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Arbuscular Mycorrhizal Fungi Enhance Growth and Increase Concentrations of Anthocyanin, Phenolic Compounds, and Antioxidant Activity of Black Rice (*Oryza sativa* L.)

Sabaiporn Nacoon [1], Wasan Seemakram [1], Jindarat Ekprasert [1], Piyada Theerakulpisut [2,3], Jirawat Sanitchon [4], Thomas W. Kuyper [5] and Sophon Boonlue [1,3,*]

[1] Department of Microbiology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
[2] Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
[3] Salt-Tolerant Rice Research Group, Khon Kaen University, Khon Kaen 40002, Thailand
[4] Department of Agronomy, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand
[5] Soil Biology Group, Wageningen University & Research, P.O. Box 47, 6700 AA Wageningen, The Netherlands
* Correspondence: bsopho@kku.ac.th; Tel.: +66-89-186-2646

Abstract: Black rice (*Oryza sativa* L.) contains high concentrations of bioactive compounds that are associated with human-health benefits. Arbuscular mycorrhizal fungi (AMF) can increase plant performance and concentrations of these bioactive compounds. In a pot experiment, the effects of four different species of AMF (*Claroideoglomus etunicatum*; *Rhizophagus variabilis*; *Rhizophagus nov. spec.*; *Acaulospora longula*) were assessed on growth performance, grain yield, concentrations of phenolic compounds and anthocyanin, and antioxidant activity of two black-rice cultivars. The experiment was a completely randomized factorial design with two factors, viz. cultivar (Niew Dam Hmong and Maled Phai) and treatment (four different species of AMF and two non-inoculated treatments, without and with mineral fertilizer). Results showed that cultivar, treatment, and their interaction were almost always significant sources of variation for both plant performance parameters and concentrations of bioactive compounds. Maled Phai showed higher performance and higher concentrations of phenolics and anthocyanins but lower antioxidant activity than Niew Dam Hmong. The non-inoculated treatment without mineral fertilizer showed the lowest performance. The non-inoculated treatment with mineral fertilizer resulted in larger root and shoot biomass than the mycorrhizal treatments, but grain yield was higher in the mycorrhizal treatments. Inoculation with *R. variabilis* resulted in the highest concentration of phenolics and anthocyanins. We conclude that *R. variabilis* was the best inoculum for increasing grain yield and bioactive compounds, especially in Maled Phai.

Keywords: AMF; *Rhizophagus variabilis*; Maled Phai; Niew Dam Hmong; phytochemical; rice productivity

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1. Introduction

Rice (*Oryza sativa* L.) is a major staple cereal that feeds more than 50% of the global population. Almost all rice (more than 90%) is being produced in Asia. The most commonly consumed rice cultivars have a white kernel. However, specialty rice cultivars exist with red, purple, and black colored kernels, and these cultivars are known as black rice [1]. Their grains contain high amounts of phenolic compounds, especially anthocyanins and antioxidant substances. These substances are considered health-promoting and are especially beneficial for memory enhancement and for strengthening the human immune system [2–4]. Black-rice cultivars contain the highest concentrations of anti-oxidants [5]. Although the demand for black rice in Thailand is rapidly increasing because of its high nutritional value, the market share of this specialty rice is still low. In Thailand, the production of black rice is clustered around the northern and northeastern parts of the country. However, rice production in these locations is characterized by low productivity because soils are mostly sandy

and have unfavorable physicochemical conditions, including low organic-matter content, amounts of available nitrogen and phosphorus, and cation exchange capacity [6]. Because of low inherent fertility, farmers have constantly and extensively used mineral fertilizer to increase rice production. Injudicious use of mineral fertilizer has caused deterioration in the physical and chemical properties of these soils as well as environmental pollution.

Because of declining soil fertility, other forms of field management are clearly needed, and these include a larger focus on the soil biota that contribute to improved performance of black rice. Such improved performance could go hand in hand with a reduced environmental footprint through reduced water demand and lower greenhouse gas emissions. Among such beneficial soil biota, there is a major role for arbuscular mycorrhizal fungi (AMF), a group of mutualistic root and soil-inhabiting fungi. The large majority (over 80%) of crops are able to form mycorrhizal symbiosis, and rice, especially when grown under aerobic conditions, also benefits from the mycorrhizal symbiosis [7,8]. AMF enhance the acquisition of growth-limiting nutrients and improve the acquisition of water and drought tolerance through various mechanisms. Moreover, AMF often increase resistance against belowground and aboveground pathogens and herbivores, although a recent study indicated increased susceptibility of mycorrhizal rice against various pests [9]. In many soils, there is sufficiency of AMF inoculum and the potential of the mycorrhizal symbiosis to contribute to enhanced plant performance then depends on the forms of management applied by the farmer. However, past injudicious management could have reduced AMF inoculum to too low levels, and in such conditions, the use of commercial inoculum could be considered. Such commercial inocula consist of one or more species of AMF, whose provenance may either be local or may have been imported from elsewhere, with the potential risk of these organisms becoming invasive in their new habitat. Experimental research towards the mycorrhizal symbiosis, on the other hand, often involves laboratory or greenhouse experiments, in which control plants are cultivated in sterilized soil and in which various AMF species, alone or in combination, are tested. Such studies allow ascertaining the relative benefits of individual species of AMF under specific conditions, and such knowledge can subsequently be used for upscaling under field conditions.

The main objective of this study was to test the effects of different species of AMF on the growth performance and concentrations of bioactive substances of black rice in a pot experiment under laboratory conditions. The effects of these AMF species were compared with two control treatments, viz. a treatment at inherent soil fertility and a treatment where mineral fertilizer has been added. By executing the experiment with two different controls, the study does allow to the assess both AMF under the inherent soil fertility and the extent to which management of AMF can be an economic substitute for the use of mineral fertilizer. As mycorrhizal management is often cheaper than the acquisition of mineral fertilizer, the study has the potential to demonstrate options to reduce farmer expenses by refraining from mineral-fertilizer use and thereby increase their income. Farmers could then also benefit from other ecosystem services from their rice fields due to improved soil quality.

2. Materials and Methods

2.1. AMF Identification

2.1.1. Isolation of Species of AMF

A total of 15 soil samples were collected from the rhizosphere of black-rice plants grown as upland (aerobic) rice in different regions in the Northeast of Thailand, including Khon Kaen Province (Nam Phong district and Muang district), Roi-Et Province (Nong Phok district) and Kalasin Province (Kuchinarai district). Fifty grams of each rhizosphere sample were thoroughly suspended in 500 mL water in a beaker and allowed to settle afterwards. AMF spores were separated from these soils by wet-sieving and decanting techniques [10] using a series of sieves that were arranged from top to bottom in the following order: 250 µm, 125 µm, and 90 µm. The trapped spores were filtered througWhatman No. 1 filter paper by repeated washing with water. Spores were gently picked using forceps and

placed on a glass slide for visualization under a stereomicroscope (Nikon SMZ745 model LC-LEDS, Zhejiang, China.

2.1.2. Multiplication of Spores of AMF

Only the spores of the most abundant AMF species obtained from each soil sample were subjected to spore multiplication. Multiplication of AMF spores was carried out using a pot culture technique as described by Boonlue et al. [11]. Briefly, soil was twice sterilized by autoclave at 121 °C for 2 h and then added to 20-cm-diameter plastic pots. Maize (*Zea mays* L.) seeds were surface-sterilized by soaking in 10% sodium hypochlorite solution for 30 min before adding them to the pots. Then, individual spores of each morphologically distinct AMF species were added to the pots containing these maize seeds. Maize was subsequently grown in a greenhouse at 30−35 °C and irrigated with tap water every day. After 90 days, irrigation was stopped, and the plants were allowed to dry out, which causes sporulation by the AMF. The plants were cut off at a position just above the soil surface. After that, the soil was air-dried and then ground into fine particles (<0.2 mm). The purity of spores and the total spore number in the soil were determined using the sucrose centrifugation method [12]. Dried soils containing AMF spores, mycelia, and colonized root fragments were then used as the inoculum in the pot experiment.

2.1.3. Identification of AMF Species

DNA of AMF was extracted from single spores obtained from the cultures described above. The spore surface was cleaned by sonication for 10 **s** and sterilized thoroughly using Chloramine–T solution (2% *w/v*) for 5 min. The spore was then rinsed with sterilized deionized water 3 times. A sterilized spore was transferred into a PCR tube containing 7 µL of TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) [13] and then broken using a sterilized microtip under a stereomicroscope. DNA extracts were submitted to a nested PCR protocol [14]. The Thermo Scientific Phire Plant Direct PCR Master Mix kit was used for a nested PCR amplification in a total volume of 20 µL. The primer pairs SSUmAf1 and LSUmAr3 were used in the first amplification step. The second amplification step was performed using the mixed primer SSUmCf3 and SSUmCf1, LSUmBr5 and LSUmBr1, which targets only glomeromycotan fungi. PCR products were purified using the QIAGEN PCR Purification Kit (Thermo Scientific, Vilnius, Lithuania). The rDNA sequences were submitted for sequencing at the U2Bio Thailand. After DNA sequencing, the DNA sequences were compared with similar sequences on GenBank. Phylogenetic analyses was executed through the program MEGA 7, using maximum likelihood [15]. The obtained sequences were deposited to the National Center for Biotechnology Information (NCBI) (for accession numbers, see Section 3.1).

2.2. Greenhouse Experiment

2.2.1. Soil Preparation for Rice Cultivation

The soil for growing black rice in this study was a sandy loam soil with a pH of 7.26, electrical conductivity (EC) of 0.043 dS m^{-1}, organic matter (OM) content of 6.4 g kg^{-1}, total nitrogen (N) content of 240 mg kg^{-1} (C:N ≈ 13), total phosphorus content of 146 mg kg^{-1}, total potassium (K) content of 428 mg kg^{-1}, available P (method Bray 2) 61 mg kg^{-1}, exchangeable K 50 mg kg^{-1}, calcium (Ca) 655 mg kg^{-1}, and sodium (Na) of 50 mg kg^{-1}. Stones, wood chips, and plant debris in a soil sample were removed. The soil samples were sterilized by autoclaving at 121 °C at a pressure of 15 psi for 120 min. The soils were left at room temperature overnight and then sterilized again at the same condition before being packed into 20-cm-diameter plastic pots (5 kg pot^{-1}).

2.2.2. Preparation of Rice Seedlings

Two different cultivars of black upland rice (*Oryza sativa* subsp. *indica*) named as Niew Dam Hmong and Maled Phai were provided by the group of Rice project, Faculty of Agriculture, Khon Kaen University, Thailand. Niew Dam Hmong is a glutinous, pigmented, upland rice with a black seed coat, light-sensitive behavior, and short life span. Generally,

it is cultivated around August. Maled Phai is a non-glutinous, pigmented, upland rice that originated from southern Thailand. It has brown-purple seed, and its cultivation period is from the beginning of June–November. Its seeds are somewhat smaller, and the cultivar is somewhat less productive. However, it has stronger bioactivities than Niew Dam Hmong [16]. Note that the authors of that publication refer to the cultivars as Ma-Led-Fy and Dam-Mong, respectively. Both rice cultivars have been developed at the Agronomy Station, Khon Kaen University and are widely distributed to the community. Rice seeds were surface-sterilized by soaking in 6% sodium hypochlorite solution for 10 min. Twice-sterilized soil was used as the plant substrate contained in 20-cm-diameter plastic pots. The rice was grown in a greenhouse at 30−35 °C and irrigated with tap water every day. Seven-day-old rice seedlings with relatively similar sizes and having true leaves were selected for the experiment.

2.2.3. Experimental Design and Pot Preparation

The experiment was conducted in enclosed greenhouses at Khon Kaen University, Khon Kaen, Thailand. The experiment was arranged as 2-factorial experiment (factors: rice cultivar–two cultivars; mycorrhiza–six treatments) in a Completely Randomized Design (CRD) with 7 replicates per treatment, resulting in a total of 84 pots. The experiment was carried out for 4 months after which the plants were harvested. The mycorrhizal factor included six treatments, viz. (T1) control, sterilized soil without AMF; (T2) control with mineral fertilizer, sterilized soil without AMF, with addition of mineral nutrients (per pot in total 150 mg N, 90 mg P, and 25 mg K, applied in three doses, at 20–25 days after planting (DAP), 45–50 DAP, and 70–75 DAP); (T3) inoculation with AMF isolate ROI-ET2-02; (T4) inoculation with isolate ROI-ET2-01; (T5) inoculation with isolate ROI-ET1-01; (T6) inoculation with isolate KS-02. The AMF inoculum was applied adjacent to plant roots at a rate of approximately 200 spores pot^{-1}.

2.2.4. Determination of Plant and AMF Performance

The following plant performance parameters were measured at 120 days after transplantation (at harvesting stage). SPAD chlorophyll meter reading (SCMR) was recorded using a chlorophyll meter SPAD-502 plus (Konica Minolta, Japan). Plant biomass (shoots, panicles with grain, roots) was determined after the samples were dried at 80 °C for 3 days. Shoot samples were also analyzed for concentrations of N, P, and K.

The following functional compounds were analyzed: anthocyanin concentration (TAC), total phenolic compound (TPC), and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). TAC, TPC, and DDPH were extracted according to Kapcum et al. [17] with some modifications. The samples of dried seed were finely ground. An amount of 1.0 g of samples was extracted with 10 mL methanol, then shaken for 2 h, and centrifuged at 3000 rpm for 10 min. The mixture was filtered (Whatman No.1 filter paper), and the residues were re-extracted twice with 5 mL methanol using the same procedure. The three aliquots were combined and stored at −40 °C in the dark until analyzed.

TAC was determined using two aliquots of 50 µL of extracts to which 3 mL 0.025 M of KCl buffer at pH 1.0 and 0.4 M sodium acetate buffer at pH 4.5 were added. The mixture was then allowed to stand for 20 min before measuring absorbance at 520 and 700 nm. Total anthocyanin concentration was calculated using the following equation and expressed as cyanidin-3-glucoside equivalent per 100 g sample [6]:

$$\text{Total anthocyanins } (\text{mg}/100\text{ g}) = \frac{\Delta A \times MW \times D \times (V/G) \times 100}{\epsilon \times L} \quad (1)$$

where ΔA is absorbance = (A520 nm–A700 nm) pH 1.0–(A520 nm–A700 nm) pH 4.5, ϵ is molar extinction coefficient of Cy-3-G = 29,600 M^{-1} cm^{-1}, L is cell path length of cuvette = 1 cm, MW is molecular weight of anthocyanins = 449.2 g mol^{-1}, D is a dilution factor, V is a final volume (mL), and G is weight of sample (g).

The TPC of the extracts was determined using 125 µL of extracts and 250 µL Folin-Ciocalteu's reagent, followed by the addition of 3 mL distilled water. The solution was mixed well and then allowed to stand for 6 min, after which 2.5 mL 7% sodium carbonate solution was added. The reaction mixture was allowed to stand for 90 min at room temperature before measuring absorbance at 760 nm (Hitachi High-Tech Science Corporation, Tokyo, Japan). Gallic acid was used as a calibration standard, and results were expressed as mg gallic acid equivalent per 100 g sample.

DPPH free radical scavenging activity was determined according to the method described by Leong & Shui [18], with some modifications. Freshly prepared solution of 0.1 mM solution of DPPH in methanol was prepared with absorbance 517 nm. An aliquot of 100 µL of each sample (with appropriate dilution) was mixed with 4.0 mL of DPPH solution, then allowed to stand at room temperature for 30 min before measurement. The percentage of radical-scavenging ability was calculated by using the formula:

$$\text{Scavenging ability } (\%) = \frac{(\text{Absorbance 515 nm of control}) \times (\text{Absorbance 515 nm of sample})}{(\text{Absorbance 515 nm of control})} \times 100 \quad (2)$$

To assess the intensity of AMF root colonization, fresh root samples were stained with 0.05% lactoglycerol trypan blue solution according to the method described by Koske & Gemma [19]. The stained root segments were observed under a microscope. Mycorrhizal colonization intensity was measured according to Trouvelot et al. [20].

2.2.5. Statistical Analysis

Data were analyzed by two-way analysis of variance (ANOVA). Data were tested for normality and homogeneity of variances. The least significant difference (LSD) test was applied to test for significant differences among the means of different treatments at p-value < 0.05. The correlation between parameters was calculated by Pearson's correlation coefficient and evaluated at p-value < 0.05. All statistical analyses were performed using the Statistix 10.0 version software.

3. Results

3.1. Molecular Identification of AMF Species and Performance on Pot Culture

Molecular identification, based on a phylogenetic analysis of the newly generated sequences, showed that isolate ROI-ET2-01 belonged to *Claroideoglomus etunicatum* (Accession No. OQ466528), KS-02 in the clade of *Rhizophagus*, with best matches to *R. variabilis* (Accession No. OQ456401) and especially with a sequence (FR873160) from the French Antilles [21], ROI-ET1-01 belonged to the genus *Rhizophagus* (Accession No. OQ755166), likely constituting a new species, conspecific with a sequence (JX683735) of Glomus Agro-03-S [22], and isolate ROI-ET2-02 to *Acaulospora longula* (Accession No. OQ455726). In the remainder of this paper, these fungal species will be indicated by their scientific names.

These four isolates, which are stored at the Mycorrhiza and Microtechnology Lab, Department of Microbiology, Khon Kaen University, were subjected to spore multiplication. *Acaulospora longula* produced the highest amount of spores, viz. 32 spores g^{-1} soil, and C. etunicatum the lowest amount, viz. 2 spores g^{-1} soil. All four species colonized roots of maize, with fractional colonization ranging between 18% (*Rhizophagus nov. spec.*) and 35% (*R. variabile*) (Table 1). Based on adequate colonization and sufficient spore production, these four species were used to study rice growth in the greenhouse experiment.

Table 1. Total number of spores (g^{-1} soil) and fractional root colonization of maize of the isolated AMF species.

AMF Isolates	Total Spore (Spore/g Soil)	Root Colonization (%)
A. longula	32	25
C. etunicatum	2	19
R. nov. spec.	6	18
R. variabilis	13	35

3.2. Fractional Root Colonization and Spore Number of AMF on Maled Phai and Niew Dam Hmong

Fraction colonization by the four AMF species in black-rice roots and the number of AMF spores are shown in Table 2. Analysis of variance showed that mycorrhizal species, rice cultivar, and the interaction were significant sources of variation (Table 2). The control plants of Maled Phai remained free of mycorrhizal colonization, whereas the control plants of Niew Dam Hmong showed low colonization, lower than the inoculation treatments. No AMF spores were found in the controls of both rice cultivars. All four AMF successfully colonized the roots of both rice cultivars. Fractional colonization and spore number were significantly higher in Maled Phai than in Niew Dam Hmong. Among the AMF species investigated, *R. variabile* exhibited the highest fractional colonization and spore number.

Table 2. The number of AMF spores in soil and the percentage of AMF colonization in roots of Maled Phai and Niew Dam Hmong rice cultivars at the harvest stage.

Treatments	Root Colonization (%)	Total Spore (Spore g^{-1} Soil)
Maled Phai		
Control	0 i	0 f
NPK fertilizer	0 i	0 f
A. longula	19 c	2 d
C. etunicatum	25 b	3 c
R. nov. spec.	14 d	3 c
R. variabile	28 a	6 a
Niew Dam Hmong		
Control	2 h	0 f
NPK fertilizer	1 h	0 f
A. longula	7 f	2 d
C. etunicatum	9 e	1 e
R. nov. spec.	5 g	2 d
R. variabile	14 d	4 b
% CV	10	18
Treatment	**	**
Rice cultivar	**	**
Treatment × Rice cultivar	**	**

Numbers followed by the same letter in each column are not significantly different according to the LSD test. **, Significant difference at $p \leq 0.01$.

3.3. Effects of AMF Species on the Promotion of Growth and Yield of Maled Phai Niew Dam Hmong

The results of plant growth and yield parameters of both rice cultivars are provided in Table 3. For all nine growth parameters, mycorrhiza (treatment) was a significant source of variation, rice cultivar was a significant source of variation for six parameters

(excluding tiller number, panicle number, and seed weight), while the interaction term was significant for five parameters (excluding harvest index [HI], the ratio of grain mass over total aboveground mass, tiller number, panicle number, and SPAD). The control treatment without fertilizer resulted in the smallest plants, but with addition of mineral fertilizer, these plants achieved largest plant height and total biomass. One exception was noted. The total biomass of Maled Phai, inoculated with *R. variabilis*, was larger than that of plants that had received mineral fertilizer. Those mycorrhizal plants were characterized by a particularly large root dry weight. The effect of mineral fertilizer, compared to the mycorrhizal treatment, was especially visible in increases in shoot and root dry weight but not in grain yield. Non-inoculated (control) plants, both without and with mineral fertilizer, had lower grain weight than AMF-inoculated plants. As a consequence, the harvest index was significantly higher for AMF-inoculated than non-inoculated plants. Nutrient concentration of shoots was lowest in the non-inoculated control without fertilizer and highest in the non-inoculated control with mineral fertilizer. The N:P ratios of all plants were below 5, indicating several N limitations. In addition, AMF, rice cultivar, and the interaction were also significant sources of variation for antioxidant activity, and concentrations of phenolic compounds and anthocyanin (Table 4). Concentrations of phenolic compounds and anthocyanin were higher in Maled Phai than in Niew Dam Hmong, consistent with previous reports [16]. Antioxidant activity showed a more variable pattern, without consistent differences between cultivars. Among the AMF species, *R. variabilis* showed the largest positive effect on phenolic and anthocyanin concentrations in both cultivars (Table 4).

Table 3. Effects of AMF on plant growth parameters of Maled Phai and Niew Dam Hmong cultivars at harvesting stage.

Treatments	Root Dry Weight (g)	Shoot Dry Weight (g)	Grain Weight (g)	Aboveground Weight (G)	Harvest Index (HI)	Number of Panicles	Number of Tillers	Height (cm)	SPAD
Maled Phai cultivar									
Control	16 i	44 e	4 g	48 g	0.08 g	3 e	8 d	92 de	35 d
NPK fertilizer	75 b	121 a	14 f	135 a	0.10 g	6 cd	18 a	102 b	45 a
A. longula	39 g	58 d	17 cde	65 de	0.23 de	6 cd	11 cd	97 bcd	45 a
C. etunicatum	46 e	60 d	18 def	78 d	0.22 e	6 cd	14 bc	90 e	42 abc
R. nov. spec.	35 h	71 c	20 bcd	91 c	0.22 e	6 cd	13 c	96 b–e	44 a
R. variabilis	104 a	94 b	25 a	119 b	0.21 e	8 a	13 bc	98 bcd	45 a
Niew Dam Hmong cultivar									
Control	7 c	23 f	5 g	28 h	0.16 f	3 e	9 d	95 cde	37 cd
NPK fertilizer	16 i	76 c	15 ef	91 c	0.17 f	6 bc	17 ab	114 a	43 ab
A. longula	50 d	43 e	22 b	65 ef	0.34 a	6 bc	14 bc	95 cde	38 bcd
C. etunicatum	42 fg	45 e	19 bcd	64 ef	0.30 bc	5 d	12 cd	99 bc	38 bcd
R. nov. spec.	33 h	43 e	20 bc	63 f	0.32 ab	6 cd	14 abc	96 b–e	45 a
R. variabilis	45 ef	51 de	18 cde	69 de	0.26 cd	7 ab	13 bc	97 b–e	42 abc
% CV	8	15	19	13	16	16	27	6	14
Treatment	**	**	**	**	**	**	**	**	**
Rice cultivar	**	**	ns	**	**	ns	ns	*	*
Treatment × Rice cultivar	**	**	**	**	ns	ns	ns	*	ns

Numbers followed by the same letter in each column are not significantly different according to LSD test. ns, not significant *, Significant difference at $p \leq 0.05$; **, Significant difference at $p \leq 0.01$.

Table 4. Effects of AMF on phenolic compounds, total antioxidant and anthocyanin of rice seeds and nutrient concentrations in shoots.

Treatments	Phenolic Compound (mg 100 g^{-1} DW)	Antioxidant (% DPPH Radical Scavenging)	Anthocyanin (mg 100 g^{-1} DW)	N (mg g^{-1})	P (mg g^{-1})	K (mg g^{-1})
			Maled Phai cultivar			
Control	138 d	25 f	40 d	1.6 g	0.5 e	4.6 de
NPK fertilizer	196 b	39 de	98 a	6.0 a	1.2 b	11.2 a
A. longula	184 c	27 f	70 c	2.3 ef	0.6 de	5.6 de
C. etunicatum	140 d	58 c	62 c	2.1 f	0.5 e	5.5 de
R. nov. spec.	188 bc	43 d	66 c	2.7 de	0.7 cde	7.3 c
R. variabilis	209 a	68 b	82 b	3.5 c	0.9 bc	9.7 b
			Niew Dam Hmong cultivar			
Control	53 h	32 ef	36 d	1.6 g	0.5 e	2.7 f
NPK fertilizer	69 g	80 a	21 e	4.7 b	1.7 a	9.1 b
A. longula	87 f	55 c	34 d	2.9 d	0.8 cd	4.4 e
C. etunicatum	71 g	41 de	23 e	2.8 d	0.8 cd	5.1 de
R. nov. spec.	70 g	58 c	36 d	2.8 d	0.7 cde	5.4 de
R. variabilis	116 e	43 d	94 a	3.6 c	0.8 cd	5.8 d
%CV	7	18	18	9	20	13
AMF	**	**	**	**	**	**
Rice cultivars	**	**	**	ns	**	**
AMF × Rice cultivar	**	**	**	**	**	*

Numbers followed by the same letter in each column were not significantly different according to LSD test. ns, not significant; *, Significant difference at $p \leq 0.05$; **, Significant difference at $p \leq 0.01$.

Correlations between AMF colonization, plant performance parameters, and concentrations of secondary compounds are shown in Table 5. Mycorrhizal colonization was significantly positively correlated with seed weight, root weight, and total biomass, whereas the correlation between mycorrhizal colonization and shoot biomass was not significant. Mycorrhizal colonization was also significantly positively correlated with concentrations of phenolics and anthocyanin. Seed weight was significantly positive correlated with root weight, and less so with shoot weight. Both shoot and root weight were positively correlated with concentrations of phenolics and anthocyanin, whereas the correlation between seed weight and concentrations of phenolics and anthocyanin were barely significant.

Table 5. Correlation between AMF and % colonization with plant growth parameters of rice at harvest stage.

Correlation	Root Colonization	No. of Spore	Root Dry Weight	Shoot Dry Weight	Grain Weight	Biomass	HI	No. of Panicles	No. of Tillers	Height	SPAD	Anti-Oxidant	Phenolic Compound	Antho-Cyanin	N	P
No. of spore	0.86 **															
Root dry weight	0.39 **	0.45 **														
Shoot dry weight	0.16 ns	0.14 ns	0.75 **													
Grain weight	0.53 **	0.66 **	0.55 **	0.28 *												
Biomass	0.34 **	0.38 **	0.94 **	0.92 **	0.54 **											
HI	0.20 ns	0.36 **	−0.04 ns	−0.41 **	0.69 **	−0.14 ns										
No. of panicles	0.47 **	0.60 **	0.64 **	0.40 **	0.86 **	0.63 **	0.46 **									
No. of tillers	−0.03 ns	0.03 ns	0.45 **	0.47 **	0.32 **	0.50 **	0.07 ns	0.39 **								
Height	−0.22 *	−0.18 ns	0.33 **	0.30 *	0.10 ns	0.33 **	−0.06 ns	0.20 ns	0.18 ns							
SPAD	0.23 *	0.21 *	0.32 **	0.36 **	0.26 *	0.37 **	0.00 ns	0.24 *	0.34 **	0.00 ns						
Antioxidant	0.19 ns	0.25 *	0.55 **	0.29 *	0.46 **	0.47 **	0.19 ns	0.43 **	0.39 **	0.26 *	0.26 *					
Phenolic	0.52 **	0.42 **	0.45 **	0.66 **	0.21 ns	0.59 **	−0.35 **	0.27 *	0.09 ns	−0.11	0.31 **	−0.13 ns				
Anthocyanin	0.41 **	0.45 **	0.41 **	0.57 **	0.23 *	0.52 **	−0.20 ns	0.38 **	0.22 *	−0.10	0.30 *	−0.13 ns	0.73 **			
N	−0.20 ns	−0.06 ns	0.69 **	0.75 **	0.28 *	0.76 **	−0.14 ns	0.44 **	0.57 **	0.51 **	0.30 *	0.37 **	0.22 *	0.37 **		
P	−0.26 *	−0.16 ns	0.58 **	0.49 **	0.24 *	0.56 **	−0.08 ns	0.38 **	0.47 **	0.65 **	0.18 ns	0.57 **	−0.09 ns	−0.05 ns	0.8 **	
K	0.11 ns	0.14 ns	0.79 **	0.89 **	0.32 **	0.88 **	−0.31 **	0.46 **	0.51 **	0.40 **	0.39 **	0.39 **	0.56 **	0.46 **	0.8 **	0.7 **

**, Significant difference at $p \leq 0.01$; *, Significant difference at $p \leq 0.05$; ns, not significant.

4. Discussion

At present, there are only a few studies that provide information on the effects of AMF species on black rice. Surendirakumar et al. [23] reported seven species of AMF associated with black rice in paddy soils in India, including one species of *Acaulospora* and two species of *Rhizophagus*. However, the very different environmental conditions in that study (paddy rice) and this study (aerobic rice) would make a direct comparison difficult. Wangiyana et al. [24] reported increased grain yield of black rice after inoculation with a commercial inoculum, whose composition was not specified but likely consists of a mixture of different species of AMF and ectomycorrhizal fungi. A similar positive mycorrhizal effect was reported by Anugrah et al. [25], again without reporting the identity of the AMF species that might have caused this yield increase. Tisarum et al. [26] reported that inoculation with AMF, a mixture of three different species, increased drought tolerance and enhanced anthocyanin concentrations of black rice. Fractional colonization in that study was approximately 30%, comparable with our results (Table 2) for Maled Phai (14–28%) but considerably higher than for Niew Dam Hmong (5–14%). Other studies reported even higher fractional mycorrhizal colonization of rice than reported here, for instance, a colonization rate of 26–40% [27], <5–40% [28], and 12–27% under flooded, and 22–43% under non-flooded conditions [29].

This study showed that colonization by AMF had a beneficial effect on the growth performance of two black-rice cultivars. Biomass production of plants, inoculated with one of four species of AMF, outperformed non-inoculated plants in the absence of mineral fertilizer. The application of mineral fertilizer also boosted plant performance, but the effect was especially noteworthy in shoot biomass. In fact, seed weight and harvest index were less for non-inoculated fertilized plants than for inoculated, unfertilized plants. These non-inoculated plants showed very low fractional colonization. Considering the differences in seed yield and harvest index, we argue that the low levels of mycorrhizal colonization had only a minor impact on plant performance. High levels of fertilizer application have been reported before to result in plant investment in vegetative growth rather than in seed production. Mycorrhizal effects on cereal yields have been reported before by Zhang et al. [30] in a meta-analysis that showed a yield increase in grain yield of rice of 17% due to the mycorrhizal symbiosis, indicating an effect that was noted both under field and greenhouse conditions.

The increase in yield in the mycorrhizal treatments, as compared with the non-inoculated fertilized treatment, coincided with lower concentrations of nitrogen, phosphorus, and potassium in shoots (Table 4). However, compared with the unfertilized control, mycorrhizal plants had higher shoot concentrations of these three macronutrients. These mycorrhizal plants also showed increases in concentrations of phenolics and anthocyanins compared with unfertilized, non-inoculated plants. Fertilizer application also increased the concentration of these bioactive compounds. In the case of inoculated Maled Phai, unfertilized mycorrhizal plants exhibited similar concentrations of phenolics and anthocyanins than non-inoculated, fertilized plants. In the case of Niew Dam Hmong, inoculated, unfertilized plants even outperformed these non-inoculated, fertilized plants, demonstrating an effect that was most conspicuous in plants that were inoculated with *R. variabilis.* This recently described fungal species also achieved highest fractional root colonization in both cultivars and also significantly increased root biomass in Maled Phai. This effect is particularly interesting considering the significant positive correlations between root biomass, root colonization, and concentrations of bioactive compounds. Plant species-specific differences between AMF species in effects on plant performance have been regularly published, whereas similar effects on different cultivars of the same species have been less frequently described. Wang et al. [31] demonstrated how two cultivars of maize, a landrace and a hybrid, responded differently to two different AMF species, with the landraces being more responsive to *Funneliformis mosseae*, and the modern hybrid showing a slight preference for *Claroideoglomus etunicatum*. In this study, *Rhizophagus variabilis* had a more positive effect than the other three AMF species on the performance of Maled Phai,

whereas Niew Dam Hmong performed equally with these four AMF species. Further studies on *R. variabilis*, which possibly has a global distribution [32], are planned.

The concentrations of phenolics and anthocyanins of Maled Phai were higher than Niew Dam Hmong, which is in agreement with the earlier study by Sripanidkulchai et al. [16], but the higher productivity of Maled Phai than of Niew Dam Hmong is contrary to study. However, comparing productivity data from field studies with those of individual plants in pots is inherently difficult. Beneficial effects of the AMF symbiosis on secondary compounds have been reported frequently [33–35]. Fiorilli et al. [36] reported upregulation of a gene involved in anthocyanin biosynthesis in mycorrhizal rice, inoculated with *R. irregularis*, compared with the non-mycorrhizal condition, but did not provide further details. Upregulation of anthocyanin biosynthesis in black rice as a consequence of the AMF symbiosis has also been reported by Tisarum et al. [26] and Wangiyana et al. [24]. Soltaniband et al. [37] reported increase in anthocyanin levels after inoculation with the AMF *R. irregularis* in strawberry (*Fragaria x ananassa*), and a similar effect was noted for anthocyanin concentrations in the berries of tempranillo grapevine (*Vitis vinifera*) [38]. Lee & Scagel [39] observed an increase approximately 35% in the concentrations of anthocyanins in leaves of *Ocimum basilicum* associated with *R. intraradices* in comparison with non-mycorrhizal controls in greenhouse cultivation. Baslam & Goicoechea [40] found that levels of anthocyanins in leaves were very sensitive to the presence of AMF colonizing roots of both cultivars of lettuce *(Lactuca sativa*), Batavia Rubia Munguía and Maravilla de Verano. AMF-species-dependent effects on anthocyanins were found in two weed species, *Solanum nigrum* and *Digitaria sanguinaria*, where inoculation with *F. mosseae* increased anthocyanin concentrations, whereas inoculation with *R. intraradices* or *R. fasciculatus* decreased it.

In this study, as in many other studies [33–35], there is a double mycorrhizal effect, both a direct effect through increased plant or seed yield and an additional indirect effect through higher concentrations of bioactive compounds such as phenolics and anthocyanins than in non-mycorrhizal plants. The underlying mechanism of enhanced synthesis of these bioactive compounds is currently unknown. In a study on *Cannabis sativa* [41], the increase in bioactive compounds was correlated with increases in phosphorus acquisition. However, in this study, mineral fertilizer plants showed the highest shoot concentration of N, P, and K, but this did not result in the highest concentrations of bioactive compounds. Both nutritional and non-nutritional factors have therefore been proposed to explain the increased production of secondary metabolites in AMF-colonized plants [35]. Nutritional mechanisms refer to the improvement of the nutritional condition of the host [41–43]. Zhao et al. [35] summarized current knowledge on potential non-nutritional mechanisms by stating that AMF colonization results in the activation of plant defense mechanisms with the production of phenolics and flavonoids. AMF also induce the production of signaling molecules, such as nitric oxide, salicylic acid, and hydrogen peroxide, which influence the activation of key enzymes such as l-phenylalanine ammonia lyase and chalcone synthase, for the biosynthesis of phenolic compounds [44]. Non-nutritional mechanisms may further involve the activation of metabolic routes [45], production of signaling molecules, alterations in the activity of key-enzymes for the production of these compounds [46–48], and hormonal alterations [49]. The results obtained in this study partly supported a nutritional mechanism, as evidenced by the correlations between nutrient concentrations and concentrations of bioactive compounds, both phenolics (for N, P, and K) and anthocyanins (for N and K) (Table 5). However, the comparison between non-inoculated mineral-fertilized plants and mycorrhizal, unfertilized plants indicates the importance of non-nutritional mechanisms as well.

5. Conclusions

Inoculation of black rice with four different species of AMF promoted plant growth compared with non-inoculated plants under unfertilized conditions. While mineral fertilization under non-inoculated conditions enhanced plant performance, this was mainly expressed through larger shoot biomass, whereas seed weight and harvest index were

smaller under fertilization than under mycorrhizal fungal inoculation. The importance of AMF was also evident by the significant correlations between mycorrhizal colonization, seed weight and concentrations of bioactive compounds. Higher correlations between seed mass and root mass than between seed mass and shoot biomass also support this important role of AMF. Plant inoculated with AMF also possessed larger concentrations of phenolics and anthocyanins compared with uninoculated controls. The largest beneficial effect was noted for the AMF *Rhizophagus variabilis*, and this species deserves further scrutiny.

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