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Acaulospora as the Dominant Arbuscular Mycorrhizal Fungi in Organic Lowland Rice Paddies Improves Phosphorus Availability in Soils

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Abstract: Flooding in rainfed lowlands greatly impairs the mutualistic relationship between indigenous arbuscular mycorrhizal fungi (AMF) and rice. In flooded soils, root colonization by AMF is arrested, but some AMF genera, defined as the core AMF, remain present. However, the core AMF in rainfed lowlands and their symbiotic roles remain unknown. Here, we showed that *Acaulospora* fungi were the core AMF in rice seedling roots of the Sangyod Muang Phatthalung (SMP) landrace rice variety grown in non-flooded and flooded paddy soils. Subsequently, indigenous *Acaulospora* spores were propagated by trap cultures using maize as the host plants. Therefore, to clarify the roles of cultured *Acaulospora* spores in a symbiotic partnership, the model *japonica* rice variety Nipponbare was grown in sterile soil inoculated with *Acaulospora* spores, and recolonized with a native microbial filtrate from the organic rice paddy soil. Our data demonstrated that the inoculation of *Acaulospora* spores in well-drained soil under a nutrient-sufficient condition for six weeks enabled 70 percent of the rice roots to be colonized by the fungi, leading to higher phosphate (Pi) accumulation in the mycorrhizal roots. Unexpectedly, the growth of rice seedlings was significantly suppressed by inoculation while photosynthetic parameters such as fractions of incoming light energy and relative chlorophyll content were unaltered. In the soil, the *Acaulospora* fungi increased soil phosphorus (P) availability by enhancing the secretion of acid phosphatase in the mycorrhizal roots. The findings of this work elucidate the symbiotic roles of the dominant *Acaulospora* fungi from lowland rice paddies.

Keywords: arbuscular mycorrhizal fungi; *Acaulospora*; soil phosphorus availability



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1. Introduction

Rice forms a mutualistic relationship with AMF to deal with nutrient deficiencies in soils, especially P. AMF symbiosis increases P acquisition efficiency through the mycorrhizal uptake pathway in roots, leading to higher P uptake in shoot and root tissues [1]. Under well-drained soil, external phosphorus availability is the major factor regulating AMF symbiosis in rice [2]. However, in lowland rice paddies, water is irrigated and/or rainfed, enabling the cultivation of rice plants in mostly flooded soil [3]. The flooded condition induces the formation of aerenchyma in the root cortex, reducing the accommodation of AMF and the degree of AMF symbiosis in rice roots [4]. Moreover, flooding displays a stronger impact on the intensity of AMF colonization than soil P availability [2,5].

Although AMF symbiosis is restricted by flooding, it remains sustained in paddies of lowland rice. While AMF colonization in roots of upland rice is well developed, with an increasing amount of mature arbuscules and intracellular hyphae, colonization in roots of lowland rice is scarcely present [6]. The colonization rate of indigenous AMF in roots is in the range of 0 to 40 percent, depending on the growth and developmental stage of

the plant [7–9]. Moreover, flooding markedly reduces AMF species richness, diversity and abundance in rice roots [6]. As indicated by the reduction of mycorrhizal growth responses [10], the role of AMF symbiosis is minimized by flooding in lowland rice paddies, but the remaining AMF still contribute sufficient direct and/or indirect benefits to the host plants.

AMF act as soil P improvers. AMF symbiosis assists the host plants to assimilate P derived from unavailable forms such as organic P and precipitated P by increasing P mineralization in the soil [11]. AMF are capable of secreting acid phosphatase and phytase from extraradical hyphae to the surrounding soils, where they convert unavailable organic P and phytate, respectively, into inorganic P available for uptake in rice plant roots [12,13]. AMF prevent P and nitrogen losses in rice paddies by reducing nutrient leaching and runoff from the soil [14,15]. In addition to improving nutrient acquisition efficiency in rice paddies, applied AMF enhance the resilience of rice to environmental stresses in paddies, such as drought, salt and heavy metals [16,17]. However, the AMF used in previous studies, such as *Funneliformis mosseae* and *Rhizophagus intraradices*, belong to the family Glomaceae. They are present in lowland rice paddies [7] but in some areas they may be absent or not the dominant AMF species due to their relatively low abundance [8,18].

Diversity of indigenous AMF in rice paddies is dependent on the rice ecosystem and water management [6,8]. *Glomus* and *Funneliformis* AMF genera are ubiquitously present in most upland and lowland rice paddies [6,18]. *Paraglomus* and *Claroideoglomus* were reported as the second most abundant AMF in upland rice paddies [19,20]. In several lowland rice paddies, *Acaulospora* is the most dominant AMF genus [18,20]. However, little is known about the symbiotic role of *Acaulospora* fungi in rice, especially the native *Acaulospora* species in lowland rice paddies.

Our previous study showed that flooding plays a major role in shaping fungal communities and significantly reduced AMF colonization and AMF abundance in the roots of the SMP landrace rice variety grown in organic rice paddy soil. Only eight amplicon sequence variants of the fungal ITS sequences belonged Glomeraceae, which seems to underestimate the total AMF species in the study site [5]. Moreover, the molecular identification of AMF at the genus or species level by the ITS region is limited. Therefore, in this study, the 1.5 kb SSU-ITS-LSU region generated by the Krüger primers was further analyzed to identify the core AMF in the SMP roots under non-flooded and flooded soils. Later, we attempted to culture the core AMF to characterize its mutualistic functions in the model *japonica* rice variety Nipponbare. We expected the indigenous core AMF cultured from organic lowland rice paddy soil to promote the growth of the host plants, and potentially be useful for improving organic rice cultivation in lowland rice paddies.

2. Materials and Methods

2.1. Determination of AMF in Rice Roots

Genomic DNA samples of roots of Sangyod Muang Phatthalung (SMP) rice plants grown in non-flooded and flooded conditions for 6 weeks were obtained from our previous study [5]. To identify the 1.5-kb SSU-ITS-LSU sequences of AMF present in the roots, nested PCR was run with Krüger primers [21]. The primary run was performed using a 25 µL reaction containing 50 ng of root gDNA, 1xPhusion HF buffer, 0.2 mM dNTPs mixture, 0.5 µM of the forward and reverse primers, and 0.25 unit of Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The primary PCR condition was programmed as follows: Initial denaturation at 98 °C for 30 s followed by 35 cycles of a 3 step PCR including denaturation at 98 °C for 10 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min. The reaction was completed with a final extension at 72 °C for 10 min. The PCR product was diluted 1:50 and used as the DNA template in the secondary PCR. The second run used a 25 µL reaction containing 1X ViBuffer S, 0.1 mM dNTPs mix, 0.4 µM each of forward and reverse primers, and 1 unit of Tag DNA polymerase (Vivantis, Shah Alam, Malaysia). The secondary PCR was carried out under the following conditions: 2 min initial denaturation at 94 °C, 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at

60 °C, 2 min elongation at 72 °C and 7 min final elongation at 72 °C. PCR products were visualized by 1% agarose gel electrophoresis under UV light.

The PCR products were purified by a QIAquick gel purification kit (Qiagen, Hilden, Germany). Equal amounts of six purified PCR products from roots grown under the same condition were pooled into a single sample, ligated using the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed in *E. coli* DH5 α . Sixty genuine transformants were selected by colony PCR using the M13 forward and reverse primers. Plasmids were extracted with the Presto™ Mini Plasmid kit (Geneaid, New Taipei, Taiwan) and sequenced.

120 SSU-ITS-LSU sequences of AMF were aligned by Clustal Omega [22] and clustered into different operational taxonomic units (OTUs) at a 97% identity threshold using the adegenet [23] and kmer [24] packages in the statistical software R (version 3.6.3). Rarefaction curves were constructed by the vegan package [25] in R to determine whether the obtained clones sufficiently represented Glomeromycotean diversity in the rice root endosphere under the non-flooded and flooded conditions.

The representative AMF OTUs were blasted in the NCBI database. Clustal Omega [22] was used to align the 21 AMF OTUs, 20 AMF reference sequences from the NCBI and MaarjAM databases and two sequences of *Chaetomium globosum* and *Myrothecium* sp. as outgroups. A neighbor-joining tree (Kimura 2 parameters, 1000 replications) was constructed using the MEGA software version 10 [26]. Sequences from this study were made available in the GenBank under accession numbers OL662890, OL672273-OL672310, OL672756-OL672834, OL679926 and OL679961.

2.2. Determination of AMF Diversity in Rice Roots

The frequency of sequences in each AMF OTU was collected. The richness, diversity, and evenness of AMF OTUs were compared between the non-flooded and flooded conditions. Shannon's (H') diversity index within the same soil condition was calculated following the formula: $H' = -\sum_{i=1}^n p_i \ln p_i$ where n is the total number of OTUs and p_i is the proportion of sequences belonging to each OTU relative to the total number of sequences. AMF OTU evenness was calculated by dividing Shannon's diversity index H by the natural logarithm of species richness, $\ln(s)$, using Microsoft Excel.

2.3. Trap Culture

In a pot 20 cm in height and 24 cm in diameter, the same batch of organic paddy soil used in our previous study [5] was mixed 1:3 (v/v) with sterile sand to be used for a trap culture using maize as the host plant. *Acaulospora* spores were isolated by the wet sieving and decanting method [27] and used to obtain a monospore culture. For the monospore culture, 300 *Acaulospora* spores were inoculated in four maize plants in the mixture of sterile sand and compost at a ratio of 1:1 (v/v) in a 1-L pot 12 cm in height and 14 cm in diameter. The culture was recolonized with 100 mL of a microbial wash, prepared from 50 g of the paddy soil suspended in 1 L of distilled water and filtered 3 times with Whatman no.1 paper. The trap culture was fertilized with 0.5X Hoagland's solution twice a week and watered daily with tap water for 10 weeks and later air-dried for 2 weeks in a greenhouse to complete the trap culture.

2.4. Identification of Cultured AMF Spores

The *Acaulospora* spores were permanently mounted in polyvinyl alcohol-lactic acid-glycerol (PVLG) solution with and without Melzer's reagent and visualized under a microscope. Ten spores were crushed in 5 μ L of 1xPhusion HF buffer by a pipette tip and heated at 95 °C for 5 min. The supernatant was used for the nested PCR and transformation as previously described. Ten SSU-ITS-LSU sequences from the spores were individually annotated in the NCBI database. The ten sequences from the spores and the representative *Acaulospora* OTUs (OTU11–21) were used to calculate sequence similarity with the seqinr package [28] in R to confirm the presence of cultured *Acaulospora* fungi in the roots grown

in the indigenous rice paddy soil. The percent similarity was demonstrated in a heatmap by the pheatmap package [29] in R. Sequences of *Acaulospora* spores from this study were made available in the GenBank under accession numbers OL661631 to OL661640.

2.5. Experimental Design for Determination the Role of *Acaulospora* fungi in Rice Seedling

Seeds of *Oryza sativa* subsp. *japonica* cv. Nipponbare were kindly provided by Prof. Dr. Supachitra Chadchawan, Chulalongkorn University, Thailand. Rice seedlings were grown in 1 L pots containing the sterile compost and sand mix, recolonized with the microbial wash described above. Each pot contained five plants. Soil was added with and without the *Acaulospora* AMF inoculum containing 1000 spores ($n = 5$ biological replicates), and five pots containing the non-inoculated soil without rice seedlings were prepared as a blank treatment to analyze the soil P status at the end of the experiment. All of the treatments were fertilized twice a week with 200 mL of $0.5 \times$ Hoagland's solution and watered daily with 200 mL of distilled water for 6 weeks.

2.6. Analysis of Soil Properties

Soil from pots in the blank treatment ($n = 5$ biological replicates) was collected to analyse soil properties. Soil organic matter was determined by the titration method [30]. Total N was determined by the combustion method using a C/N analyzer CN628 (LECO, Thailand). Total P and available P were determined by the Molybdovanadophosphate method using a spectrophotometer (Prove 300, Merck KGaA, Darmstadt, Germany) [31,32]. Total K was determined by the flame photometric method using inductively coupled plasma optical emission spectrometry (ICP-OES) (Avio 500, Perkin Elmer, Waltham, MA, USA) [33]. Soil pH and electrical conductivity were measured with a conductivity meter (Orion Star A112, Thermo Fisher Scientific, Waltham, MA, USA).

2.7. Determination of Plant Growth Performance

Photosynthetic parameters such as (Phi2), (PhiNPQ), (PhiNO) and relative chlorophyll content were measured in the first mature leaf of three rice seedlings in the same pot by the Photosynthesis RIDER protocol using the MultispeQ fluorometer (PhotosynQ, East Lansing, MI, USA) [34]. The three rice seedlings were dried at 70 °C for 3 days before measuring shoot and root dry weights.

2.8. Determination of Pi Content in Rice Seedlings

Soluble Pi in plant tissues was determined by a molybdenum blue-based quantitative assay [35]. Briefly, 10 mg of the first mature leaf and 20 mg of root from a rice seedling in each pot were ground in 600 μ L of 3% (v/v) perchloric acid. After centrifugation at 11,000 rpm for 5 min, the supernatant was collected and then mixed with 400 μ L of the assay reagent containing 1% (w/v) $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4 \text{H}_2\text{O}$ and 5% (w/v) $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ in 1 N H_2SO_4 . After shaking followed by incubation for 10 min, absorption at 720 nm was measured using a microplate reader (BIOTEX, PowerwaveX). The Pi content in each sample was calculated using a KH_2PO_4 (5–250 nmol/mL range) standard.

2.9. Determination of AMF Colonization

Root cuttings measuring 1.5 cm were cleaned in 1 mL of 10% (w/v) KOH at 95 °C for 15 min. The roots were then rinsed with water and incubated in 1 mL of 1% (v/v) HCl at room temperature for 10 min. After removal of the HCl solution, the samples were incubated overnight in 1 mL of Trypan blue staining solution containing 0.05% (w/v) Trypan blue, 33% (v/v) lactic acid and 33% (v/v) glycerol. The root samples were destained twice overnight in 1 mL of 50% (v/v) glycerol. Thirty root pieces were neatly placed on microscopic slides and mounted with 50% (v/v) glycerol. Five categories of mycorrhizal structures were quantified from 200 observations of roots under a light microscope at $100 \times$ magnification. The categories included (1) non-colonization, (2) hyphae (H), (3) hyphae and vesicles (H+V), (4) hyphae and arbuscules (H+A), (5) hyphae, vesicles and

arbuscules (H+V+A) [36]. The percentage of colonization and each mycorrhizal structure were calculated.

2.10. Determination of Soil Acid Phosphatase

One gram of soil was collected from the middle of the pot. The fresh soil was suspended in 4 mL of 5% (*v/v*) toluene and 0.5 mL of the soil supernatant was mixed with 0.4 mL of 200 mM acetate buffer at pH 5.2 and 0.1 mL of 150 mM PNPP disodium hexahydrate (TCI, Tokyo, Japan). The mixture was incubated at 37 °C for 1 h before the reaction was stopped by adding 0.5 mL of 0.5 M NaOH. Absorption at 410 nm was measured using a microplate reader (BIOTEX, PowerwaveX). The activity of acid phosphatase (nmol PNP/g soil FW/hour) was calculated using a p-nitrophenol (PNP) (200–2000 nmol/mL) standard.

2.11. Data Analysis

The data were visualized as box plots by the ggplot2 package [37] in R. Significant differences among the means of the treatments were analyzed by Student's *t*-test and One-way ANOVA following LSD by the agricolae package [38] in R.

3. Results

3.1. *Acaulospora* Is the Dominant AMF in Lowland Rice Roots under Non-Flooded and Flooded Conditions

Our previous study demonstrated that flooding reduced colonization and abundance of AMF in roots of rice seedlings grown in organic rice paddy soil [5]. To identify endophytic AMF in the rice roots, the rDNA sequences of rice roots grown in non-flooded and flooded conditions were amplified by Kruger primers and clustered into different OTUs with 97% sequence similarity. To determine whether the numbers of clones sufficiently represented AMF diversity in the rice roots, we constructed rarefaction curves and their extrapolations (Figure 1A). In the two different gravimetric regimes, rarefaction curves for OTUs reached a plateau. The data suggest that the number of sequences provided full coverage of the AMF diversity. The total OTU numbers (richness) of the endophytic AMF rDNA were 21 OTUs, 13 OTUs were detected in the non-flooded condition, 12 OTUs in the flooded condition. The Shannon–Wiener diversity index (H') and AMF rDNA sequence evenness were higher in the non-flooded condition than in the flooded condition (Figure 1B). This result indicates that flooding reduces the diversity of AMF in rice roots. The representative OTU sequences were blasted in the NCBI database and these sequences were used for the subsequent phylogenetic analyses. The result revealed the presence of four Glomeromycotean genera in the rice roots, including *Acaulospora*, *Dentiscutata*, *Glomus* and *Paraglomus*. In rice roots grown in the non-flooded condition, the major AMF were *Acaulospora* and *Dentiscutata*, while in the flooded condition, *Acaulospora* was the most dominant genus. Moreover, *Acaulospora* OTU13–14 were relatively more abundant and were found in both the non-flooded and flooded conditions. Thus, they were considered the core AMF in the lowland rice paddy condition.

3.2. *Acaulospora* Spores Are Propagated by Trap Culture

We propagated the indigenous AMF spores in a greenhouse using a trap culture with a maize host (Figure 2A). *Acaulospora* spores were isolated and used for a monospore culture (Figure 2B). The cultured *Acaulospora* spores mounted in PVLG solution with and without Melzer's reagent were visualized under a microscope. The germinal wall of spores was stained red–purple by Melzer's reagent (Figure 2C,D). Finally, to confirm whether the cultured *Acaulospora* spores were the core AMF present in the rice roots, we analyzed the sequence similarity of the SSU-ITS-LSU sequences from spores with the representative *Acaulospora* OTUs. The result showed eight of the ten spore sequences shared sequence similarity at 98.4–99.4% with OTU13–14, which were the dominant AMF OTUs in the rice roots. The other two spore sequences showed the highest similarity, with OTU14 at 95% (Figure 2E). The *Acaulospora* spore sequences were annotated with the NCBI database. They

shared the highest identities with *A. delicata* in the range of 93.50–94.29%, *A. mellea* in the range of 90.45–93.58% and *A. rugosa* in the range of 93.58–94.05.

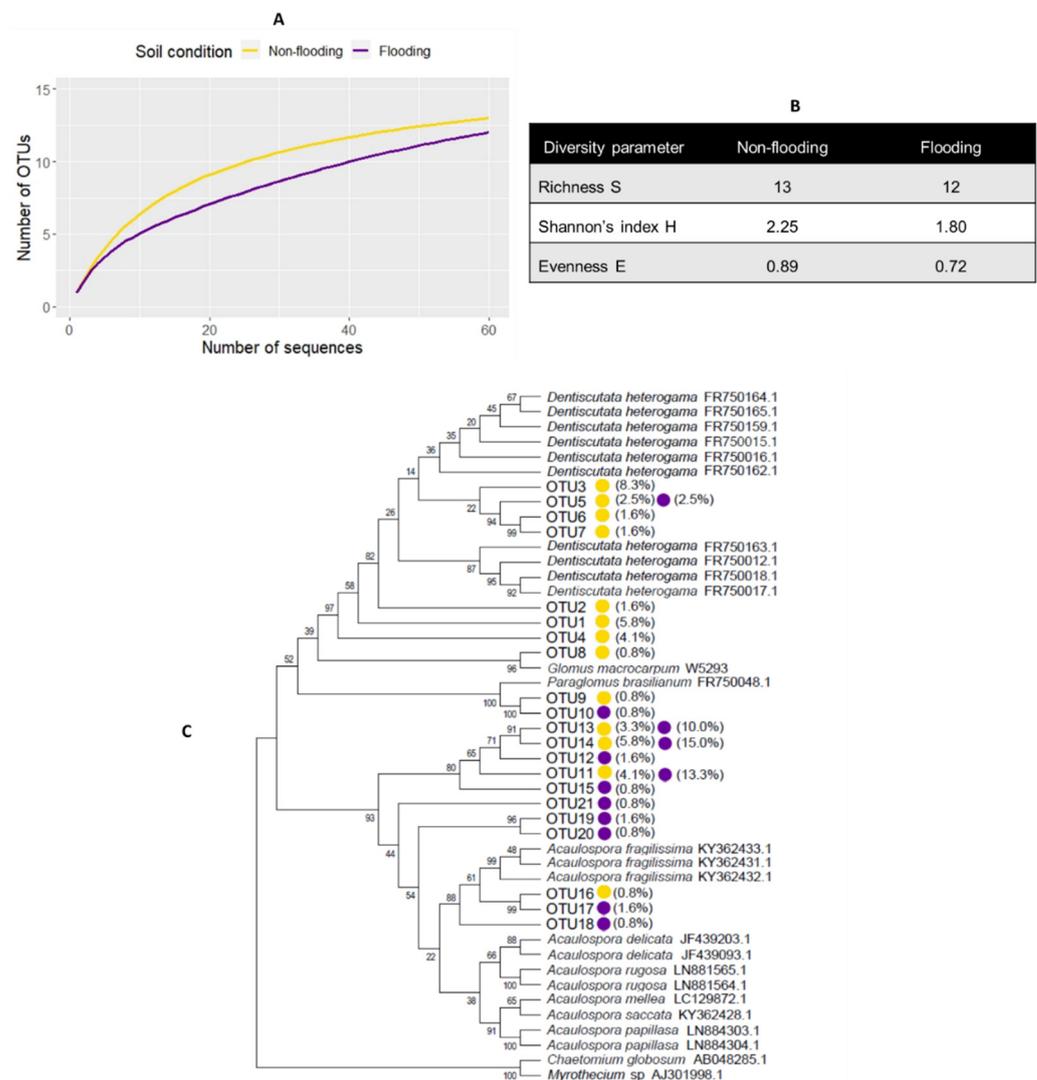


Figure 1. AMF OTUs detected in rice roots grown in non-flooded and flooded conditions. Rarefaction curves were analyzed from the SSU-ITS-LSU sequences of Glomeromycota amplified from rice roots grown in both conditions (A). Diversity indices between the two different conditions were summarized in Table (B). Phylotaxonomic analyses of representative AMF OTUs and their closest sequence similarity from the Maarjam and NCBI databases were constructed from the SSU-ITS-LSU sequences of mycorrhizal rice roots grown under non-flooded (●) and flooded (●) conditions (1000 bootstrap). GenBank accessions are placed behind AMF species reported in the databases. Relative abundances of AMF OTUs are demonstrated in percent with parentheses (C).

3.3. The *Acaulospora* Fungi Suppress Rice Seedling Growth but Not Photosynthetic Efficiency

To characterize the biological function of the cultured *Acaulospora* fungi in rice, we grew Nipponbare rice seedlings in sterile soils with and without the *Acaulospora* inoculum for six weeks in the greenhouse. The soil used in this experiment contained 347.96 ppm total nitrogen, 72.52 ppm total P, 37.38 ppm available P, 479.34 ppm total K and 28.48 g/kg organic matter. The soil pH and electrical conductivity were 7.08 and 0.24 ds/m, respectively. Therefore, we considered this a fertile and P-sufficient soil.

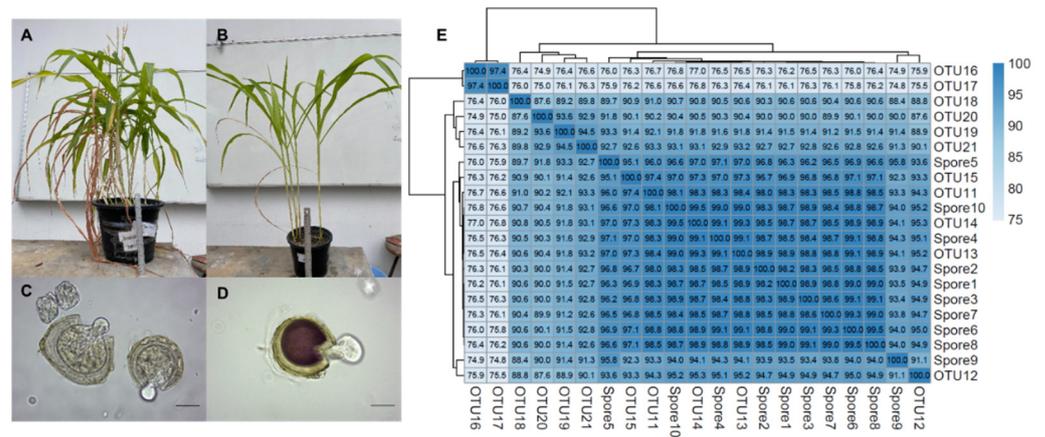


Figure 2. Trap culture of indigenous AMF spores. The trap culture using maize plants was prepared from organic rice paddy soil and sterile sand at a ratio of 1:3 (A). Later, *Acaulospora* spores were isolated based on their spore morphology for the monospore culture (B). The *Acaulospora* spores were mounted in polyvinyl alcohol–lactic acid–glycerol solution (C) and Melzer’s reagent (D), visualized under a bright field microscope. Scale bars represent 30 μm . The heatmap displays percent similarity of the SSU-ITS-LSU sequences from the *Acaulospora* spores and the representative *Acaulospora* OTUs from the rice roots (dark blue represents most similar and light blue less similar) (E).

The AMF-inoculated roots were examined by Trypan blue staining and visualized under a microscope. Mycorrhizal structures such as hyphae, vesicle and arbuscule were detected in the roots of inoculated plants (Figure 3A,B). The AMF colonization and mycorrhization of rice roots were about 70% and 40%, respectively (Figure 3C). Our result showed that AMF inoculation reduced plant height (Figure 4A) and plant biomass (Figure 4B). It did not change shoot P concentration but significantly increased root P concentration (Figure 4C). However, AMF inoculation did not change the proportion of incoming light energy for photosynthesis (Figure 4C) and the relative chlorophyll content (Figure 4D). These results suggest that AMF inoculation suppresses the growth of rice seedlings but does not alter photosynthetic activities.

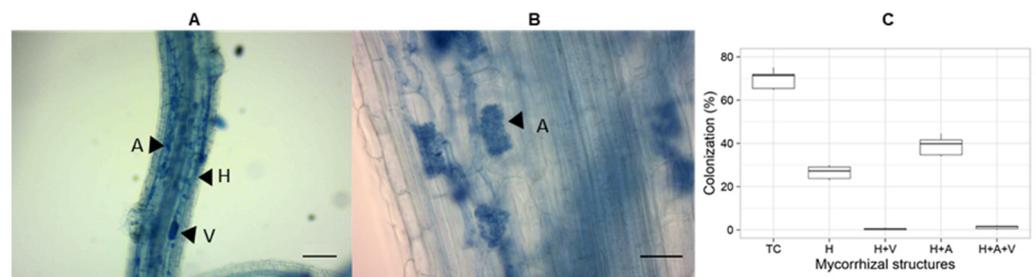


Figure 3. Photograph (A) shows the degree of colonization by *Acaulospora* fungi in AMF-inoculated roots of rice seedlings. Mycorrhizal roots containing various AMF structures were visualized under a bright field microscope. H, V, and A represent hypha, vesicle and arbuscule, respectively. The scale bar represents 100 μm . Photograph (B) shows mature arbuscules of *Acaulospora* fungi in the roots. The scale bar represents 30 μm . Degrees of AMF colonization were categorized into the following five groups: 1. Non-colonization, 2. Hyphae (H), 3. Hyphae and vesicles (H+V), 4. Hyphae and arbuscules (H+A), and 5. Hyphae, vesicles and arbuscules (H+V+A). The box plots (C) show the distribution of mycorrhizal structure (n = 5 biological replicates).

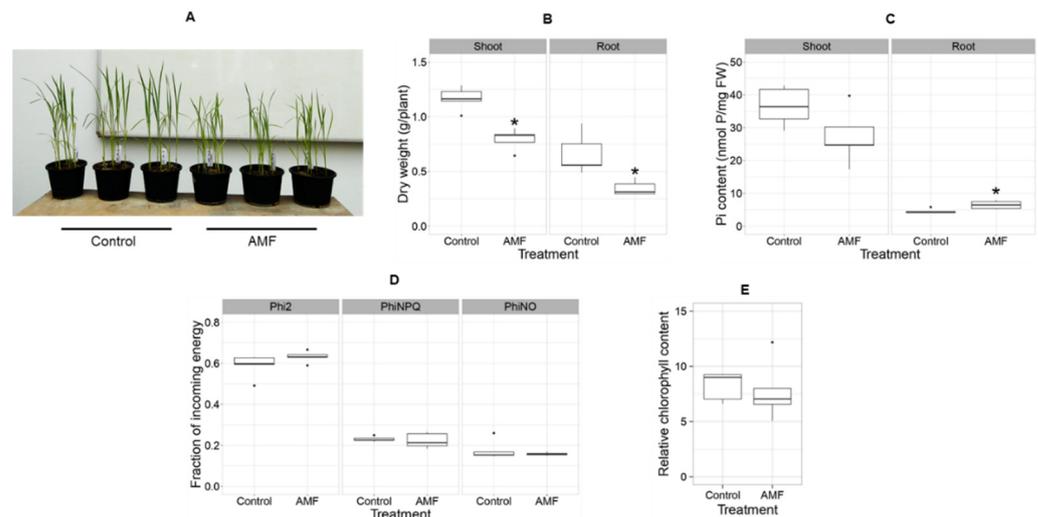


Figure 4. Effect of AMF inoculation on rice growth performance. Rice seedlings were grown for 6 weeks in sterile soil recolonized with a microbial wash (control) and with the *Acaulospora* inoculum (AMF) (A). The box plots show the distribution of shoot and root dry weight (B), shoot and root Pi content (C), fraction of incoming energy consisting of Phi2, PhiNPQ and PhiNO (D), and relative chlorophyll content (E) ($n = 5$ biological replicates). Statistical analysis was performed by Student's *t*-test. Asterisks indicate significant differences between the control plants and the AMF-treated plants ($p < 0.05$).

Inoculation with *Acaulospora* fungi improved soil P availability by enhancing soil acid phosphatase activities.

To determine the effect of *Acaulospora* AMF inoculation on soil P status, we measured total P content, available P and acid phosphatase activity in the non-inoculated and inoculated soils, and compared the data with the data from a blank treatment at the end of the experiment. The blank pots without rice seedlings contained the sterile soil recolonized with the microbial filtrate. The results showed that total P contents between the non-inoculated and inoculated soils were not different but lower than the total P content of the blank soil (Figure 5A). This suggested that some P in the soils was consumed by the rice seedlings. However, the AMF inoculation significantly increased the available P content (Figure 5B) and acid phosphatase activity (Figure 5C) in the soil. Thus, the presence of *Acaulospora* fungi in the soil improves soil P availability.

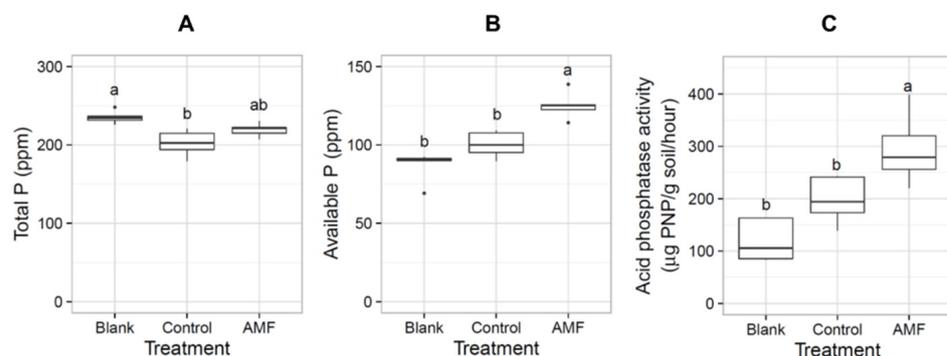


Figure 5. Effect of AMF inoculation on soil P availability. The box plots show the distribution of total P (A), available P (B) and acid phosphatase activity (C) in non-inoculated soil without rice seedlings (blank), non-inoculated soil (control) and AMF-inoculated soil (AMF) ($n = 5$ biological replicates). Statistical analysis was performed by One-way ANOVA following LSD. Different letters indicate significant differences among the treatments ($p < 0.05$).

4. Discussion

Based on the AMF sequences present in the roots of rice plants grown in non-flooded and flooded soil, flooding reduced the diversity of AMF in the roots (Figure 1B). The reduction of AMF diversity was related to the absence of *Dentiscutata* (Figure 1C). *Dentiscutata* OTU 1–7 shared the highest sequence similarity with *Dentiscutata heterogama*. Their sequence abundance was depleted in the flooded condition while *Acaulospora* OTU 11–24 were enriched. In a depressional wetland, *Dentiscutata* and *Acaulospora* are found in different hydrologic gradients. *Dentiscutata heterogama* is dominant in a predominantly dry zone while *Acaulospora* fungi are more often observed in an intermittent wet zone. Moreover, both of them rarely appear in a predominantly wet zone [39]. Furthermore, in coastal wetlands, their presence is dependent on the season. *Dentiscutata heterogama* is restricted in dry seasons while *Acaulospora* fungi are found in dry and rainy seasons [40]. *Acaulospora* species are thought to be relatively tolerant to flooding since they are reported as the dominant AMF and fungal genus in high-moisture soils [41,42]. These findings support the spatial existence of *Dentiscutata heterogama* and *Acaulospora* fungi in rice roots in non-flooded and flooded organic rice paddy soil.

Acaulospora fungi are the core AMF in the roots of rice grown in organic lowland rice paddies. While the colonization of AMF in roots was significantly reduced in flooded soil [5], *Acaulospora* fungi were found to be relatively more abundant than other indigenous endophytic AMF in rice roots (Figure 1C), and a higher abundance of *Acaulospora* fungi has been reported in some lowland rice paddies [18,20]. Although, the density of total AMF spores is relatively lower in lowland rice paddies, compared to midland and upland rice paddies, *Acaulospora* spores are the most abundant in lowland ecosystems. Up to 80% of AMF spores found in lowland rice paddy soils were from *Acaulospora* [20]. Therefore, in the present study, it is highly likely that the *Acaulospora* spores found in the organic lowland rice paddy soil were propagated by the trap cultures (Figure 2A,B).

Almost all of the cultured *Acaulospora* spore sequences shared sequence similarity at 98.4–99.4% with the dominant AMF OTU13–14 in the rice roots, and the other two spore sequences showed the highest similarity with the OTU14 at 95%. The cultured *Acaulospora* spores could be classified into two species based on the standard 97% sequence similarity for clustering of microbial rDNA sequences [43]. However, classification of AMF species using the rDNA gene sequence similarity threshold of 97% may overestimate the actual number of AMF species, especially when the sequences contain the ITS regions [44,45]. For example, the intra-genomic similarity in the ITS2 region from the ten rDNA paralogs of the *Rhizophagus irregularis*, a model AMF species, is 90.28% [45]. Thus, further identification of the cultured *Acaulospora* spores by AMF spore morphology will be performed.

To characterize the symbiotic functions of the cultured *Acaulospora* fungi, Nipponbare rice seedlings were inoculated with the *Acaulospora* inoculum. At 6 weeks post-inoculation, the AMF had colonized 70% of the rice roots and formed mature arbuscules (Figure 3C). This indicates the successful colonization of rice roots by AMF [46]. Compared with the non-inoculated plants, AMF inoculation suppressed the growth of rice seedlings (Figure 4A,B) and did not promote accumulation of P in shoots (Figure 4C). We speculate that the growth suppression due to AMF inoculation found in this study might be a result of the soil P condition. The available P content of the soil was 37.38 ppm, which is considered a high P level for rice seedlings [47]. This available P concentration is generally found in lowland rice paddies [48,49]. In a high P condition, AMF symbiosis in rice roots was inhibited [2]. However, in our previous study, the addition of P in the soil did not affect the colonization of SMP rice roots by indigenous AMF [5]. In this study, the dominance of AMF from *Acaulospora* in the SMP rice roots may be due to a tolerance of high P soil. Some AMF species, including *A. laevis*, which can aggressively colonize roots in P-deficient soils are also capable of intensively colonizing roots in P-sufficient soils, but they reduce the growth of host plants and do not enhance plant P uptake [50]. In addition, P fertilization promotes the parasitic relationship between AMF and host plants, which lowers the biomass of the mycorrhizal plants [51]. However, in our study, the parasitism between the mycorrhizal Nipponbare rice

seedlings and the *Acaulospora* AMF did not reduce photosynthetic efficiency in the leaves (Figure 4D,E). This behavior is unlike behaviors observed in certain other plants infected by fungal root parasites such as *Fusarium oxysporum* and *Setophoma terrestris* [52,53]. Growth suppression of rice seedlings by *Acaulospora* AMF might be a response to an imbalance between the cost and benefit experienced during the AMF–plant interaction.

To clarify the effect of AMF inoculation on soil P availability, a blank treatment was included in the experiment using sterile soil without rice seedlings or AMF inoculum. Compared with the control and AMF treatments, the blank soils contained the highest level of total P but the lowest level of available P (Figure 5A,B). AMF inoculation significantly increased the amount of available P and the acid phosphatase activity in the soil (Figure 5B,C). The reduction of total P in the soils was due to P uptake by the rice seedlings. Rice seedlings increased the amount of available P in non-inoculated and inoculated soil since rice roots exude acid phosphatase, which increases soil P solubility [54]. Moreover, the genes involved in acid phosphatase secretion in the host plants are up-regulated by AMF symbiosis, which increases the release of acid phosphatase from mycorrhizal roots. AMF are also able to directly release acid phosphatases from their hyphae into the soil [55]. These findings strongly indicate that *Acaulospora* AMF improve soil P availability by increasing acid phosphatase activity in the soil.

5. Conclusions

Acaulospora fungi were the core arbuscular mycorrhizal fungi in lowland rice roots grown in organic rice paddy soil. They improved P mobilization by enhancing the release of acid phosphatase in the soil. In rainfed lowland rice cultivation, especially by direct seeding of rice, rice seeds are sown in non-flooded fields, allowing the seedlings to transiently form a symbiosis with indigenous arbuscular mycorrhizal fungi. After the rice seedlings are flooded, arbuscular mycorrhizal fungal colonization and symbiosis are arrested. Thus, the usual suppression of growth by *Acaulospora* fungi in the P-sufficient condition is prevented at the beginning of rice growth and development. However, the existence of *Acaulospora* fungi could accelerate the P mineralization of rice paddies, leading to more available P in the soil, which is essential for the growth of lowland rice.

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