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Assessment of Community Dynamics of Arbuscular Mycorrhizal Fungi in the Rice (*Oryza sativa* L.) Rhizosphere and Potential Application as Biofertilizer

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Abstract: Arbuscular mycorrhizal fungi (AMF) have the potential to maintain the sustainability of rice cultivation via maintaining soil health. The objective of this study was to produce an AMF-based biofertilizer for the rice variety Bg350 using indigenous dominant species of AMF that are adapted to paddy wetland soil conditions in dry, wet, and intermediate zones in Sri Lanka and are co-inoculated with the bacterium *Azospirillum*. A pot experiment was carried out to evaluate the effectiveness of the produced biofertilizer using the rice variety Bg350. Treatments were inorganic fertilizer, compost, biochar, produced AMF-biofertilizer [1 kg of ground carrier material inoculated with 50 g of AMF propagules and 20 mL of 1.5×10^8 (CFU/mL) of *Azospirillum*], and the control. A two-factor factorial, completely randomized design was used under sterilized and non-sterilized soil conditions with four replicates. The genera *Glomus*, *Claroideoglomus*, and *Aculospora* were identified as the most common AMFs in paddy soil in all investigated sites. In the 9th week of sampling, AMF root colonization was positively correlated ($p = 0.028$) with spore density. In Sri Lanka, for the first time, the highest AMF colonization rates in rice were recorded at 36.40% in the roots of the Bg350 from the Gampaha district. AMF root colonization increased over sampling time and was different according to the interactive effect of fertilizer application and soil condition. The biometric parameters and yield-attributing characteristics were significantly higher in the rice plants grown in sterilized soil, independent of the tested treatments. The number of grains per panicle was significantly similar ($p \leq 0.05$) in the compost, AMF-biofertilizer, and inorganic fertilizer added treatments. It can be concluded that application of paddy soil adapted AMF species as a biofertilizer increased rice plant growth, productivity, and yield.

Keywords: AMF; biofertilizer; root colonization; submerged rice; yield

1. Introduction

To fulfill the rising global food demand, it is common to conduct user-friendly farming practices that intensify production processes without consideration of natural ecosystem functionality. As a result, some agricultural anthropogenic activities and the resultant land degradation due to high productivity processes cause a disturbance to natural ecosystem functionality and thereby threaten environmental sustainability and, furthermore, can be a

warning to future food security [1,2]. To compromise this issue, the establishment of sustainable agriculture is a better alternative. As a part of sustainable agriculture, organic farming and regenerative agriculture depend on the application of beneficial microorganisms [1]. Soil microorganisms have the potential to balance soil organic matter decomposition and plant nutrient status. The selection and application of beneficial soil microbial inoculants can serve as a fertilizer (termed a "biofertilizer") in sustainable agriculture [3,4]. A biofertilizer is a biological product that contains living microorganisms that have beneficial roles in soil fertility, plant growth, and productivity [5,6].

On the farming side, both biotic (pathogens) and abiotic stresses (climate, soil health, and water availability) are the major determinants that affect plant viability, functionality, and productivity [1]. More interestingly, arbuscular mycorrhizal fungi (AMF), belonging to the Phylum *Glomeromycota*, positively address all these ecological determinants and plant physiological events by symbiotically associating with crop plants. Because mycorrhizae have co-evolved with plants over a period of 400 million years and were involved in evolutionary adaptation related to the transition of plants to the land environment [2], 80% of land plants have been colonized with the formation of low host specificity [7]. Through their nutritional and non-nutritional functionality, AMF cause morphological, biochemical, and physiological alterations, including changes in gene expression levels in the plant that lead to an over-intensification of the plant productivity [8].

Rice (*Oryza sativa* L.) is an important staple food crop in Asia, Africa, and South and Central America, with over 50% of the global population consuming rice as their main calorie intake [9]. Globally, more than 75% of rice is produced in lowland areas under submerged conditions, and the rest is from upland areas under non-submerged conditions [10]. Previous studies have shown that AMF colonization in rice under waterlogged conditions is absent or very rare. However, new findings have proved that although waterlogged conditions reduced the transparent spore numbers [11], they can survive under waterlogged (anoxic) conditions through the formation of sufficient inoculum to survive in rice roots and serve a beneficial role in plant growth, productivity, and its yield components [12]. Rice was derived from a semi-aquatic ancestor with unique characteristics, and the plant can regulate AMF colonization under waterlogged conditions by influencing root architecture, anatomy, and physiology, even though AMF are obligate aerobes. In such ecosystems, rice plants develop aerenchymatous (also known as aeriferous parenchyma) tissues that enable AMF to obtain O_2 from the atmosphere [13,14].

As a better alternative to chemical fertilizer, the application of AMF-based biofertilizer for the rice cultivation system has both environmental and cost-effective benefits. AMF with an edaphic origin is mainly considered when managing AMF-based biofertilizers in agriculture. Researchers have mentioned that some indigenous AMF species are adapted to the soil environment of the paddy wetlands without considering any edaphic and climatic parameters [12]. In this research, we studied the effectiveness of the indigenous AMF groups obtained from paddy soil as a biofertilizer for rice plants grown in paddy soil under submerged conditions by comparing it with other fertilizer application types, including inorganic fertilizer, compost, and biochar in Sri Lanka.

2. Materials and Methods

2.1. Sampling Sites and Sample Collection

Paddy wetlands around Sri Lanka (dry zone: Jaffna, Anuradhapura, Vavuniya, and Trincomalee; intermediate zone: Kurunegala, Badulla, Matara, and Monaragala; wet zone: Kandy, Gampaha, Rathnapura, and Galle) were chosen as the sampling sites (Figure 1) that were controlled under compost application with the cultivation of rice variety Bg350 (3-month variety). Rice samples consisting of leaves, roots, and rhizosphere soil were collected from each site at 6-9 weeks (after the flowering stage) after plantation according to the stratified sampling method from four places in each district. For further analysis, a composite sample was used. For the AMF spore analysis process and studying the basic properties (soil type, soil pH, electrical conductivity, and moisture content) of the paddy

soil, two soil samples (0-10 cm in depth) from each paddy field were collected. The roots of each sample were immediately wrapped in pieces of moist cotton, and the whole plant was wrapped in a newspaper for transport to Rajarata University in Sri Lanka, Mihintale. In the laboratory, each root sample was thoroughly washed with tap water, and only fine lateral roots were carefully separated, cut into 1 cm pieces, and fixed in a volume ratio mix of 37% w/w formaldehyde, glacial acetic acid, and anhydrous ethanol: FAA (5:5:90 by $v/v/v$) [15] until they were used for the enumeration of AMF colonization.

Figure 1. Sample collection sites with their three climatic zones in Sri Lanka.

2.2. AMF Spore Isolation, Identification, and Calculation of Spore Density

The collected rhizosphere soil (200 g) was mixed with a substantial volume of tap water to ensure that the soil aggregates had broken apart and then decanted through a series of sieves (53, 106, and 300 µm). This washing and decanting process was repeated until the water was clear. The final washing step was done with distilled water. The trapped soil part in each sieve was transferred to the centrifuge tube, and the centrifugation was carried out at 3000 rpm for 5 min. The supernatant was removed, and the pellet was re-suspended in 60% sucrose by vigorously shaking the tightly stoppered tube. The sample was then centrifuged at 3000 rpm for 5 min [16]. For vacuum filtration, immediately after centrifugation of spores, the sucrose supernatant was poured into the pre-wetted filter paper in the Buchner funnel and washed with distilled water to remove the sucrose. The filter paper with spores was placed in a plastic Petri dish and observed under the stereo microscope (Micros-MC 30) [16]. Spore density was calculated by using the method discussed in the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) [17]. Spore identification up to genus level, using spore color, shape, size, and subcellular structure of spores (spore wall, germination wall, cicatrix) and subtending

hypha was done by using INVAM culture collection (https://invam.wvu.edu, accessed on 21 March 2022)

2.3. Assessment of AMF Colonization

The root sample that was fixed in FAA was used for the staining process. The fixed root sample was washed with distilled water and placed in 10% KOH, heated to 90 °C for 15–30 min in a water bath, and rinsed with distilled water [18]. The pigmented root samples were bleached with 3% H_2O_2 for 10–20 min at room temperature. Then, excess H_2O_2 was removed by thoroughly rinsing the roots in distilled water [18]. Thereafter, roots were acidified with 1% HCl for 1 min [19]. The root segments were stained with the preheated 0.05% trypan blue in lactoglycerol made up of a 1:1 ratio mix of 0.05% trypan blue and lactoglycerol for 5 min at 75 °C [15]. Then, the stained root sample was washed with deionized water and destained in a 1:2:2 ($v/v/v$) lactic acid: glycerol: deionized water solution.

Ten segments of stained roots were randomly selected from the sample and mounted in glycerin on a microscopic slide, gently squashed under a cover slip, and viewed under a compound microscope (Micros-MC 30) at X1000 magnification. The presence of AMF structures was scored for 100 intersections of roots per sample by selecting 10 imaginary grid lines according to the magnified intersections method discussed by McGonigle et al. [20]. The AMF structures of arbuscules, vesicles, coils, or internal hyphae (with lack of septa) were considered arbuscular mycorrhizal intersections. The AMF root colonization percentage was calculated as AMF colonized root intersects/the total number of roots intersects X100 [20].

2.4. Trap Culture Method for Increasing the Population of Specific AMF Species

The most common types of spores present in all study samples were isolated (the procedure is discussed in Section 2.2) from the mixed fungal spores and used as the inoculants. Under the stereo microscope, the single species spore separation was done by the needle and the paintbrush and placed on a separate pre-wetted filter paper in a Petri dish. The culture was maintained in 5 kg of sterilized sand in a 5000 mL plastic pot with maize (*Zea mays* L.) as the host plant. A day before the introduction of the maize seeds, 50–200 separated single-species spores were placed at 5 cm depth in sand supplemented with the mineral nutrients (Table 1) [17]. For each single species, a separate pot was maintained. The maize seeds, untreated with fungicides, were surface sterilized by first immersing them in 70% ethanol for 1 min. Afterwards, seeds were transferred to the 6% NaOCl for 3 min and rinsed with distilled water 5–6 times [21]. Blotted seeds 45–50 were then placed in each pot 2 cm in depth and kept in the plant house at the Faculty of Applied Sciences, the Rajarata University of Sri Lanka, for one month (Figure 2).

Table 1. Nutrient supplement for maize pot cultures that are grown in sandy soil.

Compound	Amount (g/L)	Stock Solution
KH_2PO_4	10.80	A
K_2SO_4	45.00	A
NH_4NO_3/2 weeks	15.00	A
$CaCl_2 \cdot 2H_2O$	45.00	B
$MgSO_4 \cdot 7H_2O$	6.00	C
$MnSO_4 \cdot H_2O$	3.00	C
$ZnSO_4 \cdot 7H_2O$	3.00	C
$CuSO_4 \cdot 5H_2O$	1.50	C
H_3BO_3	0.24	C
$CoSO_4 \cdot 7H_2O$	0.12	C
$Na_2MoO_4 \cdot 2H_2O$	0.09	C

(A–C: stock solutions were prepared by mixing each compound mentioned in sterilized distilled water).

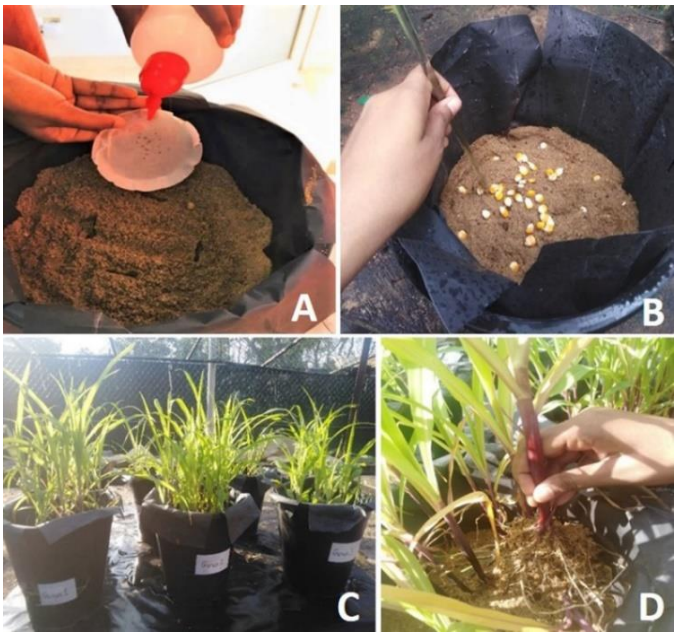


Figure 2. Established trap culture for single species AMF isolates using maize plants as hosts. (**A**) Introduction of the single-species AMF spores to the pot; (**B**) Application of the maize seeds to the top of the AMF spores after one day; (**C**) Two-week-old maize plants; (**D**) Collection of the AMF-trapped one-month-old maize roots.

2.5. Development of the Biofertilizer

The aquatic floating fern *Salvinia* sp. collected from the Mihintale tank, Anuradhapura, Sri Lanka, was used as the carrier material [22]. The ferns were oven-dried at 80 °C, ground to powder, and sieved through a 2 mm sieve. The carrier material was packed in autoclavable polythene and sterilized at 121 °C at 15 psi for 20 min. Root fragments of one-month-old maize plants, together with rhizosphere sand, were considered an AMF inoculant that was collected from trap culture pots. Three separately trapped AMF inoculants were mixed in similar proportions when applied to the biofertilizer. The pure culture of *Azospirillum* sp. was prepared using soil isolates that were collected from the faculty of applied sciences at the Rajarata University of Sri Lanka using BTB-1 medium.

For the preparation of the bio-fertilizer, each pre-sterilized 1 kg portion of ground carrier material was inoculated with 50 g of AMF propagules, and 20 mL of 1.5×10^8 (CFU/mL) of *Azospirillum* sp. were used [22]. The 1.5×10^8 CFU of *Azospirillum* sp. were prepared using 0.5 McFarland standards and by adding BTB-1 broth, and the total 20 mL volume of *Azospirillum* sp. suspension was prepared [22]. The microbial inoculants and carrier substances were packed in the polythene bag and were immediately sealed. The microbial inoculants were then mixed with a carrier material by hand until the microbial inoculum was uniformly spread into the carrier substances. Within 24 h of the preparation, biofertilizer was applied to the rice plants.

2.6. Pot Experiment

Using paddy soil, the pot experiment was carried out in a plant house at the Faculty of Applied Sciences, Rajarata University of Sri Lanka, from February to April 2022 at an annual temperature of 31–35 °C and 1000–1500 mm of annual precipitation. The paddy soil, with a pH of 6.43 and electric conductivity of 58.62 µs, was used for the experiment. The experiment was laid out in a two-factor factorial completely randomized design (CRD) with four replications. Here, the effectiveness of the produced biofertilizer was evaluated by comparing it with the current fertilizer applications in Sri Lanka using sterilized and non-sterilized soil. The five treatments: Control, without adding any fertilizer (T1), AMF-based biofertilizer (T2), biochar (T3), compost (T4), and inorganic fertilizer (T5) were applied

to the experiment. Soil sterilization was done at 121 °C with 15 psi pressure for 20 min in an autoclave. The amounts and timing of fertilizer application for each treatment are mentioned in Table 2. Non-drained pots were used to maintain the submerged conditions in all 40 pots throughout the experiment. After soaking in the water for 24 h, the pressure was applied to the seed for 48 h to initiate germination. Sprouted seeds of rice variety Bg350 (collected from Bathalagoda Rice Research and Development Institute) were grown in nursery trays separated for sterilized and non-sterilized soil respectively. The seeds used for the biofertilizer treatment were treated with 20 g/kg of soil. Ten-day-old seedlings were then transplanted into each pot with three seedlings. At the time of transplantation, 1 cm of water level was maintained.

Table 2. Amounts and time intervals of fertilizer application (produced biofertilizer, biochar, compost, and inorganic fertilizer) per pot in the pot experiment for rice plants.

Treatment	Amount of Soil (g)	Amount of Fertilizer (g)	Time of Fertilizer Application		
Control (T1)	3000	-	-	-	-
Biofertilizer (T2)	3000	30	Applied	-	Applied
Biochar (T3)	2750	250	Applied	-	Applied
Compost (T4)	2750	250	Applied	-	Applied
Inorganic fertilizer (T5)	3000	Ammonium sulphate—0.6	-	Applied	Applied
		Monocalcium phosphate—0.3	Applied	-	-
		Potassium chloride/muriate—0.3	-	-	Applied

2.7. Data Collection and Agronomic Study

The AMF colonization percentage and biometric observations, such as plant height and the number of tillers per plant, were recorded at 3, 6, 9, and 12 weeks after transplanting. Per plant, yield-attributing characteristics such as the number of effective tillers, the panicle length, the number of grains per panicle, the hundred-grain weight, the fresh and dry plant biomass, the grain yield, and the harvest index were recorded at the time of harvest (Figure 3).

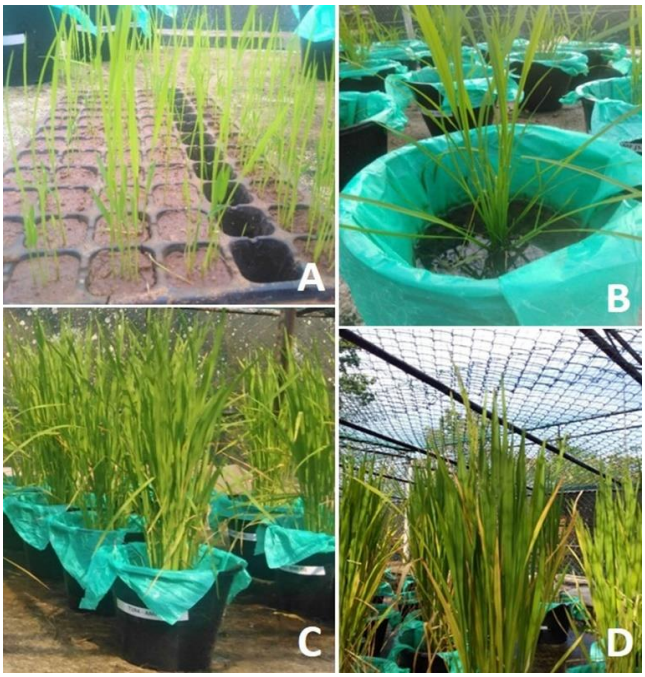


Figure 3. Different growth stages of the cultivated rice plant in the pot experiment (Bg350 variety). (**A**) Ten-day-old rice plantlets after sowing; (**B**) Three-week-old rice plants; (**C**) Six-week-old rice plants; (**D**) Nine-week-old rice plants.

2.8. Statistical Analysis

The data were analyzed using the two-way ANOVA procedure using SPSS/Minitab 17.1 statistical software (https://www.educba.com/minitab-vs-spss/ accessed on 15 October 2022). A Tukey's mean separation procedure was conducted to identify the significant differences among treatments. The relationships among the variables were tested using Pearson's test. All data were tested for normality using the Shapiro–Wilk test before analysis.

3. Results

3.1. AMF Spore Density at Different Rice Cultivation Fields in Dry, Wet, and Intermediate Zones

According to the regional location, AMF diversity, distribution, and spore density can vary (Table 3). In our study, Jaffna formed the highest spore density (Figure 4), and 12 different AMF morphotypes were identified. The highest spore diversity was found in the Matara district, with 19 different morphotypes. The lowest spore density was detected in the Rathnapura district, which belongs to the wet zone in Sri Lanka. However, there was no significant relationship between spore density and the number of AMF morphotypes that were studied. When considering the studied sites according to the ecological zones in Sri Lanka, spore density was highest in the dry zone and lowest in the wet zone. There was no significant relationship between spore density with soil pH and electrical conductivity (Table 3), but both soil pH and electrical conductivity were decreased from the dry zone to the wet zone. There was no significant relationship found between total culturable bacteria and fungi present in sampling soil with AMF, but only in the dry zone were AMF spore density and colonization positively correlated with the total fungal count at the sampling sites. In the intermediate zone, AMF spore density was negatively correlated with the total culturable bacterial count.

Table 3. Environmental conditions of the sampling sites related to dry, wet, and intermediate zones in Sri Lanka from December to January and physicochemical properties of rhizosphere soils for field-collected *Oryza sativa* L.

Zone	Sample Site	GPS Coordinate	Annual Temperature (°C)	Relative Humidity (%)	Soil Type	Soil pH	Electric Conductivity of Soil (µS/cm)
Dry	Jaffna	9°42′10″ N 80°01′14″ E	28–29	78	Grumusols	6.28	550.60
	Vavuniya	8°45′43″ N 80°30′08″ E	29–30	84	Alluvial	5.57	88.96
	Trincomalee	8°21′26″ N 81°00′29″ E	28–29	84	Alluvial	5.23	332.80
	Anuradhapura	8°16′56″ N 80°42′49″ E	29–30	84	Reddish brown earth	7.10	44.58
Intermediate	Kurunegala	7°39′09″ N 80°22′18″ E	30–31	83	Red-yellow latosols	6.54	70.30
	Badulla	6°59′37″ N 81°03′12″ E	27–28	88	Red-yellow podzolic	5.59	247.80
	Matara	5°57′55″ N 80°31′53″ E	30–31	80	Alluvial	5.25	124.60
Wet	Kandy	7°16′02″ N 80°32′56″ E	28–29	84	Reddish brown lateritic	5.61	32.58
	Gampaha	7°05′59″ N 79°59′46″ E	31–32	79	Alluvial	5.99	46.36
	Rathnapura	6°43′00″ N 80°46′22″ E	30–31	85	Alluvial	5.42	22.03
	Galle	6°20′48″ N 80°14′35″ E	29–30	79	Alluvial	5.92	123.3

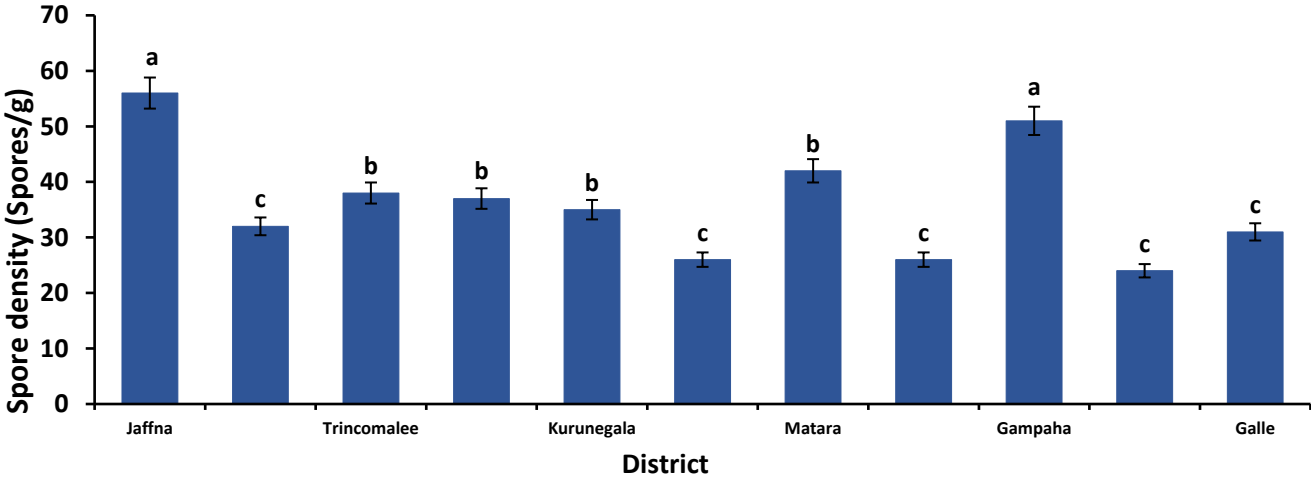


Figure 4. Arbuscular mycorrhizal fungi spore density (Spores/g) of different paddies in dry, wet, and intermediate zones in Sri Lanka. (Dry zone: Jaffna, Anuradhapura, Vavuniya, and Trincomalee; intermediate zone: Kurunegala, Badulla, and Matara; wet zone: Kandy, Gampaha, Rathnapura, and Galle). a–c indicates the significant difference between each district (Tukey pairwise comparison).

3.2. AMF Colonization

When the plant reaches the heading and ripening stages (6-9 weeks for a 3-month cultivar), the aerenchyma is fully developed, and with that sufficient O_2 supply is received by AMF to form the higher colonization with rice roots [12]. Moreover, AMF colonization can vary with geographical regions, temperature, rainfall, nutrient availability, soil pH, soil type, and altitudinal gradients [12,23–27]. In this study, the colonization was assessed using 6-9-week-old plants, assuming that at this stage all plants form a higher colonization percentage. As mentioned in the previous literature, according to regional variation, the colonization percentage varied among the studied sites. In Sri Lanka, for the first time, the highest AMF colonization was recorded in Gampaha, with 36.40% of colonization in this study (Figure 5). The lowest colonization was recorded in the Vavuniya district. A positive correlation of $p = 0.028$ was formed between spore density and the percentage of colonization. Then, the available AMF spore number in soil increased the colonization of rice roots. Only in the dry zone, the AMF colonization was positively correlated ($p = 0.046$) with the total culturable fungal count, not with the total culturable bacterial count (Figure 6).

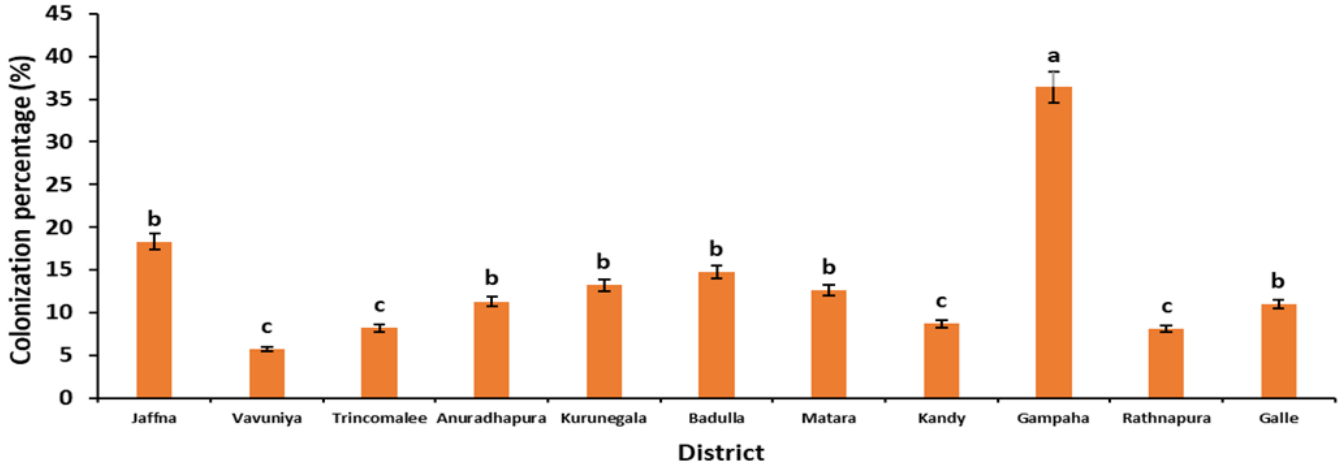


Figure 5. Colonization (%) variation of AMF among different paddies in dry, wet, and intermediate zones in Sri Lanka. (Dry zone: Jaffna, Anuradhapura, Vavuniya, and Trincomalee; intermediate zone: Kurunegala, Badulla, and Matara; wet zone: Kandy, Gampaha, Rathnapura, and Galle). a–c indicates the significant difference between each district (Tukey pairwise comparison).

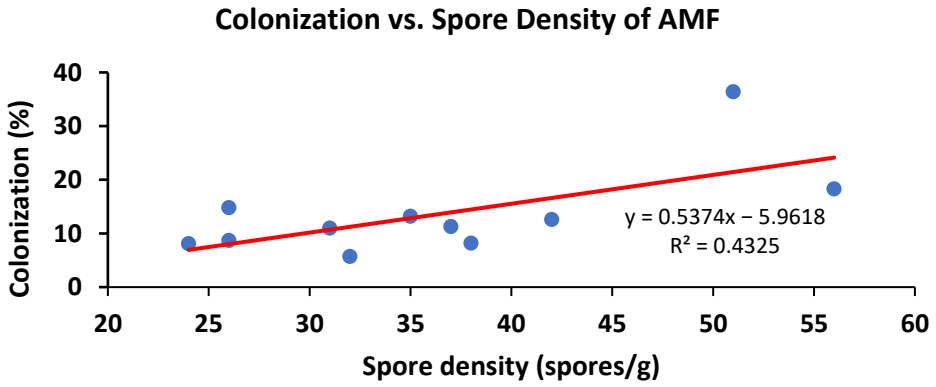


Figure 6. Relationship between AMF spore density in soil and colonization of AMF in rice roots in the different sampling sites of paddies ($p \leq 0.05$).

3.3. Identification of Adapted Indigenous AMF Species

Wang et al. [12] mentioned that some indigenous AMF species are adapted to the soil environment of paddy wetlands without considering the biotic and abiotic determinants. According to that, three different spore morphotypes were similarly distributed in all studied paddy wetlands (Figure 7), and those were identified as *Glomus* sp., *Claroideoglomus* sp., and *Aculospora* sp. using the INVAM culture collection as reference material.

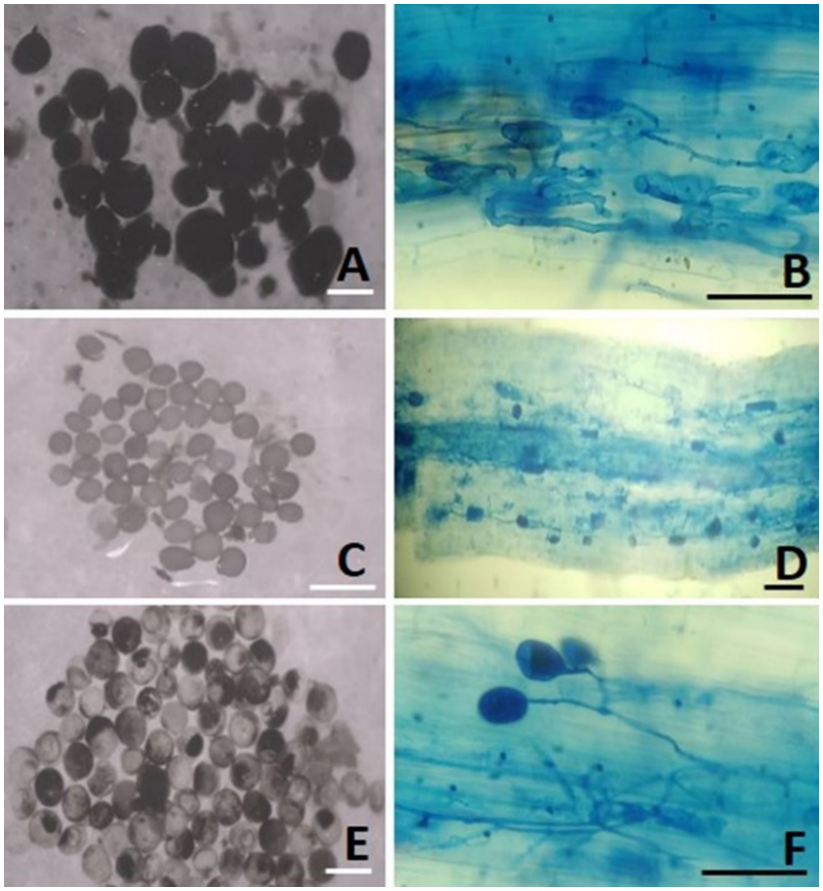


Figure 7. The paddy soil-adapted single species AMF spore isolates and their colonization patterns in one-month-old maize plant roots. (**A**) Spores of *Glomus* sp.; (**B**) Colonization patterns of *Glomus* sp. in maize roots.; (**C**) Spores of *Claroideoglomus* sp.; (**D**) Colonization patterns of *Claroideoglomus* sp. in maize roots; (**E**) Spores of *Aculospora* sp.; (**F**) Colonization patterns of *Aculospora* sp. in maize roots. Scale Bars: (**A**,**C**,**E**) = 100 µm and (**B**,**D**,**F**) = 50 µm.

For the production of the biofertilizer, these three different species were considered the AMF inocula because we assumed that the selected morphotypes were adapted to the paddy wetland conditions and could tolerate all ecological stress and confer their functional effect on rice plants. The colonization percentages of 78.6% of *Glomus* sp., 83.5% of *Claroideoglomus* sp., and 69.7% of *Aculospora* sp. in maize root inoculums were added when the fertilizer was prepared.

3.4. AMF Colonization in Rice Roots under Different Fertilizer Applications and Soil Conditions

The occurrence of AMF in rice roots was highly dependent on the growth stage of the host plant. In the 3rd week, AMF colonization was very rare or absent. When comparing the effects of the applied fertilizers and soil type at the highest colonization stage of the rice plant (9 weeks), AMF colonization differed according to the interactive effect of the fertilizer application and the soil type. The highest mean values of the AMF colonization were recorded in AMF biofertilizer applied treatments under non-sterilized soil conditions (Table 4). In the AMF biofertilizer application treatment, a significant difference between the two soil conditions was recorded. All other treatments under non-sterilized soil conditions or sterilized soil conditions do not reveal any significant difference. However, AMF colonization was different when considering the same treatment under two soil conditions.

Table 4. Effect of the different fertilizer applications on mycorrhizal root colonization of rice plant at the 9th week of the growth stage.

Treatment		AMF Colonization in the 9 Week (%)
Non-Sterilized	Control	10.425 ^{c}
	AMF-biofertilizer	26.675 ^{a}
	Biochar	8.300 ^{c}
	Compost	11.125 ^{c}
	Inorganic fertilizer	7.950 ^{c}
Sterilized	Control	0.000 ^{d}
	AMF-biofertilizer	21.275 ^{b}
	Biochar	0.000 ^{d}
	Compost	0.125 ^{d}
	Inorganic fertilizer	0.000 ^{d}

The means under each parameter followed by the same letter are not significantly different ($p \leq 0.05$) according to the Tukey pairwise comparisons. The values are the means, calculated from four replicates.

The AMF root colonization increased over the sampling time regardless of the fertilizer application and sterilized and non-sterilized soil conditions. Interestingly, the treatment with the application of AMF biofertilizer forms the highest AMF root colonization. In the AMF biofertilizer-added treatment, the applied AMF inoculums were effectively colonized in rice roots, with 23.97% average colonization rate under submerged anaerobic conditions. According to that, the application of AMF inoculum increased the AMF root colonization rate more than the normal rate of AMF colonization with rice roots.

3.5. Microbial Community Arrangement at 9 Weeks of the Rice Plant According to the Different Treatments

To test the synergistic interactions of AMF with other soil microbes and the results of those interactions, as well as the effectiveness of AMF on plant growth and productivity, two soil conditions were applied. To obtain the microbe-free soil, a set of soil was sterilized. As the formation of the highest spore density and colonization progressed, at 9 weeks, the AMF biofertilizer-treated rice pots formed the 51.5 $\pm$ 3.31 highest AMF spore density over other treatments (Table 5). When analyzing the morphotypes of the spores, the introduced paddy soil-adapted spores were more abundantly distributed than indigenous AMF species and increased the spore density compared to the spores present in the normal soil. In sterilized soil, the spore density was lower than in non-sterilized soil. In non-

sterilized soil conditions, biofertilizer treated soil formed the highest culturable fungi and bacterial count, and interestingly, the lowest fungi and bacterial count were recorded in biofertilizer-applied treatment under sterilized soil conditions.

Table 5. Total culturable bacteria, fungi, and AMF spore density in the soil 9 weeks after planting the rice plants in the pot experiment with different fertilizer applications under sterilized and non-sterilized soil conditions.

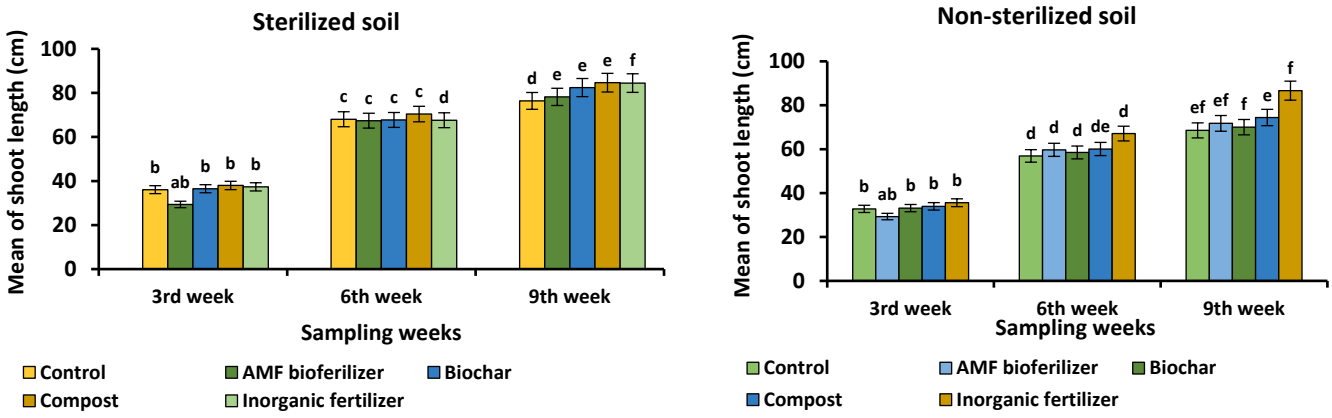
Treatment		Total Culturable Bacteria ($\times 10^{10}$)	Total Culturable Fungi ($\times 10^{5}$)	AMF Spore Density in Soil (spores/g)
Non-sterilized soil	Control	0.23 ± 0.10^{d}	15.00 ± 3.16^{b}	26.00 ± 2.38^{b}
	AMF-biofertilizer	45.50 ± 3.23^{a}	26.75 ± 2.66^{a}	51.50 ± 3.31^{a}
	Biochar	7.50 ± 1.71^{c}	4.50 ± 1.00^{c}	16.25 ± 1.38^{c}
	Compost	32.25 ± 4.59^{b}	$7.25 \pm 1.70^{b\,c}$	27.75 ± 2.46^{b}
	Inorganic fertilizer	3.00 ± 1.08^{c}	4.50 ± 0.65^{c}	29.50 ± 2.90^{b}
Sterilized soil	Control	4.25 ± 1.49^{d}	0.30 ± 0.30^{b}	0.15 ± 0.30^{b}
	AMF-biofertilizer	0.08 ± 0.20^{a}	0.14 ± 0.30^{a}	46.00 ± 6.49^{a}
	Biochar	5.50 ± 1.04^{c}	4.75 ± 1.03^{c}	4.675 ± 0.31^{c}
	Compost	5.00 ± 0.82^{b}	$4.75 \pm 0.75^{b\,c}$	6.50 ± 1.26^{b}
	Inorganic fertilizer	9.50 ± 1.71^{c}	5.25 ± 1.11^{c}	0.29 ± 0.05^{b}

Turkey pairwise comparison, Values are the means ± standard errors calculated from four replicates. a–d indicates the significant difference between each fertilizer treatment.

3.6. Effectiveness of the Produced Biofertilizer

3.6.1. Biometric Characters

In the beginning, when compared to the other fertilizer applications, AMF biofertilizer-applied plants formed the shortest plant shoot length. However, at 9 weeks, there was no significant difference found with other fertilizer applications, except for inorganic fertilizer applications. At the stage of highest plant growth (9 weeks), there was no interactive effect of fertilizer application and soil type on rice plant shoot length. The significant differences were found among treatments and the soil type, respectively, for shoot length at 9 weeks. The plants grown in sterilized soil conditions were formed with a significantly higher mean value for shoot length than non-sterilized soil (Figure 8).



(**A**) (**B**)

Figure 8. Mean values of shoot length according to different fertilizer applications at 3, 6, and 9 weeks after sowing rice seeds. (**A**) Rice plants are grown under sterilized soil conditions; (**B**) Rice plants are grown under non-sterilized soil conditions. Values are the means ± standard errors calculated from four replicates. a–f indicates the significant difference between each fertilizer treatment. (Tukey pairwise comparison).

With respect to shoot length, there was no interactive effect of fertilizer application and soil type on the number of tillers per rice plant (Figure 9). In the 9th week, inorganic fertilizer applied in pots and biofertilizer applied in pots did not form a significant difference in the number of tillers (Figure 10). The plant grown under sterilized soil conditions formed significantly more tillers than in non-sterilized soil (Figure 11).

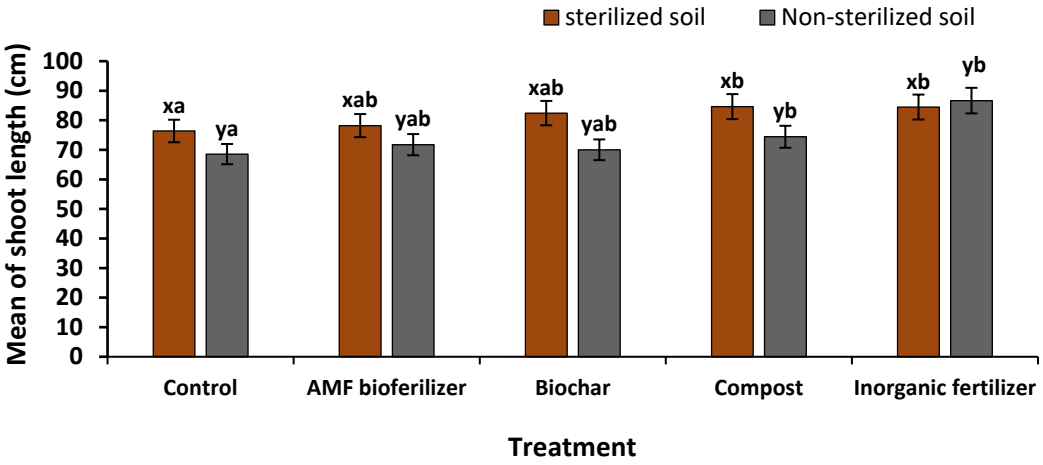


Figure 9. Mean values of the shoot length according to different fertilizer applications under sterilized and non-sterilized soil conditions at 9 weeks after sowing the rice seeds. Different letters above the columns indicate significant differences at the level of 0.05. Values are means ± standard errors. x/y indicates the significant difference in soil type, and a/b indicates the significant difference fertilizer treatments (Tukey pairwise comparison).

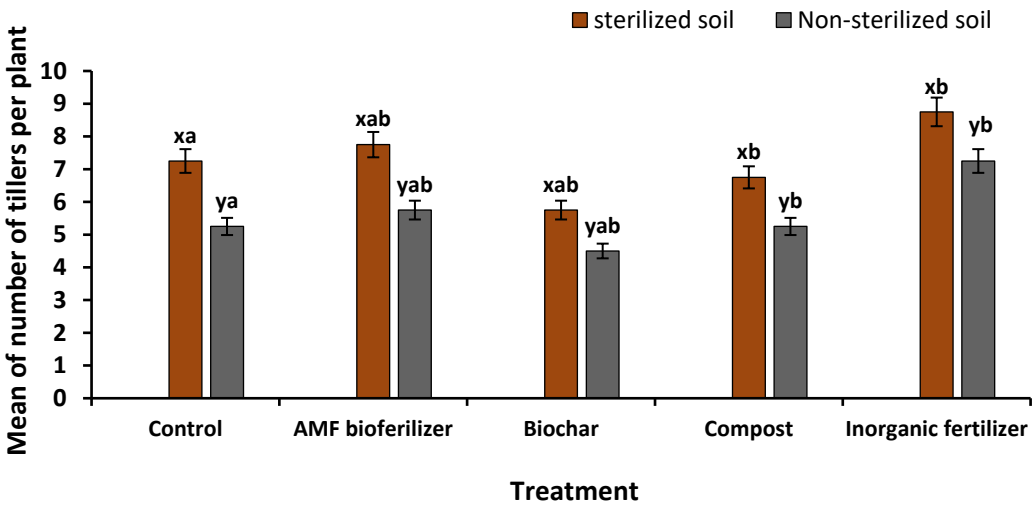


Figure 10. Mean values of the number of tillers per rice plant according to different fertilizer applications under sterilized and non-sterilized soil conditions at 9 weeks after the sowing of the rice seeds. Different letters above the columns indicate significant differences at the level of 0.05. Values are means ± standard errors. x/y indicates the significant difference in soil type, and a/b indicates the significant difference in fertilizer treatments (Tukey pairwise comparison).

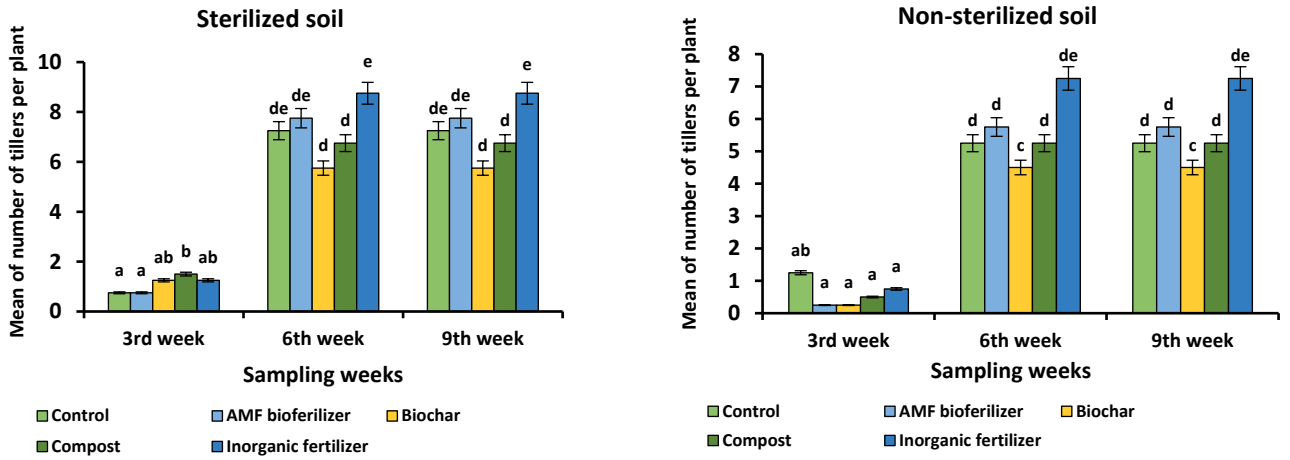


Figure 11. Mean values of the number of tillers per plant according to different fertilizer applications at 3, 6, and 9 weeks after sowing rice seeds. (**A**) Rice plants are grown under sterilized soil conditions; (**B**) Rice plants are grown under non-sterilized soil conditions. Values are the means ± standard errors calculated from four replicates. a–e indicates the significant difference between each fertilizer treatment (Tukey pairwise comparison).

3.6.2. Rice Yield

According to all investigated parameters, the plant under sterilized conditions grew significantly better than the non-sterilized soil (Table 6). Then, the final yield was higher in rice grown in sterilized soil without the effect of any fertilizer treatments that were applied (Figure 12), because there was no interaction effect between fertilization and soil type on the yield of the rice plant.

Table 6. Yield-attributing characters of the rice plant according to the different fertilizer applications under sterilized and non-sterilized soil conditions at 9 weeks.

Treatment		Number of Tillers	Number of Effective Tillers	Panicle Length (cm)	Number of Grains/Panicle	Plant Fresh Weight	Plant Dry Weight	100 Seeds Weight
Non-sterilized soil	Control	7.25 ± 0.63^{xab}	1.00 ± 0.41^{xc}	13.50 ± 4.57^{xc}	66.00 ± 22.23^{xc}	5.73 ± 0.23^{xd}	5.08 ± 0.37^{xc}	1.08 ± 0.21^{xb}
	AMF-Biofertilizer	7.75 ± 0.75^{xab}	2.00 ± 0.41^{xab}	23.90 ± 0.99^{xab}	119.75 ± 7.39^{xa}	8.53 ± 0.31^{xc}	7.1 ± 0.14^{xb}	2.51 ± 0.12^{xa}
	Biochar	5.75 ± 0.48^{xb}	1.00 ± 0.41^{xc}	15.00 ± 5.05^{xbc}	77.00 ± 25.77^{xbc}	10.78 ± 0.76^{xbc}	9.75 ± 0.61^{xab}	0.94 ± 0.19^{xb}
	Compost	6.75 ± 1.11^{xb}	1.25 ± 0.25^{xbc}	21.30 ± 0.49^{xabc}	109.75 ± 4.87^{xab}	10.85 ± 0.56^{xab}	9.08 ± 0.55^{xab}	2.25 ± 0.21^{xa}
	Inorganic fertilizer	8.75 ± 0.95^{xa}	3.25 ± 0.25^{xa}	26.10 ± 0.85^{xa}	130.25 ± 9.07^{xa}	13.3 ± 0.44^{xa}	10.55 ± 0.78^{xa}	3.26 ± 0.11^{xa}
Sterilized soil	Control	5.25 ± 0.48^{yab}	1.75 ± 0.25^{yc}	18.60 ± 0.48^{yc}	114.25 ± 2.52^{yc}	6.45 ± 0.33^{yd}	5.53 ± 0.25^{yc}	1.30 ± 0.00^{yb}
	AMF-biofertilizer	5.75 ± 0.63^{yab}	3.75 ± 0.48^{yab}	24.00 ± 0.54^{yab}	150.50 ± 10.12^{ya}	10.20 ± 0.16^{yc}	8.93 ± 2.23^{yb}	3.11 ± 0.00^{ya}
	Biochar	4.50 ± 0.50^{yb}	2.00 ± 0.41^{yc}	21.60 ± 0.96^{yabc}	117.75 ± 5.18^{ybc}	11.85 ± 0.32^{ybc}	10.18 ± 2.55^{yab}	1.80 ± 0.09^{yb}
	Compost	5.25 ± 0.48^{yb}	2.50 ± 0.5^{ybc}	21.00 ± 0.82^{yc}	155.25 ± 5.12^{yab}	12.6 ± 0.31^{yab}	11.45 ± 2.86^{yab}	3.50 ± 0.20^{ya}
	Inorganic fertilizer	7.25 ± 0.48^{ya}	4.25 ± 0.48^{ya}	27.70 ± 0.91^{yab}	172.75 ± 2.56^{ya}	14.25 ± 0.2^{ya}	12.5 ± 3.13^{ya}	3.54 ± 0.89^{ya}

The means under each parameter followed by the same letter are not significantly different ($p \leq 0.05$) according to the Tukey pairwise comparisons. Values are means ± standard errors. Values are the means ± standard errors calculated from four replicates. x/y indicates the significant difference in soil type, and a–d indicates the significant difference in fertilizer treatments.

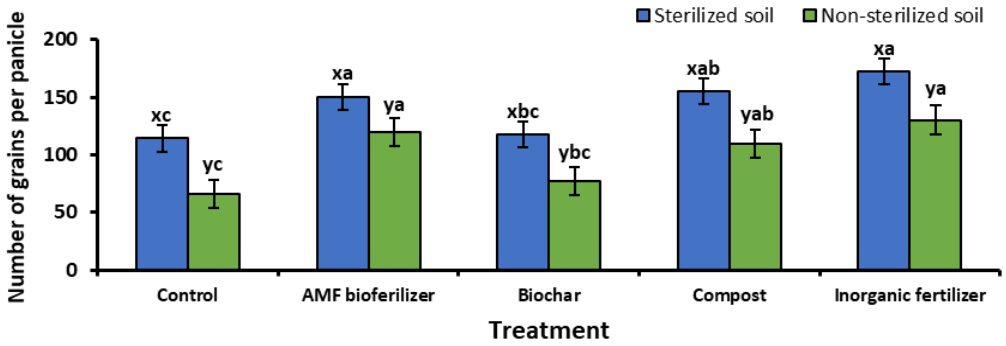


Figure 12. Mean values of the number of grains per panicle at 9 weeks after sowing the rice seeds according to different fertilizer applications and soil types. Different letters mentioned in the columns indicate significant differences at the level of 0.05. Values are means ± standard errors. x/y indicates the significant difference in soil type and a–c indicates the significant difference in fertilizer treatments (Tukey pairwise comparison).

Plants with inorganic fertilizer and biofertilizer reported the highest number of effective tillers without the formation of any significant difference. As with the control, the biochar-treated rice plants formed the fewest effective tillers (Table 6). Similar to the number of effective tillers, inorganic fertilization and biofertilizer applied plants formed the highest panicle length. Inorganic fertilizer, AMF biofertilizer, and compost treatment applied to rice plants recorded a similar mean number of grains per panicle without any significant difference. The lowest grain number per panicle was formed with both biochar and control treatments.

4. Discussion

Although transparent spore number and AMF colonization were low in rice plants, several studies have found that AMF colonization of rice plants produces a wide range of benefits to AMF plant partners, suggesting that AMF is a major component of sustainable agriculture [12,14]. In agreement with that, the application of AMF-based biofertilizer for rice plants formed a positive correlation with plant growth, productivity, and yield components. This study showed that the application of *Azospirillum* sp. provides a sufficient amount of nitrogen to plants other than what mycorrhizae provide. Mycorrhizae have wide distribution among plants and form different patterns of colonization and diversity in terms of biotic (variety of the plant, age of the host plant, rhizosphere and above-ground organisms, weeds related to the colonized host plants) and abiotic parameters (climate, moisture content, irrigation systems, latitude, C:N:P ratio in soil, soil types, soil acidity, geographical area, agricultural practices). To minimize the effect of other variables on the agro-ecological area, all samples were collected with the plant variety Bg350 under the control of organically managed fields at the flowering stage. Supporting those findings, in this study also AMF diversity, spore density, and colonization were varying among the studied agro-ecological areas; higher spore density was found in the dry zone paddies in Sri Lanka, and there was a positive correlation found between spore density and the AMF root colonization ($p = 0.046$). Baki et al. [28] mentioned that the colonization of AMF was higher under slightly acidic soil conditions. According to my study, AMF colonization does not form any significant relationship with the soil pH and electric conductivity. The soil texture is the main influencing factor for mycorrhizal growth and diversity. The clay content in soil was inversely proportionate to the spore count [29]. All of the investigated paddy fields represent soil with a higher clay content, and it can be suggested that, due to the low level of porosity in clay soil, more O_2 can't be reached the rice roots than in other soil types. It can also affect the lower colonization of AMF in rice roots. In the rhizosphere, the presence of mycorrhizae can change the soil microenvironment by producing components that are favorable to the growth of the related microbial community [30]. Negatively, in my study, there was no significant relationship with the total culturable bacterial and

fungal count in the rhizosphere soil. In the dry zone, the total fungal count was positive, and in the intermediate zone, the total bacterial count was negatively correlated with the AMF colonization.

Theoretically, AMF was effectively colonized in the vegetative stage rather than the reproductive stage because the rice plant has a role in nutrient uptake into the plant before entering the reproductive stage [14]. Moreover, with the development of the aerenchymatous tissues in the heading and ripening stages, the plant can receive more O_2, and AMF can form a higher colonization rate at that time. By supporting that theory, in this study, the colonization was increased over the sampling time without considering the treatments applied. The application of AMF inoculums to the rice plants increased the colonization rate nearly three-fold higher than the rate of natural colonization at 9 weeks. To resolve the problem of lower AMF colonization in rice, these results give a possible solution. In this study, the AMF species that are adapted to the paddy soil for the Bg 350 cultivar were selected. However, with the genotype of the plant, the diversity and distribution of AMF species can vary [31]. According to those genotypes, a specific AMF should be added. Otherwise, the application of the AMF species that are commonly distributed over all rice varieties is the best application strategy. For that, further studies are needed.

Sooksa-nguan et al. [32] reported changes in microbial communities in which differences in rice cultivation systems affected the structures of bacterial and archaeal communities. At the highest root colonization and AMF spore density stage of rice plants (9 weeks) under non-sterilized soil conditions, the total culturable bacteria and fungi count were highest in AMF biofertilizer-added pots, and the microbial count was lowest under sterilized soil conditions. Under the microbe-free condition of sterilized soil, AMF became the dominant species after the introduction of AMF as a biofertilizer. Then, AMF competes with exogenously added bacteria and fungi, thereby lowering the other bacterial and fungal communities. Supporting this point, the spore density of sterilized AMF biofertilizer added soil formed the highest spore number of other fertilizer application treatments. Under non-sterilized soil conditions, the AMF formed a synergistic interaction with microbes that were already present in the soil and formed the highest culturable bacterial and fungal count. Under non-sterilized soil conditions, AMF formed a higher AMF spore density and colonization percentage than in sterilized soil conditions, and this happened due to the synergistic interaction.

Application of compost in the field could change factors such as pH, nutrient content, total soil C and N, and temperature in the soil and confer favorable conditions for AMF and increase the diversity and hyphal growth [33]. In this study, the root colonization in compost-treated plots did not show any significant difference from the control treatment. There was no correlation between AMF root colonization and fertilizer application alone other than the interactive effect of both fertilization and soil type. Interestingly, in the pot experiment, the plants maintained under sterilized soil conditions formed greater plant growth in terms of plant height and the number of tillers and maintained better greenness without considering the fertilizer applications when compared to the non-sterilized soil. Mahmood et al. [34] demonstrated that soil sterilization significantly increased the electric conductivity and water-soluble carbon, and further, root biomass and rhizosheath soil biomass were increased. Herein, we can suggest that the plant-unavailable nutrients present in the soil can be converted into plant-available forms with the applied temperature and pressure for the soil autoclaving. As a morphological observation, revealed that without the effect of the soil condition, AMF biofertilizer-treated plants maintained better greenness than all other rice plants. Diagne et al. [35] and Sagar et al. [36] stated that when AMF colonizes plants with a low photosynthesis rate, the absorption of the Mg^{2+} ions increases and upregulates the chloroplast genes' (*RppsbA* and *RppsbD*) expression in the PSII system to increase the rate of photosynthesis.

The high level of AMF colonization and spore density in the AMF biofertilizer added to rice plants positively correlated with a clear increase in yield components, such as the number of effective tillers per plant, panicle length, and the number of grains per panicle.

AMF inoculation increased the allocation of additional N and P to panicles. The rice seedlings inoculated with AMF increased the rice yield, which is related to the increase in the panicle number [37]. With that additional access to receiving the nutrition to panicles, the plant can increase productivity and grain yield [38]. Interestingly, inorganic fertilizer, AMF biofertilizer, and compost-added plants under sterilized soil conditions formed the highest yield in rice plants, and this work will positively support the establishment of green agriculture instead of chemical fertilizer applications.

5. Conclusions

This study confirmed that AMF spore density, colonization, and diversity differed according to the ecological zones in Sri Lanka, and root colonization was positively correlated with the spore density. Application of paddy soil-adapted AMF isolates highly increased the root colonization and spore density in rice plants under submerged soil conditions, thereby clearly increasing the plant's growth, productivity, and yield. Thereby, AMF has the potential to optimize the yield of rice plants under sustainable conditions. The application of AMF biofertilizer increased the rice yield two-fold when compared to the control treatment. Plants cultivated under sterilized soil conditions increased all growth parameters and yield components without the effect of fertilization in rice cultivation. With the absence of a significant difference between AMF biofertilizer and inorganic fertilization, this work added value to green agriculture. From our point of view, screening the effectiveness of the produced biofertilizer through field experiments is much needed. AMF forms both positive and negative relationships with the nearest plants, including weeds, above-ground organisms, and microbial communities. Interestingly, AMF forms the underground common mycorrhizal network (CMN) that enhances the effect of the mycorrhizae on the host plant. If researchers can find the AMF species that are adapted to the paddy soil and all varieties of rice is widened, the industrial applicability of biofertilizers produced will be island-wide.

In the present study, we applied a one-day-old, prepared biofertilizer to the rice. However, further studies are needed to find out how to increase the storage time of the produced biofertilizer and what strategies can be applied to increase the viability of AMF inoculum. For industrial applications, mass production of AMF inoculums will be required. As a better alternative to the trap culture establishment, the application of the callus culture methods used in tissue culture can be applied to trap the AMF into cells present in the callus. As a development step, the addition of the other bacteria (PGPR) and fungi (PGPF) with the AMF as a consortium of microbes into rice can positively increase the effectiveness of the biofertilizer. The studies have shown that AMF doubles its effect on the plant when they are added in a consortium with other microbes.

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