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Research Paper

Diversity of Arbuscular Mycorrhizal Fungi Associated with Six Rice Cultivars in Italian Agricultural Ecosystem Managed with Alternate Wetting and Drying

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Abstract: Alternate wetting and drying (AWD) system, in which water has been reduced by approximately 35% with an increased occurrence of beneficial arbuscular mycorrhizal (AM) symbiosis and no negative impact on rice yield, was proposed to utilize water and nutrients more sustainable. In this study, we selected six rice cultivars (Centauro, Loto, Selenio, Vialone nano, JSendra and Puntal) grown under AWD conditions, and investigated their responsiveness to AM colonization and how they select diverse AM taxa. In order to investigate root-associated AM fungus communities, molecular cloning-Sanger sequencing on small subunit rDNA data were obtained from five out of the six rice cultivars and compared with Next Generation Sequencing (NGS) data, which were previously obtained in Vialone nano. The results showed that all the cultivars were responsive to AM colonization with the development of AM symbiotic structures, even if with differences in the colonization and arbuscule abundance in the root systems. We identified 16 virtual taxa (VT) in the soil compartment and 7 VT in the root apparatus. We emphasized that the NGS analysis gives additional value to the results thanks to a more in-depth reading of the less represented AM fungus taxa.

Key words: alternate wetting and drying system; arbuscular mycorrhizal fungi; rice; molecular diversity; virtual taxa

Rice (*Oryza sativa* L.) is the most important staple food for humans, and its demand is rising due to the increasing world population in the last decade (Bin Rahman and Zhang, 2023). Usually, rice is cultivated under wetland conditions in anaerobic environments, and this agricultural practice requires about 2 500 L of water per kilogram yield, depending on the rice ecosystem and local climate (Bouman and Tuong, 2001), and moreover limits the presence of arbuscular mycorrhizal fungi (AMF). The rice production method, referred to as alternate wetting and drying (AWD), has been receiving increased attention in recent years. AWD consists in switching from a continuously flooded field to a field with different dry periods during the rice growing season (Lampayan et al, 2015). AWD combines the beneficial side effects of anaerobic rice cultivation and aerobic cultivation practices, allowing reduction in water use by about 35% (Price et al, 2013; Lampayan et al, 2015). Moreover, AWD system can better allow exploitation of the beneficial effects of the arbuscular mycorrhizal (AM) symbiosis that are strongly reduced under wetland conditions (Vallino et al, 2014). In addition, Watanarojanaporn et al (2013) studied the native AMF community in rice growth

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under both flooded and AWD systems and reported that rice cultivated in the AWD condition shows more diversification of AMF community.

AM symbiosis is a beneficial interaction between most land plants, including major crops, and soil fungi, belonging to the phylum Glomeromycota (Tedersoo et al, 2018). AM symbiosis plays several key nutritional functions: through the AM hyphal network, host plants gain preferential access to soil water, phosphorus and nitrogen. In exchange, plants supply AMF with photo-synthesized sugars and lipids (Keymer et al, 2017; Luginbuehl et al, 2017; Bago et al, 2020; Genre et al, 2020).

Rice is a monocotyledon model plant for studies of AM symbiosis and its colonization by AMF has been reported across different continents (Wang et al, 2015; Chen et al, 2017; Bernaola et al, 2018; Sarkodee-Addo et al, 2020). Vallino et al (2009) reported that different rice cultivars grown in experimental fields, located in the northern regions of Italy, are colonized by AMF in aerobic conditions. There is a lack of agreement about the effects of AM symbiosis on rice growth and different studies reported both positive and negative impacts, depending also if the rice plants belong to modern or traditional varieties (Parvin et al, 2021). Some studies demonstrated the increase of AM colonized plant biomass, grain yield and phosphorus uptake under flooding conditions (Gewaily, 2019; Wang et al, 2021). By contrast, other studies reported that AM symbiosis decreases rice dry biomass in anaerobic conditions (Bao et al, 2019). In addition, AM symbiosis can confer plant tolerance to biotic and abiotic stresses. It has been reported that AMF enhances resistance to blast fungus *Magnaporthe oryzae* in a rice cultivar Senia (Campos-Soriano et al, 2012). Recently, Campo et al (2020) demonstrated that the AMF-induced responses to blast resistance and the rice productivity are dependent on host genotypes and fungus identity. Different rice genotypes also have diverse tolerances to phosphorus starvation due to the different abilities to absorb phosphorus and efficiently use it (Goncharova and Kharitonov, 2016). So far, few studies have investigated if there are differences in AM colonization between rice cultivars. Vallino et al (2009) studied the different AM colonization in 13 Italian cultivars grown in the field and reported that the colonization ranged between 4% and 28%, without any correlation between the compositions of the root fungal community and plant genotype. In addition, Suzuki et al (2015) studied the growth responses of 64 rice cultivars to an AMF, *Funneliformis mosseae*, reporting a wide difference (from -4% to 119%). Recently, a study of rice root microbiota, focusing on prokaryotes (Bacteria and Archaea) by performing in six rice cultivars, showed that the associated microbial communities in the rhizosphere are significantly influenced by the rice genotype (Edwards et al, 2015). Similarly, a study of both bacterial and fungal microbiota of four rice cultivars highlighted that the fungal communities are significantly affected by the cultivars. However, no deep investigation has been carried out on AMF components (Santos-Medell ń et al, 2017).

Based on the literature above cited, we hypothesized that rice cultivars could recruit different AMF taxa from the native AMF pools thriving in soil under AWD conditions, since such pools are slightly different from those present in lowland soil (Lumini et al, 2011; Chialva et al, 2020). The aims of this study were to evaluate the AMF field colonization of six rice cultivars grown under AWD conditions in an Italian experimental site (Vercelli, Italy), to identify changes in the root-associated AMF communities by comparing the data from molecular cloning-Sanger sequencing with the data previously obtained for Vialone nano by the Next Generation Sequences (NGS) (Chialva et al, 2020), and to explore how rice cultivars select different AMF taxa from a common soil pool.

RESULTS

Mycotrophic status of rice roots grown in field under AWD conditions

We evaluated the AMF colonization level of rice roots grown under AWD conditions, caused by the native AM endophytes of the Vercelli field experiment (Italy). We focused on six cultivars (Loto, JSendra, Puntal, Selenio, Vialone nano and Centauro) that were susceptible to mycorrhization and showed typical AM structures, like extra- and intra-cellular hyphae, hyphopodia and well developed arbuscules (Fig. 1-A). Except for Vialone nano, the other five cultivars were consistently colonized, even with low levels. In detail, the percentage of colonization frequency ranged from 5% to 20% and the percentage of arbuscule abundance from 1% to 4% (Fig. 1-B).

Sequencing and classification

A total of 454 data processing provided 33 Glomeromycota operational taxonomic units (OTUs) in the 6 samples corresponding to a subset of soil and root of plants at

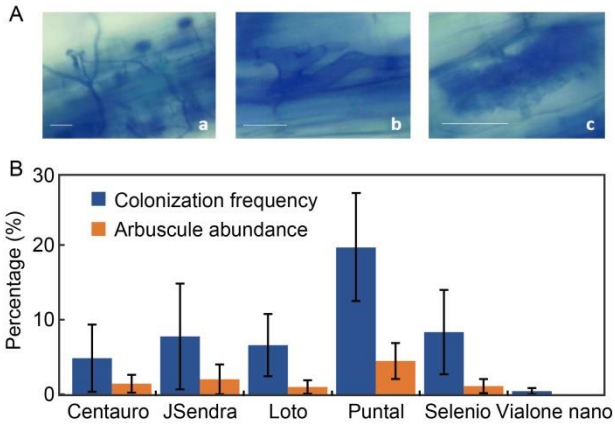

Fig. 1. Typical structures of arbuscular mycorrhizal fungi (AMF) in rice roots and their colonization levels.
A, Typical structures of AMF in rice roots: extra- and intra-radical hyphae (a), hyphopodia (b) and arbuscules (c). Scale bars are 80 μm. **B**, AMF colonization levels in six rice cultivars. Data are Mean ± SD (n = 4).

the tillering stage, grown under AWD management, as described by Chialva et al (2020). According to the MaarjAM database original classification, the 33 taxa were assigned to 16 virtual taxa (VT), and the most abundant of which is VTX00065 (35.1%, 3 OTUs classified as *Funneliformis*), followed by VTX00225 (17.1%, 4 OTUs classified as *Claroideoglomus*) and VTX00143 (16.9%, 2 OTUs classified as *Glomus sensu lato*) (Fig. 2-A).

Sixteen VT were present in the soil compartment, while seven were present in the root compartment. Nine VT (VTX00004, VTX00005, VTX00069, VTX00114, VTX00193, VTX00278, VTX00280, VTX00380 and VTX00418) were exclusively found in soil, while the remaining seven (VTX00054, VTX00065, VTX00067, VTX00093, VTX00143, VTX00225 and VTX00281) were shared by both soil and root compartments (Fig. 2-B). Table 1 summarizes the VT identified in the rice field with original MaarjAM taxonomic affiliation, updated nomenclature, OTU composition and relative frequency. Table S1, in addition, reports the name of each VT OTU along with sequence abundance retrieved in soil and root.

The Evolutionary Placement Algorithm (EPA)-based approach for taxonomic affiliation (Berger et al, 2011) allowed to analyze together sequences representative of the OTUs (33) and the restriction fragment length polymorphism (RFLP) types detected (53), despite the differences in size. Overall, 85 sequences were assigned to 30 nodes of the reference tree (Fig. S1). Only one sequence failed this step and was discarded. The results of taxonomic affiliation are summarized in Table S1. The identified taxa spread among the four orders of Glomeromycota, represented nine described genera. For eight taxa, it was possible to reach the identification at species level. Few sequences were assigned at family/order level. In the case of Glomeraceae, 12 recently described genera lack the 18S characterization (Błaszkowski et al, 2018a, b, 2021; Corazon-Guivin et al, 2019a, b; Jobim et al, 2019). For this reason, the taxon identified as *Glomus microcarpum*, probably belongs to a different genus. The EPA and VT analyses produced similar results regarding the identification of the sequences from the 454 data. The most represented taxon in rice field, here investigated, was *Funneliformis* sp. In the MaarjAM database, it was represented by VTX00065, with *Glomus caledonium* (synonym *Funneliformis caledonium*) as species type, despite the

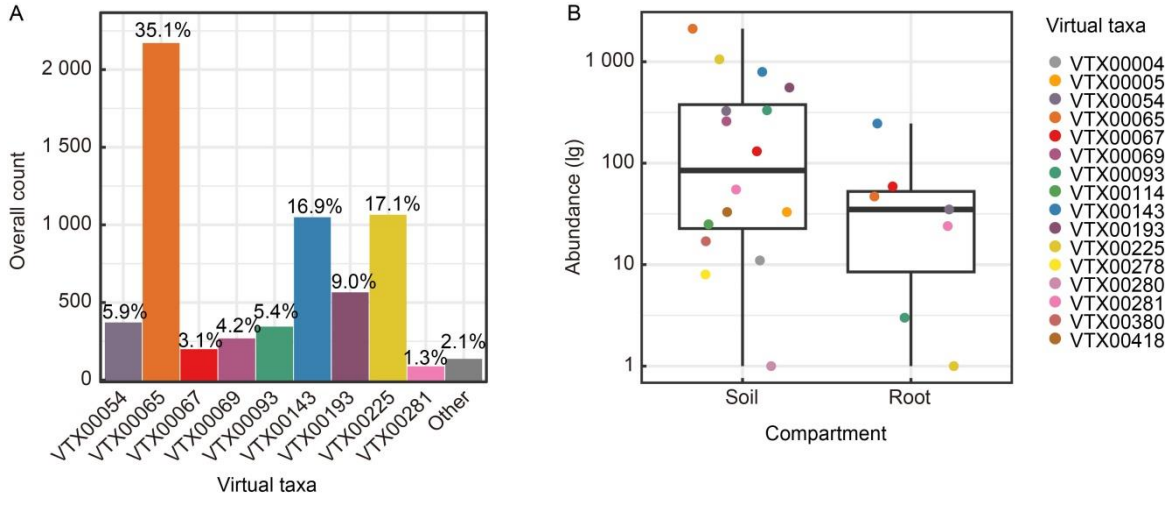

Fig. 2. Arbuscular mycorrhizal virtual taxa abundance.
A, Overall count. **B**, Relative compartment abundance (soil and root). Operational taxonomic units with less than 50 reads were merged as 'Other'.

Table 1. Virtual taxa (VT) identified in Vercelli (Italy) rice fields under alternate wetting and drying management.

Virtual taxa	No. of OTUs	MaarjAM taxonomy [a]	Current taxonomy [b]	Abundance	Relative frequency (%)
VTX00065	3	*Glomus*	*Funneliformis*	2 162	35.06
VTX00225	4	*Claroideoglomus*	*Claroideoglomus*	1 056	17.12
VTX00143	2	*Glomus*	*Glomus sensu lato*	1 040	16.86
VTX00193	3	*Claroideoglomus*	*Claroideoglomus*	556	9.02
VTX00054	3	*Diversispora*	*Diversispora*	362	5.87
VTX00093	1	*Glomus*	*Glomus sensu lato*	335	5.43
VTX00069	3	*Glomus*	*Sclerocystis*	259	4.20
VTX00067	2	*Glomus*	*Funneliformis*	190	3.08
VTX00281	1	*Paraglomus*	*Paraglomus*	79	1.28
VTX00005	3	*Archaeospora*	*Archaeosporales*	33	0.54
VTX00418	2	*Glomus*	*Glomus sensu lato*	33	0.54
VTX00114	1	*Glomus*	*Rhizoglomus*	25	0.41
VTX00380	1	*Diversispora*	*Diversispora*	17	0.28
VTX00004	2	*Archaeospora*	*Archaeospora*	11	0.18
VTX00278	1	*Claroideoglomus*	*Claroideoglomus*	8	0.13
VTX00280	1	*Glomus*	*Rhizoglomus*	1	0.02

[a] Taxonomy names derived from Öpik et al (2010). [b] Current taxonomy names according to Wijayawardene et al (2022). OTUs, Operational taxonomic units.

VT is also hosting other *Funneliformis* species (*F. geosporus*, *F. fragilistratus* and *F. verruculosus*). The second and the third more represented taxa were recognized as *Claroideoglomus* sp. and other *Glomeraceae* spp. (i.e. *Glomus sensu lato* and *Sclerocystis*; *Rhizoglomus*).

Fig. 3 showed a heatmap based on the AMF presence in the six rice cultivars, by means of 454 data and RFLP types. Specific AM fingerprints were detected in each cultivar, with a few sharing AM taxa. Only *Claroideoglomus* (Cl3) was common to all the isolates with the exception of Vialone nano. JSendra revealed the highest biodiversity with seven associated OTUs (Cl3, Cl2, Acau, Cl6, Cl7, Cl4 and Rh), while Centauro only revealed two OTUs (Cl3 and Cl5). Interestingly, irrespectively of the very low colonization percentage, Vialone nano was associated with seven diverse AMF (Fun1, Cl10, Glom2, Par, Fun3, Div and Glom1). By contrast, Puntal, which resulted the highest colonized plant, was hosted only four OTUs (Cl3, Cl2, Glom2 and Cl1).

The Shannon diversity index indicated that some AMF species (Cl3 and Cl2, i.e. *Claroideoglomus etunicatum* and *Claroideoglomus* sp., respectively) were more frequently associated with rice roots than others. While Centauro, JSendra, Loto and Selenio showed an equitability index (E) close to 1, suggesting an equitable distribution of species. Puntal and Vialone nano, whose E index was in the medium, could host a more variable species distribution (Table 2).

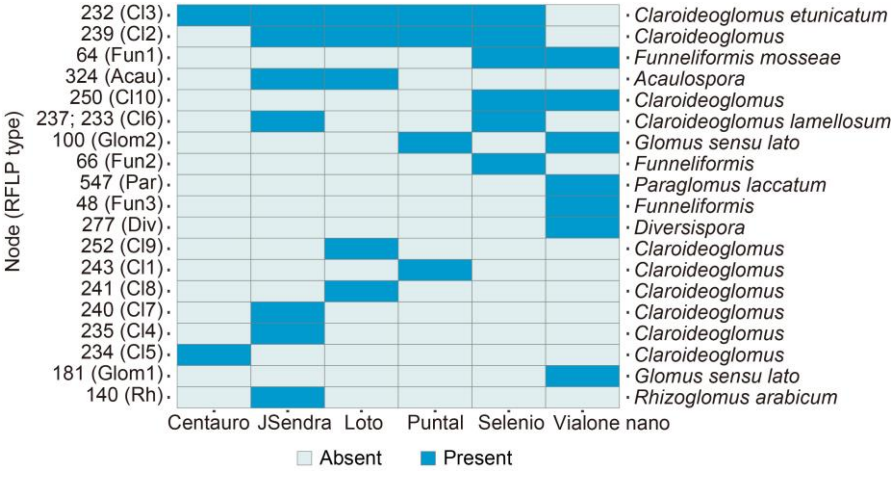

Fig. 3. Heatmap of presence/absence of arbuscular mycorrhizal fungus taxa retrieved from six rice cultivars.

Main tree nodes with related phylogenetic assignment (in brackets) are reported. RFLP, Restriction fragment length polymorphism.

Different methods and platforms: Cloning-Sanger sequencing, NGS and MaarjAM database

One of the goals of this study was to evaluate the possibility to use data originated from different methods. Indeed, we have identified changes in the root-associated AMF communities by comparing the data from molecular cloning-Sanger sequencing with the data previously obtained for Vialone nano (Chialva et al, 2020). As a further step, the identified OTUs were compared with the MaarjAM database, which is considered one of the most important platforms for

Table 2. Arbuscular mycorrhizal fungus diversity (H) and equitability (E) in six rice cultivars.

Rice cultivar	Shannon diversity index (H)	Shannon equitability index (E)
Centauro	0.56	0.81
JSendra	1.65	0.84
Loto	1.36	0.85
Puntal	0.83	0.60
Selenio	1.58	0.88
Vialone nano	1.09	0.56

the identification of Glomeromycota (Öpik et al, 2010), since there are also relevant associated metadata including information about AMF geographical distribution, habitat occurrence and host plant association. This database allows comparisons among research projects, however, it reports some genus names that are outdated according to Wijayawardene et al (2022). For this reason, we reported the most updated names of the taxa in Tables 1 and S1. We referred to the more recent taxonomy when we detailed the components of each VT.

DISCUSSION

Several studies have demonstrated that different plant species and different genotypes recruit specific AMF communities, starting from a common soil pool (Sawers et al, 2008; Diedhiou et al, 2016; Parvin et al, 2021; Huang et al, 2022). In the present study, to detect the potential links between AMF colonization and rice cultivars, we investigated the presence of AMF within the roots of six rice cultivars sampled at the tillering stage and grown under AWD conditions. It is known that AMF communities are influenced, within natural or agronomic ecosystems, by different factors, such as edaphic characteristics, seasonal changes, physiological stages of the host plant (i.e. seedling, tillering, heading and ripening) and agricultural management (Chialva et al, 2020). Our results revealed that each rice cultivar had a specific AM fingerprint, confirming previous results, which demonstrated that rice cultivars differed from those studied here (except for Vialone nano), had a different AM susceptibility upon flooding management (Vallino et al, 2009). Taken in the whole, these data revealed the relevance of the plant genotype, irrespectively of the cultivation management.

Interestingly, under AWD conditions, the less responsive cultivar Vialone nano is one of the most cultivated Italian rice, which is poorly susceptible to mycorrhization, also according to Vallino et al (2009). In this study, only extraradical hyphae were detected, while intraradical hyphae and arbuscules were very rare (Fig. 1). The different responses to AM colonization were also demonstrated by Davidson et al (2019), who revealed that 334 cultivars from the Rice Diversity Panel 1, inoculated with a single AMF isolate (*Rhizophagus irregularis*), show significant variations in hyphal colonization, ranging from 21% to 89%, explained by rice genotypes. In addition, it is known that rice has a heterogenous root system, where the thin lateral roots do not host AMF (Fiorilli et al, 2015). The low susceptibility of Vialone nano to AMF could be related to a different architecture of its root system.

The two different approaches (Cloning-Sanger sequencing and NGS) were used to study AMF communities in soil and rice roots, led to slightly different results, especially in the detection of low abundant or patchy AMF taxa. With reference to the soil data, under AWD conditions, we detected a higher Shannon index (H), equal to 2.13, if compared with that obtained in lowland corresponding to 1.00 (Chialva et al, 2020). This result demonstrated how different agronomic management conditions (AWD vs lowland) can intensely modify soil fungal biodiversity. Comparable soil biodiversity indices, ranging from 1.96 to 3.68, were recorded in different Bangladeshi rice fields, under similar management (Ibne Baki et al, 2021). Indeed, 16 VT were identified by 454 data, representing a good level of AMF biodiversity for agroecosystem. However, only 7 out of the 16 VT colonized the rice roots under the AWD management, again with a certain degree of selectivity (Fig. 2). Centauro revealed the lowest OTU richness, while Vialone nano resulted the richest with 7 OTUs, notwithstanding the limited mycorrhization success resulting from the morphological analysis. Seven OTUs were also detected in JSendra, however, five out of them belonged to the genus *Claroideoglomus*. Interestingly, *Acaulospora* was not recovered from the soil and was exclusively detected by RFLP analysis in the roots of Loto and JSendra. We can speculate that propagules of this taxon in soil were probably less abundant than other AMF, which were preferentially amplified, while Loto and JSendra roots could have represented a more convenient niche for this taxon. Even if *Acaulospora* is acknowledged as a taxon with a global distribution (da Silva et al, 2022), members of *Acaulosporaceae* family show low soil colonization levels (Hart and Reader, 2002; Hempel et al, 2007). More specifically, low presence of *Acaulospora* has been observed in rice varieties grown in Bangladesh

fields, where 8% of OTUs was recorded, only belonging to the Acaulosporaceae family (Parvin et al, 2021). In addition, *Acaulospora* presence has been demonstrated to be strongly influenced by ecosystem types and phenology (Xavier Martins and Rodrigues, 2020). These researchers reported that *Acaulospora* is more diffused in lowland with the maximum species richness recorded at the harvesting stage, while a limited number was observed at the vegetative stage, which corresponds to the time when we sampled rice cultivars. Lastly, the limited presence of *Acaulospora* in this study could be explained by the poor competition of these AMF in some contexts, as suggested by Schreiner and Mihara (2009).

In this study, *Claroideoglomus* resulted to be the most represented genus in terms of different OTUs (Cl1–Cl10, Fig. 3). *Claroideoglomus* taxa affiliated to Cl2 and Cl3 OTUs, covered all rice cultivars. This genus is reported as one of the most widespread genera in different agroecosystems (Carballar-Hernández et al, 2017), comprising several Bangladeshi rice fields, cultivated with both modern and traditional rice varieties (Parvin et al, 2021). Glomeraceae is usually considered the most dominant AMF family associated with rice roots (Wang et al, 2015; Parvin et al, 2019; Chialva et al, 2020; Sarkodee-Addo et al, 2020). Here, taxa belonging to these families (*Glomus*, *Funneliformis* and *Rhizoglomus* species) were recorded with a limited number of clones occurring in a few cultivars (Puntal, Selenio and JSendra).

In conclusion, notwithstanding that the six rice cultivars grow in the same soil, they seemed to be able to recruit specific AMF isolates, with the exception of the *Claroideoglomus* genus. The capacity to recruit specific AMF did not depend on the geographical origin of cultivars. Indeed, we investigated the AM responsiveness in both Italian (Centauro, Loto, Selenio and Vialone nano) and Spanish (Puntal and JSendra) cultivars, but the native cultivars do not seem to be more prone to be colonized than the non-native ones.

The mechanisms behind the recruitment of AMF and of other microbiota components are not fully known (Arnault et al, 2023). The researchers suggested that stochastic or deterministic factors may play a relevant role. Among the deterministic factors, we can hypothesize again the architecture of the rice root system, which develops three types of roots: crown roots, large lateral roots and fine lateral roots. Large lateral roots are the most susceptible to AMF colonization, while fine lateral roots are not colonized (Fiorilli et al, 2015). In addition, AMF stimulates the formation of large lateral roots, but not of crown roots and fine lateral roots (Chiu et al, 2018). We can hypothesize that different rice cultivars can differently develop their root system, modulating colonization success and AMF selection. From a functional point of view, it is well known that strigolactones regulate the establishment of AMF symbiosis (Yoneyama et al, 2007; Lanfranco et al, 2018). Variations in strigolactone exudation have been detected in different rice varieties (Huang et al, 2022). A *japonica* rice variety Azucena secretes higher levels of strigolactones when compared with an *indica* rice variety Bala, indicating the strigolactones as a key factor regulating AM colonization in different rice subpopulations (Cardoso et al, 2014). The root strigolactone profile of the six rice cultivars was not investigated here. However, we suggest further investigating whether the AM specific fingerprint is related to the exudation of specific strigolactones, which have a key role in stimulating hyphal branching in native AMF.

As a general conclusion in the recent years, soil biodiversity studies have progressed exponentially thanks to omics techniques that have helped to better decipher what has been defined the dark soil matter. A better knowledge of the plant microbiota, i.e. the microbial communities which support the plant growth (Chialva et al, 2020) is at the basis of new, less invasive and more sustainable cultivation systems.

Based on morphological and molecular approaches, this study provides some information on rice cultivars which are suitable for growing under AWD and in the meantime benefit from the resource offered by the AMF community naturally present in the soil. In addition, it represents a further contribution to understanding how the AMF community is active in a low-water management system, such as AWD, and how the genetics of the different cultivars is a major factor in selecting AMF.

METHODS

Rice plant sampling and morphological observation

Six rice cultivars (*O. sativa* L. cv. Vialone nano, Centauro, Loto, Selenio, JSendra and Puntal) belonging to the GreenRice panel (Oliver et al, 2019), comprising 12 accessions (Fig. S2), were sampled under the AWD conditions at the tillering stage following the rice BBCH-scale (Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie; Lancashire et al, 1991) at the experimental site of Research Centre for Cereal and Industrial Crops in Vercelli, Italy (coordinates 45°19′204″

N, 8º22′25,35″ E, 134 m above the see level), belonging to the Council for Agricultural Research and Economics. Soil of this area is classified as the loam type and its physicochemical characteristics were reported by Oliver et al (2019). Rice plants were grown under the AWD system to provide aerobic conditions. Watering under upland conditions was provided by three flushing irrigation treatments during the cropping season (May to October): one in June (tillering), one in July (panicle initiation) and one in August (late flowering) depending on climatic conditions. Rainfall and mean monthly temperature in summer were 1 300 mm and about 23 ℃, respectively.

We considered four biological replicates belonging to the four blocks (Fig. S2) consisting of five pooled plants of each cultivar. Rice plants were stored in polyethylene bags and transported to the laboratory at 4 ℃. Roots were extensively and carefully washed to remove the soil and blotted on paper. Half of the root material was immediately stored at -20 ℃ for further molecular analysis, while the remaining was used for morphological AMF and analyzed as follows: stained overnight with 0.1% cotton blue in lactic acid, destained three times in lactic acid for 3 h, and finally, 1-cm-long root fragments mounted onto microscope slides for a total of 20 root pieces per slide, and observed with an optical microscope. Quantitative AM colonization parameters were determined on 1 m of root per plant as described by Trouvelot et al (1986).

DNA extraction and amplification

To identify the AMF diversity associated with the six different rice cultivars, DNA was extracted from 0.25 g of root material, belonging to the four biological replicates of each cultivar (Vialone nano, Centauro, Loto, Selenio, JSendra and Puntal), with Nucleospin Plant II kit by Macherey-Nagel (Düren, Germany), following the manufacturer's instructions. The concentration of DNA was spectrophotometrically determined using a NanoDrop 1000 instrument (Thermo Scientific, Waltham, MA, USA) and standardized at 2 ng/μL for further PCR amplification. Due to a very low colonization level observed in Vialone nano roots, we decided to perform a deeper analysis on its DNA by using NGS analysis as described by Chialva et al (2020). By contrast, DNA extracted from the other five cultivars was processed as follows: Two sets of primers were used to amplify a region of the small subunit (SSU) of the Glomeromycota ribosomal DNA. Partial SSU ribosomal RNA gene fragments were amplified using nested PCR (Casazza et al, 2017) with the universal eukaryotic primers NS1 and NS4 (White et al, 1990), and a following amplification round with Glomeromycota-specific primers AML1 and AML2 (Lee and Lee, 2008). Although longer and higher discriminating regions are available (Krüger et al, 2012), the AML1/AML2 SSU region was targeted because most Glomeromycota diversity data were obtained using this region, which provides a larger comparative DNA sequence dataset. PCR was carried out in three replicates with 20 μL PCR reaction mix, containing 2 μL of template DNA, 0.2 mmol/L of dNTPs, 1.5 mmol/L of $MgCl_2$, 0.5 μmol/L of each primer, 2 U of GoTaq (Promega, Madison, USA), and the supplied reaction buffer. Amplifications were carried out in 0.2 mL PCR tubes using a Biometra T Gradient thermocycler, according to the following steps: 5 min initial denaturation at 94 ℃, 35 cycles of 1 min at 94 ℃, 1 min at 55 ℃ and 58 ℃ for the two nested PCR rounds, respectively; 1 min at 72 ℃; and a final elongation of 10 min at 72 ℃. All the PCR products were checked using 1.5% agarose gel stained with ethidium bromide (Sigma-Aldrich, Milan, Italy). The three nested PCR product replicates were pooled and purified using Wizard® SV Gel and PCR Clean-Up System kit (Promega, Madison, USA). Before ligation, the quantity and quality of the PCR amplicons were checked using a spectrophotometer (NanoDrop Technology, Wilmington, USA). Cloning was done using the pGEM-T vector system (Promega, Madison, USA), and was transformed into *Escherichia coli* (Xl1 blue). At least 40 recombinant clones per amplicon library (n = 5 corresponding to DNA from roots of Centauro, Loto, Selenio, JSendra and Puntal rice plants) were screened for the AML1/AML2 fragment (750 bp) on agarose gels. Thirty-two positive clones of the five root clone libraries were tested for RFLP. RFLP analysis was carried out by independent digestion with *Hinf* I and *Hsp92*II, according to the manufacturer's instructions (Promega, Madison, USA), and analyzed on 2.5% agarose gel electrophoresis. At least two representative samples of each RFLP type were chosen for Sanger sequencing.

Sequence analysis and classification

Pre-processing 454 data from Vialone nano was performed according to Chialva et al (2020). Briefly, 18S raw GS-FLX AMF sequencing outputs were processed by the sffinfo tool from 454, keeping separate root and soil libraries. Analyses were performed using QIIME (Quantitative Insights into Microbial Ecology) v1.8.0 (Caporaso et al, 2010). The script splitlibraries. py was used to bin reads to samples, to remove primers, linkers and barcodes, and to filter reads for quality and length (minimum average phred score of 25 over a sliding window of 25 nucleotides, and a minimum sequence length of 250 bp). Demultiplexed sequences were screened for chimeras using *de novo* detection (abundance based) with usearch61 (v6.1.544). Non-chimeric sequences were clustered into OTUs with the usearch61, using a 97% similarity threshold and retaining only clusters larger than 10 reads. The longer sequence in each cluster was designated as the representative sequence, giving the name of OTU, and the taxonomic affiliation was performed via an open-reference approach on the MaarjAM database (Öpik et al, 2010), using the pick_closed_reference_otus.py script.

All statistical analyses were performed under the R programming environment (R Core Team, 2021): data were handled and rendered with functions implemented in the tidyverse v1.3.2 package (Wickham et al, 2019). The AMF communities were analyzed with the phyloseq v1.42.0 package

(McMurdie and Holmes, 2013), after merging the root and soil datasets. Shannon diversity index was calculated using the 'estimate richness' function in 'phyloseq' (McMurdie and Holmes, 2013). Taxonomic affiliations were confirmed using BLAST v.2.13.0 (Camacho et al, 2009) against the NCBI nt database, discarding sequences not matching Glomeromycota hits.

Sequencing data from the other five rice cultivars (Centauro, Loto, Selenio, JSendra and Puntal) were analyzed together with representatives of each OTU as follows: The taxonomic affiliation of the sequences was obtained using an EPA approach, performed with RAXML v8.2.10 (Berger et al, 2011) and the Glomeromycota classification referred to Wijayawardene et al (2022). The sequences (queries) were aligned in MAFFT (Katoh and Standley, 2013) together with a 18S reference dataset that includes 276 sequences representing 4 orders, 10 families, 24 genera, 98 described species and 45 undescribed isolate/phylotaxa in the Glomeromycota. The 18S reference dataset was used to obtain a maximum likelihood (ML) reference tree via CIPRES Science Gateway 3.1 (Miller et al, 2010), using RAxML-NG 1.0.1 (Kozlov et al, 2019) with a ML/1000 bootstrapping run, GTR + I + G (the general time reversible model with a proportion of invariant sites and gamma-distributed site-to-site rate variations) as the nucleotide substitution model (Abadi et al, 2019), and ML estimated the proportion of invariable sites and base frequencies. The reference tree and the alignment were used as input in the EPA analysis to allow the affiliation of the queries. The EPA output was analyzed in Gappa (Czech et al, 2020) for the placement mass (likelihood weight ratio) accumulation of the placements of each sequence upwards the reference tree with threshold 0.8.

The 454 raw sequencing data belong to BioProject PRJNA638897, deposited in the NCBI Sequence Read Archive (SRA-NCBI; https://www.ncbi.nlm.nih.gov/sra). Sanger sequences were deposited in NCBI under accession ON901878–ON901929.

Diversity analysis

The Shannon diversity (H) index was calculated as an additional measure of AMF diversity. The following formula was used: $H = -\Sigma(pi \times \ln pi)$, where pi is the proportion of the entire community made up of species i. Moreover, the equitability index (E) was calculated applying the following formula: $E = H / \ln S$, where H is the Shannon diversity index and S is the total number of unique species.

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SUPPLEMENTAL DATA

The following materials are available in the online version of this article at http://www.sciencedirect.com/journal/rice-science; http://www.ricescience.org.

Fig. S1. Phylogenetic placement of operational taxonomic units (OTUs) and restriction fragment length polymorphism (RFLP)-type representative sequences on 18S rDNA maximum likelihood reference tree used in analysis.

Fig. S2. Split plot experimental design for alternate wetting and drying (AWD) system.

Table S1. Sequence abundance retrieved in soil and root with the respective operational taxonomic units (OTUs) assignment and virtual taxa.

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