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Improvement of growth and phosphorus utilization efficiency in Thai rice (*Oryza sativa* L. ssp *indica* cv. Chaew Khing) by inoculation of arbuscular mycorrhizal fungi under high phosphorus supply

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ABSTRACT: Arbuscular mycorrhizal fungi (AMF) provide benefits to host plants mainly by improving nutrition. In rice, as in other plants, AMF colonization is promoted under phosphorus (P) deficiency, and the symbiosis can lead to an improvement in P uptake and plant biomass. While there is economic potential for the fungi to substitute chemical fertilizers for sustainable rice production, the current dogma holds that their benefits are nullified when used in fertile soil. Here we show that inoculation of an AMF mixture in non-sterilized lowland paddy soil with high P availability increased the degree of fungal colonization and the development of arbuscular mycorrhizal structures in 'Chaew Khing' rice seedlings. The increase in AMF colonization was correlated with higher shoot growth in the AMF-inoculated plants. Analysis of plant nutrient status indicated that the AMF treatment caused a reduction in the total P and Fe concentrations in the shoot tissues and a reduction in the total N concentration in the root tissues of the mycorrhizal plants. Nevertheless, the enhanced mycorrhizal colonization led to an increase in P utilization efficiency as well as N:P and C:P ratios in the AMF-inoculated seedlings. These results indicate that, under high P supply, AMF can benefit the host plant by enhancing more efficient use of P, rather than improving its uptake. This work highlights the potential benefit of AMF inoculation for rice cultivation under high P availability.

KEYWORDS: arbuscular mycorrhizal fungi, rice, phosphorus utilization efficiency

INTRODUCTION

Rice is the main diet for nearly half of the world population, and its production must increase to meet the rising global consumption amid extreme climate and water scarcity. While intensive, high-input agriculture has contributed substantially to the increase in rice yields over the past decades, the over-use of pesticides and synthetic chemical fertilizers of nitrogen (N) and phosphorus (P) also deteriorates the global agroecosystem [1]. To meet increased demands for rice and other grain cereals while conserving natural resources, policies and measures have been implemented by different organizations and governments to steer agricultural practices towards sustainable intensification [2]. Increasing nutrient use efficiency and improving soil health are principal components of this endeavor.

Thailand is one of the top rice exporters in the world. Apart from the world-renowned jasmine rice 'Khao Dawk Mali 105' (KDML105) and its derivatives, there are several other aromatic rice cultivars with distinguished cooking quality grown by farmers in different regions within the country. One of these is 'Chaew Khing' rice, a landrace found in the region bordering Songkhla and Pattani provinces in southern Thailand. This cultivar is well-known for its superior flavor and nutritional values, including a higher fiber, iron and cobalamin content as compared to other varieties. The production of 'Chaew Khing' rice is currently operated by a local community enterprise, and thanks to its organic and environment-friendly farming practice, the variety has gained popularity in niche markets, pricing at about two times higher than typical jasmine rice. The cultivation area for 'Chaew Khing' rice has expanded as consumer demand increases, but its light-sensitive and long-duration nature limits the production to only once a year. Furthermore, relying on rainfed and traditional low-input organic farming, poor growth and low grain yield have been a major challenge for its production.

Plant interactions with arbuscular mycorrhizal fungi (AMF) have long attracted interests from researchers and farmers for their practical use in agriculture, as the fungi offer a promising means to improve efficient use of mineral resources and to increase crop productivity with less dependence on chemical fertilizers [3]. This is especially relevant in organic farming, providing that AMF can serve as biofertilizers [4], while their extensive external hyphal network also makes a significant contribution to the improvement of soil structure [5]. In rice, AMF symbiosis has been shown to enhance foliar Pi accumulation and to increase seedling growth, grain yield, and plant tolerance to biotic and abiotic stresses [6–8].

The major and most well-known advantage of AMF symbiosis is an improvement of P efficiency in the host plants. P efficiency consists of P acquisition efficiency (PAE) by root P uptake and internal P utilization efficiency (PUE) [9]. P uptake by AM plants involves two pathways: the direct uptake pathway and the AM uptake pathway. The direct uptake pathway occurs when Pi is directly taken up from the rhizosphere by Pi transporters localized in the root epidermal cells. The AM uptake pathway depends on AMF-specific Pi transporters in arbuscule-containing cells of the root inner cortex [10]. The two pathways coordinate to achieve maximum rates of P uptake. In rice, the mycorrhizal pathway can contribute up to 70% of P acquired by the plant through the AMF-specific Pi transporter OsPT11 [11]. Beside P uptake, AMF also increase soil P availability by preventing P loss and by releasing acid phosphatases to utilize organic P in the paddies [12, 13].

AMF symbiosis also increases the accumulation of other macro- and micro-nutrients, such as N, potassium (K), sulfur (S), iron (Fe), calcium (Ca), magnesium (Mg) and zinc (Zn) [14]. In rice, mycorrhizal plants receive at least 40% of N through the AM uptake pathway [15]. AMF can use both inorganic forms of N as nitrate and ammonium and organic forms of N as amino acids. Root infection by AMF induces the expression of the AMF-specific ammonium transporter gene *OsAMT3;1* while the gene mutation causes a reduction in N and P uptake from the AM uptake pathway [16]. AMF colonization also specifically activates the nitrate transporter gene *OsNPF4.5* in the arbuscule-containing cells, leading to a higher N uptake in the host plant [15]. Less is known about how AMF facilitate the acquisition and uptake of other nutrients, but it is believed that they may manipulate the ionome of the host plants to balance the nutrient demand and supply.

Application of AMF for improving growth and yield has been widely explored in crop plants, but their role in sustaining rice production is less studied due to a misconception that AMF symbiosis is limited under lowland rice agroecosystem [17]. In rice, as in other crops, high supply of P in soil generally diminishes AMF colonization [18]. Nevertheless, there are instances where plant growth is enhanced by AMF even though soil P availability is high [19]. As is the case with plant microbiomes, successful utilization of AMF for rice production depends on plant genotype-by-environment-by-AMF-by-management interactions [20]. A positive growth and yield response may occur when a single AMF species or AMF mixture is used for a specific rice variety under a particular agroecosystem and farming practice, but this could result in a neutral or negative outcome when one factor is altered. A study on 12 *japonica* rice varieties showed that the AM fungus *Rhizophagus irregularis* has higher root colonization rates than *Funneliformis mosseae*, but both of which cause significant varietal differences in growth response of the host plant, from negative to neutral to positive growth. Pre-inoculation of *R. irregularis* before transplantation of seedlings into flooded fields enhances grain yields of the Loto and Gines rice varieties [6]. Similarly, pre-colonization of *F. mosseae* before transplantation into upland fields increases the percentage of ripened grain and 1000-grain weight in the *indica* rice variety ARC5955 but not in the *japonica* rice varieties Nipponbare and Koshihikari [21]. Another field study with the lowland rice varieties Magitolngar and Tox-728-1 showed that inoculation of endogenous AMF increases plant height, tillering and grain yields of both rice varieties [22]. These studies illustrate that, if properly managed, AMF can be utilized to maintain sustainability and profitability in rice production, although the knowledge about rice-AMF interaction under different cultivation methods is yet to be gained. It is therefore of value to identify the right combination of rice and AMF genotypes for their use in rice production.

In this study, we tested whether AMF can be used as biofertilizers to improve P efficiency and enhance growth of 'Chaew Khing' rice. Non-sterilized natural paddy soil with relatively high P content was inoculated with an AMF mixture, then growth parameters and accumulated nutrients were determined in the seedlings. In contrast to previous studies showing that high P supply in soil inhibits AMF colonization [13, 18], our data demonstrate that AMF inoculation increases root colonization in 'Chaew Khing' rice seedlings. The increase in mycorrhizal colonization is accompanied by shoot growth promotion in the mycorrhizal plants. While mycorrhizal P uptake is compromised, AMF symbiosis helps improve PUE under high P supply.

MATERIALS AND METHODS

Soil property analysis

Soil was collected 0–30 cm below the surface from 3 locations in a local field, then pooled and mixed thoroughly before using for the study. Soil organic matter was determined by the titration method [23]. Total N was determined by the combustion method using a C/N analyzer CN628 (LECO, Thailand) [24]. Total P, citric acid insoluble P, and available P were quantified by the Molybdovanadophosphate method using Spectrophotometer Prove 300 (Merck KGaA, Germany) [25]. Total K was determined by the flame photometric method using ICP-OES Avio 500 (Perkin Elmer, USA). Soil pH and electroconductivity (EC) were measured by a conductivity meter Orion Star

A112 (Thermo Fisher Scientific, USA).

Experimental design and measurement of growth parameters

'Chaew Khing' is an *indica* rice cultivar indigenous to Songkhla province in the south of Thailand. The seeds were obtained from a local farmer in Songkhla. Seeds pre-soaked in distilled water for 24 h were sown in a 1-l pot (10 seeds per pot) containing non-sterilized soil with or without 50-g AMF inoculum mixture (SV Group, Thailand). The 50-g inoculum contains approximately 1500 AMF spores, composed of *Glomus geosporum*, *G. etunicatum*, *G. mosseae* and *Acaulospora foveata* with an approximate ratio of 3:3:1:3, where the number of AMF spores for each species were 1200, 1106, 320 and 920, respectively. Seedlings were thinned to 5 plants per pot at 7 days after sowing. The experiment was performed in five biological replicates ($n = 5$). The seedlings were watered daily with tap water for 5 weeks, without additional fertilization. The experiment was performed in a greenhouse during May to June 2021. The temperature and relative humidity, recorded by a data logger (HOBO® Pro v2, USA), ranged from 25–35 °C and 50–90%, respectively. In each replicate, plant growth parameters, including shoot length, root length, shoot biomass and root biomass, were measured from three individual plants five weeks after sowing.

Determination of AMF colonization

Individual plants were used for determining AMF colonization as previously described [26]. Briefly, root samples (1.5-cm each), collected from the middle region of the whole root lengths toward the tip, were cleared in 10% (w/v) KOH and boiled at 95 °C for 15 min. The root samples were rinsed with deionized water, and then incubated in 1% (v/v) HCl at room temperature for 10 min. The HCl solution was replaced with 1 ml of the Trypan blue staining solution [0.05% (w/v) Trypan blue, 33% (v/v) lactic acid, 33% (v/v) glycerol]. The samples were vacuumed for 3 min using a concentrator plus (Eppendorf), and subsequently de-stained overnight in 1 ml of 50% (v/v) glycerol. After destaining, 15 root samples were placed on a microscopic slide and mounted with 50% (v/v) glycerol. The slide was sealed using transparent nail polish. Mycorrhizal structures were quantified under a light microscope (100 views per slide) using a 10 × magnification objective lens. AMF colonization was classified into five categories: non-colonization; hyphae; hyphae and vesicles; hyphae and arbuscules; and hyphae, vesicles and arbuscules.

Determination of nutrient uptake

Dry tissues from three plants were pooled into one sample. Dry weight was measured using a 4-digit scale. For each sample, approximately 30 mg of ground tissues were incubated in 2 ml of 65% (w/v) HNO_3 at 95 °C for 1 h before adding 1 ml of 30% (v/v) H_2O_2 to complete the digestion. The solution was filtered through Whatman paper No. 1. The volume was then adjusted to 10 ml by distilled water. The total P and Fe concentration (% w/w) was measured by ICP-OES (AVO 500, Perkin Elmer). Thirty milligrams of ground tissues were processed by combustion to determine the total N and C concentrations (% w/w) using a C/N analyzer CN628 (LECO, Thailand). The N:P ratio, C:P ratio and P use efficiency (PUE), which indicates the biomass produced per unit of accumulated P (kg DW/g P) [27], were calculated.

Statistical analyses

All the statistical analyses and data visualization were performed in R (version 3.6.3). Means of AMF colonization, shoot and root lengths, shoot and root biomass, nutrient concentrations and the N:P ratio, C:P ratio and PUE between the AMF-inoculated and non-inoculated (control) samples were compared with Student's t-test using the agricolae package [28]. Data were visualized using the ggplot2 package [29].

RESULTS

AMF inoculation in P-sufficient soil enhances AMF colonization and mycorrhization in rice roots

The soil used in this study was analyzed for the total and available P content as well as other physical properties. On average the soil contained 72 ppm of total P, approximately 64% (46 ppm) of which was in an available form (Table 1). With reference to the standard soil P status for crop production [30], this is considered high P as the available P is greater than 40 ppm. Also, the soil contained over 1000 ppm of total N, 496.7 ppm of total K, and 2.32% of organic matter. Overall, this was considered nutrient-rich and fertile soil [31]. Although high P availability in soil is known to inhibit the establishment of AMF symbiosis [18], we asked whether inoculation of an AMF cocktail could still improve AMF colonization and growth of 'Chaew Khing' rice seedlings despite high P supply. To this end, rice seeds were sown in non-sterilized soil with or without an AMF inoculum. AMF colonization and mycorrhization and plant growth parameters under the greenhouse settings were evaluated after five weeks post germination.

The degrees of colonization and mycorrhization in rice roots were examined under a light microscope. AMF colonization was classified into five categories: no colonization (Fig. 1A), hyphae (Fig. 1B), hyphae and vesicles (Fig. 1C), hyphae and arbuscules (Fig. 1D), and hyphae, vesicles and arbuscules (Fig. 1E). Notably, fully developed arbuscules were also detected in the roots (Fig. 1F). Since the soil was used without prior sterilization, AMF colonization and mycorrhization were detected in both the AMF-inoculated and

Table 1 The properties of soil used in this study.

Organic matter (% w/w)	Total N (ppm)	Total P (ppm)	Citrate insoluble P (ppm)	Available P (ppm)	Total K (ppm)	pH	EC (dS/m)
2.32 ± 0.09	1093.5 ± 122.0	72.1 ± 7.5	25.8 ± 3.0	46.4 ± 6.9	496.7 ± 50.2	6.37 ± 0.13	0.11 ± 0.06

Data represent the mean and standard deviation (SD) of three replicates.

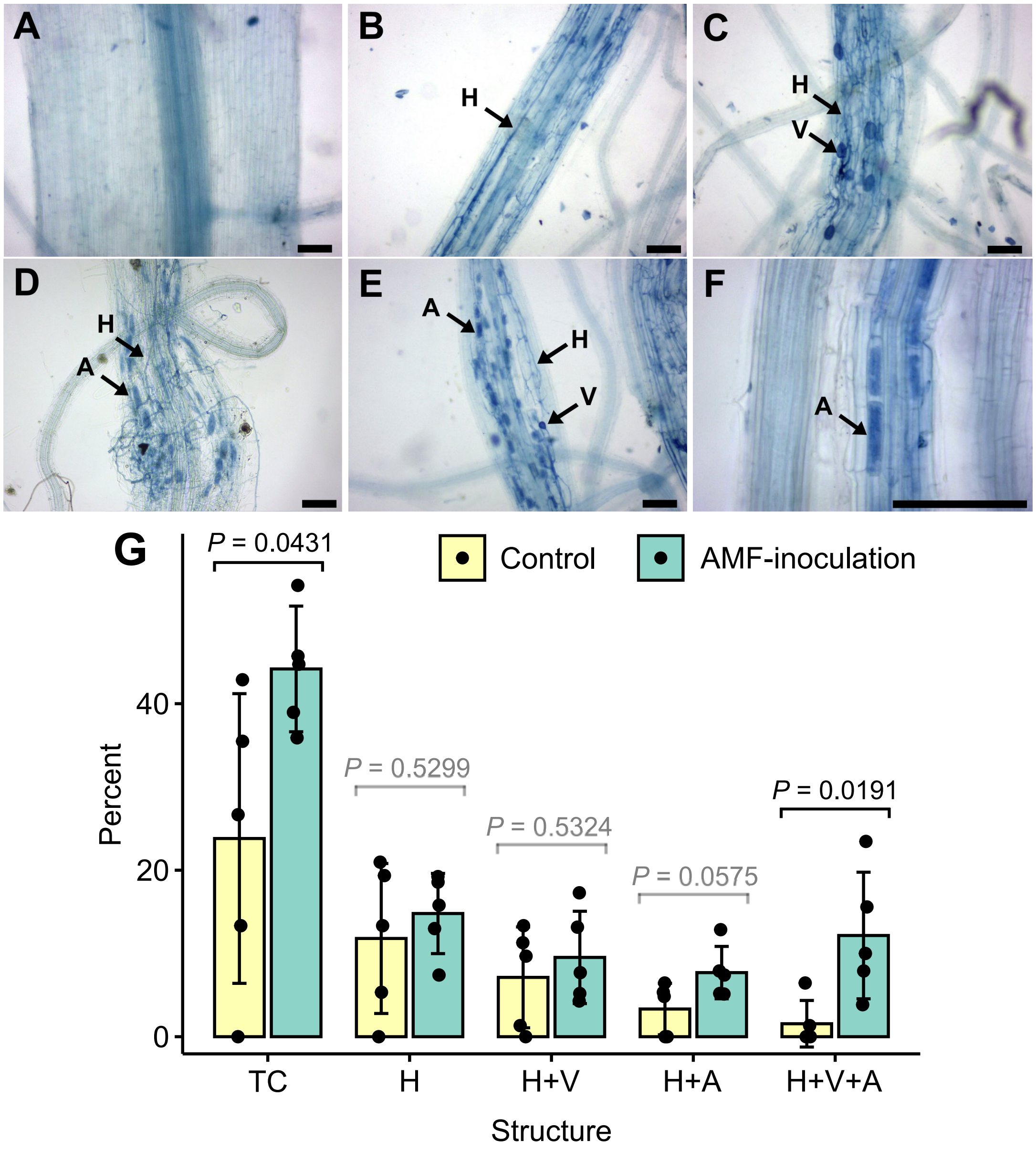

Fig. 1 The degree of AMF colonization in rice roots is more intensified in the AMF-inoculated seedlings than in the non-inoculated control plants. Mycorrhizal structures and colonization rate were compared in the roots of 5-week-old seedlings grown in the soil with or without AMF inoculation. The roots containing AMF structures were categorized into five classes: (A) no colonization, (B) hyphae (H), (C) hyphae and vesicles (H+V), (D) hyphae and arbuscules (H+A), and (E) hyphae, vesicles and arbuscules (H+V+A). (F) Magnified image of fully developed arbuscules. Scale bars are 100 µm. The degree of total colonization (TC) and proportion of different AMF categories are presented as a percentage of each category of AMF structure from the total observed root segments (G). Data correspond to the means of five biological replicates ($n = 5$); error bars represent SD. Statistical analysis was performed with a Student's t-test, with p-value for each comparison provided.

non-inoculated control plants. However, comparison of different mycorrhizal structures showed that AMF inoculation significantly increased the degree of total root-length colonization, particularly the proportion of arbuscules in the roots (Fig. 1G). This finding indicates that inoculation of AMF mixture in non-sterilized high P soil can further enhance AMF colonization and subsequent mycorrhization in 'Chaew Khing' rice. It also demonstrates that the rice-AMF symbiosis can be established in both the AMF-inoculated and control treatments within five weeks post inoculation.

AMF inoculation increases growth performance of rice seedlings

To determine whether AMF inoculation affects growth of 'Chaew Khing' rice seedlings, shoot and root growth parameters, including the length and dry weight, were compared between the AMF-inoculated and control treatments. We found that shoot length and shoot dry weight of the AMF-inoculated seedlings were significantly higher than those of the non-inoculated control plants (Fig. 2A-C). On the other hand, root length and root dry weight were not significantly different between the two treatments (Fig. 2A-C), indicating AMF inoculation could enhance growth of the shoot but not the root tissues. Overall, these data indicate that, under high P supply, the treatment of AMF inoculum during seed germination promotes growth of the above ground tissues of 'Chaew Khing' rice seedlings.

AMF inoculation improves phosphorus use efficiency in rice seedlings

Growth promotion as a result of plant-AMF symbiosis is generally associated with an improvement in nutrient acquisition or nutrient use efficiency. To test whether AMF symbiosis improves nutrient uptake in the mycorrhizal 'Chaew Khing' rice, the concentration of total P, N, and Fe was measured in the shoot and root tissues of 5-week-old plants. Interestingly, while P and Fe concentrations in the root tissues were not different between the two groups, shoot P and Fe contents in the AMF-inoculated seedlings were significantly lower than those in the non-inoculated plants (Fig. 3A and Fig. 3C). The total shoot N content was similar between the AMF-inoculated and the control seedlings, but the total root N content was lower in the AMF-inoculated plants than in the control group (Fig. 3B). These results indicate that the higher shoot biomass in AMF-inoculated 'Chaew Khing' rice seedlings is not related to higher P, N or Fe accumulation in the plant tissues.

Next, we examined whether P utilization in the plant tissues was altered by AMF symbiosis. Comparison of PUE between the AMF-inoculated and the control plants revealed that AMF inoculation significantly increased PUE in the shoot but not in the root tissues of the mycorrhizal plants (Fig. 4A). AMF symbiosis also increased the shoot N:P and C:P ratios in AFM-inoculated seedlings (Fig. 4B-C). These data

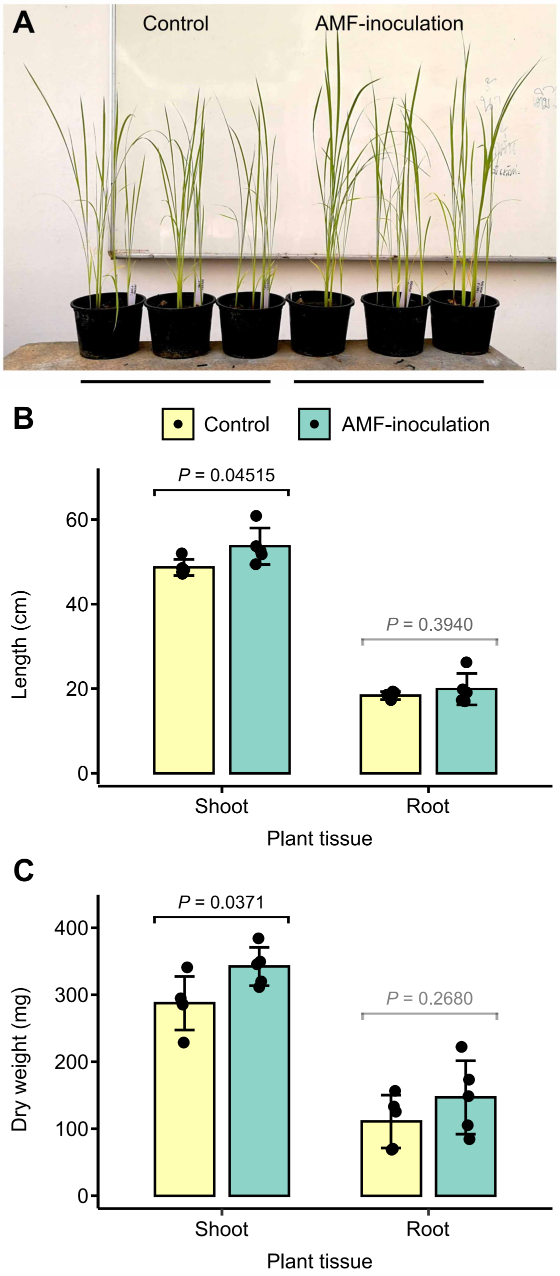

Fig. 2 AMF inoculation enhances shoot growth of 'Chaew Khing' rice seedlings. (A) Representative image of 5-week-old 'Chaew Khing' rice grown in non-inoculated (control) and AMF-inoculated soil. Growth parameters, including shoot and root length (B) as well as shoot and root biomass (C) were compared between the two treatments. Data represent the means of five biological replicates ($n = 5$); error bars represent SD. Statistical analysis was performed with a Student's t-test, with p-value for each comparison provided.

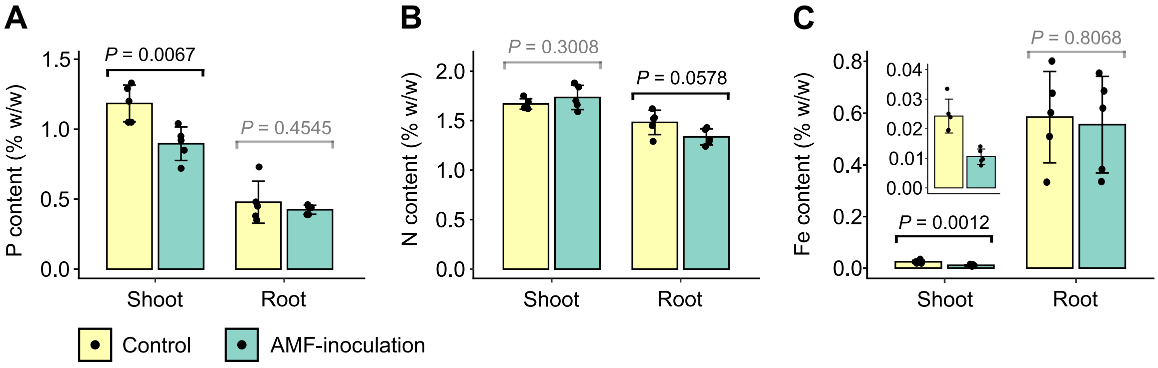

Fig. 3 AMF inoculation alters nutrient contents in 'Chaew Khing' rice seedlings. Concentration (% w/w) of selected nutrients, including P (A), N (B) and Fe (C), was determined in shoot and root tissues of the non-inoculated control and AMF-inoculated plants. Data correspond to the means of five biological replicates ($n = 5$) and error bars represent SD. Statistical analysis was performed with a Student's t-test. p-value for each comparison is provided.

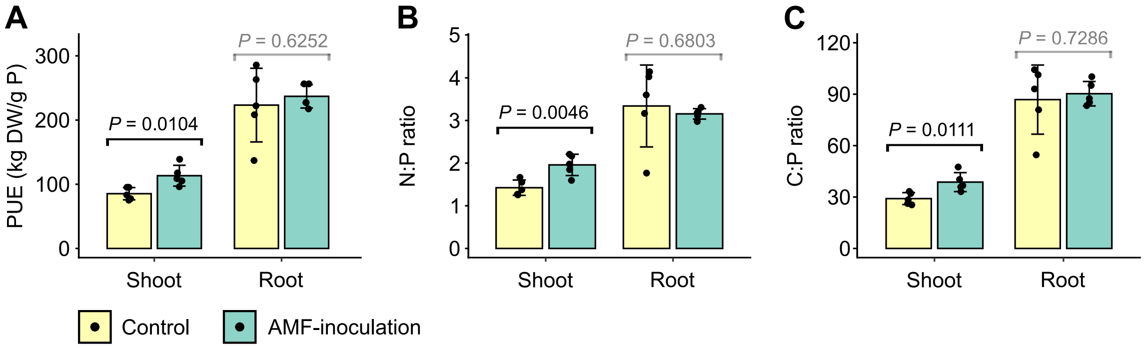

Fig. 4 AMF inoculation increases PUE and plant C:N:P ratio. PUE (A), N:P ratio (B), and C:P ratio (C) in shoot and root tissues were compared between the non-inoculated control and AMF-inoculated plants. Data correspond to the means of five biological replicates ($n = 5$) and error bars represent SD. Statistical analysis was performed with a Student's t-test. p-value for each comparison is provided.

illustrate that the AMF-inoculated rice seedlings consume a lower amount of P for the production of shoot biomass and for the accumulation of N and C. Thus, instead of enhancing P acquisition, the application of AMF inoculum in the fertile and high P soil appears to promote the growth of rice seedlings by improving the efficiency of P utilization.

DISCUSSION

AMF symbiosis plays a crucial role in plant nutrient efficiency. In the face of P deficiency, rice roots can form symbiotic associations with AMF to increase P acquisition. The rate of AMF colonization in rice paddies ranges from 10 to 40%, depending on soil properties and rice developmental stages [32]. The degree of AMF colonization can be intensified by AMF inoculation in a dose-dependent manner [33]. While P deficiency promotes AMF associations, high content of available P in soil has been shown to negatively impact AMF colonization in rice [18]. Compared to the standard nutrient profile of fertile soil in paddy fields [31], the soil used in this study has relatively high P content and is optimal for rice growth. Nevertheless, here we showed that the application of AMF inoculum increases AMF colonization in 'Chaew Khing' rice seedlings (Fig. 1). Therefore, it is possible that higher AMF colonization can be achieved by the introduction of AMF inoculum even if the soil has a high P supply.

Plant performance as a result of AMF inoculation depends on the interactions between the AMF identity, the plant genotype, and the soil property and environmental conditions. For a given host species, growth response to AMF inoculation can vary markedly not only between the fungal taxa but also among isolates of the same AMF species [34]. In rice, growth responsiveness to AMF also varies significantly depending on the rice variety [35]. It has been proposed that plant genetic factors affect fungal growth, which in turn proportionately impacts the performance of the host itself [36]. For instance, superior growth promotion is correlated with extensive development of the AM extraradical hyphal density and higher P uptake by the AM plants [37]. In this study, by using non-sterilized fertile soil inoculated with a mixture of four AMF species, including *G. geosporum*, *G. etunicatum*, *G. mosseae* and *A. foveata* at the ratio 3:3:1:3, we found that an increase in root colonization and mycorrhization in 'Chaew Khing' rice seedlings is associated with growth promotion of the plant above ground tissues (Fig. 2). It has yet to test whether and how an alteration in the AMF species composition and/or ratio affects rice performance. In some other Thai rice varieties, application of AMF fertilizer containing *F. mosseae*, *F. geosporus*, *Claroideoglomus claroideum*, *G. microaggregatum*, and *R. irregularis* does not affect shoot and root growth, but helps reduce yield loss under drought conditions [8]. Similarly, individual inoculation of *G. etunicatum*, *G. geosporum* and *G. mosseae* in the upland rice cultivar 'Leum Pua' does not increase P uptake even though the plants are grown in low P soil, yet they enhance net photosynthesis and grain yield under salt stress [7]. It remains to be determined whether growth promotion in the AM 'Chaew Khing' rice seedlings is translated into higher biomass and grain yield at maturity. It might be more suitable to address this question under field conditions, as it has been demonstrated that AMF-mediated growth promotion at the seedling stage increases grain yield under field but not greenhouse conditions [38]. Furthermore, as the benefit of AMF symbiosis is also influenced by the environment and soil properties, it would be interesting to examine how this fungal mixture affects rice production under different soil types and environmental conditions. It is also important to understand how the interaction between the inoculant and indigenous microbes across soil types affects plant performance.

Improvement of plant growth and fitness by AMF symbiosis involves the direct benefits of enhancing nutrient and water efficiency, re-structuring the rhizosphere, and altering plant gene expression and phytohormone balance [39]. Of these, enhancing nutrient efficiency by increasing P acquisition, known as PAE, has been documented in multiple species. However, despite shoot growth promotion, our analysis of nutrient content in the seedling tissues showed a significant reduction in the amount of total P and Fe in the shoot and total N in the root of AMF-inoculated seedlings, as compared to the control plants (Fig. 3). In line with our finding, a decrease in leaf and stem P contents has also been observed in AM rice in the field [40]. Since our experiment was performed using high P soil, it can be interpreted that shoot growth promotion in the AMF-inoculated seedlings was not due to an increase in PAE. However, given that P uptake of AM plants occurs via both the direct and mycorrhizal uptake pathways and that the symbiosis remarkably diminishes the direct P uptake by the root [41], whereas the contribution of the mycorrhizal pathway in P uptake is reduced as the soil P content increases [42], it is possible that the reduced P accumulation in the shoot tissues of the AMF-inoculated seedlings is associated with the imbalanced contributions of an increase in the fungal P uptake versus a decrease in P uptake by the direct pathway. If this is the case, the extent to which the AM uptake pathway contributes to the total shoot P content needs further investigation. The reduction in shoot P and Fe concentrations could also be interpreted that P and Fe were more effectively utilized for the production of shoot biomass in the mycorrhizal plants.

Apart from PAE, improving P efficiency can also be achieved by increasing PUE, the ability to produce biomass or yield with the acquired P [9]. The relative contribution of PAE and PUE to P efficiency varies with soil P content. When P supply is limited, PAE contributes more to P efficiency than PUE. On the other hand, PUE is more important than PAE with adequate P supply [9]. Consistently, our data showed that while P uptake was reduced, an increase in PUE was observed in the shoot tissues of the AMF-inoculated seedlings (Fig. 4). PUE can be related to the amount of P utilized for the amount of N and C in biomass production. The decrease in shoot P concentrations contributing to the greater N:P and C:P ratios might result from a dilution effect due to an increase in shoot biomass in the AMF-inoculated seedlings. The alteration of plant C:N:P stoichiometry due to AMF symbiosis has been observed in several plant species [43]. In rice, increased C:P ratios due to environmental factors result from the reduction of tissue P concentration while the C concentration is constant [44].

In summary, this study shows that inoculation of an AMF cocktail in P sufficient soil promotes the aboveground growth of 'Chaew Khing' rice seedlings. The AMF-mediated growth stimulation is associated with increased fungal colonization and further development of mycorrhizal structures. The mycorrhizal symbiosis enhances P efficiency of the shoot tissues by increasing PUE. While it is generally thought that the benefits of AMF in crop nutrient efficiency is limited to P-deficient soil, our findings present a premise that AMF could be used as biofertilizers to further enhance crop performance in fertile soil for organic or low-input production of 'Chaew Khing' and possibly other lowland *indica* rice.

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