallow (p = 0.03), while the other treatments did not show differences. Principal Coordinates Analysis (PCoA) explained 18.8 % of the data variation (Supplementary Material 3).

However, when soil chemical properties were incorporated into the distance-based Redundancy Analysis (dbRDA), the method explained a significantly higher proportion of variation (65 %; Figure 4). That analysis highlighted the effects of calcium, phosphorus, and organic matter on soil AMF diversity, and demonstrated the influence of soil chemical properties on the dynamics of those fungal communities.

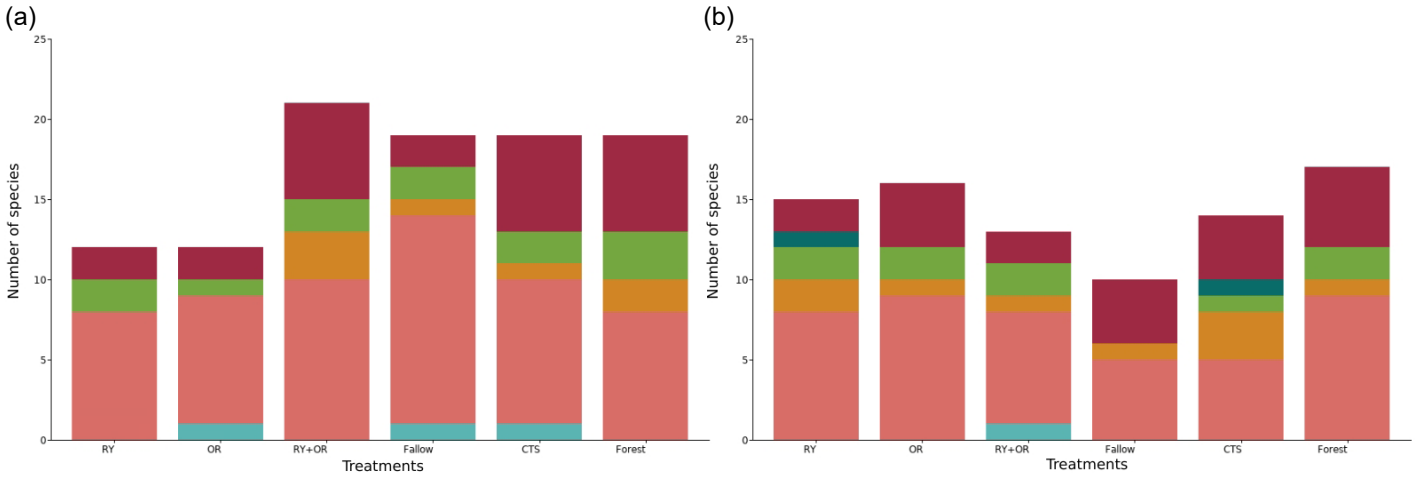

Figure 3. Number of AMF species per family during the cover crop in winter (a) and during the onion in spring (b) in soil covered with rye (RY), oilseed radish (OR), rye + oilseed radish (RY+OR), fallow in the No-Tillage Vegetable System (NTVS) for onions, and in the Conventional Tillage System (CTS) and Forest.

In the dbRDA, there was a noticeable distinction between the Forest and the other treatments, highlighting the unique effects of plant communities on specific AMF taxa. Species such as *Rhizophagus sp4* (Rsp4), *Acaulospora foveata* (Af), *Acaulospora sp1* (Asp1), *Gigaspora sp* (Gisp), and *Glomus sp1* (Gsp1) increased their frequency in spring (Figure 4). Moreover, the dbRDA was able to better differentiate the seasons. This was evidenced by the separation of species such as *Cetraspora* (Ce), *Acaulospora scrobiculata* (Asc), *Diversispora* (Dh), and *Glomus sp9* (Gsp9), all displaying distinct patterns of prevalence in each season. That shows how environmental and temporal factors influence AMF diversity in different cropping systems and seasons.

Second study: High-throughput sequencing of soil arbuscular mycorrhizal fungi

We identified 75 operational taxonomic units (OTU), from a total of 570,237 sequencing reads, belonging to the genera *Glomus*, *Claroideoglomus*, *Diversispora*, *Acaulospora*, *Gigaspora*, and *Paraglomus*. The OTU are predominantly distributed in the classes Glomeromycetes (99.6 %) and Paraglomeromycetes (0.4 %) (Figure 5a). The majority belong to the order Glomerales (92 %), with the remainder divided between Diversisporales (7 %) and Paraglomerales (1 %) (Figure 5b). The families include Glomeraceae (76 %), Claroideoglomeraceae (17 %), Acaulosporaceae (4 %), Diversisporaceae (2.2 %), Gigasporaceae (0.5 %), and Paraglomeraceae (0.3 %) (Figure 5c). The predominant genera were *Glomus* (76 %), followed by *Claroideoglomus* (17 %), *Acaulospora* (4 %), *Diversispora* (2.2 %), *Gigaspora* (0.5 %), and *Paraglomus* (0.3 %) (Figure 5d).

Analysis of AMF relative abundance across different treatments and seasons revealed a marked dominance of the genus *Glomus* (Figure 6). At the end of the cover crop cycles in 2020 and 2021, in treatments including cover crops (BO, OR, BO+OR), the genera *Glomus*, *Claroideoglomus*, *Acaulospora*, *Diversispora*, and *Paraglomus* were identified. In contrast, the fallow areas had higher diversity, including the genus *Gigaspora* (Figures 6a and 6d). In subsequent onion crops, the proportion of Claroideoglomus increased, particularly in

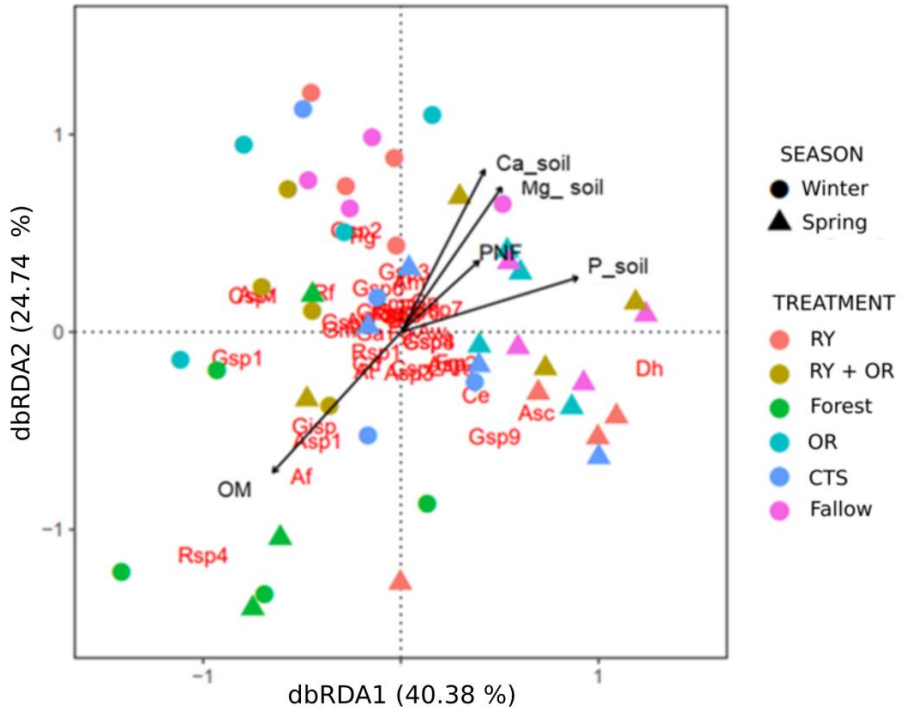

Figure 4. Distance-based Redundancy Analysis (dbRDA), with Jaccard dissimilarity matrix, between the AMF diversity and soil chemical properties (PNF - phosphatase activity; P - soil Phosphorus; Ca - Calcium; Mg - Magnesium; MO - Organic Matter) in soil covered with rye (RY), oilseed radish (OR), rye + oilseed radish (RY+OR), fallow in the No-Tillage Vegetable System (NTVS) for onions, and in the Conventional Tillage System.

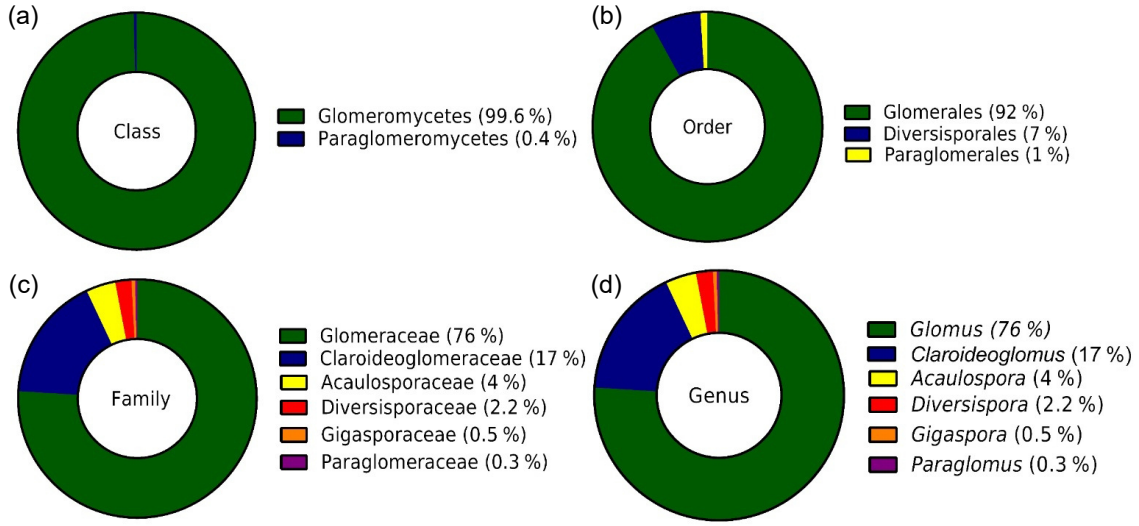

Figure 5. Distribution (%) of the molecular AMF community evaluated in the no-tillage vegetable system (NTVS) for onions with different winter cover crops and temporal analyses by class (a), order (b), family (c), and genus (d).

treatments with oilseed radish, which showed a higher occurrence of the genus *Acaulospora* (Figure 6b). In the case of the velvet bean (*M. aterrima*) crop, there was a reduction in the dominance of *Glomus*, which fell below 55 %, and an increase in the occurrence of *Acaulospora*. Additionally, *Claroideoglomus* increased its occurrence in treatments with black oats and oilseed radish during winter (Figure 6c).

Phylogenetic tree showed alignment rates higher than 80 % (Figure 7), allowing for the classification of OTU into small clades, primarily at the genus level. However, there are notable exceptions, such as OTU 3, which was consistently classified up to the phylum Glomeromycota with 100 % certainty, and OTUs 61 and 69, which were classified up to the order Glomerales.

PERMANOVA revealed an interaction between cover crops and the seasons (p-value of 0.03), indicating that the combination of different types of cover and the seasons affected the diversity of AMF. The PCoA was able to explain 24.35 % of the variation in the data. Although no clear clustering trend was observed in most evaluations, an exception occurred at the end of the onion cycle, when the data showed less dispersion and were predominantly concentrated in the first quadrant of the PCoA (Figure 8).

DISCUSSION

The identification of arbuscular mycorrhizal fungi (AMF) species based on spore morphology, which in some cases reached the species level, showed that the families Glomeraceae and Acaulosporaceae are predominant in the long-term experiment and adjacent areas (Figures 2 and 3). The genera *Glomus* and *Acaulospora* are commonly found in tropical regions (Stürmer et al., 2018; Vieira et al., 2018), a pattern also observed in this study in subtropical conditions. The consistent presence of Glomeraceae in various land-use systems can be attributed to their high capacity to tolerate environmental changes, their ability to form anastomoses, and the formation of robust mycelial networks in plant communities, distinguishing features of this family (De-La-Providencia et al., 2005). *Acaulospora*, in turn, was the second most frequent genus and was also noted as significant in Brazilian forest ecosystems (Stürmer and Siqueira, 2008).

In winter, growing a single cover crop species resulted in a reduction in the number of AMF species (Figure 2). This occurred with oilseed radish, which is a non-mycorrhizal

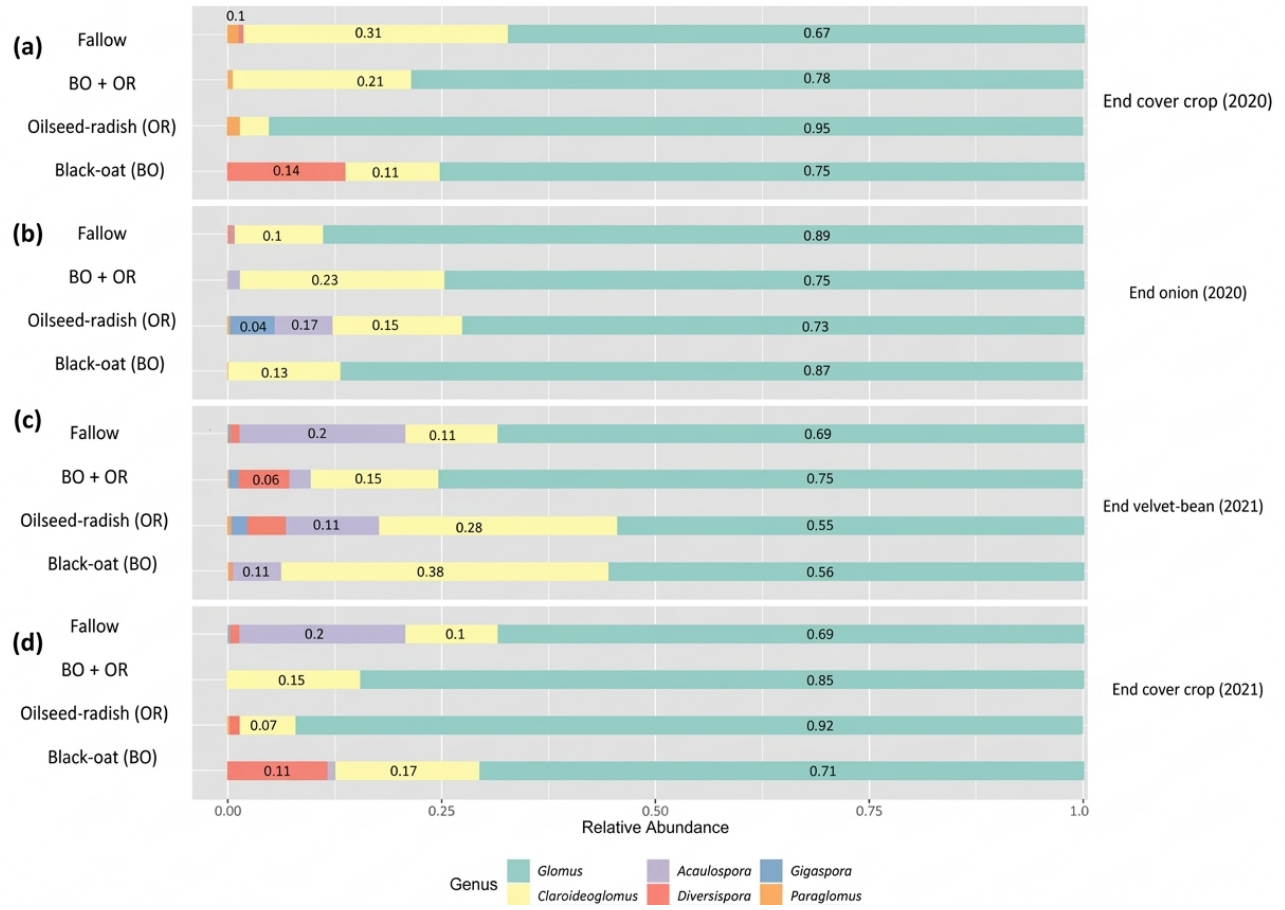

Figure 6. Relative abundance (1.0 = 100 %) of AMF genera identified from the rhizospheric soil at different times end cover crop (2020) (a), end onion (2020) (b), end velvet-bean (2021) (c) and end cover crop (2021) in the No-Tillage Vegetable System (NTVS) for onions evaluated across different seasons in 2020 and 2021 with different winter cover crops.

plant, but also with rye, which is mycorrhizal. The decreases in spore production may be associated with reduced plant diversity. This phenomenon was observed after growing oilseed radish and rye for 3 years, leading to a reduction in the AMF community composition compared to the consortium of rye with pea or rye with clover (Cloutier et al., 2020).

Archaeospora sp. (Archaeosporaceae) was detected exclusively in rye and in the conventional tillage system, which has a history of using millet, a mycorrhizal Poaceae (Figures 2 and 3). Although we found no studies directly associating *P. glaucum* with this AMF species, it has been shown that *Archaeospora* predominates in systems with Poaceae (Channabasava and Lakshman, 2015). The dominant species in our long-term experiment can be explained by their generalist nature (Chiomento et al., 2019). For example, *Claroideoglomus* was identified as dominant in all treatments (Figures 2 and 3). A study on soil microbial diversity and colonization in onion after mycorrhizal and non-mycorrhizal cover crops showed that the presence of non-mycorrhizal species did not affect spore diversity, but 23 species were found in soil around onion roots, most of which belonged to the genera *Glomus* and *Claroideoglomus* (Pakarinen et al., 2021).

The dbRDA (Figure 4) showed that AMF diversity was affected by soil cover type and season. Those results are in agreement with proposed models of geographic distribution for AMF species, emphasizing climate as a relevant abiotic factor and the plant community as a biotic factor (Eom et al., 2000; Chaudhary et al., 2008).

Despite being an established and low-cost technique, the morphological assessment of the AMF in ecosystems or areas impacted by human activities has limitations. Those limitations include variable sporulation among species, morphological ambiguities, and varying spore integrity (Kehri and Akhtar, 2018). Therefore, it is recommended to

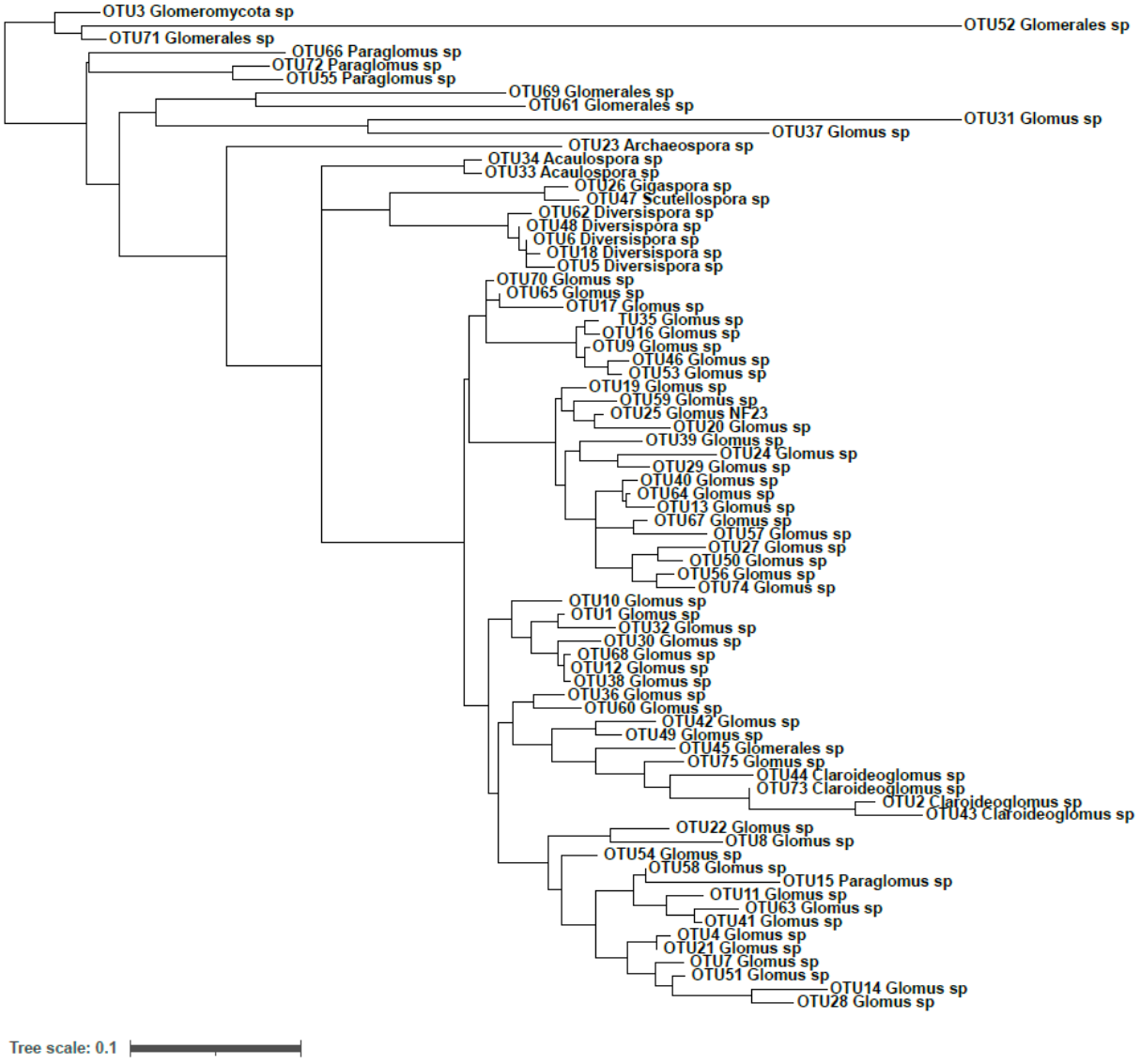

Figure 7. Phylogenetic tree based on sequences from the 18S region for AMF from rhizospheric soil of onion crops in the No-Tillage Vegetable System (NTVS) evaluated across different seasons in 2020 and 2021 with different winter cover crops. Clades based on alignments of domains (>97 %) with the maximum likelihood method, and 999 bootstrap replicates.

complement such surveys with molecular techniques, such as high-throughput sequencing (Noreen et al., 2023). Molecular approaches allow precise differentiation among similar species, detection of non-sporulating AMF, and reliable, detailed characterization of the AMF community structure (Hart et al., 2015), as observed in our study.

When comparing the results of our first survey, based on spore morphology, with the data obtained from DNA sequencing some years later, in the same area, all the families identified in 2017 were confirmed, including Glomeraceae, Acaulosporaceae, Gigasporaceae, Claroideoglomeraceae, and Paraglomeraceae, with a clear dominance by Glomeraceae (Figures 2, 3, 5, and 6). The molecular technique additionally allowed the identification of the family Diversisporaceae (Figures 5 and 6). This difference can be attributed to the identification method used since genetic sequencing, unlike the morphological approach, does not depend on a relatively abundant production of spores and the preservation of their structures for identification (Noreen et al., 2023). So far, to our knowledge, there are no studies on AMF diversity in no-tillage vegetable production using high-throughput sequencing.

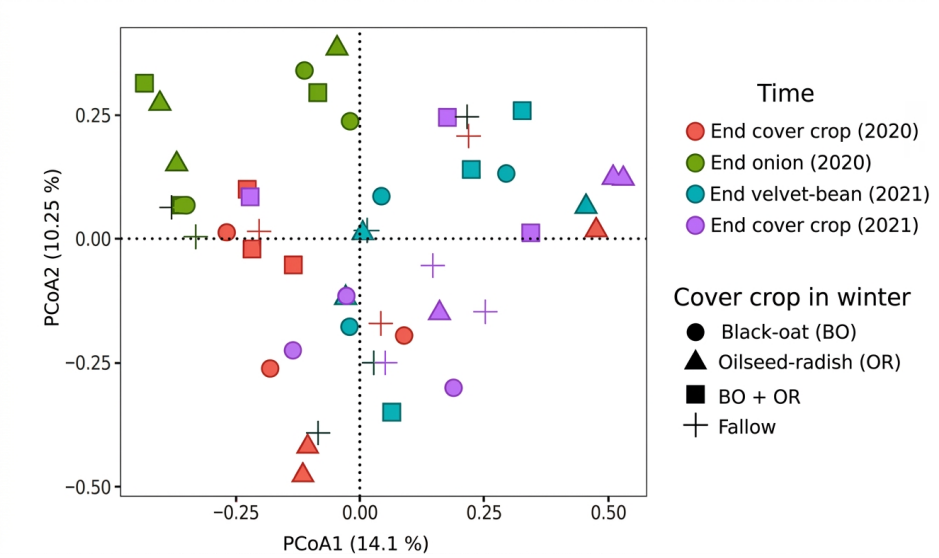

Figure 8. Principal Coordinates Analysis (PCoA) based on Bray-Curtis distance for the AMF community present in the No-Tillage Vegetable System (NTVS) for onions with different cover crops evaluated across different seasons in 2020 and 2021.

The use of various cover crops did not result in significant changes in arbuscular mycorrhizal fungi diversity, likely due to the stability of the system, which had already been using onions in the spring and velvet-bean in the summer for 11 and 12 years, respectively, by 2020 and 2021. Fallow had higher AMF diversity (Figure 6), and this increase can be attributed to the presence of diverse weeds in this area (Souza et al., 2020), as observed in tropical conditions (Mhlanga et al., 2022).

Onion growth increases the proportion of the genus *Claroideoglomus*. Notably, in the treatments with oilseed radish, the dominance of the genus *Acaulospora* was also observed (Figure 6). In comparison, a study using conventional onion tillage and high-throughput sequencing, identified 14 phylotypes, with distributions of 79, 14, and 7 % for the genera *Glomus*, *Archaeospora*, and *Paraglomus*, respectively (Galván et al., 2009). Those differences can be attributed to various factors, including environmental conditions (Cardoso and Andreotte, 2016), the plant species, and the management system adopted (Angelini et al., 2012).

Velvet-bean increases in the occurrence of the genera *Claroideoglomus* and *Acaulospora* in treatments that use black oats and oilseed radish (Figure 6). That underscores the effect of cover crops on AMF diversity, as evidenced by a previous study reporting the presence of Acaulospora, Glomus, and Scutellospora following velvet-bean cultivation (Benedetti et al., 2005). This observation highlights the role of cover crops in modulating AMF communities, determining their composition and diversity.

We decided to classify fungi up to the genus level to include all identified OTU, given that classification up to the species level resulted in numerous unclassified OTU. This limitation arises because the MaarjAM database (Öpik et al., 2010), a global reference for AMF classification used in its 2019 version for this study, still contains a limited number of sequences, particularly those of Brazilian origin. This situation underscores the importance of studies like ours for enriching the database and contributing to the scientific community (López-García et al., 2020). Some OTU were classified only up to higher levels, such as the phylum (OTU 3) and the order (OTU 61 and 69) (Figure 7). This indicates that those AMF have not yet been adequately classified in the genetic bank used, suggesting opportunities for further investigations in this field.

The PCoA in the second study did not reveal clear clustering trends, except at the end of the onion cycle (Figure 8). This specificity may be associated with the dominance

of the genus *Glomus* and the low diversity of genera observed and cataloged. This phenomenon highlights a possible impact of monoculture and the phenological state of the onion on the structure of arbuscular mycorrhizal fungi communities, as discussed by Prates et al. (2021). Such a situation underscores the complex interaction between agricultural practices and soil biodiversity, which affects the composition and dynamics of microbial communities.

Our study hypothesized that the presence of a non-mycorrhizal species, such as oilseed radish, would decrease the diversity of AMF present in the environment, but this was not evident from the spore taxonomy or high-throughput sequencing. This is linked to the consolidation of the management system, which has a history of growing onion and velvet-bean mycorrhizal species after winter cover crops since 2009. That management system may have maintained or reestablished AMF species in the system (Caproni et al., 2018), suggesting that the resilience of AMF communities to changes in crop composition, and highlighting the complexity of interactions between plants and microorganisms in the soil.

CONCLUSIONS

Morphological identification of arbuscular mycorrhizal fungi (AMF) spores revealed a dominance of the families Glomeraceae and Acaulosporaceae. This was confirmed by high-throughput sequencing of the rhizospheric soil, which yielded 75 operational taxonomic units, predominantly of the genus *Glomus*. The hypothesis that the presence of a non-mycorrhizal plant, such as oilseed radish, would decrease the occurrence of AMF species was not supported by either morphological assessments or molecular techniques, showing that AMF communities are affected by the management system as a whole. Use of various cover crop species in a long-term succession system maintains the natural mycorrhizal community in terms of occurrence. This suggests that crop diversity within rotational systems plays a critical role in sustaining the health and stability of soil microbial communities.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://www.rbcsjournal.org/wp-content/uploads/articles_xml/1806-9657-rbcs-50-e0250039/1806-9657-rbcs-50-e0250039-suppl01.pdf

DATA AVAILABILITY

The data will be provided upon request.

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