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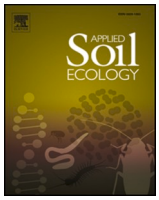

Arbuscular mycorrhizal fungi originated from soils with a fertility gradient highlight a strong intraspecies functional variability

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ABSTRACT

Characterization and selection of arbuscular mycorrhizal fungal (AMF) taxa to design inocula tailored to meet a spectrum of needs is a crucial first step to achieve specific beneficial agronomic functions. Commonly, commercial microbial inocula are based on generalist single AM fungal taxa, having low genetic variability and not offering efficiency and stability when applied in agroecosystems. In this study, we investigated the AMF functional variability at inter- and intra-species levels by characterizing colonization traits, host growth, and mineral uptake of single-spore AM fungi isolated from soils with a fertility gradient. Nineteen single-spore cultures, showing high spore density and AMF colonization, were phylogenetically assigned to different isolates of 3 AMF species (i.e. *Entrophospora claroidea*, *Funneliformis mosseae* and *Archaeospora trappei*). A higher functional variability in infectivity and effectiveness was detected among isolates within AMF species (25 % of total variance) than among AMF species. Most of AMF isolates of *F. mosseae* have a better outcome in terms of plant growth, although with a performance gradient, while the isolates of *E. claroidea* showed a variable functional pattern, and those of *A. trappei* a less variable pattern. Overall, isolates originating from the soil of the conventional arable field with higher pH and phosphorous availability promoted the uptake of plant nutrients, while those originating from soils with higher SOM and plant diversity promoted plant growth. On the contrary, the infectivity traits of the AM fungi were more conserved, as they were not affected by the environmental parameters of the soils of origin. Finally, we highlighted that soil pH played an important role in shaping the pattern of AMF functionality. Boosting the isolation and cultivation of AMF taxa, originating from agricultural and natural soils, is shown to be a key step in exploiting AMF diversity and designing the new generation of microbial inoculants.

1. Introduction

Arbuscular mycorrhizal fungi (AMF) are the most widespread and oldest root symbionts that establish an association with 73 % of vascular plants (Öpik et al., 2013; Strullu-Derrien et al., 2018), including the large majority of crops (Soudzilovskaia et al., 2020). They provide many benefits, such as improving soil fertility and aggregate stability (Bedini et al., 2009; Soudzilovskaia et al., 2019; Wilson et al., 2009), promotion of plant growth, yield, and nutrient uptake (Lekberg and Koide, 2005; Zhang et al., 2018) in return for photosynthetically fixed carbon (C) (Wipf et al., 2019). Furthermore, AMF positively affect plant tolerance to different abiotic and biotic stresses (Marro et al., 2022; Veresoglou and Rillig, 2012). These results have promoted the application in agriculture of AM fungal inoculants, and increasing numbers of commercial inoculants have been registered as amendments or biostimulants, and released onto the market in the last few decades (Benami et al., 2020; Sudheer et al., 2023).

Therefore, soil AM fungal inoculants are regarded as a potential alternative or integration to traditional mineral fertilization with beneficial effects, which depend on soil nutrient availability resulting from agricultural managements (Hoeksema et al., 2010; Mäder et al., 2000). However, successful inoculant designs are still a challenge and biostimulants result as not a fully tapped resource to support agricultural sustainability (French et al., 2021). In this context, the characterization and selection of AM fungal taxa to design single or multiple inocula, tailored to meet a spectrum of needs, is the first crucial step in

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achieving specific beneficial agronomic functions. Commonly, large-scale production of inocula for commercial purposes are based on generalist single AM fungal taxa, heavily sporulating, easily propagated and having r-strategies of reproduction (Sýkorová et al., 2007). However, these AM fungal inocula, showing low genetic variability, do not appear to offer both efficiency and stability when applied in different agroecosystems (Bender et al., 2019; Säle et al., 2021; Salomon et al., 2022). Therefore, increasing the isolation and cultivation of unexplored AM fungal taxa, originated from agricultural and natural soils, is a prerequisite to exploit the diversity of AMF and to design the new generation of microbial inocula.

During secondary succession following farmland abandonment, large positive changes in soil properties and increases in plant diversity and biomass occur (Kardol et al., 2006; Kuramae et al., 2010, 2011). Nutrients and organic matter increase in the soil surface layer, together with changes in vegetation composition and increases in plant diversity. These changes in vegetation and soil characteristics have been identified the main drivers of the abundance and diversity of microbial communities (e.g. bacteria, AMF). In natural and managed grasslands and agricultural soils, the diversity of the AM fungal community is modified by identity of plant species and is promoted by increases in the diversity of the plant community, soil pH (from 3.5 to 7.1), soil organic carbon (SOC) (from 1.5 to 5.5 %), and time since disturbance (Deveautour et al., 2019; Fitzsimons et al., 2008; Johnson et al., 1992; Kohout et al., 2015; Zhu et al., 2020). However, AM fungal diversity is reduced by soil N fertilization and fungicide application (Egerton-Warburton et al., 2007; Hartnett and Wilson, 2002). Therefore, agricultural fields converted to natural vegetations (e.g. grasslands) offer great opportunities, serving as model systems, for the isolation, characterization, and propagation of taxa of AMF adapted to specific environmental conditions and having tailored functions (Kuramae et al., 2011; Hannula et al., 2017).

Although the AM symbiosis interaction is generally positive for the host in terms of plant growth and yield, a certain variability in the outcome has been reported, ranging from highly beneficial to detrimental (Johnson and Graham, 2013; Johnson et al., 1997; Klironomos, 2003; Smith and Smith, 2013). Variability is typically assessed by studying different traits, e.g. plant growth and rate of nutrient transfer to plants (Avio et al., 2006; van der Heijden and Scheublin, 2007). Differences in AM fungal colonization traits (e.g. hyphal and spore density, size and turnover, intra- and extraradical growth), plant growth, and nutrient patterns (Hart and Reader, 2002; Marro et al., 2022; Munkvold et al., 2004) have been evidenced among AM fungal taxa, but the factors that drive functionality in terms of infectivity and effectiveness are still not fully understood (Hoeksema et al., 2018; Koch et al., 2017; Säle et al., 2021). Functional variability among AM fungal taxa was reported to be determined by the fungal-host combination (Arcidiacono et al., 2023; Yang et al., 2016), fungal phylogeny (Maherali and Klironomos, 2007; Säle et al., 2021), and local adaptation processes (Ji et al., 2013; Johnson et al., 2010). The magnitude and direction of these effects are also highly variable within fungal orders (Marro et al., 2022), families (Arcidiacono et al., 2023; Horsch et al., 2023; Hart and Reader, 2002), genera (Dodd et al., 2000), species (interspecies variability) as well as isolates within species (intraspecies variability) (Avio et al., 2006; Koch et al., 2006, 2017; Munkvold et al., 2004; Marro et al., 2022). A great intraspecific variability has been demonstrated by differential responses of the same host (Munkvold et al., 2004; de Novais et al., 2014; Mensah et al., 2015) or different hosts (Koch et al., 2017; Schoen et al., 2021). This was also supported by the occurrence of a high genetic variability among isolates of the same AM fungal species (Corradi and Sanders, 2006; Mathieu et al., 2018). Other studies demonstrated that genetic diversity (i.e. nucleotypes frequencies) occurs during the clonal growth of a single spore of an AM fungus (Angelard et al., 2010, 2014; Angelard and Sanders, 2011). This was observed among single-spore lines (i.e. multiple generation) and even among initial lines of a single AM fungal isolate, and was linked to phenotypic variation. Regarding local adaptation, local-adapted AM fungal taxa were reported to produce more extraradical hyphae in their native soil, being more mutualistic compared with non-local AMF (Johnson et al., 2010; Ji et al., 2013). Finally, genetic structure in terms of inter-isolate variability in nuclear ratios, following successful hyphal fusion (i.e. anastomosis), could also play a driving role in AM fungal functional variability (e.g. establishment of the symbiosis and plant growth) (Croll et al., 2009; de Novais et al., 2017; Kokkoris et al., 2020).

In this study, we investigated the functional variability of AMF at the interspecies and intraspecies level of single-spore AMF isolated from soils with a fertility gradient by characterizing colonization traits, host growth, and mineral uptake. Additionally, we investigated how the environmental parameters of the soils of origin drive functionality of AMF. We hypothesized that: 1) there is higher functional variability in infectivity and effectiveness among isolates within AM fungal species than among AM fungal species; 2) abiotic and biotic factors of the site of origin, such as soil chemical parameters, plant diversity and soil prokaryotic diversity, drive AM fungal functionality and can guide the isolation of AMF with specific beneficial functions. To test these hypotheses, we first trap-cultured AMF from soils with varying soil fertility, and then we established representative single-spore cultures. The gradient of soil fertility was achieved utilizing four different sites: grasslands last plowed and fertilized 22, 36, and 66 years ago (series of secondary succession: W1, W2, and W3) and a conventional arable field (W0). Nineteen single-spore isolates that showed high spore density and AM fungal colonization and morphologically identified as belonging to *Entrophospora* spp., *Funneliformis* spp., *Funneliformis mosseae* and *Archaeospora* spp. were molecularly characterized and used to study AM fungal functional variability. Non-mycorrhizal controls were also set up. Finally, we tested the relationship between the environmental parameters recorded in the soil of origin by Kuramae et al. (2010, 2011) and the infectivity and effectiveness of the AMF, allowing to identify the major environmental drivers that select AMF with targeted beneficial functions.

2. Material and methods

2.1. Characterization of the soil of origin and setting up of AM fungal single-spore trap cultures

Three grasslands where soil was last plowed and fertilized 22, 36 and 66 years ago (site: Wrakelberg 1, 2, 3, W1, W2, and W3, respectively) were selected in the nature reserve at Wrakelberg, Limburg, The Netherlands (50° 50′ N lat; 5° 54′ E long and 215 m a.s.l.) to represent a chronosequence of a secondary succession of chalk grasslands (Fig. 1) (Kuramae et al., 2010, 2011). Averaged over a period of three years (2004–2006), maximum, minimum and average annual air temperature was 13.4 °C, 6.4 °C and 9.9 °C, respectively, while annual precipitation was 422 mm (https://www.visualcrossing.com/weather-data). The chronosequence was located along a slope (15–20° inclination) and exposed to the South West. Plant species richness per 1 m^2 at 13 ± 0.7, 11 ± 1.6 and 24.0 ± 1.6 in W1, W2, W3, respectively. A total of 52 different plant species were observed: *Arhenatherum elatius*, *Avenula* sp., *Avenula* sp. (hard), *Brachypodium pinatum*, *Briza media*, *Campanula* sp., *Carex flacca*, *Carex cariophyllaceae*, *Cirsium* sp., *Centaurea scabiosa*, *Convolvulus arvensis*, *Crataegus* sp., *Dactylis glomerata*, *Daucus carota*, *Euphrasia stricta*, *Festuca ovina*, *Galium mollugo*, *Genista tinctoria*, *Gymnadenia conopsea*, *Hieracium pilosella*, *Hypericum perforatum*, *Inula conyza*, *Knautia arvense*, *Leontodon hispidus*, *Leucanthemum vulgare*, *Linum catharticum*, *Lolium perenne*, *Lotus corniculatus*, *Medicago lupulina*, *Ononis repens*, *Oreganum vulgare*, *Pimpinella saxifraga*, *Plantago lanceolata*, *Poa* sp., *Poligola comosa*, *Potentilla* sp., *Prunella vulgaris*, *Prunus avium*, *Ranunculus* sp., *Reseda lutea*, *Rhinanthus minor*, *Rosa* sp., *Rubus* sp., *Sanguisorba minor*, *Scabiosa columbaria*, *Senecio* sp., *Thymus pulegioides*, *Trifolium pratense*, *Trifolium* sp., *Vicia* sp. and *Viola* sp. Furthermore, an adjacent conventional arable field with winter wheat (*Triticum aestivum* L.) was used as a reference site (Wrakelberg 0, W0). Soil samples were

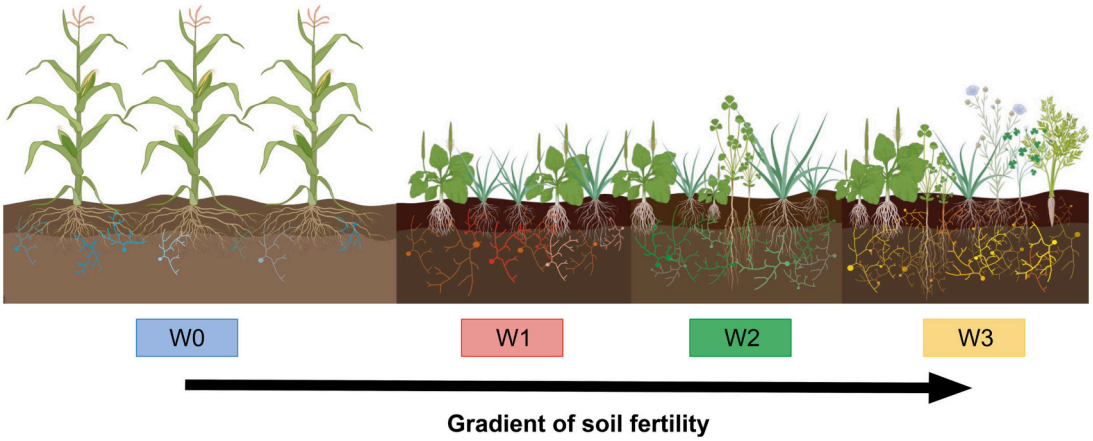


Fig. 1. Graphical scheme of the study sites representing a soil fertility gradient and from which the arbuscular mycorrhizal fungi (AMF) were sampled, trap cultured, and single-spore trap cultures were set up. Three grasslands representing chronosequences of secondary succession of chalk grasslands that were last plowed and fertilized 22, 36 and 66 years ago (W1, W2, W3, respectively). An adjacent conventional arable field with winter wheat (*Triticum aestivum* L.) was utilized as reference site (W0). All the sites were located in the nature reserve at Wrakelberg, Limburg, Netherlands (43° 84' N lat; 5° 91' E long and 215 m a.s.l.) (Kuramae et al., 2010, 2011). The image was created with BioRender.com.

taken in February 2007 at the four sites along 20 m linear transects (Fig. S1). At each of the four sites, five (A, B, C, D, and E) within-field replicate composite soil samples were made by combining two cores (10 cm soil depth, 2 cm diameter) taken <10 cm apart. This yielded a total of 20 soil samples. The soil samples were sieved at 3 mm diameter, thus excluding larger root fragments and chalk pieces. Soil samples were characterized for pH, ammonium (NH_4^+), nitrate (NO_3^-), soil organic matter (SOM), and available phosphorus (P_{avail}) and plant and prokaryotic diversity was determined (Kuramae et al., 2010, 2011). Details about the analyzes and results are given in Materials and Methods S1 and data are reported in Tables S1, S2, and S3.

Using soil samples (mycorrhizal inoculum), obtained by pooling the five soil (composite) samples from each of the four sites (W0, W1, W2, and W3), subsequent cycles of trap cultures (Oehl et al., 2003) allowed to obtained single-spore inocula. Single-spore inocula were based on the diversity pattern of AM fungal morphotypes at the different sites. The trap cultures were grown in a greenhouse at the Department of Microbial Ecology of The Netherlands Institute of Ecology (Wageningen, The Netherlands). Nineteen single-spore inocula were morphologically identified as *Entrophospora* spp., *Funneliformis* spp., *Funneliformis mosseae*, and *Archaeospora* spp., and the density of spores was checked ($\geq$ 10 spore g^{-1}; Table S4) (Daniels and Skipper, 1982). Healthy-looking spores were mounted on slides with polyviniyl-lactic acid-glicerol (Koskey and Tessier, 1983) or polyviniyl-lactic acid-glicerol mixed 1:1 (v:v) with Melzer's reagent (Brundrett et al., 1994). The spores were examined under a light microscope (Leitz, Laborlux, Wetzlar, Germany) at a magnification of up to 400×. Identification was based on species descriptions available on the Web site of the International Collection of Vesicular Arbuscular Mycorrhizal Fungi, previously hosted at West Virginia University and actually at the University of Kansas (https://invam.ku.edu).Furthermore, the infectivity of the 19 single-spore AM fungal inocula was evaluated measuring the AM fungal root colonization rate of leek (*Allium porrum* L.; cv. Lungo della Riviera (Royal Seeds, S.r.l, Mirandola, MO, Italy), The pot cultures were grown in the greenhouse of the Arbuscular Mycorrhizal Fungal Bank of the Crop Science Research Center of the Scuola Superiore Sant'Anna (Pisa), located at 'Società Agricola del Bambù' (San Giuliano Terme, Pisa, Italy). Pot cultures were set up using a as substrate consisting of a 2:2:1 (v:v:v) mixture of sterilized quarz sand (0.7–3 mm grain size), TerraGreen (calcinated clay, OILDRI, Chicago, IL, USA) and 4-mm sieved top soil of a not fertilized grassland (steam-sterilizing cycle: 121 °C 25 min, on two-consecutive days) (Pellegrino et al., 2011). In detail, among the 19 AM fungal morphotypes, three, eight, three and five belonged to W0, W1, W2 and W3, respectively. After three months of plant growth, roots of leek were sampled for AM fungal root colonization rate (Phillips and Hayman, 1970; McGonigle et al., 1990) (Table S4). In addition, a sub-sample of roots were oven-dried at 50 °C and stored at −20 °C before molecular characterization.

2.2. Molecular characterization of the single-spore AMF

Genomic DNA was extracted from the roots of leek (200 mg dry weight) of AM fungal single-spore cultures using the DNeasy Plant Mini Kit (Qiagen, Germany) and following the manufacturer's instructions. Fifty-seven (19 morphotypes × 3 replicates) plus three non-mycorrhizal controls were used for DNA extraction. The quantity and quality of DNA were checked with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The quantity of DNA was on average 75.8 ± 3.9 ng μl^{-1}, while the 260:280 ratio was on average 1.81 ± 0.01. Then, PCR amplifications were performed using the primer pair SSUmAf-LSUmAr, followed by a nested PCR with SSUmCf-LSUmBr (Krüger et al., 2009), targeting approximately 1.8-kb of SSU-ITS-LSU fragment and resulting in a 1.5-kb amplicon, covering approximately 250 bp of the SSU, the whole ITS region and ca. 800 bp of the LSU. The Krüger set of primers was selected because they have deep resolution (Krüger et al., 2009). Details about PCR conditions are given in Materials and Methods S2. PCR amplicons were purified using QIAquick (Qiagen, Venlo, The Netherlands), cloned into the PCR 2.1 vector (Invitrogen, Carlsbad, CA, USA) and transformed into One shot© TOP10 chemically competent *Escherichia coli* cells. After plasmid isolation from transformed cells, the cloned DNA fragments were sequenced with SSU_seq1 (5'-AACGAGGAATCCCTAGTAAG-3′; one direction) using an ABI 3730 XL Applied Biosystem. The taxonomy assignment of the sequences is described below.

2.3. Evaluation of the AM fungal infectivity and effectiveness

2.3.1. Experimental design

After molecular characterization, the 19 single-spore isolates were used for the evaluation of the functional inter- and intra-species variability in AMF. Research was carried out in a climatic chamber, where sterilized seeds of leek (cv. Lungo della Riviera) were sown in plastic pots (350 ml volume, 7x7x8 cm) containing a mixture 2:2:1 by volume of quartz sand, TerraGreen, and soil collected in a low input agricultural field (Fig. S2a,b). The mixture was steam sterilized (121 °C for 25 min, for two consecutive days) to kill naturally occurring AMF. The soil used

in the mixture was collected in San Piero (Pisa, Italy). Details about the physical and chemical characteristics of the soil are given in Materials and Methods S3. The experiment was set up according to a completely randomized design with 19 AM fungal morphotypes and the non-mycorrhizal control as treatments and five replicates, for a total of 100 pots (Fig. S3). Each pot was inoculated with 40 ml of crude inoculum (mycorrhizal roots and soil containing spores and extraradical mycelium) of the 19 fungal single-spore AMF or with 40 ml of a mixture of the five non-mycorrhizal controls obtained from propagation culture. Ten seeds of leek per pot were planted and after emergence (Fig. S2c) the seedlings were thinned to five per pot. The plants were grown at a temperature of 23/18 °C day/night, 16/8 h light/dark cycle, 65/50 % day/night humidity (Fig. S2d). The photosynthetic photon flux density at the top of the plant canopy was 300 μmol m^{-2} s^{-1}. The plants were supplied with deionized water twice per week (50 ml and 70 ml, respectively) and monthly fertilized with 1/4-strength Hoagland solution (60 ml per pot) until harvest in October 2019.

2.3.2. Plant measurements

Fifteen weeks after sowing (October 2019), leek plant shoots were harvested by cutting plants 1 cm above the soil level and the root system was removed from each pot. Fresh subsamples were used for the evaluation of the AM fungal infectivity traits. Finally, for each sample, the remaining shoots and roots were used for dry weight and nutrient determination. The percentage of AM fungal root colonization, as well as the percentage of root length containing arbuscules and vesicles, was determined under a light microscope (Leitz Laborlux S, Wetzlar, Germany), after clearing and staining with lactic acid instead of phenol (Phillips and Hayman, 1970), following the magnified intersection method (McGonigle et al., 1990). In detail, twenty fragments of roots per pot were mounted on a microscope slide and examined at the light microscope for hyphae, arbuscules, vesicles and not mycorrhizal intersections. A magnification of x125–500 was used, and then a magnification of x1250 was applied to verify the structures. The total AM fungal colonized root length was calculated by multiplying the percentage of AM fungal root colonization by the root length.

The dry weight of the shoot and root was determined by oven drying at 65 °C for three days. The shoot length of each plant was measured (five plants as subreplicates per pot). Root length was measured using the newly developed *HyLength* tool (Cardini et al., 2020). Images of root systems were taken with a mirrorless camera (alpha-6000, Sony).

For the determination of the concentration of nutrients in the shoots and roots, the samples were ground and oven dried at 50 °C for four days. Potassium (K), calcium (Ca), magnesium (Mg), copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) concentrations were evaluated by a microwave-assisted acid digestion system (COOLPEX Smart Microwave Reaction System, Yiyao Instrument Technology Development Co., Ltd., Shanghai, China) and a Microwave Plasma Atomic Emission Spectroscopy (4210 MP-AES, Agilent Technologies, Santa Clara, CA, USA). Details of the methods for nutrient analysis are given in Materials and Methods S4.

2.4. Bioinformatics and statistical analysis

The newly generated sequences were aligned using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) and the detection of chimeric sequences was performed using Chimera Check version 11 (Cole et al., 2014). The newly generated sequences (at least three sequences for each AM fungal single-spore culture) were aligned with the AM fungal reference sequences and with the closest AM fungal species from NCBI searches. The reference AM fungal alignments were databases composed by sequences of morphologically characterized and described AM fungal isolates belonging to the detected species (Schüßler and Walker, 2010). Alignments were performed using the ClustalW algorithm in Bioedit (Hall et al., 2011), followed by manual editing and exclusion of unambiguously aligned regions. For the outgroup, we chose a lineage that falls outside the detected AM fungal species, but that was closely related. Phylogenetic trees were inferred by Maximum Likelihood (ML) analysis using MEGA11 (Stecher et al., 2020; Tamura et al., 2021) and the Kimura 2-parameter model (Kimura, 1980). Branch support bootstrap values are derived from 1000 bootstrap replicates. The phylograms were drawn with MEGA11 and edited with Adobe Illustrator CC 2022. The newly generated AM fungal sequences were assigned to operational taxonomic units (OTUs) based on the phylogenetic placement with a bootstrap value ≥90. All newly generated sequences were deposited in the NCBI Sequence Read (SRA) database as SUB13754036 (accession numbers from OR454500 to OR454508), SUB13728350 (accession numbers from OR389383 to OR389421), and SUB13844335 (accession numbers from OR559617 to OR559625).

A nested analysis of variance (ANOVA) was performed after the necessary transformations (e.g. log10, arcsen) on the AM fungal infectivity traits, plant growth, and mineral concentration, with the AM fungal species as a fixed factor and isolate as a random factor nested within the AM fungal species. The Tukey-B procedure was used to test the differences among AM fungal species (interspecies diversity). The means and standard errors given in the tables and figures are for untransformed data. Furthermore, variance components were calculated from the nested model and used to calculate the relative contribution of variation among AM fungal species (interspecies diversity) and among isolates (intraspecies diversity). The overall effect of AMF was compared with non-mycorrhizal controls by treating all mycorrhizal treatments as one group in a one-way ANOVA, followed by the *t*-test to identify the differences between groups. All statistical analyzes were performed using the SPSS 25.0 software package (SPSS Inc., Chicago, IL, USA).

A multivariate approach based on permutational analysis of variance (PERMANOVA) was applied to investigate the effect of AM fungal species and AM fungal isolate within AM fungal species on infectivity (AM root colonization, percentage of root length containing arbuscules, and percentage of root length containing vesicles) and effectiveness traits (shoot and root growth and mineral concentrations). The AM fungal species was used as a fixed factor and the isolate was used as a random factor nested within the AM fungal species ($n = 3$). Data were square root transformed, normalized, and a Euclidean distance matrix of similarity was calculated. Data were visualized using a Draftsman plot [based on Pearson's correlations (r), −1 to +1]. The significance of the correlations was calculated using the SPSS software package (version 25.0, SPSS Inc., Chicago, IL, USA) and a correlation map was drawn and edited by Adobe Illustrator 2022. The P values in the PERMANOVA were calculated using 999 permutations (Anderson and Braak, 2003). The explained variance was calculated and partitioned among the sources of variations (AM fungal species, AM fungal isolate, and unexplained). Since the analysis is sensitive to differences in multivariate location (average community composition of a group) and dispersion (within-group variability), the analysis of homogeneity of multivariate dispersion (PERMDISP; Anderson, 2006) was performed to check the homogeneity of dispersion among groups (β-diversity) and within groups (α-diversity) (Anderson et al., 2006). When PERMANOVA was statistically significant, principal coordinate analysis (PCO) was performed to visualize the most relevant patterns in the data. The circle in the PCO biplot, whose diameter is 1.0, allows the reader to understand the scale of the vectors in the vector plot.

To study the effect of environmental parameters (i.e. soil chemical parameters, plant diversity, and soil prokaryotic diversity) recorded at the soil of origin by Kuramae et al. (2010, 2011) on the infectivity and effectiveness traits of the isolated AMF, we first performed a PCO to visualize the most relevant patterns in the environmental data. The data were previously square root transformed, normalized, and a Euclidean distance matrix of similarity was calculated. Then, a cluster analysis, based on hierarchical agglomerative clustering with group average linkage, was performed on the Euclidean distance matrix of similarity of

the environmental data. The similarity profiles (SIMPROF) analysis was performed using 999 permutations and a significant level of 5 % to objectively define the groups within the dendrogram, and thus to infer the effect of the soil of origin. In the dendrogram and in PCO biplot, slices/clouds were drawn at statistically supported resemblance levels. A RELATE analysis (Clarke and Warwick, 2001), based on Spearman rank and 999 permutations, was used to test the significance of the relationships. The ρ equal to 1 represents a perfect relationship. Then, BEST analysis, based on BioEnv methods (all combinations), Spearman rank and 999 permutations, allowed to find by the Global test the ρ, the significant level of sample statistics (*P*) and the best descriptor(s) of the relationship together with correlation values (Clarke et al., 2008). Finally, the Distance-based Linear Method (DistLM) analysis, using a stepwise selection and Akaike's information criterion (AICc), was applied to measure the significance and variance explained by the best descriptor/s (Knorr et al., 2000), and Distance-based Redundancy Analysis (dbRDA) was used to plot the significant axes of DistLM (Legendre and Anderson, 1999). All multivariate analyzes were performed using PRIMER 7 and PERMANOVA+ software (Clarke and Gorley, 2006; Anderson, 2008).

3. Results

3.1. Molecular characterization of AMF

The BLAST of ca. 1.5-kb-long SSU-ITS-LSU gene fragments flanked by the PCR primers SSUmCf and LSUmBr (Krüger et al., 2009) allowed to assign the sequences with sufficient phylogenetic resolution to three different AM fungal species, *Entrophospora claroidea*, *Funneliformis mosseae* and *Archaeospora trappei* (Table S5). These species belong to three different orders (i.e. Entrophosporales, Glomerales, and Archaeosporales, respectively) and to the AM fungal families, Entrophosporaceae, Glomeraceae, and Archaeosporaceae, respectively. Overall, among 19 isolates, 16 %, 68 %, and 16 % were assigned to *E. claroidea*, *F. mosseae*, and *A. trappei*. In detail, 3 AM fungal single-spore cultures that originated from the conventional arable field with winter wheat and the grassland that was last plowed and fertilized 66 years ago (i.e. W0–4, W0–6 and W3–73; Table S5) belonged to *E. claroidea*. Looking at the ML trees (Fig. 2a), the *E. claroidea* sequences clustered in three supported groups and separately from other *E. claroidea* isolates currently available in NCBI (i.e. *E. claroidea* isolates: Att1063–4, Nr360, Nr672 and Nr133). Thus, the ML phylogram allowed to identify within *E. claroidea* three distinct AM fungal isolates. Furthermore, the AM fungal isolates W0–4 and W0–6 were more similar to each other than to W3–73. Regarding the AM fungal morphotypes assigned to *F. mosseae*, one originated from the conventional arable field (W0–12), while seven, one and four from

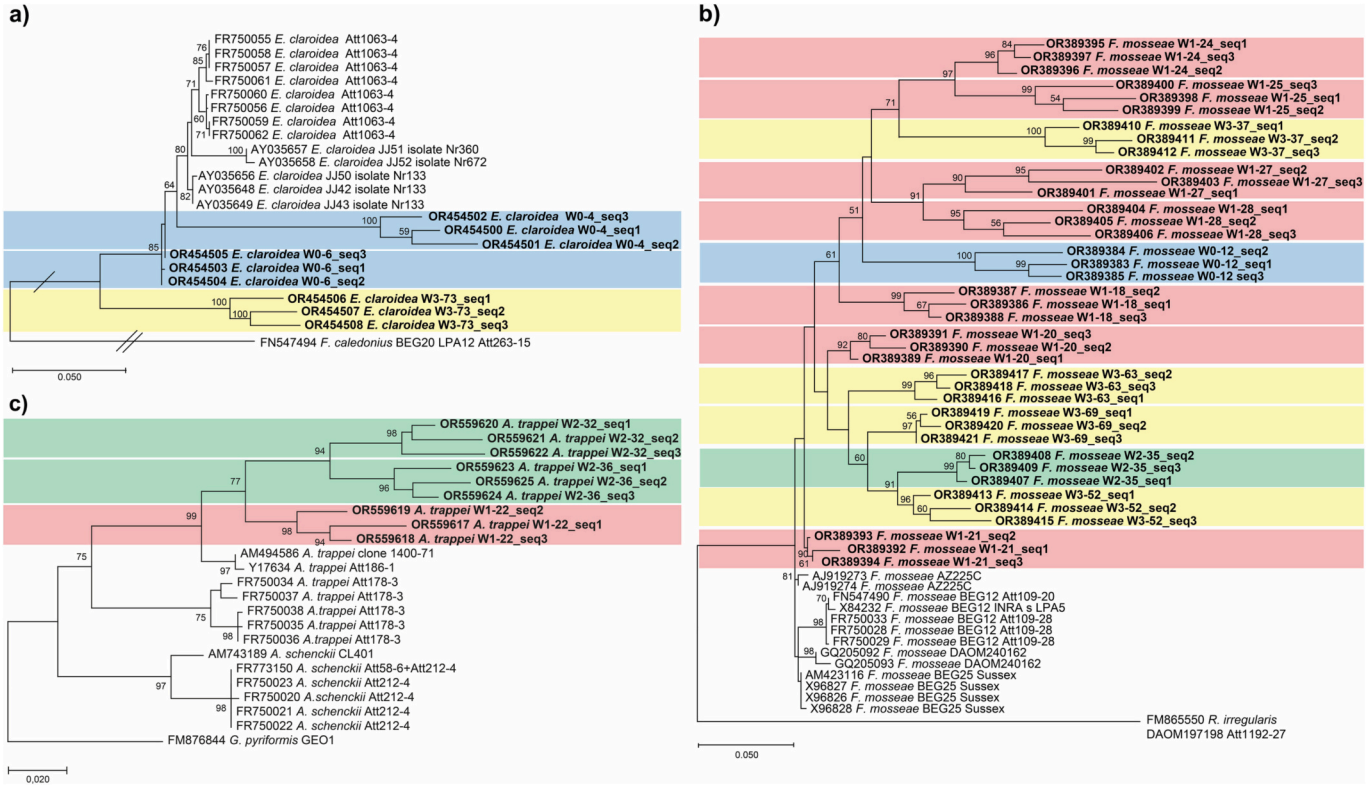


Fig. 2. Maximum likelihood phylogenetic trees inferred from nuclear ribosomal rDNA sequences of (a) *Entrophospora claroidea*, (b) *Funneliformis mosseae*, and (c) *Archaeospora trappei* originating from soils of three grasslands that were last plowed and fertilized 22, 36 and 66 years ago, respectively (W1, W2 and W3) and from a conventional arable field with winter wheat (*Triticum aestivum* L.) (i.e., reference site). The reference sequences in each tree are from NCBI. Partial sequences of the entire nuclear ribosomal rDNA cistron (ca. 1010 aligned sites) were used. The sequences are composed of the 3' end of the small subunit ribosomal RNA gene (SSU; 200 bp), the inter internal transcribed spacer (ITS) 1, 5.8S and ITS2 (ca. 500 bp) and the 5' end of the large subunit ribosomal RNA gene (LSU: ca. 140 bp). The sequences *Funneliformis caledonius* BEG20, *Rhizophagus irregularis* DAOM197198, and *Gesiphon pyriformis* GEO1 were used as outgroup to root the tree of *E. claroidea* (a), *F. mosseae* (b), and *A. trappei* (c), respectively. Branch support values are based on bootstrapping with a number of replications equal to 1000, using the Kimura-2-parameter model (Kimura, 1980). In each tree, clades formed by sequences of the AM fungal single-spore cultures are enclosed with rectangles and colored according to the origin sites: W0=blue; W1=red; W2=green and W3=yellow. Newly generated sequences are highlighted in boldface, and their accession numbers are prefixed by the isolate /clone identifier. Sequences were obtained from colonized roots of leek (*Allium porrum* L.) used as trap plants. The analyses were done in MEGA11 (Stecher et al., 2020; Tamura et al., 2021) and phylograms were edited by Adobe Illustrator CC 2022.

grasslands last plowed and fertilized 22, 36 and 66 years ago, respectively (Table S5). Looking at the ML phylogenetic tree (Fig. 2b), the *F. mosseae* sequences clustered in 13 supported groups and separately from other *F. mosseae* isolates available in NCBI (i.e. AZ225C; BEG12, BEG25, DAOM240162). This ML phylogram allowed to identify 13 distinct AM fungal isolates of *F. mosseae*. However, some AM fungal isolates were more phylogenetically similar, such as W1–24, W1–25 and W3–37. Likewise, other AM fungal isolates were phylogenetically more similar to each other: W1–27 and W1–28; W3–69, W2–35, and W3–52. Finally, three AM fungal morphotypes that originated from grasslands last plowed and fertilized 22 and 36 years ago (i.e. W1–22, W2–32, W2–36) belonged to *A. trappei* (Table S5). Looking at the ML tree (Fig. 2c), the sequences clustered in three distinct AM fungal isolates of *A. trappei*. These isolates were similar to two sequences already available in NCBI, *A. trappei* Att186–1 and an uncultured *Archaeospora* sp. The isolates W2–32 and W2–36 were phylogenetically more similar to each other than to W1–22 and were all distant from the sequences belonging to some isolates of *A. schenckii* (i.e. Att212; Att58–6 + Att212–4 and CL401).

3.2. AM fungal infectivity and effectiveness

Microscopic measurements allowed to visualize the AM fungal root colonization, as well as arbuscules and vesicles within the roots of leek inoculated from 15 weeks by the 19 AM fungal isolates (Fig. 3d,e). All fungal isolates established well in the root system and colonized >70 % of the root length (Fig. 3a), while in non-mycorrhizal controls AM fungal colonization was not detected. All infectivity traits significantly varied at AM fungal isolate level, while only the percentage of root length containing arbuscules varied at AM fungal species level (Table S6). The AM fungal isolate explained from 33 % to 37 % of the total variability in arbuscule and vesicle traits, respectively, while AM fungal species explained only 16 % of the total variability in arbuscules (Fig. S4a). Inter- and intra-species variation in the infectivity traits is reported in Fig. 3. With respect to AM fungal variation at the species level, the leek plants inoculated with *E. claroidea* and *F. mosseae* showed a significantly higher percentage of root length containing arbuscules compared to those inoculated with *A. trappei* (38 % vs. 5 %) (Fig. 3b). As examples of intraspecific variation, the percentage of root length containing arbuscules varied within *F. mosseae* from 0 % to 57 %, while within *E. claroidea* from 24 % to 74 % and within *A. trappei* from 0.7 % to 9 % (Fig. 3b). Futhermore, the AM fungal root colonization varied within *F. mosseae* from 31 % to 97 %, within *E. claroidea* from 55 % to 91 % and within *A. trappei* from 36 % to 56 % (Fig. 3a), while the percentage of root length containing vesicles varied within *F. mosseae* from 0 % to 41 %, within *E. claroidea* from 9 % to 48 % and within *A. trappei* from 8 % to 14 % (Fig. 3c).

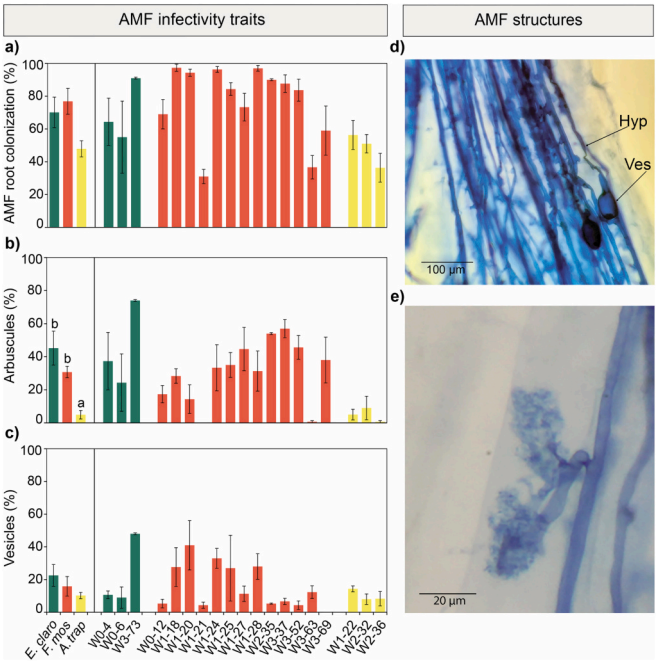


Fig. 3. Effect of arbuscular mycorrhizal fungal (AMF) species and isolate on fungal infectivity (inter- and intra-species functional diversity, respectively). AMF colonization traits are: (a) percentage of AMF root colonization, (b) percentage of root length containing arbuscules and (c) percentage of root length containing vesicles. Host plant was leek (*Allium porrum* L.). Nineteen AMF isolates were propagated from three grasslands whose soils were last plowed and fertilized 22, 36 and 66 years ago (W1, W2 and W3: 22, 36, 66 years) and from a conventional arable field as reference (W0). AMF species used as fixed factor: *Entrophospora claroidea*, *Funneliformis mosseae* and *Archaeospora trappei*. Three AMF species belonging to three orders. AMF isolate used as random factor (nested factor within AMF species): three isolates of *E. claroidea*; 13 isolates of *F. mosseae* and three isolates of *A. trappei*. AMF isolate code i.e. W0-4, W0 refers to the site and 4 to the number of the AMF isolate. Images of AMF intraradical root colonization structures: (d) hyphae (Hyp) and vesicles (Ves); (e) arbuscule.

Although the vast majority of AM fungal isolates significantly increased shoot and root dry weight, as well as shoot length compared to controls (+M vs NM: +51 %, +22 %, and + 18 %, respectively; $P \leq 0.016$) (Fig. 4), no significant variability ($P = 0.733$) was observed in root length (Fig. 5f). Furthermore, no variation was observed among AM fungal species in all plant traits (Table S6; Fig. 4). On the contrary, the identity of the AM fungal isolate within the species significantly explained the dry weight and length of the shoot (28 % and 24 % of total variability, respectively) (Fig. S4b). As an example of pronounced intraspecific variations, the dry weight of the shoot varied within *F. mosseae* from 0.42 g plant^{-1} in W2–35 to 0.74 in W1–20, within *E. claroidea* from 0.42 g plant^{-1} in W0–6 to 0.67 in W0–4, and within *A. trappei* from 0.58 g plant^{-1} in W2–36 to 0.73 in W2–32. In addition, the length of the shoot varied similarly within AM fungal species (Fig. 4). In Fig. 6f, examples of AM fungal intraspecies variability in plant growth are shown.

The vast majority of the AM fungal isolates significantly increased the concentration of Zn, Cu, and K in the shoots compared to the control (+M vs. NM: +87 %, +67 %, −5 %, respectively; $P \leq 0.034$), while the other plant nutrients did not change (Table S7 and S8; Fig. 6). Despite the relatively uniform mineral concentration in the shoots at the species level, with the exception of Cu, a great intraspecies variation was recorded (Table S6). The variance explained by isolate (intraspecies variation) ranged from 20 % to 30 % of the total variability depending on the nutrient (Fig. S4c). Indeed, shoot nutrient concentration significantly varied for Cu, Mn, K, Ca, and Mg, accounting for 23 %, 30 %, 20 %, and 24 % of the total variance (Fig. 6a-e; Table S6 and S7). Significant differences were observed among species for some nutrients in roots, such as Cu, K and Ca (Table S6), accounting for 17 %, 13 % and 9 % of the total variance (Table S6; Fig. S4d). Similar to the uptake of shoot nutrients, a high intraspecies variation was observed for Cu, Mn, K, and Mg concentration in roots (Fig. 5a-e, Table S6 and Table S8), accounting for 31 %, 31 %, 19 %, and 23 % of the total variance (Fig. S4d). Moreover, the variation within *A. trappei* was in some cases lower (i.e. shoot: Cu, K; root: Cu, Mn, K, Mn) than within *F. mosseae* and *E. claroidea*.

PERMANOVA allowed to summarize the pattern of AM fungal functionality and to evidence statistically significant differences at the intraspecies level (Table S9). Indeed, the AM fungal isolate explained 25 % of the total variance (Fig. 7c). On the contrary, the AM fungal species did not significantly affect AM fungal infectivity and effectiveness. This pattern is evident in the PCO biplot (Fig. 7a). The AM fungal isolates within the species clustered separately along the first axis, which explained 34 % of the total variation. Plant growth traits (shoot and root dry weight and shoot length) and some nutrients (e.g. Cu, K, Mg in

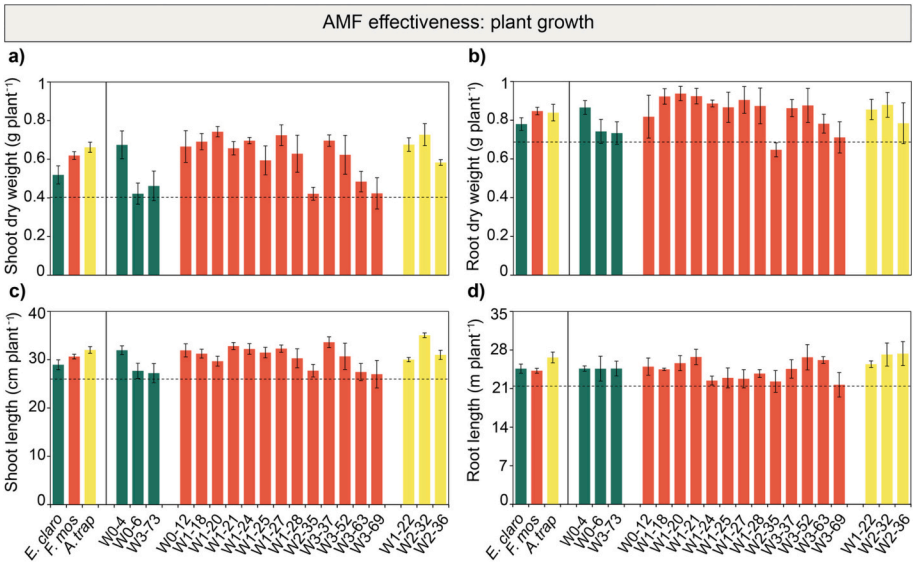


Fig. 4. Effect of arbuscular mycorrhizal fungal (AMF) species and isolate on fungal effectiveness (inter- and intra-species functional diversity, respectively). Plant growth traits are: (a) shoot dry weight, SDW; (b) root dry weight, RDW; (c) shoot length, SL; (d) root length, RL. Host plant was leek (*Allium porrum* L.). Nineteen AMF isolates were propagated from three grasslands whose soils were last plowed and fertilized 22, 36 and 66 years ago (W1, W2 and W3: 22, 36, 66 years) and from a conventional arable field as reference (W0). AMF species used as fixed factor: *Entrophospora claroidea*, *Funneliformis mosseae* and *Archaeospora trappei*. Three AMF species belonging to three orders. AMF isolate used as random factor (nested factor within AMF species): three isolates of *E. claroidea*; 13 isolates of *F. mosseae* and three isolates of *A. trappei.* AMF isolate code i.e. W0-4, W0 refers to the site and 4 to the number of the AMF isolate. The dotted horizontal line represents non-mycorrhizal control.

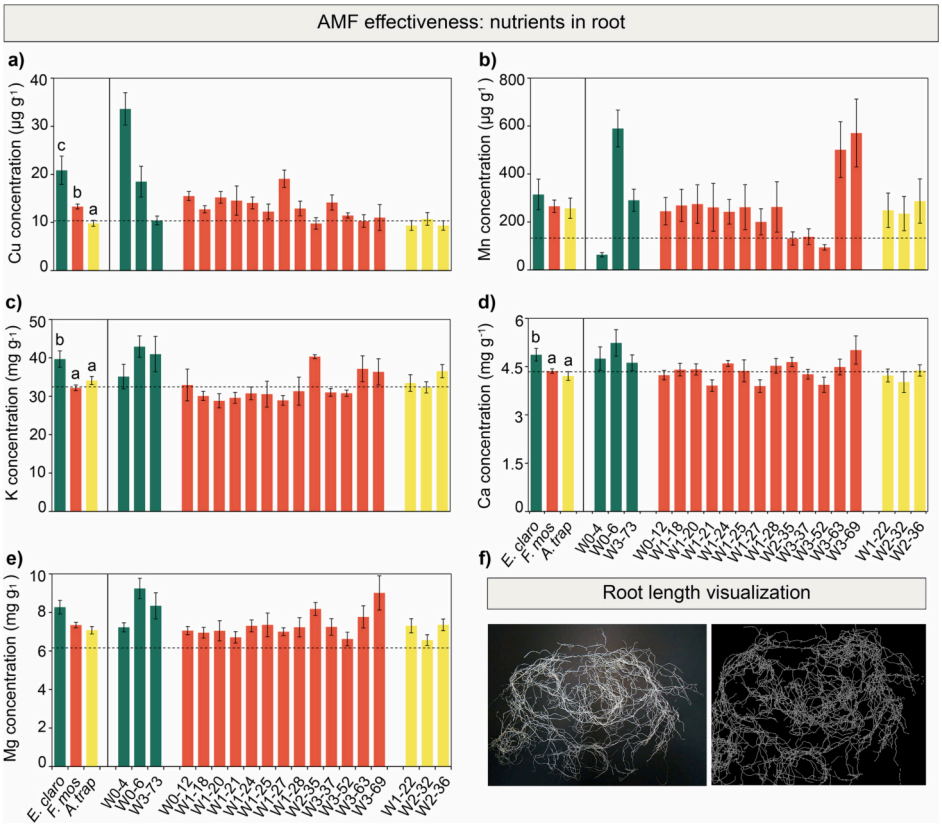


Fig. 5. Effect of arbuscular mycorrhizal fungal (AMF) species and isolate on fungal effectiveness (inter- and intra-species functional diversity, respectively). Plant nutrient concentration in shoots: (a) Cu, (b) Mn, (c) K, (d) Ca, (e) Mg. Host plant was leek (*Allium porrum* L.). Nineteen AMF isolates were propagated from three grasslands whose soils were last plowed and fertilized 22, 36 and 66 years ago (W1, W2 and W3: 22, 36, 66 years) and from a conventional arable field as reference (W0). AMF species used as fixed factor: *Entrophospora claroidea*, *Funneliformis mosseae* and *Archaeospora trappei*. Three AMF species belonging to three orders. AMF isolate used as random factor (nested factor within AMF species): three isolates of *E. claroidea*; 13 isolates of *F. mosseae* and three isolates of *A. trappei.* AMF fungal isolate code i.e. W0-4, W0 refers to the site and 4 to the number of the AMF isolate. The dotted horizontal line represents non-mycorrhizal control. (f) Example of root length visualized by *HyLenght* (Cardini et al., 2020): picture of the root system of *A. porrum* (left) and skeletonized picture of the root system (right).

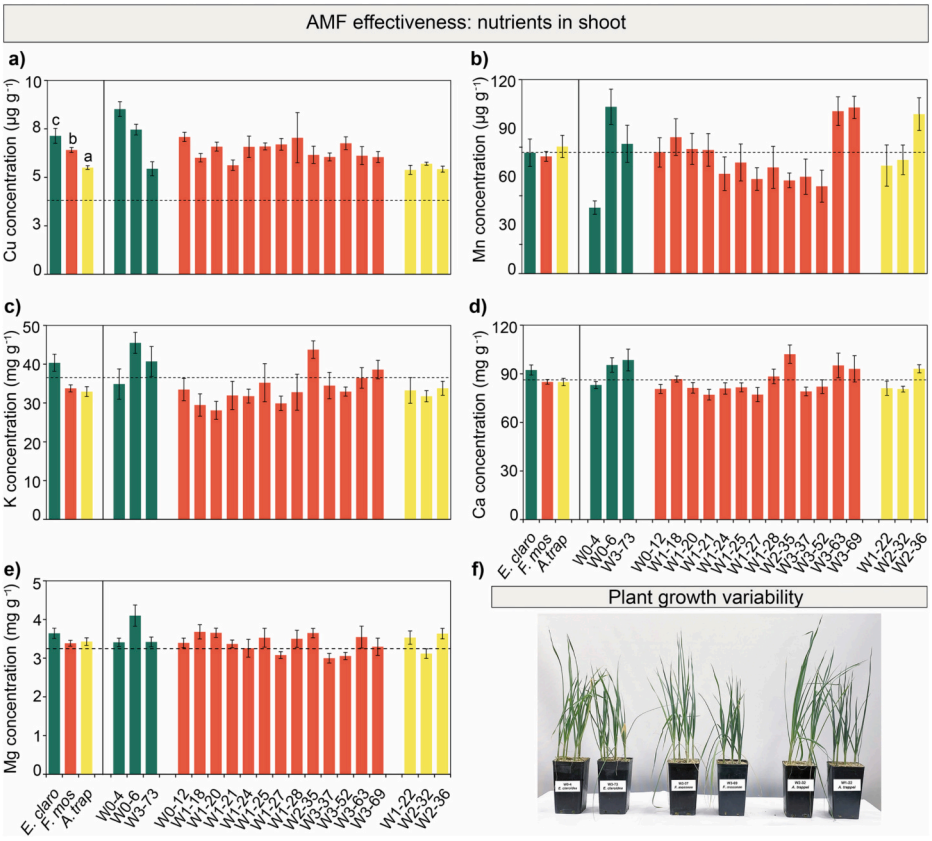


Fig. 6. Effect of arbuscular mycorrhizal fungal (AMF) species and isolate on fungal effectiveness (inter- and intra-species functional diversity, respectively). Plant nutrient concentration in roots: (a) Cu, (b) Mn, (c) K, (d) Ca, (e) Mg. Host plant was leek (*Allium porrum* L.). Nineteen AMF isolates were propagated from three grasslands whose soils were last plowed and fertilized 22, 36 and 66 years ago (W1, W2 and W3: 22, 36, 66 years) and from a conventional arable field as reference (W0). AMF species used as fixed factor: *Entrophospora claroidea*, *Funneliformis mosseae* and *Archaeospora trappei*. Three AMF species belonging to three orders. AMF isolate used as random factor (nested factor within AMF species): three isolates of *E. claroidea*; 13 isolates of *F. mosseae* and three isolates of *A. trappei.* AMF fungal isolate code i.e. W0-4, W0 refers to the site and 4 to the number of the AMF isolate. The dotted horizontal line represents non-mycorrhizal control. (f) Example of plant growth variability: *E. claroidea* W0-4 and W3-73 (left); *F. mosseae* W3-37 and W3-69 (centre); *A. trappei* W2-32 and W1-22 (right).

shoots and roots and Ca in shoots) were the stronger drivers of functional variability along the first axis. The distribution of the samples along the first axis shows that: (i) most AM fungal isolates of *F. mosseae* have a greater result in terms of plant growth, although with a gradient of performances; (ii) the isolates of *E. claroidea* species are more spread; (iii) the isolates of *A. trappei* are closely grouped. Furthermore, the percentage of root length containing arbuscules and AM fungal root colonization are better discriminants for variability at the species level.

The correlation analysis describing the pairwise relationships of all AM fungal infectivity and effectiveness parameters showed, as expected, significant positive associations between all plant growth parameters (Fig. 7e; Tables S10 and S11). Furthermore, the correlation analysis showed significant negative associations between plant growth parameters and root and shoot concentrations of many plant nutrients. In detail, with Mn, K, Ca, and Mg in roots and all nutrients in shoots. On the contrary, significant positive associations were found between most of the concentrations of nutrients in the shoots and in the roots, as well as between each other. In fact, some correlations were evidenced between nutrients in roots and many more in shoots. Several positive correlations were also highlighted between nutrients in roots and shoots. Furthermore, as expected, the AM fungal infectivity traits were positively related to each other.

3.3. Predictors of AM fungal variability

The PCO biplot based on the environmental parameters (i.e. soil chemical parameters, plant diversity, and soil prokaryotic diversity) of the soils of origin well separated the AM fungal isolates propagated from soils with higher pH and P_{avail} belonging to W0 from the others having higher SOM and plant diversity (W1, W2, and W3) (Fig. 7b). This was also supported by the distance thresholds obtained by SIMPROF cluster analysis (Fig. S5) that were plotted in the PCO biplot (Fig. 7b). The significance of the relationship between the AM fungal infectivity and effectiveness matrix and the environmental parameter matrix of is supported by the RELATE analysis ($\rho = 0.187$; $P = 0.009$) (Fig. 7d). Overall, AM fungal isolates originated from soils with higher pH and P_{avail} (W0) promoted nutrient uptake in shoots and roots, while those of soil having with higher SOM and plant diversity (W1, W2 and W3) promoted plant growth (Fig. 7a,b and 8b,c). In contrast, the environmental parameters of the soil of origin did not affect the infectivity traits and were negatively correlated with the soil prokaryotic diversity. The BEST analysis highlighted pH, S_{plant} and H'_{plant} as good predictors ($\rho = 0.301$; $P = 0.01$) (Fig. 8a), while the DistLM analysis supported the main role played by pH in determining the pattern of AM fungal functional diversity (Fig. 8b).

4. Discussion

In this study, the propagation of AMF from four sites with a soil fertility gradient allowed the isolation of 19 single-spore cultures of four morphotypes (i.e. *Entrophospora* spp., *Funneliformis* spp., *F. mosseae*, *Archaeospora* spp.). Therefore, three, thirteen and three single-spore cultures from all sites were phylogenetically identified as different isolates belonging to *Entrophospora claroidea*, *Funneliformis mosseae*, and

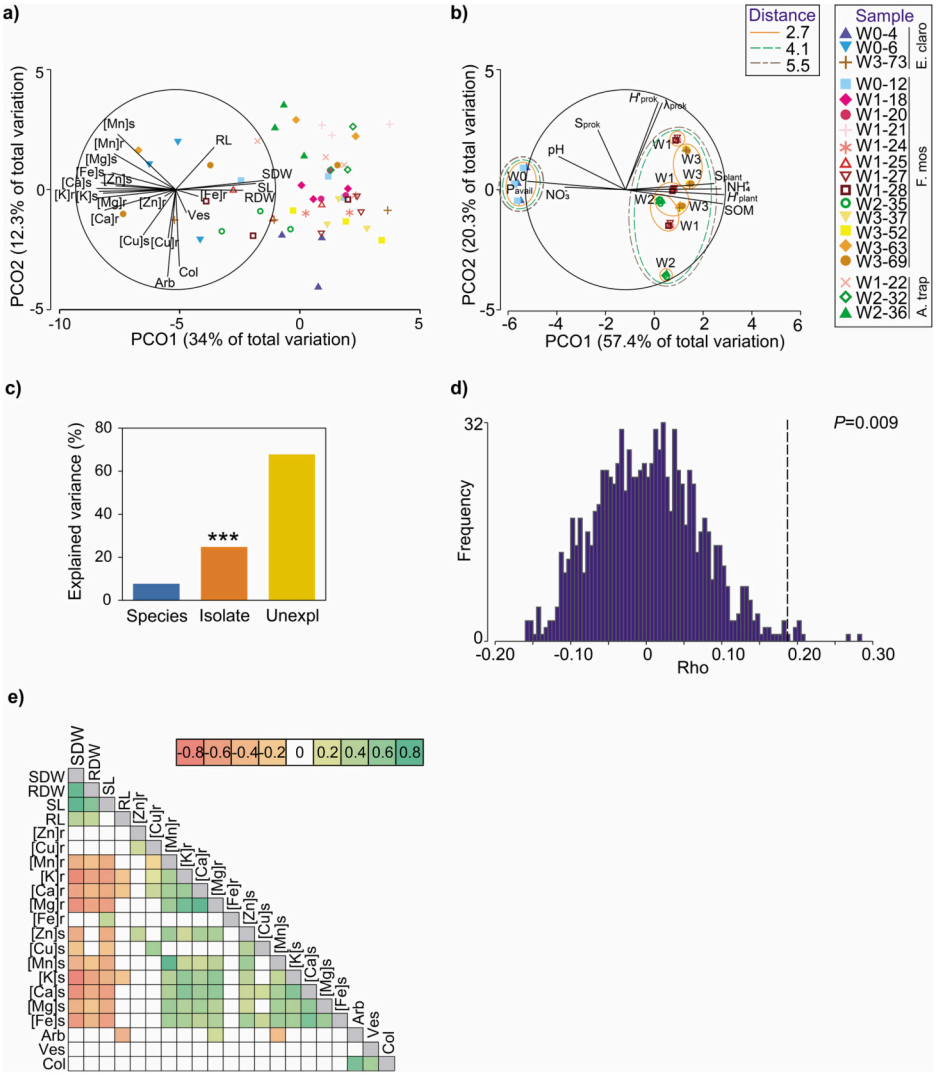


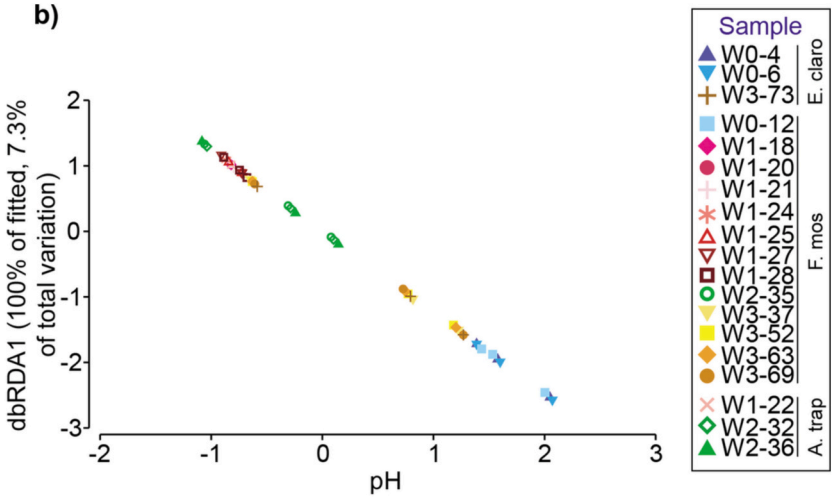
Fig. 7. (a) Principal coordinate analysis (PCO) biplot based on the Euclidian distance matrix of similarity calculated on the square root and normalized parameters of arbuscular mycorrhizal fungal (AMF) infectivity and effectiveness, which highlights the significant effect of the AMF isolate within species (intraspecies functional diversity), accordingly to the significance value of the permutational analysis of variance (PERMANOVA; Table S9). AMF infectivity parameters are: percentage of AMF root colonization (Col), percentage of root length containing arbuscules (Arb) and percentage of root length containing vesicles (Ves). AMF effectiveness parameters are: shoot dry weight, (SDW), root dry weight (RDW), shoot length (SL), root length (RL) and plant nutrient concentration (i.e., Z, Cu, Mn, K, Ca, Mg, Fe) in shoots (s) and roots (r). Replicates are three per each AMF isolate. Host plant was leek (*Allium porrum* L.). Nineteen AMF isolates were propagated from three grasslands whose soils were last plowed and fertilized 22, 36 and 66 years ago (W1, W2 and W3: 22, 36, 66 years) and from a conventional arable field as reference (W0). AMF species used as fixed factor: *Entrophospora claroidea*, *Funneliformis mosseae* and *Archaeospora trappei*. Three AMF species belonging to three orders. AMF isolate used as random factor (nested factor within AMF species): three isolates of *E. claroidea*; 13 isolates of *F. mosseae* and three isolates of *A. trappei*. AMF fungal isolate code i.e. W0-4, W0 refers to the site and 4 to the number of the AMF isolate. (b) Principal coordinate analysis (PCO) biplot based on the Euclidian distance matrix of similarity calculated on the square root and normalized environmental parameters of the soils of origin of the AMF isolates. Environmental parameters are: soil pH, ammonium (NH_4^+), nitrate (NO_3^-), organic matter concentration (SOM), available P (P_{avail}); plant species richness (S_{plant}) and Shannon-Weaver Index (H'_{plant}); soil prokaryotic species richness (S_{prok}) and Shannon-Weaver Index (H'_{prok}), Simpson Index (λ_{prok}). Replicates are three per each type of soil of origin. Samples are grouped into clusters based on their similarity/homogeneity of parameters according to distance thresholds obtained by the Similarity Profile (SIMPROF) cluster analysis (Fig. S6). (c) Variation partitioning according to the results of PERMANOVA testing the effect of the AMF species and isolate within species on fungal infectivity and effectiveness (inter- and intra-species functional diversity, respectively; ***: P(perm) = 0.001; Table S9). (d) RELATE analysis based on Spearman rank and 999 permutations for testing the significance of the relationship between the two matrices: matrix of AMF infectivity and effectiveness and matrix of environmental parameters ($\rho = 1$ perfect relationship) (Clarke and Warwick, 2001). P values are reported. (e) Pearson (r) correlation map among AMF infectivity and effectiveness parameters.

Archaeospora trappei, respectively. These AM fungal isolates were used to establish the study of functional variability among AMF at inter- and intra-species level. A greater functional variability in infectivity and effectiveness occurs among isolates within AM fungal species than among AM fungal species. AM fungal isolates from soils of the conventional arable field (W0) with higher pH and P_{avail} promoted plant nutrient uptake, while those originated from soils with higher SOM and plant diversity (chronosequences of secondary succession of grasslands: W1, W2, and W3) promoted plant growth. However, since negative significant correlations were highlighted between plant growth and nutrient uptake, plant nutrient uptake responses can be a direct effect of plant growth, and thus our categorization could be modified following this interpretation. Moreover, the infectivity traits were more conserved since they were not affected by the environmental parameters of the soil of origin. Finally, we highlight the main role played by soil pH in determining the pattern of AM fungal functional diversity.

a)

BEST results

	ρ	P
Global test	0.302	0.01
No.Vars	Corr.	Selections
2	0.302	pH, Splant
3	0.302	pH, Splant, H'plant
3	0.301	pH, Pavail, Splant
4	0.301	pH, Pavail, Splant, H'plant
3	0.301	pH, Splant
4	**0.301**	**pH, SOM, Splant, H'plant**
2	0.299	pH, SOM
5	0.299	pH, SOM, Pavail, Splant, H'plant
2	0.299	pH, H'plant
3	0.298	pH, SOM, H'plant



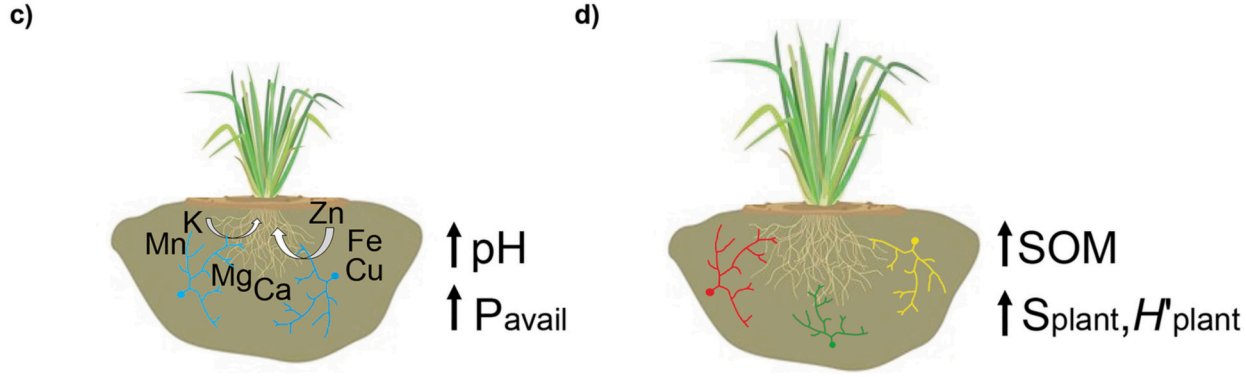


Fig. 8. Results of the BEST analysis based on BioEnv methods (all combinations), Spearman rank and 999 permutations: (a) ρ and *P* of the Global test (ρ = 1 perfect relationship) (Clarke and Warwick, 2001) and BEST descriptor(s) of the relationship together with the correlation values (Clarke et al., 2008). (b) Distance-based redundancy analysis (dbRDA) plot used to visualize the first axis of the Distance-based linear method (DistLM) analysis applied to measure the significance and the variance explained by the best descriptor(s). Graphical scheme of the results of Principal coordinate analysis (PCO) highlighting that the environmental parameters of the soils of origin of the AM fungal isolates promoted AM fungal functional diversity: (c) AM fungal isolates originated from soils with higher pH and P_{avail} (W0) promoted plant nutrient uptake, (d) whereas those originated from soils having higher SOM and plant diversity (W1, W2 and W3) promoted plant growth.

4.1. Molecular characterization of AMF

In this study, the phylogeny of the 19 AM fungal single-spore cultures belonging to four morphotypes (i.e. *Entrophospora* spp., *Funneliformis* spp., *Funneliformis mosseae* and *Archaeospora* spp.) was revealed using the AM fungal specific primer pairs developed by Krüger et al. (2009). In fact, three isolates of *E. claroidea*, 13 isolates of *F. mosseae*, and three isolates of *A. trappei* were discriminated by high bootstrap supports (≥ 90). These results highlighted the presence of molecular variation among isolates of the same species from close sites. We applied the approach developed by Krüger et al. (2009) targeting a ca. 1.5 kb region, covering the 3′ part of the SSU gene, the full ITS region, and the 5′ part of the LSU. However, since the applied Sanger sequencing was based on the use of a forward primer and not on the use of additional reverse and central primers, the phylogeny was actually based on ca. 1010 aligned sites. The region covering a portion of the 3′ end of the SSU (200 bp), and the entire ITS region (ca. 500 bp) (ITS1, 5.8S, and ITS2) and a portion of the 5′ end of the LSU (ca. 140 bp), showed to have a good resolution for the delimitation of AM fungal isolates within three species, *E. claoridea*, *F. mosseae*, and *A. trappei*. However, the fragment we used does not cover the variable V4 and V5 regions of the SSU that are generally applied for the characterization of AM fungal communities in metabarcoding studies (Dumbrell et al., 2011; Lekberg et al., 2018; Mhlanga et al., 2022; Öpik et al., 2009). By contrast, the fragment covers, in addition to the full ITS and a portion of the LSU, a quite conserved region of the 3′ end of the SSU, located before ITS1. Moreover, the fragment we utilized does not cover both the well-resolving hypervariable regions of the LSU D1 and D2 (Delavaux et al., 2020), but only the D1. However, the output of our phylogenetic trees supports the use of the full ITS together with portions of SSU and LSU for a reliable identification of taxa within some AM fungal species. We acknowledge that the success of this approach could have been facilitated by focusing the phylogenetic assignment on the AM fungal species separately. However, the presence of genetic variation within an isolate and within species has previously been evidenced on few AM fungal species (Chen et al., 2018; Sanders, 2004; Thiéry et al., 2012, 2016). We are also aware that an approach based on Sanger sequencing of a larger number of clones, PacBio sequencing (Egan et al., 2018; Kolaříková et al., 2021) or whole genome sequencing (Sahraei et al., 2022) could be applied to integrate the information about taxonomy.

In other studies, different molecular approaches were applied to discriminate the intraspecies variation of AMF. Pellegrino et al. (2012, 2022) found molecular variation among isolates of *F. mosseae* and *R. irregularis* using the set of primers NS31/LSUGlom1. Similarly, intraspecies variation was detected in the AM fungal species *R. irregularis* and *Glomus aggregatum*, using the mitochondrial large ribosomal subunit (mtLSU) (Börstler et al., 2008; Raab et al., 2005; Sýkorová et al., 2012). Furthermore, the RNA polymerase II gene (RPB1) and the P-type IID

ATPase were also successfully used for the intraspecies discrimination of some AMF (Redecker et al., 1997; Thioye et al., 2019). Recently, Säle et al. (2021), using the PCR primer pairs designed by Krüger et al. (2009), found a great molecular variation among isolates of some AM fungal species belonging to five orders. In addition to DNA-based analysis, the proteomic-based chemotaxonomic biotyping using MALDI-TOF mass spectrometry has recently been reported to be a reliable technique to discriminate AM fungal isolates within species of *Enthrophospora etunicata*, *Rhizophagus clarus*, and *E. claroidea* (Crossay et al., 2017) and could be applied together with other approaches to study AM fungal intraspecies variation.

The fact that the most abundant taxa isolated from the four sites belonged to *F. mosseae* confirms the superior capacity of this species to adapt to different environments, its generalist behavior, high infectivity potential, and short life cycle (i.e. time of sporulation) and reduced sensitivity to disturbance (e.g. Oehl et al., 2003, 2017; Öpik et al., 2006). Indeed, members of the genus *Funneliformis* were the most abundant AMF in various types of land use, ranging from arable fields to grasslands, uncultivated soils, wetlands, and woodlands/forests in a wide range of soil pH (Ciccolini et al., 2016; Douds and Millner, 1999; Oehl et al., 2003; Větrovský et al., 2023). The lack of detection of *A. trappei* under W0 (conventionally cultivated site) is consistent with the results of other studies which reported that species belonging to the order of Archaeosporales are not detectable or scarcely present in arable soils (Alguacil et al., 2014; Borriello et al., 2012; Větrovský et al., 2023). On the contrary, Archaeosporales were larger in grasslands (Větrovský et al., 2023). In fact, members of Archaeosporaceae are slow root colonizers, although they have a fast life cycle due to the short sporulation time (Oehl et al., 2003, 2005). This may explain the lack of *A. trappei* in arable lands subjected to intensive farming practices, such as tillage, chemical weeding, monocropping with short vegetation and prolonged fallow periods. Consistent with our study, where most of the *E. claroidea* isolates came from the conventional field (W0), members of the genera *Entrophosphora* were commonly found in intensively managed arable lands together with *Funneliformis* and *Glomus* taxa (Alguacil et al., 2014; Hontoria et al., 2019; Oehl et al., 2003, 2005). The fact that the family of Entrophosphoraceae together with Glomeraceae shows a higher preference for biomes dominated by herbaceous and graminoid vegetation was also recently supported by a meta-analysis of high-throughput sequencing data (Větrovský et al., 2023). In our study, we also observed a closer relationship among isolates that originated from the same site. Previously, Stukenbrock and Rosendahl (2005) and Rosendahl and Matzen (2008) identified distinct genotypes in *F. mosseae*, *Funneliformis caledonium*, and *Funneliformis geosporum* spores collected from agricultural sites. This may result from the selection of genotypes by soil parameters that may determine subdivision within a single species (Rosendahl, 2008). Furthermore, our results can be explained by a limited dispersal of spores, low hyphal propagation and no or low formation of anastomosis between mycelia of the same genotype/isolate (Croll et al., 2009; Giovannetti et al., 2004).

The fact that 19 AM fungal isolates were molecularly characterized and made available in a culture collection represented a great starting point for the characterization of functional variation within AM fungal species. So far, the functional characteristics of the AMF detected in field samples are largely unknown, because most of the taxa have not been isolated in culture and are not cultivable (Fitter, 2005; Helgason et al., 2002). Even information regarding the cultured species is scarce. Furthermore, understanding the genetic basis of AM fungal functional intraspecific variation is made even more complicated by the fact that spores and hyphae contain multiple and genetically different nuclei within a common cytoplasm (Chen et al., 2018; Kokkoris et al., 2020) that could allow local adaptation of AMF to environmental/host variation (Angelard et al., 2014; Hoeksema et al., 2010).

4.2. Arbuscular mycorrhizal fungal functional variability

In this study, we have shown a larger variation in fungal infectivity and effectiveness within species than among species. Earlier, Daniels and Duff (1978) and Dodd et al. (1996) reported differences in spore germination and isozymes pattern among several isolates of *F. mosseae*, respectively, suggesting for the first time within-species differences. Hart and Reader (2002) and Maherali and Klironomos (2007) reported a strong variation at family level (Acaulosporaceae, Gigasporaceae, and Glomeraceae) on AM fungal colonization strategy. Furthermore, some authors evidenced a great variation at the species level regarding sporulation investment, root colonization, extraradical mycelium (ERM) architecture, amount of C extracted from host, plant biomass, P uptake and allocation of host C to storage versus nutrient uptake (Hart and Reader, 2005; Pearson and Jakobsen, 1993; Smith et al., 2004; Violi et al., 2007). Later, Munkvold et al. (2004) highlighted no intraspecific variation in AM fungal colonization, but prominent differences in plant growth (ca. 70 % of the total variance) by screening isolates of four species (*F. mosseae*, *E. claroidea*, *F. caledonium*, and *F. geosporum*) inoculated on subterranean clover (*Trifolium subterraneum* L.). Consequently, Koch et al. (2006) found a large variation in root length, hyphal length, and spore density among isolates of *R. irregularis* grown with transformed carrot roots (*Daucus carota*), and this pattern was observed irrespective of P availability in the medium. They also found a great intraspecific variation in total plant dry weight under non stress and dry conditions. Moreover, Avio et al. (2006) detected a functional variability at species and within-species level by studying two isolates of *F. mosseae* and *R. irregularis* inoculated on alfalfa (*Medicago sativa* L.). This variability was supported by plant growth and nutrient traits (i.e. N and P shoot and root uptake) and AM fungal traits (i.e. extent, structure, and interconnectedness of ERM). Later, a higher functional variability was detected within species than between species (de Novais et al., 2014; Schoen et al., 2021). However, Schoen et al. (2021) analyzed a large collection of tropical AM fungal isolates belonging to several families (i.e. Acaulosporaceae, Entrophosphoraceae, Gigasporaceae, and Glomeraceae), while previous works studied only a small number of isolates and few AM fungal species (Avio et al., 2006; Koch et al., 2006). In contrast, some authors did not report significant differences in anastomosis formation, ERM traits, and P plant uptake between isolates of *R. irregularis* and *F. mosseae* (De La Providencia et al., 2005; Jansa et al., 2005). However, recently, a high-resolution phylogeny was used to show that fungal genetic variation occurs within the AM fungal species *R. irregularis* driving the plant fatty acid pathway that regulates symbiotic exchange (Savary et al., 2020). Peculiar differences in plant growth and significant differences in the transcription of several genes (e.g. pheromone perception; formation of mating tubes; sexual sporulation; and mating regulators) were detected when different isolates of *R. irregularis* were co-inoculated (Mateus et al., 2020). Thus, it was demonstrated for the first time that non-self-interacting isolates of the same species co-existing inside the root are responsible for differences in the up-regulation of some genes and consequently for plant-fungus functional variability.

In the present work, AM fungal intraspecies variability was investigated, including also the less studied order of Archaeosporales (Větrovský et al., 2023). Isolates of *A. trappei* were associated with a large variability of plant growth responses, similar to those of *F. mosseae* and *E. claroidea*, while the variability of root colonization and various plant nutrients was small. However, we have to consider that three isolates were analyzed for both *E. claroidea* and *A. trappei*. Contrary to what has recently been reported for isolates belonging to Archaeosporaceae (Säle et al., 2021), *A. trappei* showed a high benefit in terms of plant growth and nutrient uptake, comparable to *F. mosseae* and *E. claroidea*. However, similar to Säle et al. (2021), low AM fungal colonization was observed. Our results agree with Koch et al. (2017), who observed conserved fungal traits and no effects of fungal phylogeny on plant growth. Furthermore, we were unable to observe any

relationship between intraradical fungal volumes and plant performance. This supports previous findings that mycorrhizal function is not truly related to the degree of colonization (Burleigh et al., 2002; Graham and Eissenstat, 1998). However, since the variation in the response of plants to AMF appears to be related to the interaction between the evolutionary history of plants and that of fungi (Hoeksema et al., 2018; Säle et al., 2021), the inconsistencies we found may be related to ecological factors (biotic and abiotic factors) driving plasticity within particular combinations of plants and AMF (Hoeksema et al., 2010).

Finally, in the present work for the first time, the evidence of AM fungal intraspecies variability was supported by a wide spectrum of plant nutrients. Previous works highlighted inter- and intra-species variability focusing only on P and N uptake and with isolates of some AM fungal families (i.e. Acaulosporaceae, Claroideoglomeraceae, Diversisporaceae, Glomeraceae, Gigasporaceae) (interspecies: Jansa et al., 2007; Wang et al., 2018; Smith et al., 2004; intraspecies: Avio et al., 2006; de Novais et al., 2014; Munkvold et al., 2004; Schoen et al., 2021). In these studies, plant nutritional differences were related to the extent and interconnectedness of the ERM and to hyphal distribution. Although Zn, Cu, Fe, and, to a limited extent and under specific conditions, K, Mn and Ca increased in inoculated plants (Arcidiacono et al., 2023; Cardini et al., 2021; Cavagnaro, 2008; Lehmann and Rillig, 2015), to our knowledge no study focused on these nutrients to investigate intraspecies variability. We expected that the variability we observed at the intraspecies level was more related to AM fungal intraradical colonization traits than to plant traits. However, we hypothesized that the large spectrum of nutrients studied allowed to identify plant traits as discriminants of intraspecies variability. The identification of discriminants was supported by a robust data set, as shown by the positive association between all the characteristics of the AM fungal infectivity and the negative associations between plant growth and mineral nutrients. Furthermore, the pattern of association among plant nutrients suggests that the balance of plant chemical elements may be regulated by AM fungal symbiosis, in addition to the need to maintain fixed ratios of nutrients to maintain physiological processes and to scale the size of plants (Elser et al., 2010).

Thus, our data strengthened the concept that it is not possible to generalize functional traits from a single isolate to a species. The application of the species concept to glomeromycotan fungi has been challenging (Stockinger et al., 2010), and as suggested by van der Heijden et al. (2004) it should be based on a number of characters, such as phylogenetic, physiological, and biochemical parameters. Indeed, in evolutionary terms, the high-level intraspecific divergence may be as important as the low-level phylogenetic nodes in generating the variation of traits that can affect agroecosystem processes. However, intraspecific variation can be further affected by the environmental context (Hoeksema et al., 2010), making it difficult to assign a single trait value to a species and to determine which trait is fundamental for the discrimination of functional variability.

4.3. Predictors of AM fungal functional variability

In this work, AM fungal isolates propagated from conventional arable soils with winter wheat promoted plant nutrient uptake, while those propagated from grasslands mainly promoted plant growth. Higher soil pH and available P in soils of origin drove AM fungal isolates beneficial for plant nutrition, whereas lower soil pH and available P drove AM fungal isolates beneficial for plant growth. Furthermore, higher SOM and plant diversity drove AM fungal isolates beneficial for plant growth. In general, it is known that soil chemical parameters affect soil AM fungal colonization traits and community composition (Wang et al., 1993; Hazard et al., 2013; Verbruggen et al., 2012). Firstly, soil pH is well known to modulate nutrient availability (Barrow, 2017; Hartemink and Barrow, 2023) and many authors highlighted its effect on AM fungal spore germination, fungal growth (Wang et al., 1993), root colonization, and phosphate activities of the ERM (Skipper and Smith, 1979; van Aarle et al., 2002; Verbruggen et al., 2012). Soil pH was also reported to be the main factor in determining the composition of AM fungal communities (Dumbrell et al., 2010; van Aarle et al., 2002). Furthermore, in a large landscape study, the composition of the AM fungal community was strongly influenced by abiotic variables (rainfall and soil type) and not by land use or geographical distance, regardless of plant species (Hazard et al., 2013). The diversity of AMF was also reduced with a high soil available P, despite the fact that different plants contain different AM fungal communities (Ciccolini et al., 2016; Gosling et al., 2013; Van Geel et al., 2016; Verbruggen et al., 2012). In an eight-year field experiment, the relative abundance of *Gigaspora gigantea*, *Gigaspora margarita*, *S. calospora*, and *Paraglomus occultum* decreased, whereas *R. irregularis* increased in response to the application of mineral fertilizers (Johnson, 1993). Furthermore, AM fungal colonization was lower in the soil subjected to fertilization compared to the unfertilized soil. Similarly, our results suggest that fertilization selects for less mutualistic AMF in terms of plant growth. Interestingly, similarly to our findings, Louis and Lim (1987) reported that a strain of *Rhizophagus clarus* isolated from low P soils improved soybean (*Glycine* max) plant growth and nitrogenase activity, whereas a strain isolated from high P soil was not effective. Furthermore, supporting our results, AM fungal traits positively linked to the degree of mutualistic association are affected by increases of organic C in soil (Jiang et al., 2021) and high plant diversity in soils of origin (i.e. inoculum source plot) can increase the response in terms of biomass of the plant host (Burrows and Pfleger, 2002).

4.4. Perspectives and constraints for inoculum production for large-scale field application

The relationship recorded between chemical parameters of the soil of origin and infectivity and effectiveness of AMF supports the fact that the model system we applied (i.e., a chronosequence of secondary succession of chalk grasslands) for the isolation of AMF with targeted beneficial functions was successful. This method developed in a cold area could also be implemented for the isolation of AM fungal taxa adapted to other climatic contexts, with the aim to build large microbial banks able to boost the advancement of AM fungal inoculum production. However, some of the isolates found efficient can be only propagated using pot culturing or soilless culture systems and not by in vitro mass propagation (i.e. isolates belonging to *F. mosseae*). This could represent a limitation for the AM fungal inoculum production at large scale, together with shift/change of the AM fungal function over the time of propagation (Kokkoris and Hart, 2019). Nevertheless, a recent work of Kameoka et al. (2019) showed that fatty acids can boost AM fungal growth and sporulation under asymbiotic conditions. In the same direction, Sugiura et al. (2020) and Tanaka et al. (2022) reported successful axenic cultivation of AMF using metabolites, such as myristate or plant hormones. This may support a new generation of AM fungal propagation and isolation methods of high-efficient taxa having targeted functions. This advancement could guarantee valid alternatives to the present wide use of few AM fungal taxa in inoculum formulation. Despite that, in the field, the environmental and host-plant interactions can affect the survival of the AM fungal isolates in soil and roots or change the AM fungal function which would make the AM fungal isolate only applicable for certain scenarios (Rosa et al., 2020).

5. Conclusions

In general, our results promote the understanding of the intraspecific functional variation of AMF and point to higher functional variability in infectivity and effectiveness among isolates within the AM fungal species than among the AM fungal species. Furthermore, we gave new insights into the functionality of the ancient lineage of Archaeosporaceae. Different soils with a gradient of soil fertility allowed the selection of isolates with specific effects on plant growth and nutrient uptake.

Overall, the relationships between isolated AMF and efficiency traits are related to the availability of the nutrients in the soils of origin. The results depicted here reveal the complexity of the process of isolation of AMF with targeted beneficial functions and highlight some theoretical links that underlie that complexity. However, our evidence represents an important starting point for the selection of AMF and production inoculants with targeted functions (i.e. high effectiveness in plant growth or nutrient uptake) for specific agricultural contexts.

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CRediT authorship contribution statement

Valentina Marrassini: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Laura Ercoli:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Eiko E. Kuramae:** Writing – review & editing. **George A. Kowalchuk:** Writing – review & editing. **Elisa Pellegrino:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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

Data will be made available on request. Moreover, DNA sequences are available in the NCBI Sequence Read (SRA) database as SUB13754036, SUB13728350 and SUB13844335.

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Appendix A. Supplementary data

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