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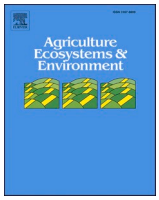

Durum wheat-lentil relay intercropping enhances soil mycorrhizal activity but does not alter structure of arbuscular mycorrhizal fungal community within roots

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ABSTRACT

In intercropping systems, crop species select host-adapted microorganisms and influence the associated plant-microbial interactions like in the case of arbuscular mycorrhizal fungi (AMF). Attempts to assess the impact of intercropping on the activity, diversity, and community composition of AMF remain inconclusive, more so in intercropping systems involving traditional Mediterranean crops such as durum wheat and lentils. We carried out field experiments in Central Italy to assess the impact of relay intercropping durum wheat (*Triticum durum* Desf. cv. Minosse) and lentil (*Lens culinaris* Medik. cv. Elsa) on soil mycorrhizal inoculum potential (MIP) (2019 and 2020), AMF root colonization (2019, 2020, and 2021), and root AMF diversity and community composition (2020 and 2021), compared to the respective sole crops. Results showed that relay intercropping enhanced lentil grain yield and durum wheat grain protein concentration but marginally reduced durum wheat grain yield and lentil grain protein concentration. In addition, relay intercropping enhanced soil mycorrhizal activity but differentially influenced mycorrhizal root colonization compared to sole cropping. Sequencing analyses generated a total of 234 amplicon sequence variants belonging to Glomeromycota, which were assigned to 31 virtual taxa using the MaarjAM reference database. Glomeraceae and Claroideoglomeraceae were the most abundant taxa but had contrasting abundances in 2020 and 2021. The overall changes in AMF species diversity and community structure were affected by the interaction between crop species and year, and not by intercropping. *Claroideoglomus* and *Septoglomus* showed a strong association with lentil roots while *Rhizophagus* and *Paraglomus* were associated with durum wheat roots in 2020, affirming host genotype-AMF preferences. The principal component analysis showed that grain protein concentration was associated with selected mycorrhizal parameters such as community richness and AMF root colonization. Further studies on the functional analysis of the different AMF communities selected by the crop genotype and year may reveal the importance of intercropping in maintaining soil functionality and productivity under low-input systems.

1. Introduction

Relay intercropping involves the simultaneous growing of two or more crops on the same field for part of their growing cycle and is considered more advantageous over monocultures (Tanveer et al., 2017). For instance, it has been proposed to promote the above- and below-ground biodiversity that jointly regulate the agroecosystem's multifunctionality at different spatial and temporal scales, especially in low-input cropping systems, where microbial communities including arbuscular mycorrhizal fungi (AMF) and plant growth promoting rhizobacteria (PGPR) mediate below-ground complex interactions important in maintaining soil fertility and plant fitness (Amossé et al., 2014; Pivato et al., 2021; Lu, 2022). Although relay intercropping increases soil biodiversity, investigations on such practice have been limited to a small number of crop species combinations and, to a lesser extent, have taken into account plant density configuration or the role of AMF in affecting crop yield (Amossé et al., 2013; Yang et al., 2017; Raza et al., 2019).

Most of the previous intercropping studies have reported a number of benefits linked to the integration of legumes into cereal cropping

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systems, i.e., yield stability, pest and disease reduction, reduced inter-specific competition (temporal and spatial), enhanced resource use and harvesting efficiency, among others (Latati et al., 2019; Fan et al., 2020; Gong et al., 2020; Kammoun et al., 2021). A few intercropping studies have examined the contribution of microbial communities such as AMF under low input conditions in contributing to intercrop yield advantages (Lu, 2022; Lee et al., 2023). Contemporary intercropping, although very common globally, may not be suitable in certain geo-climatic areas due to temperature sensitivity and biological cycle of specific crops such as spring lentil (*Lens culinaris* Medik.) and winter durum wheat (*Triticum durum* Desf.). Due to environmental constraints, relay intercropping of spring legumes like lentils in already established winter cereals such as durum wheat is commonly practiced in some parts of the Mediterranean area. The fluctuating weather patterns (cold stress and rainfall), which affect the sowing time and yield stability of winter durum wheat i.e., in some extreme cases the sowing can shift from October to February, has become a concern to the Mediterranean durum wheat farmers (Yang et al., 2020). This calls for new sustainable alternative practices that can improve the stability of durum wheat production in the area. Few studies on durum wheat-legume relay intercropping managed under rain-fed Mediterranean low-input conditions have been carried out, yet this practice offers sowing flexibility and potential yield advantages (Bedoussac and Justes, 2010; Mariotti et al., 2012).

Over the years, efforts have been channeled to assess the impact of intercropping on the diversity and composition of PGPR through field and greenhouse studies (Schmid et al., 2019; Dang et al., 2020; Tang et al., 2020; Pivato et al., 2021). Still, studies focusing on the impact of intercropping cereals and legumes such as durum wheat and lentils on AMF communities, more so in the Mediterranean alkaline soils with limited P, remain scarce (Guzman et al., 2021; Pires et al., 2021). Crop species select for host-adapted microorganisms thus shaping plant-associated microbial communities such as AMF (Njeru et al., 2017; Hontoria et al., 2019). Increasing plant species diversity impacts soil quality, AMF diversity, and agroecosystem multifunctionality (Ryan and Graham, 2018; Guzman et al., 2021), by enhancing root–root interactions, soil C (an energy source for microbes) littering and rhizodeposition (Sánchez-Galindo et al., 2021), and modifying the rhizosphere environment, for instance, pH (Khlifa et al., 2017). Considering that cereals are more competitive for soil inorganic nutrients such as N and P than legumes (Jensen et al., 2020), there is a need to examine (under the Mediterranean low input conditions) the interaction of cereal-legume intercrops with soil microorganisms such as AMF important in nutrient acquisition and mobilization.

Studies on changes in the diversity and composition of mycorrhizal community induced by various intercrop species have yielded variable results. Jefwa et al. (2006) reported a reduced AMF diversity in a *Sesbania sesban*-maize intercrop, while Zhang et al. (2020) showed that maize-soybean intercropping promoted the diversity and shaped the structure of AMF communities in soil and root compartments, compared to the respective sole crops. Using wheat-soybean and *Eucalyptus grandis-Acacia mangium* intercrops, Lu (2022) and Bini et al. (2018), respectively, showed that intercropping increases AMF activity and diversity in the soil, thanks to the intercrops that modified soil biochemical properties. Besides the influence of crop species, AMF community diversity and composition changes due to crop cultivars have been detected. For instance, different wheat cultivars may select specific AMF phylotypes colonizing the roots, signifying a complex pattern of plant-AMF interactions even at the genotype level (Mao et al., 2014; Stefani et al., 2020). Thus, the choice of the intercrop partner in relay intercropping could be critical. Ideally, those plant species or cultivars that complementarily stimulate early root colonization, fasten spore germination, and boost the functionality of the local AMF communities should be considered. In this study, we prioritized the selection of cultivars based on their functional traits, i.e., resource use complementarity in the intercrop, growth habit, maturity time, seed size, and microbial association. Based on our functional approach, field experiments were designed to explore the intercropping effect of the selected cultivars on AMF abundance, composition, and diversity.

Despite being a minor cereal crop globally, durum wheat is widely cultivated in the Mediterranean basin, often as a monoculture. Integration of legume partner crops such as chickpea, faba bean, soybean, field bean, lentil, and peas is being increasingly adopted (Bedoussac and Justes, 2010; Mariotti et al., 2012; Latati et al., 2019; Kammoun et al., 2021). Lentil is a highly nutritional legume and a hardy crop that can withstand adverse Mediterranean growing conditions but suffers considerable yield losses due to lodging and weed infestation (Koskey et al., 2022; Sellami et al., 2021). These crops have different functional, morphological, phenological, and biochemical traits, i.e., root structure, root exudate composition, nutrient acquisition strategies, growth habit and cycle, allelopathic substances, and mycorrhizal dependencies, which can differentially influence the AMF recruitment, community composition and diversity (Gunes et al., 2007; Ryan and Kirkegaard, 2012; Leoni et al., 2021; Koskey et al., 2022). Legume crops such as lentils and peas are more mycotrophic than cereal crops such as wheat and tend to depend more on AMF symbiosis for nutrient acquisition (Bainard et al., 2014). In addition, lentils and peas can stimulate early recruitment of specific AMF and *Rhizobium* species to form endosymbioses that facilitate P and N transfer to the non-legume partner and consequently reduces the inter-specific plant competition through facilitation (Bastolla et al., 2009; Wahbi et al., 2016; Hontoria et al., 2019). Therefore, because of trait complementarity between the two companion crops, we expect that the durum wheat-lentil association could be successful in enhancing below- and above-ground interactions important in maintaining soil health and crop productivity (Bedoussac and Justes, 2010; Costanzo and Bàrberi, 2014).

To our knowledge, there are no studies focusing on the impact of durum wheat-lentil intercrops on AMF communities found in Mediterranean alkaline soils. Furthermore, the effect of varying the intercrop plant density on AMF richness has not been exhaustively studied. Intercrop densities can indirectly affect AMF abundance by interfering with N and P balance in the soil rhizosphere. For instance, high sowing density of legumes can increase N input and may decrease P availability, hence stimulating AMF activity to counteract the P stress (Xiao et al., 2019). AMF characterization remains a challenge considering that it is difficult to grow them in pure lab cultures due to their obligate biotrophic nature (Kim et al., 2022). Nonetheless, they should be studied and researched to understand better their presence and contribution in nutrient mobilization under different cropping systems. The activity of the indigenous AMF has been assessed using the soil mycorrhizal inoculum potential (MIP) bioassay, which evaluates the infectivity of soil AMF inoculum on young roots (Bedini et al., 2013; Njeru et al., 2014). Meanwhile, the diversity and AMF community structure have been studied on plant roots using next-generation sequencing techniques (Hontoria et al., 2019; Lu, 2022).

In this study, we carried out a field experiment, to investigate the impact of relay intercropping durum wheat with lentils on the activity (soil MIP and root colonization), diversity, and community structure of AMF colonizing the plant roots. We also assessed the association between the selected agronomic and mycorrhizal parameters as influenced by intercropping. We hypothesized that (i) the soil from the intercropped fields will have higher mycorrhizal inoculum potential (MIP) than that of the sole crops, (ii) intercropped durum wheat will have a higher root AMF colonization than the sole crop, with lentil partner facilitating the AMF colonization increment, (iii) AMF diversity and structure will significantly differ depending on the crop species (durum wheat vs. lentil), cropping type (high- and low-density intercrop vs. sole crop) and year (2020 vs. 2021). The differences in mycotrophic levels of the two hosts and root architecture and biochemistry will probably influence AMF root colonization and hence the detected AMF communities. Thus, the intermingling of roots in the intercropped plots will host a higher AMF community diversity and composition than that from the

sole cropped plots, with lentils hosting more diverse AMF communities than durum wheat. These differences will also be affected by the field location and environmental conditions, indicated herein as the 'year' factor. (iv) Intercropping will enhance the overall crop productivity and grain nutritional quality (protein concentration), thanks to the facilitative and complementary functional traits of the intercrops.

2. Materials and methods

2.1. Experimental site, soils, and climate

Field experiments were carried out in 2019, 2020, and 2021 growing seasons at the Interdepartmental Centre for Agri-Environmental Research Enrico Avanzi (CiRAA) of the University of Pisa (43°40'48.0''N, 10°20'45.5" E) in three separate (200–700 m apart) but adjacent field sites (Fig. S1) managed as a rain-fed, low input system with no use of chemical fertilizers, herbicides, or pesticides. The three experimental fields had characteristic alkaline sandy loam soil (49–54 % sand, 37–41 % silt, and 8–10 % clay), classified as Typic Xerofluvent by USDA (Soil Survey Stuff, 1999) and as Fluvisol by FAO (IUSS Working Group WRB, 2015), with pH (1:1 H_2O) of 8.28–8.35, total Kjeldahl N 1.17–1.27 g kg^{-1} (Bremner, 1960), Walkley-Black organic carbon 1.14–1.24 % (Nelson and Sommers, 1996), and available Olsen P_2O_5 14.35–17.70 mg kg^{-1} (Olsen, 1954) (Table S1). Seedbed was prepared by moldboard ploughing to 25 cm depth followed by a shallow (10 cm depth) disc harrowing. The preceding crops were lucerne (*Medicago sativa* L.), corn (*Zea mays* L.), and common wheat (*Triticum aestivum* L.) in 2019, 2020, and 2021, respectively. The total rainfall during the first six months (November to April) critical for durum wheat and lentil growth was 385 mm, 560 mm, and 660 mm in 2019, 2020, and 2021, respectively (Fig. S2).

2.2. Planting materials, cropping pattern, and experimental design

The trial involved relay intercropping of autumn-sown durum wheat (*Triticum durum* Desf. cv. Minosse) with early spring-sown lentil (*Lens culinaris* Medik. cv. Elsa). Seeds of both crop cultivars were procured from Agroservice SpA. (Marche, Italy), and were chosen because of their growth habits, phenological trait complementarity, and environmental adaptability to the Mediterranean conditions. Lentil cv. Elsa has a medium-late growth cycle that synchronizes well when relay intercropped in early spring with autumn-sown durum wheat cv. Minosse (https://www.agroservicespa.it/media/pages_file/37/Catalogo_Web_EN_ISEA_2021.pdf). The cropping pattern involved row planting of winter durum wheat (November – January) in small plots (3 × 8 m), followed by relay intercropping with lentils within the wheat rows at the end of February in each year. Crop seeds were sown in 18 cm spaced rows using a small plot precision seeder. We used a randomized complete block design (RCBD) with four replications in the first year and five replicates in the second and third year. The design consisted of plots of (i) sole durum wheat-100 % density, (ii) sole lentils-100 % density, (iii) relay intercropped durum wheat-33 % + lentils-100 % (33 %Mix-low density), and (iv) relay intercropped durum wheat-100 % + lentils-100 % (100 %Mix-high density). Considering that wheat has a more aggressive growth and is more competitive than lentils, we chose to vary durum wheat density, and this was based on the knowledge gained from our previous pilot trials. Therefore, the target crop densities were 350 plants m^{-2} for durum wheat (sole cropped and 100 %Mix) and 116 plants m^{-2} for 33 %Mix durum wheat, and 180 plants m^{-2} for all the lentil treatments (Fig. S1).

2.3. Soil mycorrhizal inoculum potential (MIP) bioassay

Soil samples were collected from a depth of 0 – 20 cm in three randomly selected points within each plot using a 5-cm diameter soil probe. The three sub-samples were mixed to form a homogenous representative plot sample. Only 2019 and 2020 fields were considered for MIP analyses. The first sampling was done at wheat tillering (BBCH 29) stage (Meier et al., 2009), just one day before lentil sowing, and the second after wheat harvesting (BBCH 92). The pooled soil samples were used to assess mycorrhizal activity in the soil following the soil MIP bioassay protocol described by Njeru et al. (2014). *Cichorium intybus* L. cv. Zuccherina di Trieste seeds were used as host plants and sown in three replicates of 50 ml sterile Falcon tubes filled with 45 g of each plot soil sample. The plants were maintained in a growth chamber (16/8 h light/dark daily cycle, 24/20 °C and watered twice a week) until harvest. The whole root system of each plant was harvested 35 days after emergence by carefully washing away the soil to minimize root disturbance. The *C. intybus* roots were stained with acidified trypan blue dye (Phillips and Hayman, 1970) and the proportion of colonized root length was determined under a dissecting microscope at × 40 magnification using the gridline intersect method (Giovannetti and Mosse, 1980).

2.4. Durum wheat and lentil root sampling

Root sampling of both crops was done at the same time following durum wheat phenology 50 % wheat flowering BBCH 60 (Meier et al., 2009), and inner rows were considered. Roots were sampled per host in each treatment, i.e., (wheat root on sole wheat, lentil root on sole lentil, wheat root in 33 %Mix, lentil root in 33 %Mix, wheat root in 100 %Mix, and lentil root in 100 %Mix. Six plants of each host in each plot were chosen randomly and dug out gently with a hand spade at 20 cm depth and 15 cm radius. In intercrops, a whole root system composed of wheat + lentil was dug out and separated by host species. The aboveground portion of each plant was cut and discarded. The roots were washed in water at the site and stored at 4 °C in portable cooler boxes. Finally, fine roots (≤ 1 mm thick) were cut into 3-cm long fragments and 10 aliquots of the fragments from each of the six plants were pooled to make a composite plot sample, which was stored at − 80 °C for subsequent AMF root colonization assessment and DNA extraction. Root samples from 2019, 2020, and 2021 fields were considered for AMF root colonization assessment while the samples from 2020 and 2021 fields were selected and screened for AMF diversity and community composition (Fig. S1).

2.4.1. AMF root colonization assessment

Ten aliquots of 3-cm long pieces of fine roots of durum wheat and lentils from each composite sample were randomly chosen for mycorrhizal staining following the same procedure described in the soil MIP bioassay Section 2.3 above.

2.4.2. DNA extraction, amplification, and Illumina library preparation

Thirty six (36) wheat and lentil root samples collected in 2020 and 2021 were selected for molecular analyses. i.e., [1 (sole wheat) + 1 (sole lentil) + 1 (100 %Mix wheat) + 1 (100 %Mix lentil) + 1 (33 %Mix wheat) + 1 (33 %Mix lentil)] × 3 replicate blocks × 2 years. Aliquots (250 μg) of the root fragments in each composite plot sample were homogenized in liquid nitrogen and ground to a fine powder. Total community DNA was extracted using the DNeasy® PowerSoil® Pro Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The quality and quantity of the extracted DNA were checked using agarose gel electrophoresis and fluorometric quantification (Qubit 2.0 fluorometer, Invitrogen, USA), respectively. Three amplicon libraries for each treatment and plant species were prepared by PCR amplification of the 18 S rRNA gene according to the Illumina 18 S metagenomic sequencing library protocol. PCR amplification of the AMF communities was performed with primers AML1/AML2 (Lee et al., 2008) which targets the V3–V5 variable region of the 18 S small subunit (SSU) rRNA gene using an iCycler Thermal Cycler (Bio-Rad Laboratories, Inc., Milan, Italy). Amplification was done in 50 μl reaction mix composed of: 1 μl genomic DNA (10–20 ng), 5 μl *Ex Taq* buffer 10X, 4 μl (0.2 mM each) dNTP mixture, 1 μl (0.5 μM) of each primer (AML1/AML2), and 1.25 U of *TaKaRa Ex Taq* (5 U μl^{-1}) DNA Polymerase (Takara Bio Inc., Shiga,

Japan). Thermocycling conditions used were a hot start initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 58 °C for 40 s, extension at 72 °C for 55 s, and a final extension at 72 °C for 10 min. PCR amplicons were visualized in a GelRed® (Biotium, USA) stained 2 % agarose gel and samples were run at 80 V for 60 min. Amplicons with 800 bp were selected for downstream purification and concentration.

Before library preparation, a nested PCR using a new primer set nu-SSU-0450–5′ (5′- CGCAAATTACCCAATCCC-3′) and nu-SSU-0899–3′ (5′-ATAAATCCAAGAATTTCACCTC-3′) that targets the V3–V4 region of the nuclear 18 S ribosomal RNA gene and yields a 490 bp length amplicon, was carried out to comply with the sequencing length capacity of the 2 × 300 bp Illumina MiSeq® Reagent Kit v3 (Stefani et al., 2020). In the nested PCR, purified amplicons from the initial PCR which used AML1/AML2 primers were used as templates and the thermocycling conditions remained the same except for the number of cycles that were reduced to 20.

Illumina library preparation, using the purified products from the nested PCR, was done following the protocol described by Stefani et al. (2020). Briefly, the PCR products were purified using Agencourt AMPure XP (LABPLAN; Naas, Ireland) according to the Illumina metagenomic sequencing library protocol. Purified PCR products were normalized to 1–2 ng μl^{-1} with the SequalPrep™ Normalization Plate kit (ThermoFisher Scientific, Waltham, USA) according to the manufacturer's instructions. Nextera index kit (Illumina, San Diego, USA) was used for library indexing according to the manufacturer's protocol. Indexed purified and normalized amplicons were quantified by qPCR using the Illumina KAPA library quantification kit (KAPA Biosystems, USA). A paired-end 2 × 300 bp sequencing was carried out on the final high-quality libraries using an Illumina MiSeq® sequencer for 500 cycles at the BMR Genomics S.r.l (Padova, Italy).

2.4.3. Bioinformatic analyses

Processing and analysis of raw demultiplexed sequences were carried out in QIIME2 (Bolyen et al., 2019) version 2020.2 pipeline, following the protocol described by Stefani et al. (2020). Briefly, the high throughput paired-end sequence reads were pre-processed using Cutadapt v.10 (Martin, 2011) included in the QIIME2, then denoised, dereplicated, and filtered for chimeras using the DADA2 plugin (Callahan et al., 2016). Amplicon sequence variants (ASVs) with a frequency less than 0.1 % of the mean sample sequencing depth were removed. De-novo clustering at a 100 % similarity threshold was performed using the QIIME2 vsearch plugin (Rognes et al., 2016). Taxonomic assignment and classification of ASVs were grouped into virtual taxa (VT) against the MaarjAM (Öpik et al., 2010) and NCBI GenBank databases. Hits with the highest pairwise similarity (>97 %) and query coverage (>95 %) were selected and sequences belonging to Glomeromycota were only considered. A phylogenetic tree was inferred using cultured and un-cultured AMF reference sequences deposited in the MaarjAM and NCBI GenBank databases to refine and complement the taxonomic classification of each ASV. The Bootstrap value was set at 999 and Tamura 3-parameter (T92) + gamma distribution (G) substitution model was chosen. The relative abundance (%) of each ASV was calculated from the total number of sequence reads. The raw sequence data were submitted (submission number SUB12294999) to the NCBI GenBank database under the accession numbers OP894122 - OP894355.

2.4.4. Diversity analyses

AMF alpha-diversity was calculated using the R *vegan* package ver. 4.1.0 (R Core Team, 2021) and presented as Hill numbers, taking virtual taxa as a proxy of species richness (Hill, 1973; Chao et al., 2014). Bray-Curtis dissimilarity index (Bray and Curtis, 1957) was used to estimate the AMF community dissimilarity between different treatments.

2.5. Statistical analyses

Depending on the type of data and error distribution, generalized linear mixed-effect models (GLMM) with a Gaussian or log link distribution functions in 'Lme4' R package (Bates et al., 2015) were fitted to determine the effect of crop species (durum wheat and lentil) and/or crop stand type (high- and low-density intercrops and sole crops) on soil MIP and AMF root colonization comparing with that of the sole crops. Year (2019, 2020, and 2021) was treated as a fixed factor only if there were significant year × crop or year × crop stand type interaction effects. Blocks and plot pseudo-replicates were used as random factors when available. Kolmogorov–Smirnov test of normality in the 'DHARMa' R package (Hartig and Lohse, 2021) was used to assess the goodness of fit of the chosen model based on the scaled residuals. Tukey's post hoc test using R/emmeans was used to check for the significant differences of means at $p \leq 0.05$ (Lenth, 2019). Differences in the AMF community structures between host crop species, crop stand types, and year were examined using the Permutational Multivariate Analysis of Variance (PERMANOVA, nperm = 999) in R *vegan* package (Oksanen et al., 2022) and illustrated using a non-metric multidimensional scaling (NMDS) based on the Bray-Curtis's dissimilarity matrices (nperm = 999). Indicator species analysis (Hill et al., 1975), was used to identify the AMF VT groups associated with a given crop species and year. The indicator value (IndVal) from the R *indcspecies* package indicates the strength of association while the statistical significance of association is tested using a permutation test (nperm = 999) at $p < 0.05$ (De Cáceres et al., 2010).

3. Results

3.1. Effect of crop stand type and year on soil MIP

Soil MIP bioassay showed a common level of mycorrhizal colonization (35–41 % in 2019 and 36–44 % in 2020) in all the plots before relay intercropping (Fig. 1; Table S2). At the end of the crop cycle, MIP values increased at different measures (40–52 % in 2019 and 42–55 % in 2020) depending on the crop stand type and year, upon a significant ($p <$ 0.0001) crop stand type × year interaction (Fig. 1; Table S3). Notably, intercropped fields consistently but interchangeably recorded a higher soil MIP in 2019 (52.8 ± 0.9 % and 47.2 ± 1.0 % in 100 %Mix and 33 % Mix, respectively) and 2020 (48.1 ± 0.7 % and 54.9 ± 0.9 % in 100 % Mix and 33 %Mix, respectively) compared to the sole lentil fields which had the lowest MIP values at the end of the crop cycle in both years (40.2 ± 1.6 % and 41.9 ± 1.1 % in 2019 and 2020, respectively) while the sole wheat fields showed a higher MIP (47.2 ± 0.8 %) in 2019 (Fig. 1; Table S4).

3.2. Effect of crop stand type and year on AMF colonization in durum wheat roots

Averaged among all the crop stand types, the proportion of root length colonized by AMF in durum wheat was lower at tillering (40.5 ± 1.8 %) compared to the colonization level at flowering (50.4 ± 2.5 %) (Fig. 2). At flowering, multiple significant interactions were observed between the crop stand type × year ($p = 0.014$), crop stand type × sampling stage ($p < 0.0001$), and crop stand type × sampling stage × year ($p < 0.0001$) (Fig. 2; Table S5 and S6). The intercropped durum wheat roots recorded a consistently higher AMF root colonization at flowering stage than the sole-cropped wheat in 2020 (58.6 ± 1.6 % and 62.7 ± 2.1 % in 100 %Mix and 33 %Mix, respectively vs. 48.3 ± 0.9 % in sole wheat) and 2021 (54.6 ± 1.2 % and 55.4 ± 1.4 % in 100 %Mix and 33 %Mix, respectively vs. 44.1 ± 1.5 % in sole wheat), regardless of the intercropping density (Fig. 2; Table S6).

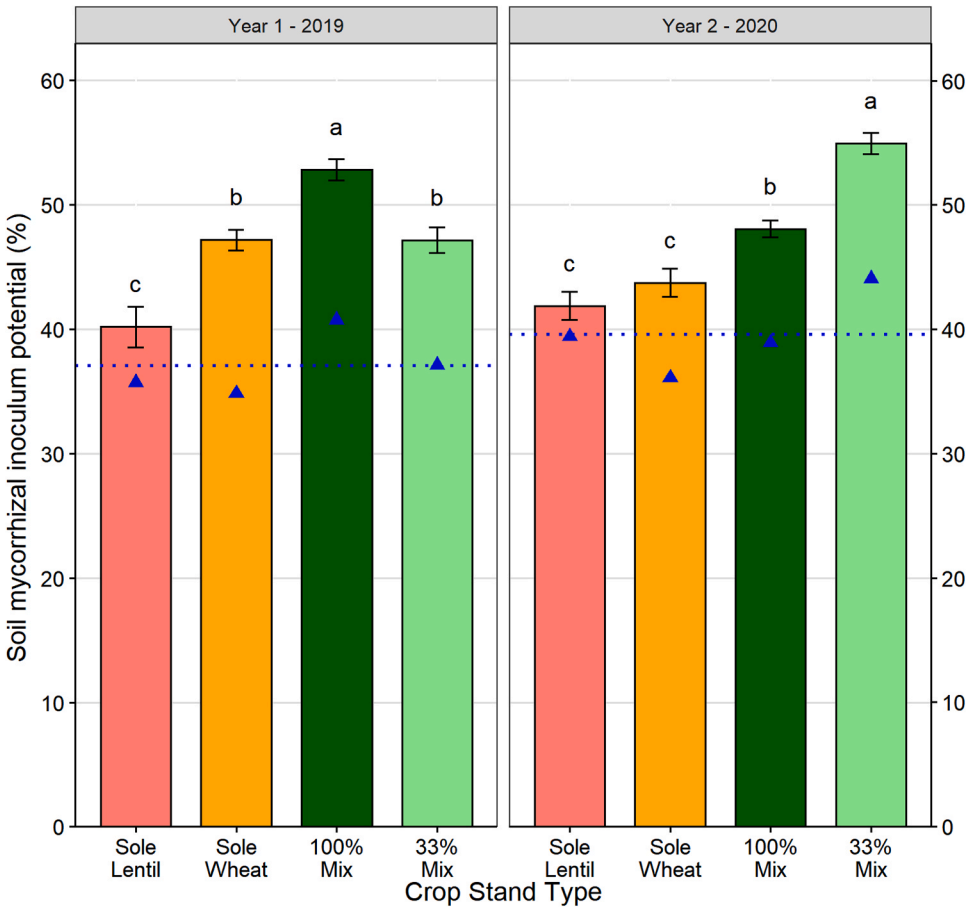

Fig. 1. Effect of crop stand type and year on soil mycorrhizal inoculum potential (MIP) activity before relay intercropping and at the end of the crop cycle. Different lowercase letters indicate statistically significant differences among the crop stand type treatments (Tukey's post hoc test $p \leq 0.05$) within each year. The bars indicate the mean MIP values after crop harvesting while error bars represent the standard errors of the means. The blue triangles indicate the mean MIP values of each crop stand type, and the dotted horizontal blue line indicates the baseline MIP averaged among the crop stand type treatments before sowing lentils for sole crops or undersowing lentils on to the already established durum wheat for the intercrops. 100 %Mix, high-density wheat + lentil intercropping; 33 %Mix, low-density wheat + lentil intercropping.

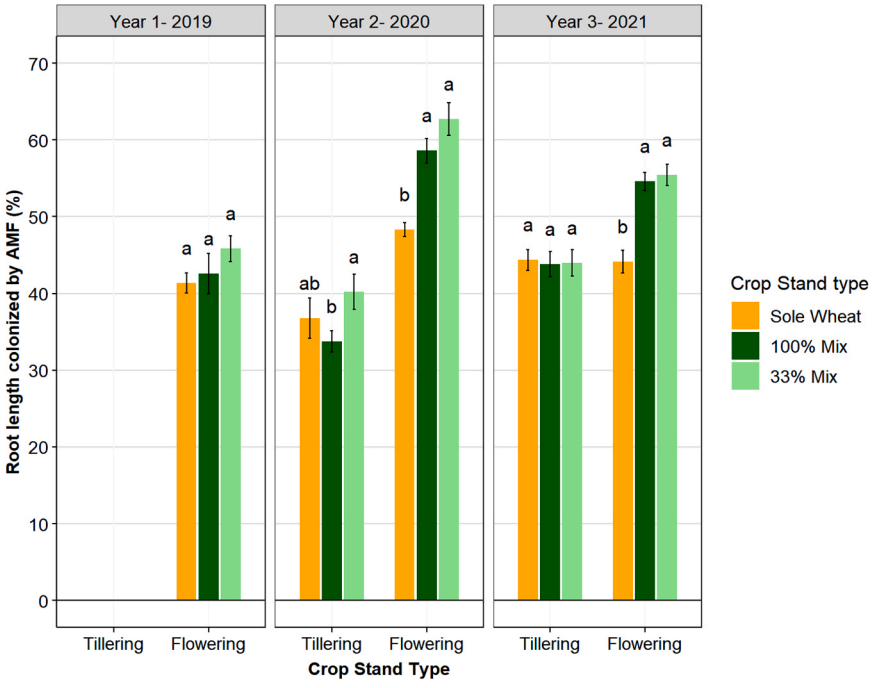

Fig. 2. Effect of crop stand type and year on durum wheat AMF root colonization at tillering and flowering stages. Different lowercase letters indicate statistically significant differences among the crop stand type treatments (Tukey's post hoc test $p \leq 0.05$) within each sampling stage in each year. The bars indicate the percentage root length colonized by AMF while error bars represent the standard errors. The 2019 data at tillering stage are not available. 100 %Mix, high-density wheat + lentil intercropping; 33 %Mix, low-density wheat + lentil intercropping.

3.3. *Effect of crop stand type and year on AMF colonization in lentil roots*

Averaged across the years, the proportion of the root length colonized by AMF was generally higher in lentils (64.1 ± 1.5%) than in durum wheat (50.9 ± 1.2 %) at the flowering stage (Table S7). Among the lentil treatments, AMF colonization significantly differed depending

on the crop stand type ($p < 0.0001$), year ($p < 0.0001$), and crop stand type × year ($p = 0.046$) interaction (Fig. 3; Table S8). Root colonization was lower in the intercrops (59.6 ± 6.6 % and 65.0 ± 2.8 % in 100 % Mix and 33 %Mix, respectively, in 2019, 57.3 ± 2.0 % and 72.2 ± 3.4 % in 100 %Mix and 33%Mix, respectively, in 2020, and 54.6 ± 1.2 % and 55.1 ± 2.1 % in 100 %Mix and 33 %Mix, respectively, in 2021) depending on the year and wheat density, compared to the sole crops (72.2 ± 2.2 %, 76.2 ± 2.0 % and 66.0 ± 1.6 % in 2019, 2020 and 2021, respectively). Moreover, in 2020, root colonization was significantly lower in lentil roots intercropped with high-density wheat (57.3 ± 2.0 %), compared to the lentil roots intercropped with low-density wheat (72.2 ± 2.2 %) (Fig. 3; Table S9).

3.4. Effect of crop species, crop stand type, and year on root AMF taxonomic composition and distribution

Sequencing analyses produced 761,833 and 720,078 reads in 2020 lentil and durum wheat root samples, respectively, and 704,261 and 754,133 reads in 2021 lentil and durum wheat root samples, respectively (Table S10, S11). Approximately, 41 % and 34 % of the 2020 lentil and durum wheat reads, respectively, and 40% and 41 % of the 2021 lentil and durum wheat reads, respectively, passed merging, trimming, and chimera filtering steps, and were analyzed for amplicon sequence variant (ASV) AMF search (Table S10).

In total, 234 ASVs which matched the Glomeromycota 18 S small subunit rRNA gene sequences (similarity ≥ 97 %) from the MaarjAM database (Öpik et al., 2010) were generated from the final sequence reads and were clustered into 31 virtual taxa (VT) (Fig. S3, Table S11) represented by the families Glomeraceae (22 VTs, 164 ASVs), Claroideoglomeraceae (4 VTs, 41 ASVs), Diversisporaceae (3 VTs, 21 ASVs), Archaeosporaceae (1 VT, 4 ASVs)and Paraglomeraceae (1 VT, 4 ASVs) (Fig. 4, S4, Table S11). The most abundant AMF taxa VTX00193 and VTX00067 corresponded to *Claroideoglomus lamellosum* and *Funneliformis mosseae*, respectively, based on BLASTN and represented 38.0 % and 34.5 % of all the reads (Fig. 4). The abundance and distribution of the dominant taxa varied between the two years and crop species, whereby, ASV assigned to the VTX00193 were the most abundant in 2020 root samples (83.7 % and 40.3 % in lentil and durum wheat, respectively) while those of the VTX00067 taxa dominated in 2021 root samples (61 % and 52.8 % in lentil and durum wheat, respectively). Each of the remaining VT represented less than 10 % of the total relative abundance: though, in 2020, VTX00113 which corresponds to *Rhizophagus intraradices* showed higher occurrence in durum wheat (25 %) than in lentil root samples (3.3 %). Only a few other minor taxa were present at higher than 5 % abundance in some crop stand type treatments; for instance, VTX00064, VTX00143, and VTX00065, which correspond to *Septoglomus constrictum*, *Glomus* sp., and *Funneliformis caledonius*, respectively (Fig. 4). Remarkably, most of the VT were commonly (18 VT in 2020 and 22 VT in 2021) identified in lentil and durum wheat as illustrated by the Venn diagrams (Fig. S5).

3.5. Effect of crop species, crop stand type, and year on AMF diversity

Crop stand type did not significantly influence alpha diversity indices of the AMF communities in wheat and lentil roots in 2020 and 2021. However, crop species and year differently affected the AMF diversity (Hill numbers and Pielou's evenness), as attested by the significant year × crop interactions (Table 1). AMF richness in lentils did not significantly change between years (15.1 ± 0.46 in 2020 and 15.9 ± 1.11 in 2021), while in durum wheat, a higher AMF richness was detected in 2020 (17.1 ± 1.24) than in 2021 (14.9 ± 1.11). Pielou's AMF evenness were lower in lentils in 2020 than in durum wheat while no differences in AMF evenness were detected between the two crops in 2021. The unbalanced distribution of species was confirmed by the analysis of the Hill numbers. In durum wheat Hill N1 was higher (5.01 ± 0.68) than in lentils (2.14 ± 0.24) in 2020, but no significant differences were detected in 2021 (3.23 ± 0.39 and 3.55 ± 0.41, respectively). The same trend was observed for Hill N2 (Table 1).

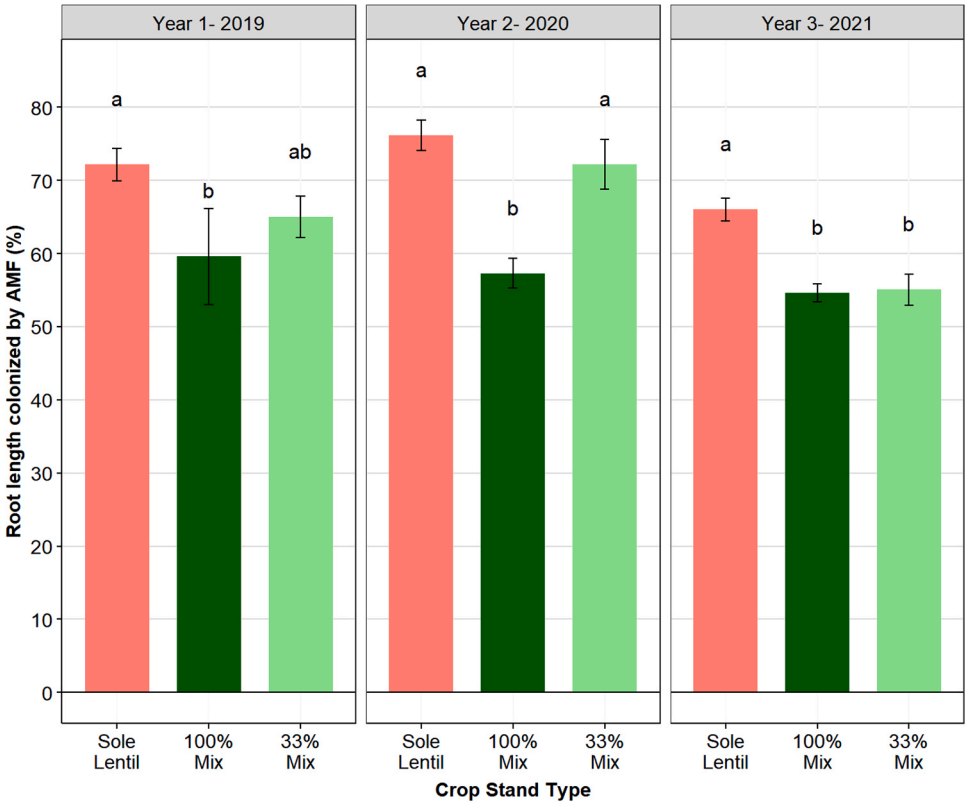

Fig. 3. Effect of crop stand type and year on lentil AMF root colonization at flowering stage. Different lowercase letters indicate statistically significant differences among the crop stand type treatments (Tukey's post hoc test $p \leq 0.05$) within each sampling stage in each year. The bars indicate the percentage root length colonized by AMF while error bars represent the standard errors. 100 %Mix, high-density wheat + lentil intercropping; 33 %Mix, low-density wheat + lentil intercropping.

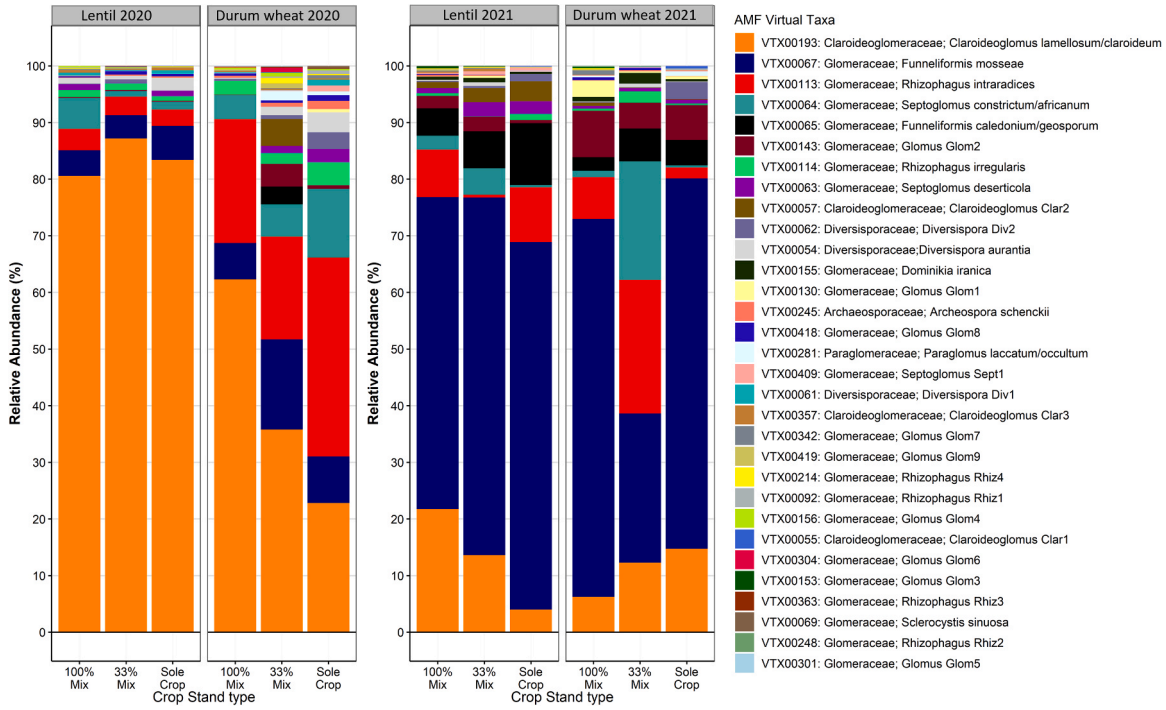

Fig. 4. Mean relative abundance of 31 AMF virtual taxa (VT) detected according to the MaarjAM database. The grouping is based on the crop species (durum wheat and lentil), crop stand types (sole crops and intercrops), and years (2020 and 2021). 100 %Mix, high-density wheat + lentil intercropping; 33 %Mix, low-density wheat + lentil intercropping.

Table 1
Alpha diversity of AMF communities collected from the roots of durum wheat and lentil sown in different intercropping stand types in two separate but adjacent field sites in 2020 and 2021 based on 18 S rRNA Illumina Miseq sequencing.

Year	Crop stand type (CST)	Crop species	Hill N1 (exp. Shannon H')	Hill N2 (Inverse Simpson 1/D)	Average AMF richness	Pielou's evenness
2020	100 %Mix	Lentil	2.41(0.69) aA	1.61(0.36) aA	14.00(0.01) aA	0.30(0.10) aA
		Durum wheat	3.16(0.62) aA	2.31(0.44) aA	14.00(2.31) aA	0.42(0.07) aA
	33 %Mix	Lentil	1.86(0.15) bA	1.32(0.09) bA	16.33(0.88) aA	0.22(0.03) bB
		Durum wheat	5.55(1.15) aA	3.72(0.87) aA	19.33(0.33) aA	0.56(0.07) aA
	Sole crop	Lentil	2.17(0.33) bA	1.45(0.15) bA	15.00(0.58) aA	0.27(0.05) bB
		Durum wheat	6.33(1.08) aA	4.48(1.11) aA	18.00(2.31) aA	0.64(0.09) aA
2021	100 %Mix	Lentil	3.77(0.99) aA	2.78(0.75) aA	17.67(0.67) aA	0.43(0.09) aA
		Durum wheat	3.76(1.11) aA	2.33(0.54) aA	17.33(2.03) aA	0.42(0.10) aA
	33 %Mix	Lentil	3.51(0.19) aA	2.26(0.10) aA	16.33(2.18) aA	0.45(0.04) aA
		Durum wheat	2.93(0.43) aB	2.04(0.25) aB	13.33(1.45) aB	0.41(0.12) aA
	Sole	Lentil	3.37(0.97) aA	2.37(0.63) aA	13.67(2.33) aA	0.41(0.13) aA
		Durum wheat	3.00(0.39) aB	2.11(0.33) aB	14.00(2.00) aA	0.41(0.10) aA
p-values	Year		0.670	0.610	0.456	0.659
	CST		0.719	0.664	0.614	0.777
	Crop		**0.004****	**0.007****	0.605	**0.011***
	Year × CST		0.152	0.231	**0.017***	0.596
	Year × Crop		**0.0003*****	**0.0003*****	0.129	**0.004****
	CST × Crop		0.337	0.253	0.690	0.485
	Year × CST × Crop		0.153	0.384	0.487	0.459

Values are treatment means (S.E); Different lowercase letters within columns indicate statistically significant differences between crop species (Tukey's post hoc test $p \leq 0.05$) within each crop stand type (CST) treatment in each year. Different uppercase letters within columns in each crop species and CST indicate statistically significant differences between years. Hill N, Hill number; 100 %Mix, high-density wheat + lentil intercropping; 33 %Mix, low-density wheat + lentil intercropping. *p*-values in bold character are significant at the 0.05 probability level. * , * *, * **Significant at $p \leq 0.05$, 0.01 and 0.001 respectively.

3.6. Effect of crop species, crop stand type, and year on root AMF community structure

NMDS ordination showed that the 2020 AMF community structure was clearly separated from that one of 2021 (Fig. 5). The PERMANOVA test (nperm = 999) from the combined 2020 and 2021 data revealed a significant (Pseudo-F = 4.22, R^2 = 0.062, $p = 0.013$) crop × year interaction effect on AMF community structure. However, crop stand type did not significantly influence the AMF community structure in both years (Pseudo-F = 0.68, R^2 = 0.02, $p = 0.66$) (Table 2). A separate analysis of 2020 data showed that crop species (Pseudo F = 10.96, R^2 = 0.407, $p = 0.0001$) structured the AMF communities while no effect was detected in 2021 (Pseudo F = 0.89, R^2 = 0.054, $p = 0.534$) samples (Table S12).

3.7. Indicator AMF VT groups associated with lentil and durum wheat roots

Indicator VT groups analyses showed that in 2020, VTX00193 which corresponds to *Claroideoglomus lamellosum* (Indval = 0.81, $p = 0.001$)

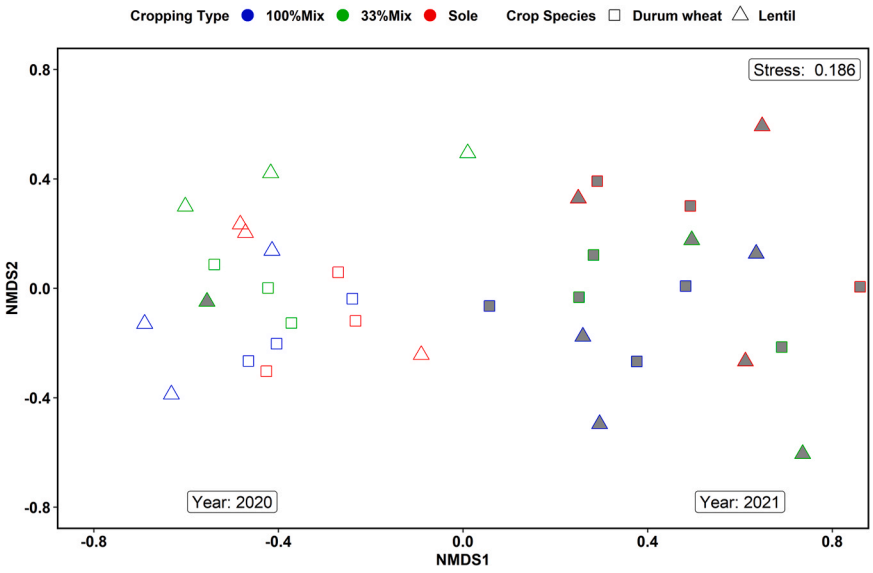

Fig. 5. Non-metric multidimensional scaling (NMDS) ordination diagram of the community composition of arbuscular mycorrhiza fungi (AMF) in durum wheat and lentils cultivated in 2020 and 2021. The ordination is based on Bray-Curtis dissimilarity matrices between samples (Stress = 0.186). Assemblages from different years are represented as open symbols for 2020 and solid symbols for 2021, while the assemblages from the different crop species are represented by squares for durum wheat and triangles for lentils. Assemblages from the different crop stand types are represented by different line colors (blue, 100 %Mix i.e., high-density wheat + lentil intercropping; green, 33 % Mix i.e., low-density wheat + lentil intercropping; red, sole crop).

Table 2
Permutational multivariate analysis of variance (PERMANOVA) testing the effects of crop species (durum wheat and lentil), crop stand type - CST (100 %Mix, 33 %Mix, and sole crop), year (2020 and 2021), and interaction of the treatments on root AMF community composition (number of permutations = 999).

Treatments	Df	Sum of Squares	R^2	F	Pr (>F)
Crop	1	0.4889	0.0655	4.4751	**0.009****
Year	1	3.0908	0.4139	28.2941	**0.0001*****
Crop stand type (CST)	2	0.1487	0.0199	0.6807	0.66
Crop × Year	1	0.4607	0.0617	4.217	**0.013***
Crop × CST	2	0.0992	0.0133	0.4538	0.863
Year × CST	2	0.1881	0.0252	0.8607	0.503
Crop × Year × CST	2	0.369	0.0494	1.6887	0.127
Residual	24	2.6217	0.3511	-	-
Total	35	7.467	1	-	-

p-values in bold character are significant at the 0.05 probability level. * , * *, * **Significant at $p \leq 0.05$, 0.01 and 0.001 respectively

was more prone to be found in lentil roots while VTX00113 and VTX00281, which correspond to *Rhizophagus intraradices* (Indval = 0.69, $p = 0.005$) and *Paraglomus laccatum* (Indval = 0.45, $p = 0.015$), respectively, were more associated with durum wheat (Table 3). In 2021, VTX00063 (Indval = 0.63, $p = 0.01$) and VTX00409 (Indval = 0.60, $p = 0.008$) which correspond to *Septoglomus deserticola* and *Septoglomus* sp., respectively, showed strong specificity to lentil roots while no VT group was significantly associated with durum wheat (Table 3).

Table 3
Significant indicator AMF VT groups associated with lentil and durum wheat roots.

Year	Crop species	AMF VT groups	Indicator value index (Indval)	*p*-value
2020	Lentil	VTX00193	0.808	0.001***
	Durum wheat	VTX00113	0.695	0.005**
		VTX00281	0.451	0.015*
2021	Lentil	VTX00063	0.629	0.01**
		VTX00409	0.603	0.008**
	Durum wheat	-	-	-

* , * *, * **Significant at $p \leq 0.05$, 0.01 and 0.001 respectively.

3.8. Effect of crop stand type and year on grain yield and protein concentration

Crop stand type significantly ($p = 0.002$) affected durum wheat grain production but not grain protein concentration ($p = 0.132$). Lentil grain yield was significantly ($p < 0.0001$) affected by the interaction between crop stand type and year while lentil grain protein concentration varied depending on the crop stand type and year (Table 4). Both intercropping systems significantly reduced the grain yield production in durum wheat by an average of 37 % in 2020 and 16 % in 2021 compared to that of the sole durum wheat. On the contrary, both intercropping systems enhanced lentil grain production by an average of 315 % in 2020 (but not in 2021) and reduced lentil grain protein concentration by an average of 5 % in 2020 and 4 % in 2021. Averaged across all the treatments, lentil grains had a higher protein concentration in 2020 (27.1 ± 0.3 %) compared to that of 2021 (23.8 ± 0.2 %) (Table 4).

3.9. Principal component analysis of agronomic and mycorrhizal traits

PCA biplots were used to represent the variability of agronomic data such as grain yield and grain protein of durum wheat and lentil and microbiological features (percent mycorrhizal length colonization and AMF diversity indices such as Hill N1, Pielou's evenness and richness) as influenced by the crop stand type (Fig. 6). The variance explained by the two principal components was larger than 70 % for both crops, making PCA data very reliable. In the durum wheat PCA, AMF diversity indices Hill N1 and Pielou's evenness correlated with PC1 while AMF root colonization at flowering correlated with PC2 (Fig. 6a). In the lentil PCA, grain protein concentration correlated with PC1 while grain yield correlated with PC2 (Fig. 6b). AMF community richness associated with durum wheat grain protein concentration while AMF root colonization at flowering associated with lentil grain protein concentration. In both PCAs, there was a clear separation of variables between 2020 and 2021 (Fig. 6).

4. Discussion

In agricultural settings, the AMF community composition and diversity can change over time depending on the crop species and management practice in place, highlighting the need to adopt cropping systems that stimulate the proliferation of AMF communities important in providing essential soil ecosystem services (Xiao et al., 2019; Lu, 2022). This study showed that durum wheat-lentil relay intercropping

Table 4
Durum wheat and lentil grain yield and protein concentration as influenced by the different crop stand types and year.

		Grain yield (t ha^{-1})		Grain protein concentration (%)	
Year	Crop stand type (CST)	Durum wheat	Lentil	Durum wheat	Lentil
2020	100 %Mix	1.79(0.15) bA	1.39(0.12) aA	13.73(0.14) aA	26.41(0.22) bA
	33 %Mix	1.78(0.15) bA	1.27(0.10) aA	13.99(0.16) aA	26.83(0.25) bA
	Sole	2.83(0.18) aA	0.32(0.11) bB	14.01(0.13) aA	28.10(0.41) aA
2021	100 %Mix	1.88(0.19) bA	1.19(0.12) aA	10.56(0.24) aB	23.34(0.26) bB
	33 %Mix	1.91(0.16) bA	1.30(0.10) aA	10.95(0.30) aB	23.61(0.23) bB
	Sole	2.26(0.21) aA	1.42(0.12) aA	11.06(0.22) aB	24.45(0.27) aB
p-value	CST	**0.002****	**0.002****	0.132	**<0.0001*****
	Year	0.534	**0.006****	**<0.0001*****	**<0.0001*****
	CST × Year	0.204	**<0.0001*****	0.866	0.569

Values are treatment means (S.E); Different lowercase letters within columns indicate statistically significant differences among crop stand type treatments (Tukey's post hoc test $p \leq 0.05$) within each year. Different uppercase letters within columns in each crop stand type indicate statistically significant differences between years. 100 %Mix, high-density wheat + lentil intercropping; 33 %Mix, low-density wheat + lentil intercropping. *p*-values in bold character are significant at the 0.05 probability level. * , * *, * **Significant at $p \leq 0.05$, 0.01 and 0.001 respectively.

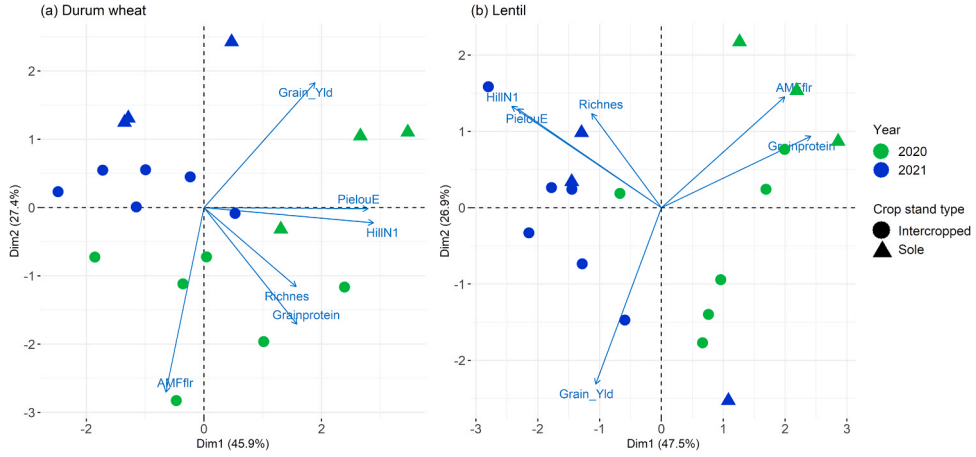

Fig. 6. Principal component analysis (PCA) representation of agronomic and mycorrhizal variables as influenced by crop stand type on **(a)** durum wheat, **(b)** lentil. AMFflr, mycorrhizal root length colonization; Richnes, AMF richness; HillN, Hill number; PielouE, Pielou's evenness; Grain_Yld, grain yield; Grainprotein, grain protein concentration. The intercrop data are merged between the two crop stand types (100 %Mix and 33 %Mix).

enhances soil mycorrhizal activity but differentially influences the AMF root colonization compared to the sole cropping systems. In addition, AMF community composition and diversity were driven mainly by the interaction between crop species and year and not by the relay intercropping practice. Nonetheless, intercropping effectively enhanced lentil productivity, thanks to the ability of the intercrops to control weeds as reported in the previous study (Koskey et al., 2022). Generally, the findings of this study are of great interest in examining the impact of plant-AMF symbiosis in intercropping systems.

4.1. Relay intercropping enhances the soil mycorrhizal inoculum potential

Soil MIP bioassay before intercropping showed a uniform mycorrhizal activity, suggesting the presence of a balanced distribution of mycorrhizal inoculum able to establish rapidly plant-mycorrhizal symbiosis. Compared to the sole lentil fields, intercropped fields consistently recorded a higher soil MIP at the end of the crop cycle in 2019 and 2020, which is in line with our first hypothesis. Intercropping may have enhanced the fine-root length density and distribution (Gong et al., 2020) and C rhizodeposition (Cong et al., 2015), therefore enriching soils with a significant amount of mycorrhizal inoculum and energy source. Wiseman and Wells (2004) observed that soils with higher root length densities have a higher soil MIP than soils with lower root densities. This finding reiterates that AMF colonized roots are good sources of mycorrhizal inoculum. Increasing plant diversity, particularly mycotrophic plants such as legumes, enhances the number of potential host-fungal pairings and boost soil mycorrhizal activity (Burrows and Pfleger, 2002). Notwithstanding, in our study, the sole lentil fields had the lowest MIP values at the end of the crop cycle probably due to heavy infestation by less mycotrophic weeds, such as *Chenopodium album* (Vatovec et al., 2005), which was among the most persistent weeds in our experimental fields (Koskey et al., 2022). Different weed species may alter the abundance, diversity, and activity of the resident AMF depending on their mycotrophic status (Kabir and Koide, 2000). Therefore, improved weed management through intercropping can be an important factor in maintaining soil mycorrhizal activity in arable fields.

4.2. Relay intercropping differentially affects mycorrhizal root colonization

At the flowering stage, the colonization level of durum wheat ranged between 41 % and 62 %, which is in line with the colonization percentages reported by Stefani et al. (2020) in the sole-cropped durum wheat cultivars Arnautka (57.8 ± 14.8 %) and Hercules (59.8 ± 6.7 %). Intercropping increased the mycorrhizal colonization levels of durum wheat (although not significantly in 2019) compared to the sole-cropped durum wheat. This finding is consistent with our second hypothesis and is supported by the results of Shukla et al. (2012), Hage-Ahmed et al. (2013), and Lu (2022) who found that intercropping enhanced AMF abundance, activity, and colonization compared to those of the sole crops. The authors associated the mycorrhization effect with the more extended root system (length, branching, biomass, density, and volume), the greater availability of C substrates for AMF use, and the

extensive hyphal networks that can facilitate cross-infectivity of AMF in the intercropping system. The advantage of intercropping over sole cropping on durum wheat root colonization was evident with time (flowering vs. tillering) in both years. The exchange of biochemicals from the more mycotrophic legumes in the intercropping system could partly explain the observed influence on the cereal (Ingraffia et al., 2019). In contrast, the non-significant colonization differences detected in our study between the high- and low-density intercropped durum wheat suggest that the intercropping density may not be as important as the cropping type in influencing mycorrhizal root colonization.

Interestingly, the proportion of lentil root length colonized by AMF was reduced in the presence of durum wheat as an intercrop partner compared to sole lentil. As observed in our previous study (Koskey et al., 2022), competition by durum wheat may have induced growth stress on lentils causing the legume to reduce the energy-demanding plant-AMF symbiotic investment, thus affecting lentil colonization. Similarly, Yang et al. (2016) observed reduced mycorrhizal root colonization in *Robinia pseudoacacia* legume seedlings intercropped with ryegrass (*Lolium perenne* L.). Likewise, the authors associated the reduction in legume root colonization with the competitive advantage of the grass over the legume in canopy growth, nutrient acquisition, and biomass accumulation traits. Unbalanced competition can lead to a reduction in the leaf net photosynthetic rate of the 'weaker' intercrop partner. To compensate for the low nutrient availability, the 'weaker' plant substantially lowers the proportion of photosynthates allocated to the AMF during symbiosis (Douds et al., 2000).

4.3. Crop species and year but not intercropping influence AMF community distribution and diversity

Sequencing results showed that crop species and year but not intercropping influenced AMF community composition and diversity, which is partly in agreement and partly contrary to our third hypothesis. Based on the NMDS ordination and PERMANOVA test, the distribution of the dominant AMF communities varied between the crop species (durum wheat vs. lentils) and years (2020 vs. 2021), which affirms the significance of AMF-plant host specificity and environment in shaping AMF communities. Contrary to the third hypothesis, relay intercropping did not affect the AMF community distribution.

The AMF VT distribution analyses showed that VTX00193 (Claroideoglomeraceae; *Claroideoglomus*), VTX00067 (Glomeraceae; *Funneliformis*), and VTX00113 (Glomeraceae; *Rhizophagus*) were the most frequent AMF VTs. These taxa have also previously been found to dominate in agricultural fields grown with maize-soybean intercrops (Zhang et al., 2020) and in wheat, maize, and soybean monocultures (Turrini et al., 2016; Renaut et al., 2020). Claroideoglomeraceae and Glomeraceae are versatile and generalist in nature (Mao et al., 2014), and their dominance in this study provides evidence that members of these families have a ruderal life strategy, supporting the model by Chagnon et al. (2013). In addition, the varied abundance and distribution of these dominant VTs could be partly associated with the variation in mycorrhizal propagule and spore distribution in the soils of the 2020 and 2021 fields considering the differences in the preceding crop species (corn in 2020 and wheat in 2021). Indeed, biotic factors such as crop host genotype are known to differentially select for specific AMF taxa (Turrini et al., 2018; Kaidzu et al., 2020; Lu, 2022). In a different field but close to our experimental sites and with similar soil conditions, a previous study by Avio et al. (2020) revealed that the aggregated distribution of most AMF species was influenced by soil heterogeneity at a small spatial scale.

Notably, this study did not detect AMF communities belonging to the families Gigasporaceae and Acaulosporaceae, whose natural occurrence is reported to be shaped by distinct environmental drivers such as soil bulk density, pH, and precipitation (Veresoglou et al., 2013). Unlike Glomeraceae, Acaulosporaceae isolates have been described as acidophilic and occupy a distinct niche, hence their preferential establishment in acidic soils, and this was not the case in our study. In addition, Hart and Reader (2002) reported that members of Acaulosporaceae have a low colonization strategy on both soil and roots. Therefore, their absence in our study may reason well with the above-mentioned trait. On the other hand, Glomeraceae produces many small spores and hyphal fragments that readily re-establish a network in highly disturbed soils and colonize plant roots (Higo et al., 2015). Barceló et al. (2020) found a higher relative abundance of Glomeraceae in the root compartments than in the soil environment. By contrast, Gigasporaceae are known to produce fewer large spores that proliferate more abundantly in the soil environment than in the roots (Hart and Reader, 2002), and their dominance decreases in high soil pH environment (Stürmer et al., 2018). This adaptation to different environments may explain the dominance or absence of some taxa in AMF communities. It is important to note that our field trials were conducted in part of the Mediterranean region with sandy-loam alkaline soils and typically low and poorly distributed rainfall. Moreover, the sequencing analysis was done on root samples and not the soil.

The root AMF species diversity, richness, and evenness significantly varied due to the interaction between crop species and year but not among crop stand type treatments. In 2020, durum wheat had a higher AMF diversity than lentils while no difference was detected between the two crops in 2021. This was contrary to the observation of Bainard et al. (2014) who reported a significantly higher AMF richness and diversity in lentil roots than in wheat roots. Unlike our study, the authors tested the effect of crop species on AMF diversity under monocropping without including intercropping. In addition, it is important to note that in our study, the identified AMF originated from the root compartment, which is more affected by the AMF-host specificity and environmental conditions than those from the soil compartment (Torrecillas et al., 2012). It cannot be excluded that analysis on soil compartment would demonstrate a different pattern. On a positive note, intercropping did not decrease the AMF diversity as observed in other studies (Jefwa et al., 2006). However, in contrast to our findings, Chifflot et al. (2009) and Zhang et al. (2020) reported a significant positive effect of poplar-soybean and maize-soybean intercropping, respectively, on the diversity of AMF species colonizing the plant roots. Both studies attributed the observed effect to the strong interspecific interactions between the crop species and soil and the interspecific functional complementarity of the plant species to promote synergy and support diverse AMF communities.

It was evident from the NMDS analysis that the AMF community structure of the root samples collected in 2020 clustered separately from that of the 2021 samples. This observation was supported further by the results of the PERMANOVA test, which revealed the differences in the AMF community structure of durum wheat from that of lentil roots in 2020. The NMDS and PERMANOVA tests confirmed the results from the alpha diversity indices, which reiterate the fact that small-scale geospatial soil variation of the Mediterranean fields can influence the distribution of the local AMF communities at the field scale or even at the sub-meter scale (Turrini et al., 2018; Avio et al., 2020). Likewise, crop stand type (high- and low-density intercropping vs. sole crop) did not affect the AMF community structure in both years. Consistent with our findings, Guzman et al. (2021) found that soil properties structured the AMF community composition stronger than the cropping system (polyculture vs. monoculture). Similarly, Li and Wu (2018) did not find any significant difference in fungal community structure between the sole crops and intercrops of cucumber, clover, wheat, mustard, ryegrass, and alfalfa.

Overall, the results of this investigation point to the possibility that, in our experimental setting, small-scale soil heterogeneity and host genotype could be important drivers influencing the diversity and community structure of the root AMF, rather than the cropping system. Considering that the fields used had varying history of cultivated crops, i.e., corn and wheat preceded the 2020 and 2021 trials, respectively. Therefore, the varying composition and abundance of AMF communities

in 2020 and 2021 may have been contributed partly by the selective effect of the preceding crop species. In agreement with our findings, the results of Higo et al. (2015) revealed a significant effect of different cover crops (wheat and rapeseed) on AMF community structure when cultivated in rotation with soybean. Similarly, Kim et al. (2022) attributed the difference in AMF communities detected in two different fields to the contrasting cropping histories of the two sites.

Indicator species analyses showed that distinct AMF VT groups preferentially occurred in different crop species and years, regardless of the cropping pattern. For instance, the genera *Claroideoglomus* and *Septoglomus* were the top taxa associated with lentil roots in 2020 and 2021, respectively, while *Rhizophagus* and *Paraglomus* were associated with durum wheat in 2020. Stefani et al. (2020) have also demonstrated the presence of *Rhizophagus* and *Paraglomus* in the roots of different durum wheat cultivars. These suggest that, given the available pool of local AMF communities, a particular crop species probably chooses specific AMF taxa (Turrini et al., 2018; Avio et al., 2020; Zeng et al., 2021).

4.4. Impact of intercropping on durum wheat and lentil grain components

Grain yield results showed that intercropping can be effectively used to enhance lentil grain production, especially in weed-infested fields, as attested by the enhanced lentil grain yield in 2020, which increased by 315 % compared to the sole crops, although with a minimal loss (−5 %) in its protein quality. In our previous study (Koskey et al., 2022), we showed that both intercropping systems (100 %Mix and 33 %Mix) have yield advantages (based on land equivalent ratio assessment ranging from 1.64 to 6.48) over the sole crops. These results partially confirm our fourth hypothesis, which states that crop yield can be increased by utilizing intercrops with facilitative and complementing functional features. However, the result on the slight decline in lentil grain protein quality in intercrops is in opposition to our fourth hypothesis, and this may be due to the competition of durum wheat with the lentils (Koskey et al., 2022).

Intercropping systems involving wheat-chickpea (Banik et al., 2006) and oat-forage legumes (Gecaitė et al., 2021) combinations have been shown to promote legume production compared to the sole legumes by suppressing the growth of aggressive weeds that induce competition against legumes. In fact, the weed infestation assessment carried out in our previous study (Koskey et al., 2022) showed that the 2020 field had a higher level of infestation than that of 2021. This may have led to the low grain production in the sole lentil treatments recorded in 2020. In addition to weed suppression, Lu (2022) linked the improved performance of the wheat-soybean intercropping system to the complementary use of resources such as water, solar radiation, and soil nutrients and the microbial enrichment of the soil rhizosphere. Although this study found a positive influence of relay intercropping on crop yield performance, we acknowledge that we did not find any association between the mycorrhizal parameters and grain yield. However, AMF community richness was associated with durum wheat grain protein concentration while AMF root colonization was associated with lentil grain protein concentration (Fig. 6). A positive effect of AMF diversity on soybean grain protein content has also been observed by Marro et al. (2020), although qualitative changes in seed proteome driven by mycorrhizal symbiosis remain poorly understood (Bona et al., 2016; Mamontova et al., 2019). Therefore, further studies (proteomics, transcriptomics, and metabolomics) in this area are needed to reveal the contribution of various mycorrhizal parameters in improving crop yield quality.

5. Conclusion

This study highlights the potential impact of durum wheat-lentil intercropping on AMF activity, diversity and community structure, and consequently, in supporting crop productivity and nutrition. Results showed that durum wheat-lentil relay intercropping enhanced soil mycorrhizal activity but differentially influenced the mycorrhizal root colonization compared to the sole cropping. In addition, the overall changes in AMF species diversity and community structure were affected by the interaction between the crop species and year and not by relay intercropping. Glomeraceae and Claroideoglomeraceae were the major taxa in this study but had contrasting abundances in 2020 and 2021. *Claroideoglomus* and *Septoglomus* showed strong associations with lentil roots while *Rhizophagus* and *Paraglomus* were associated with durum wheat roots in 2020, affirming host genotype-AMF preferences. Grain protein concentration associated with selected mycorrhizal parameters such as community richness and colonization. Further studies on the functional analysis of the different AMF communities selected by the crop genotype and year may reveal the importance of intercropping in maintaining soil functionality and productivity.

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CRediT authorship contribution statement

All authors contributed to the Conceptualization and designing of the experiment. Material preparation, data collection and analysis were performed by **Gilbert Koskey, Luciano Avio, Alessandra Turrini** and **Cristiana Sbrana**. The first draft of the manuscript was written by Gilbert Koskey. All the authors reviewed and commented on the manuscript drafts. Project fund acquisition was done by **Paolo Bàrberi.** Supervision was done by **Paolo Bàrberi** and **Luciano Avio**. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2023.108696.

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