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The use of soil biostructures created by soil fauna ecosystem engineers fed with different organic materials as inoculum source of arbuscular mycorrhiza fungi on cocoa seedling

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ABSTRACT

Soil fauna as ecosystem engineers has the ability to create soil biostructures, with the capacity to save arbuscular mycorrhizal fungi (AMF) spores. This study, therefore, aimed at investigating the AMF spore density in the biostructures created by cooperation between earthworms and ants with a different organic matter composition, and to analyze the biostructures' potential as a source of AMF inoculum on cocoa seedlings. In the first experiment, a combination of earthworms (0, 10, or 20 pieces) and ants (0, 10, or 20 pieces) composition, as well as a mixture of *Gliricidia sepium* leaves (GSL), cocoa shell bean (CSB), and sago dregs (SD)(w/w/w) was tested. Meanwhile, in the second experiment, the effect of biostructures on cocoa seedlings grown in unsterile soil was examined. According to the results, the highest (46.67±13.65) AMF spore density was obtained using 20 earthworms+10 ants with 50%GSL+50%CSB + 0%SD treatment, the lowest (12.67±3.78) spore count was obtained using 20 earthworms+10 ants with 25%GSL+25%CSB+50%SD. The total AMF spores were positively correlated (r^2 = 0.74) with the total P, but negatively correlated (r^2 = -0.53) with the C/N ratio. Therefore, biostructure application increased AMF spores number in the rhizosphere and the percent infection. Furthermore, biostructures resulting from the collaborative activity between different soil fauna ecosystem engineers were able to facilitate the germination of AMF spores and infect plant roots growing in non-sterile soil.

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1. INTRODUCTION

Soil fauna as an ecosystem engineer has the capacity to create a new aggregate of soil containing numerous microbes useful for agriculture, forestry, and the environment (Jouquet et al., 2012). A study by Taylor et al. (2019) categorized earthworms and ants as soil ecosystem engineers. New aggregates, also known as biostructures, are created from these organisms' activities while mixing organic matter with soil mineral particles (Zanella, Ponge, Topoliantz, et al., 2018). The form of the biostructure created depends largely on the type of soil fauna concerned (Forey et al., 2018). Biostructures formed from the activity of earthworms are known as cast, while counterparts generated by the activity of ants are known as nests or soil mounds (Bottinelli et al., 2015; Cunha et al., 2016). Both types have the ability to provide energy-rich substrates containing signaling molecules, to trigger soil microbial activity and growth of arbuscular mycorrhiza fungi (Kilowasid et al., 2015; Lavelle et al., 2016; Schultz et al., 2015; Shukla et al., 2016; Wills & Landis, 2018). The symbiotic-mutualistic association between AMF and crop roots contributes significantly to expansion in the range of rooting, in order to access soil nutrients and water, and consequently, help plants meet growth needs (Powell & Bagyaraj, 2017).

The use of AMF as biological technology, in soil quality and fertility improvement, has the potential to minimize commercial N and P fertilizer use in the production of food crops, plantation seedlings, and horticulture (Cobb et al., 2018). Indonesia is known as one of the world's cocoa bean producers, where cocoa beans are produced from small farmer plantations. Generally, the farmer's cocoa trees are old, and production continues to decline (Directorate General of Estates, 2019). Thus, there is a need to replace aging plants, with seedlings that have the ability to adapt to poor soil fertility and low groundwater availability. According to Bahrun et al. (2018), most small farmers grow cocoa seedlings in polybags filled with unsterilized. Adaptation to poor soil fertility and water deficit conditions of cocoa seedlings can be further improved by the application of AMF spore inoculum (Moreira et al., 2018; Seutra Kaba et al., 2021). Azizah Chulan (1991), reported the inoculation of AMF spores (*Scutellospora calospora*) produced from a mixture of soil and infected root pieces significantly increased nutrient absorption and growth in cocoa seedlings grown in sterilized soil.

The supply of soluble carbon in the substrate significantly determines AMF spores' ability to germinate and produce infective spores under conditions in the absence of plant roots (Kameoka et al., 2019; Rillig et al., 2020; Sugiura et al., 2020). In addition, AMF's sporulation and ability to colonize the roots are largely determined by the ratio of N:P substrates (Mei et al., 2019). Earthworms and ants are able to jointly create soil biostructures with higher microbial activity, compared to the soil not influenced by the organisms' activity (Franco et al., 2017; Kilowasid et al., 2015) The dissolved carbon, N, total P, pH, and soil microbial population contents from biostructures produced by the activities of earthworms and ants, are strongly influenced by the type of organic matter consumed by the organisms (Ehrle et al., 2019; Wang et al., 2019; Zanella, Ponge, & Briones, 2018). Fresh biostructures created by earthworms (*Lumbricus terrestris* L.) and black ants (*Camponatus compressus* Fabr.) contain populations of infective mycorrhiza spores (Harinikumar & Bagyaraj, 1994; Lee et al., 1996). However, further studies on the biostructure potential of ecosystem-engineered soil fauna to develop inoculum mycorrhiza arbuscular infective production system options for effective application at the farmer level in tropical environments, are required. Thus, this study aimed to study the AMF spore density in biostructures created by cooperation between earthworms and ants on soil enhanced with a composition of different organic matter types, as well as to analyze the biostructures potential, as an inoculum source of AMF spore on cocoa seedlings.

2. MATERIAL AND METHODS

Two experiments were conducted in this study. The first was to study the AMF spore density in biostructure created by cooperation between earthworm and ants on soil enhanced with a composition of different organic matter types. On the other hand, the second experiment aimed to analyze the biostructures' potential, as an inoculum source of AMF spore on cocoa seedlings.

2.1. First Experiment

2.1.1. The site and experimental design

This first experiment was performed under standing cocoa trees in smallholder plantations from October to January 2018, located at 122°31'10.0" E; 04°08'20.5" S, and 60 m above sea level, in Tanea Village, Konda District, Konawe Selatan Regency, Southeast Sulawesi, Indonesia. In this experiment, fifteen different treatments (Table 1) of earthworm and ant (individual/individual) proportions, and with a mixture of three different organic matter (OM) types (weight: weight: weight) were tested. Organic matter includes *Gliricidia sepium* leaves (GSL), cocoa bean shell (CBS), and sago dreg (SD) on three levels. Soil fauna had three levels: 0, 10, or 20 individuals per reactor while OM type had also three (0, 25, and 50%) levels. Each treatment had three replicates, following a simple randomized complete block design (RCBD).

2.1.2. Preparation and application of the treatments

Earthworms (*Peryonex* sp.) were obtained from riverbanks around the field experiment station, Faculty of Agriculture, Halu Oleo University (located in the Kampus Hijau Bumi Tridharma, Jl. H.E.A. Mokodompit, Kendari 93232 Indonesia), while ants (*Dorylus* sp.) were collected from smallholder cocoa plantations (located in the Dusun 3, Tanea Village, Konda District, Konawe Selatan Regency, and owner of the plantation is Pak Sapari), both through hand sorting techniques. Sago dregs were collected from a sago processing area (located in the Moramo district, Konawe Selatan Regency), cocoa bean shells were obtained from Kalla Kakao Industry (located in the Ranomeeto district, Konawe Selatan Regency), and gliricidia (*G. sepium*) leaves were pruned from the home garden located in the Kambu district, Kendari City.

Table 1. The treatment combinations of soil fauna proportions and mixtures of three different organic matter types.

Symbol of treatments	Description
F0B0	without soil fauna and organic matter
F1B0	10 earthworms+20 ants and without organic matter
F1B1	10 earthworms+20 ants with 25% GSL+50% CSB+25% SD
F1B2	10 earthworms+20 ants with 50% GSL+25%CSB+25%SD
F1B3	10 earthworms+20 ants with 25% GSL+25% CSB+50% SD
F1B4	10 earthworms+20 ants with 50%GSL+50%CSB+0% SD
F1B5	10 earthworms+20 ants with 50% GSL+0%CSB+50%SD
F1B6	10 earthworms+20 ants with 0% GSL +50% CSB+50% SD
F2B0	20 earthworms+10 ants and without organic matter
F2B1	20 earthworms+10 ants with 25% GSL +50% CSB+25% SD
F2B2	20 earthworms+10 ants with 50% GSL +25% CSB+25% SD
F2B3	20 earthworms+10 ants with 25% GSL+25%CSB+50%SD
F2B4	20 earthworms+10 ants with 50% GSL+50%CSB + 0%SD
F2B5	20 earthworms+10 ants with 50% GSL +0% CSB + 50% SD
F2B6	20 earthworms+10 ants and 0% GSL +50% CSB+50% SD

Remarks: GSL = *G. sepium* leaves; CSB = cocoa bean shell; SD = sago dreg.

All the collected materials (sago dregs, cocoa bean shells, and leaves of *G. sepium*) were oven-dried, pulverized using a kitchen blender, and sieved in a wire mess with a <2 mm pore opening (Sanjaya et al., 2020). One sample of each OM was analyzed for chemical attributes include organic carbon using Walkley-Black method, total-N using the Kjeldahl method, C/N ratio, total-P, and total-K prepared using the wet-digestion method (Vogt et al., 2015). Table 2 shows the chemical attributes of each collected organic material.

Furthermore, soil (0-10 cm depth) was obtained from a smallholder farmer cocoa plantation aged over 15 years old in the Konda District, South Konawe regency. Subsequently, the soil samples were wind-dried and sieved using a < 4mm sieve pore opening. A total of 100 g of each OM composition (treatment) was then mixed with 1.5 kg of soil in a block-shaped reactor comprised of a multiplex board, measuring 25 cm x 21 cm x 21 cm (Figure 1). The substrate in each reactor was then watered with tap water until saturated and left until no water drips through the five small holes on the reactor's bottom surface (Sanjaya et al., 2020). It took about 24 hours to get the substrate moisture condition that allow the mobility of ants on the substrate surface in the reactor.

Earthworms measuring 5-6 cm in length were released on tissues paper surface moistened with tap water and left until no more casts were released from the anus. After emptying stomach contents, earthworms with an individual number according to the treatment (Tabel 1) were released into each soil surface's of the mixture of SD, CBS, and GSL referred onward as organic material. Subsequently, the number of individual ants according to the treatment (Table 1) were released and allowed to enter into the substrate, after the earthworms' bodies were wholly entered into the substrate. Each reactor's entire surface was then covered with a <2 mm wire mesh green plastic gauze to prevent earthworms and ants from escaping to leave the reactor and to prevent other organisms from accessing the reactor (Figure 1). This was followed by placing all reactors on plank pads 40 cm above the ground in a simple structure with a sago leaf roof built under ± 10-year-old cacao stands (Figure 2). Moisture content (about 35% measured with a soil moisture meter, Lutron PMS 714 type) was maintained by spraying 50 ml of tap water on the entire surface of the soil mixture's every two days.

2.1.3. Estimation of total AMF spore in the biostructures

After 28 days of incubation, the soil was removed from the reactor, then earthworms and ants were separated from the soil by hand-sorting technique. The soil was air-dried under room conditions, where soil samples were taken to assess the total AMF spores and each reactor's structural chemistry. In addition, a total of 50 g of biostructure from each reactor was poured into a container containing 500 ml of tap water and stirred to obtain a homogeneous mixture. The suspension was then filtered using 3 series of sieves with sizes of 2 mm, 200 µm, and 38 µm, from top to down, respectively. Materials retained on the 200 µm and 38 µm filters were poured into the tubes containing 20%/60% concentration of sugar solution. At the end of the spin, the supernatant was poured onto the 38 µm sieve and rinsed with water until the sugar solution was drained from the sieves. The retained spores were transferred into plastic Petri dishes (with 85 mm in diameter) with an arrangement of gridline (size 1 mm x 1 mm), and counting of AMF spores was done under a dissecting microscope (INVAM, 2019). The total number of AMF spores in each biostructure was then estimated following instructions stated in the INVAM.WVU.EDU. Briefly, the number of AMF spores was counted in 20 randomly selected field views.

Figure 1: Reactor used for the experiment

Figure 2. Reactors placed inside sago hut under ± 10-year-old cacao stands

Table 2. Chemical attribute of organic matter.

Organic matter type	Parameters				
	Organic Carbon (%)	Total-Nitrogen (%)	C/N ratio	Total-Phosphorus (%)	Total-Potassium(%)
Cocoa shell bean	2.62	0.58	4.52	5.32	106.42
G. sepium leaf powder	2.52	1.03	2.45	5.09	114.16
Sago dregs	2.73	0.53	5.16	3.57	8.08

Table 3. Soil physicochemical character of soil growing medium where cocoa seedlings were planted.

Parameter	Method	Unit	Value
Soil fraction:			
Sand	Pippete	%	38.19
Silt	Pippete	%	36.69
Clay	Pippete	%	25.21
$pH_{H2O\ (1:5)}$		-	6.81
$pH_{KCl\ (1:5)}$		-	6.14
C-org	Walkey & Black	%	2.77
Total-Nitrogen	Kjeldahl	%	0.49
Total-Phosphorus	HCl 25%	Ppm	871.32
Available-Phosphorus	Olsen	Ppm	130.40
Ca	NH4OAc (pH 7.0)	$cmol^{(+)}/kg$	8.27
Mg	NH4Oac (pH 7.0)	$cmol^{(+)}/kg$	2.77
K	NH4Oac (pH 7.0)	$cmol^{(+)}/kg$	2.46
Na	NH4Oac (pH 7.0)	$cmol^{(+)}/kg$	0.21
CEC	NH4Oac (pH 7.0)	$cmol^{(+)}/kg$	16.52
Base saturation		%	82.98
Al	KCl 1N	$cmol^{(+)}/kg$	not detected
H	KCl 1 N	$cmol^{(+)}/kg$	0.13
Fe	DTPA	Ppm	65.18
Cu	DTPA	Ppm	3.60
Zn	DTPA	Ppm	32.48
Mn	DTPA	Ppm	69.99

The total field views were determined based on the ratio of the area of the plastic petri dish to the view field area of the ocular dissecting microscope at a magnification, where the AMF spores can be distinguished from other objects. The total AMF spores in suspension were estimated utilizing the average of AMF spores per field view multiplied with the total field views in plastic Petri dishes used. Furthermore, the total AMF spores were expressed as total AMF spore per biostructure weight where the spores were extracted.

2.1.4. Chemical characterization of the soil biostructures

The remaining biostructures' in the reactors were wind-dried for 48 hours inside a well-ventilated room, then sieved using a <4 mm sieve pore opening, then subjected to chemical analysis, including pH using a pH meter with a 1:5 (w/v) soil-water ratio, C-org using the Walkley-Black method,total-N using the Kjeldahl method, C/N ratio, total-P and total-K using a 25% HCl extractor (Vogt et al., 2015).

2.2. Second Experiment

2.2.1. The site and experimental design

The second experiment was conducted in Tanea Village, Konda District, Konawe Selatan Regency, Southeast Sulawesi, from February to May 2019. In this experiment, physiologically ripe criollo cocoa pods were obtained from local farmers' cocoa nurseries in Konawe Selatan Regency, Southeast Sulawesi. Cocoa beans were removed from the pods, and pithy, healthy, large beans (about 2.5 cm in length) were selected. The seeds were mixed with ashed rice husk, kneaded until the pulp of the seed's surface coating was exposed, cleaned under running water, then the cleaned seeds were laid on the carbonized rice husk to germinate, and left to grow until roots emerged.

Table 3 shows the physicochemical characteristics of the soil used as a growing medium in this study. For this experiment, a total of 500 g of soil from the cocoa plantation was mixed with goat manure in a 2:1 ratio (v/v), placed in a polybag, and soaked in a container filled with water, until the entire surface had been inundated. The bags were removed from the container after air bubbles had stopped emerging from the soil and then left overnight. A total of 100 g of each biostructure produced from the first experiment was spread on the soil surface on each polybag. The germinated three-day-old cocoa seedlings were transplanted into polybags filled with soil+goat manure and biostructure, produced from the reactor. The experiment was established in the nursery following RCBD with three replicates. The nursery is made of wood, with a roof made of woven sago leaves, and <2 mm mesh nylon net walls. The seedlings were maintained for 12 weeks.

2.2.2. Growth measurement of cocoa seedling

At 2, 4, 6, 8, 10, and 12 weeks after planting (WAP), the plants' height was measured, and the leaves were counted. At the end of the experiment, the seedlings were separated into shoot and root parts. The shoots were oven-dried at 60°C for 48 hours, and the dry weight was measured.

2.2.3. Estimation of total AMF spore from rhizosphere and root infection

Soil samples were obtained from the rhizosphere area for total AMF spore counting, using the procedure mentioned above. Meanwhile, the roots were washed with tap water, soaked in 10% KOH, and oven-dried at 90°C for 20 minutes. The roots were then rinsed with water, immersed in H_2O_2 for 12 hours, and the procedure was repeated once again. Roots washed from the H_2O_2 were stained in 0.05% aniline blue solution, and AMF infection on stained root pieces were observed under a microscope (Dhar & Mridha, 2012).

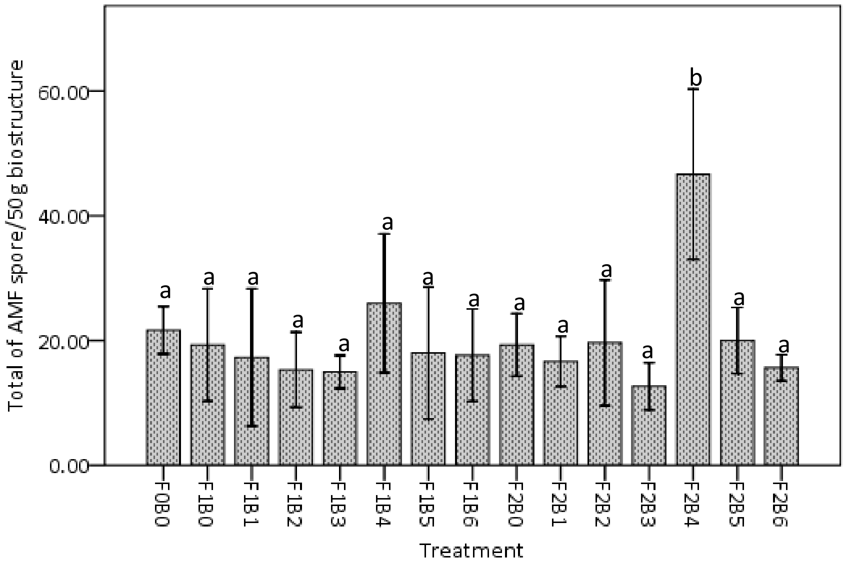

Figure 3. Number of AMF spores in biostructure produced from different treatments.

Table 4. Bivariate Spearman's r^2correlation (p < 0.05) between the AMF spore number and the biostructure's chemical attribute.

	pH	Organic C	Tot-N	C/N	Tot-P	Tot-K	AMF spore
pH		-0.41	-0.41	0.19	0.00	-0.11	-0.12
Organic C			0.46	0.00	0.33	0.16	0.24
Tot-Nitrogen				-0.84	0.29	0.46	0.40
C/N					-0.28	-0.49	-0.53
Tot-P						0.35	**0.74**
Tot-K							0.25

2.3. Statistical Analysis

The data were subject to analysis of variance, and the means of treatments were compared using Duncan's Multiple Range Test (DMRT) also correlation test, at a significance level of p <0.05.

3. RESULTS

The results showed a significant difference in the number of AMF spores within biostructures created by collaborative activity between earthworms and ants fed with a mixture of three types of OM (Figure 3). Soil from the cocoa plantation treated with F2B4 (20 earthworms+10 ants with 50%GSL+50%CBS and no SD) treatment produced a biostructure containing the highest total AMF spores (47 spores / 50 g of biostructure) (Figure 3). The total AMF spores in biostructures produced from the F2B4 treatment differed significantly from other treatments (Figure 3). The lowest spore count was obtained in F2B3 (20 earthworms+10 ants with 25%GSL+25%CSB+50%SD) (Figure 3).

Spearman's rs bivariate correlation showed the number of AMF spores had a significant positive correlation with total P (r^2 = 0.74 at p <0.05), but was insignificant with pH (r^2= -0.12), total N (r^2= 0.40), C-org (r^2 = 0.24), total K (r^2 = 0.25), and with negative (r^2 = -0.53) correlation with C/N ratio at p> 0.05 level (Table 4).

The height of cocoa seedlings treated with soil without soil fauna and organic matter (control) was higher, as compared to counterparts treated with soil from biostructure (Table 5). At 2 and 4 weeks after application, the height of cocoa seedlings inoculated with F0B0 was the tallest (9.88±2.14cm and 20.54±2.69cm, respectively) and this differed significantly (Table 5) with F1B5 (10 earthworms+20 ants with 50% GSL+ 0% CSB + 50% SD) and F1B0 (10 earthworms + 20 ants and without organic matter) treatments, but comparable with the other treatments (Table 5). At 6 weeks after biostructure application, there was a significant difference (Table 5) in height, between F0B0 (control) seedlings, applied with biostructures in treatments F1B5 (10 earthworms+20ants with 50% GSL+0%CSB+50%SD) and F2B1 (20 earthworms+10 ants with 25%GSL+50%CSB+25%SD) (Table 4). At 8 weeks after application, a significant difference in seedling height was observed between the control, as compared to F1B5 (10 earthworms+20ants with 50% GSL+0%CSB+50%SD) and F2B1 (20 earthworms+10 ants with 25%GSL+50%CSB+25%SD) treatments. This observation on F1B5 and F2B1 treated seedlings was carried over at weeks 10 and 12.

The number of leaves of cocoa seedlings at 2, 6, 8, 10, and 12 weeks after biostructure application followed the same trend as in plant height (Table 6). Cocoa seedlings treated in F0B0 biostructure, gave consistently the highest number of leaves throughout the 12 week observation period except at week 4 where there was no significant effect of the treatment on the number of leaves of cocoa seedling grown in the 15 treatment combinations (Table 6).

Table 5. Cocoa seedling height (cm) during the twelve weeks growth period, after biostructure application.

Biostructure	week after biostructure application					
sources	2	4	6	8	10	12
F0B0	**9.88±2.14c**	**20.54±2.69b**	**21.74±2.14c**	**21.94±1.76c**	**22.88±2.03c**	**25.17±2.33c**
F1B0	8.44±0.58bc	19.08±0.70b	19.47±1.00bc	20.11±1.07bc	21.10±1.24bc	22.22±1.70bc
F1B1	7.92±2.92bc	18.61±3.06b	18.88±3.00bc	19.00±3.00bc	20.18±3.04bc	22.17±4.02bc
F1B2	7.04±1.03abc	18.90±1.15b	19.00±1.09bc	20.00±1.52bc	20.55±0.58bc	21.98±1.00bc
F1B3	6.84±0.63abc	18.08±0.57b	18.50±0.50bc	19.22±0.50bc	20.33±0.60bc	21.36±1.04bc
F1B4	6.28±1.93abc	17.50±2.28ab	17.83±1.04bc	18.88±1.38bc	19.78±1.41bc	21.93±2.69bc
F1B5	**4.08±1.17a**	**13.13±6.06a**	**13.22±6.03a**	**13.55±6.43a**	**14.33±6.80a**	**15.03±7.41a**
F1B6	7.92±2.17bc	17.80±0.40ab	18.44±0.91bc	19.05±0.58bc	19.25±0.50bc	20.77±0.62bc
F2B0	7.83±0.79bc	17.55±1.45ab	17.66±1.52bc	18.22±1.50bc	18.75±1.50bc	19.36±1.84abc
F2B1	5.94±1.77abc	15.22±3.50ab	16.17±4.04ab	16.55±4.28ab	16.90±4.25ab	17.97±4.55ab
F2B2	7.12±2.06abc	17.16±3.23ab	18.00±2.64bc	19.11±1.01bc	20.31±1.08bc	21.56±2.03bc
F2B3	6.22±3.53abc	16.23±3.20ab	17.76±1.18bc	18.22±0.96bc	19.21±0.76bc	20.24±0.88bc
F2B4	5.66±2.39ab	17.51±1.45ab	18.16±1.69bc	18.22±1.07bc	18.84±1.49bc	19.96±1.44bc
F2B5	6.70±0.30abc	17.33±0.51ab	17.83±0.92bc	18.22±0.50bc	19.05±0.25bc	20.60±0.82bc
F2B6	7.30±0.87abc	16.75±1.43ab	17.20±0.80bc	17.33±0.88bc	18.58±1.60bc	20.23±2.28bc

Remarks: Numbers (mean ± sd. n = 3) followed by different letters in the same column indicate significant differences, according to Duncan's Multiple Range Test (DMRT)at the p <0.05 level.

Table 7 shows the shoot dry weight, AMF spores count in rhizospheric soil and percentage of infected roots at 12 weeks after biostructure application. The highest (38±7.40 AMF spores per 50 g biostructure)number of AMF spores were obtained from the soil treated with F1B6 biostructure (10 earthworms + 20 ants with 0% GSL+50% CSB+50%SD), and this differed significantly from those in the F0B0 [(no fauna and OM) (16±4.70 spores per 50 g soil)], F1B0 [(10 earthworms+20 ants and without OM)(19±4.63 spores per 50 g soil)] and F1B4 [(10 earthworms+20 ants with 50% GSL+50%CSB+0% SD)] which had the lowest(15±7.58 AMF spores per 50 g soil) (Table 7). AMF infected roots and shoot dry weights obtained from each biostructure treatment were not significant from each other (Table 7).

4. DISCUSSION

In this study, the total number of AMF spores was observed to vary between the biostructures created, and this variation is probably related to variations in physicochemical properties (Alimi et al., 2021; Asano et al., 2021). These variations reflect the quality of organic material consumed by the soil fauna (Briones, 2014). The availability of organic C, N, P, and K is possibly suitable to stimulate spore AMF growth in these biostructures (Salim et al., 2020). The correlation analysis shows the total number of AMF spores was negatively insignificant, compared to the biostructures' pH. The correlation with organic C, total-N, C/N ratio, and total-K is positively insignificant (Table 4). Several studies also reported an insignificant relationship between total AMF spore density with soil pH, organic C, total-N, C/N ratio (Sivakumar, 2013; Verzeaux et al., 2017). Wang et al. (2015) reported the relationship between AMF spore density and total-P soil was insignificant, whereas in this study a positive significant correlation was found (r^2 = 0.74) at the p < 0.05 level (Table 4). There is a positive significant relationship in this study, possibly related to the ecological character of the AMF taxa in the soil biostructure (Melo et al., 2019).

Table 6. Periodic number of cocoa seedling leaves during the 12 weeks growth period after biostructure application.

Biostructure	Week after biostructure application					
sources	2	4	6	8	10	12
F0B0	**2.77±0.69c**	**3.55±0.83a**	**6.00±0b**	**7.66±0.88b**	**7.77±1.07b**	**9.55±1.57b**
F1B0	2.55±1.01bc	3.88±0.38a	5.66±0.33b	6.88±0.50ab	6.88±0.50ab	7.88±2.21ab
F1B1	1.44±1.50abc	3.77±0.38a	5.88±0.50b	6.55±1.07ab	7.33±0.88b	7.77±1.64ab
F1B2	1.00±0.00abc	3.55±0.19a	5.55±0.50b	5.77±0.69ab	6.77±1.34ab	7.11±2.00ab
F1B3	0.55±0.50a	3.66±0.33a	5.33±0.57b	6.44±0.69ab	7.00±1.33ab	8.11±2.69ab
F1B4	0.88±0.83abc	3.66±0.33a	5.44±0.50b	7.11±1.17b	7.22±1.01b	8.11±1.50ab
F1B5	0.33±0.33a	2.88±1.64a	3.88±2.03a	4.88±2.91a	5.00±2.88a	5.22±3.15a
F1B6	1.44±1.26abc	4.00±0.33a	5.66±0.88b	6.66±0.66ab	7.22±0.69b	8.22±0.69ab
F2B0	1.88±0.50abc	3.88±0.69a	5.22±0.69ab	6.00±0.66ab	5.55±0.69ab	6.44±1.07ab
F2B1	1.88±1.07abc	3.22±0.83a	4.88±1.01ab	5.55±1.26ab	5.33±0.57ab	6.33±0.88ab
F2B2	1.66±1.45abc	3.66±0.00a	4.66±0.88ab	6.22±0.38ab	6.44±0.38ab	7.22±1.83ab
F2B3	0.66±0.57ab	3.55±0.83a	5.33±0.66b	6.44±0.83ab	7.33±0.66b	7.77±1.01ab
F2B4	1.00±1.00abc	3.77±0.19a	5.88±0.38b	6.22±0.69ab	6.66±0.88ab	7.33±1.20ab
F2B5	1.22±0.69abc	3.66±0.57a	5.33±0.88b	7.00±1.33b	7.44±1.50b	8.33±1.15ab
F2B6	1.55±1.01abc	3.44±0.69a	5.44±0.50b	6.00±0.66ab	6.66±1.00ab	8.00±1.85ab

Remarks: Numbers (mean ± sd. n = 3) followed by different letters in the same column indicate significant differences, according to Duncan's Multiple Range Test (DMRT) at the p <0.05 level.

Table 7. Spore AMF count, root infection and shoot dry weight of cocoa seedlings.

Biostructure sources	AMF spore count (no. 50 g soil^{-1})	Root infection (%)	Shoot dry weight (g)
F0B0	16±4.70a	16.66±17.64a^{ns}	2.71±0.46a^{ns}
F1B0	19±4.63a	36.66±20.28a	2.14±0.09a
F1B1	30±5.56ab	33.33±15.28a	2.58±0.52a
F1B2	33±9.89ab	24.44±13.47a	1.91±0.37a
F1B3	26±9.33ab	27.77±21.17a	1.88±0.46a
F1B4	15±7.58a	21.11±25.24a	2.08±1.06a
F1B5	24±12.60ab	30.00±18.56a	2.38±0.43a
F1B6	**38±7.40b**	32.22±13.47a	2.42±0.61a
F2B0	23±5.34ab	28.88±25.47a	1.88±0.45a
F2B1	23±7.21ab	13.33±3.33a	2.24±0.55a
F2B2	28±12.72ab	33.33±31.80a	1.86±0.92a
F2B3	32±7.18ab	10.00±5.77a	2.09±0.53a
F2B4	32±12.25ab	13.33±12.02a	1.77±0.22a
F2B5	28±7.50ab	22.22±10.72a	1.86±0.81a
F2B6	29±11.57ab	8.88±1.92a	2.85±1.44a

Remarks: Numbers (mean ± sd. n = 3) followed by different letters in the same column indicate significant differences according to Duncan's Multiple Range Test (DMRT) at the p <0.05 level.

The highest total AMF spore count in the biostructure was obtained using earthworm and ant populations in a 20:10 (individual/individual) ratio on *G. sepium* leaf and cocoa bean shell in a 50%:50% composition (Figure 3). This indicates the AMF spore density increased with increasing earthworm-ant ratio, confirming the presence of ants plays an important role in improving the soil pH, organic-C, N, and P contents (Almeida et al., 2019; Boots et al., 2012). Therefore, improved pH, organic-C, N, and P through bioturbation by ants,creates-more suitable conditions for earthworm activity to form new biostructures (Sankar & Patnaik, 2018; Taylor et al., 2019). In addition, the biostructures' environmental condition derived from the treatments modulates the growth of AMF spore populations (de Menezes et al., 2018; Lucas et al., 2017). The high density of AMF spores in the biostructure formed from a mixed composition of the organic matter types indicates the quality of food resources and the available conditions are suitable for collaboration between *Peryonix* sp. and *Dorylus* sp. In the present work, the addition of sago dregs to the organic matter mixture tended to decrease the spore density (Figure 3). Regarding the chemical attributes of the three organic matter types tested, sago dreg has a higher total-C and C/N ratio, but lower total-N, total-P, and total-K contents, compared to cocoa shell beans and *G. Sepium*leaves (Table 1). This is comparable with the report by Syaf et al. (2021), where the AMF spore density in the biostructure containing biochar decreased with the increasing earthworm population. Biochar is known to have high total-C and C/N and low nutrient content (Prasad et al., 2020), therefore, these results reaffirm that organic quality (total-C, C/N ratio, total-N, P, and K) has the capacity to affect AMF spore populations in soil biostructure created through activities of soil fauna ecosystem engineers (Medina-Sauza et al., 2019).

This study's results discovered the total number of spores in rhizospheric soil from cocoa seedlings subjected to biostructure treatments, tend to be more, compared to seedlings subjected to soil applications without the ecosystem engineering soil fauna's activities, while the percentage of infected roots differed insignificantly (Table 7). This indicates AMF spores carried by biostructures formed through the collaborative activity of ants and earthworms in soil from the smallholder cocoa plantation mixed with different types of organic matter, are able to germinate and produce hyphae infective to the cocoa seedlings' roots. The highest number of spores in rhizospheric soil was obtained from the application of biostructures containing the highest amount of AMF spore. A study by (Harinikumar & Bagyaraj, 1994) also found earthworm casts and ant nests contained viable AMF spores infective to *Alllium cepa* roots grown in sterilized soil. In this study, the inoculation of spores carried by the biostructure was performed under the conditions of cocoa seedlings grown on un-sterilized soil.

The large number of spores carried by the biostructure is the expected percentage of infected roots, but is also more, in some cases (Verbruggen et al., 2013). However, the results found another case, where the percentage of infected cocoa seedling roots differed insignificantly between treatments. This value ranged from 8.88% - 36.66% in all treatments, and was lower, compared to roots infected by arbuscular mycorrhizae from cocoa seedlings grown on sterilized soil (Aggangan et al., 2019). The success of AMF spore inoculation under unsterilized growing media is determined by biotic factors through an inhibitory mechanism (Fukami, 2015), the spore community inhabiting the growth media has the potential to suppress the performance of pores carried by inoculum source material to infect roots (Werner & Kiers, 2015). Furthermore, the inhibitory effect's strength is supported by the nutrient availability in the growth media (Cely et al., 2016; Hayashi et al., 2018)

The percentage of infected roots did not differ significantly between the control and biostructures treatments. However, the plant height and the number of leaves were higher in the control, compared to the biostructure treatments (Table 5 and 6), but the seedling's shoot dry weight was insignificant (Table 7). A study by Bagy Araji and Powell (1985) showed AMF application increased the height and stem diameter of marigolds grown in pots filled with unsterilized mineral soil with a characteristic pH and available P of 5.4 and 9µg/l, respectively. Meanwhile, Mau and Utami (2014) reported mycorrhizal spore inoculation did not show an increase in height for maize plants grown on un-sterilized soil dominated by sandy loam with a 7.02 pH. Also, Aggangan et al. (2019) reported the soil-based inoculant powder containing AMF spores increased the dry weight of inoculated cacao seedlings on non-sterilized soil enhanced with NPK fertilizer. In a report by Kim et al. (2017), inorganic fertilizer led to a higher increase in the dry weight of plants without spore inoculation, compared to counterparts inoculated with spores on sterile soil. Thus, this study's results reaffirm the stoichiometry of soil ecology largely determining the mutualistic-parasitic symbiosis continuum in interactions between the spores and plant roots grown on soil under field conditions (Mandyam & Jumpponen, 2015).

5. CONCLUSION

The AMF spore density in the biostructure was affected by the composition of earthworms and ants, as well as the composition of organic matter mix added to the soil. In addition, the spore abundance was positively correlated with total, P, and negatively with the C/N ratio. The biostructure applied has the capacity to increase the total number of spores in the rhizospheric soil and roots infection from cocoa seedlings growing on non-sterile soils. However, further studies are required to understand soil quality factors with the most contribution to a positive association between AMF spores carried through biostructure treatment.

Declaration of Competing Interest

The authors declare no competing financial or personal interests that may appear and influence the work reported in this paper

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