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The Impact of Biological Control Agents on Soil Microbial Communities in Oil Palm Plantation Soils

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ABSTRACT

Soil microbial community plays an important role in the soil ecosystem and the diversity of microbial community may act in check and balance to various plant pathogens. However, soils microbes are very sensitive to changes in soil condition. The use of Biological Control Agents (BCAs) is regarded as a promising measure to control Basal Stem Rot (BSR) of oil palm disease. However, its potential effect on other soil microbes is not clear. This paper reports the impact application of two BCAs-based products in the soil microbial population and diversity on oil palm plantation soil. Isolation of microbes from the soil was carried out using plate counts techniques on various media such as Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Nutrient Agar (NA). The presence of viable microbes on cultured media was observed and counted using Colony Forming Unit (CFU) method and later identified using Biolog and molecular techniques. The CFU for bacteria and yeast after application of these microbial treatments were remains unchanged, which varied between 10^3 to 10^6 cfu/g and 10^2 to 10^6 cfu/g of soil respectively. However, CFU for fungi has increased to 10^4 cfu/g of soil. Identification based on Biolog and molecular technique shows that new species arose and overcome the predominated species after application of these microbial products. The new identified species were *Enterobacter* spp., *Microbacterium* spp., *Burkholderia* spp., *Yarrowia* spp., *Streptomyces* spp., *Trichoderma* spp., etc. Application of BCAs to soil could possibly enhance the richness and evenness of microbial distribution in soil.

Keywords: oil palm, Biological Control Agents, soil microbial community

INTRODUCTION

Soil microbes are important components of the soil habitat, ecologically their role is to control the nutritional cycle in maintaining soil fertility, stabilizing the ecosystem and consequently, preventing excessive growth of soil borne pathogens [9]. However, soil microorganisms are very sensitive to agricultural activities which often associate with land clearing and intensive application of chemicals such as pesticides, fungicides and fertilizers [11]. Adaptation of agricultural practices has been proposed as a means to decrease the soil microbial community and biodiversity loss which kills most of the beneficial microorganisms. This has lead to an unbalanced equilibrium in soil and leaves the soil community weaker, leading to the emerging of soil-borne pathogens. Soil in undisturbed area has a high composition of microbial community compared to agricultural soil [24].

In Malaysia, agricultural activities such as rubber and oil palm plantations were intensively developed and have been thought to have impacts on the soil ecosystem [15]. To date, the total oil palm planted area had increased to 5.1 million hectares covering 15% of the total land area in Malaysia [17]. However, within this figure, the oil palm industry faces huge losses due to one major disease which is Basal Stem Rot (BSR) caused by *Ganoderma boninense* [5]. Recent studies to control this disease are now focused on the use of biological control agents (BCAs) which have shown some early promise in nursery and field conditions [23,2,25]. However, even though there are many published studies on this aspect but the impact of BCAs on the microbial community after applied to soil microhabitats is not fully understood [1].

Therefore, understanding in the existence of some microbial species in the soil ecosystem as well as the effect of the existence after the BCAs treatments is important to be further explored. This

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study was designed to investigate the changes of microbial population and diversity in oil palm plantation soil after the application of two BCAs products.

Materials and Methods

2.1. Study site:

The study site was located at oil palm plantation in Sapi Nangoh, Sandakan, Sabah. The chosen plot was reported to have high incidence of Basal Stem Rot disease. The experimental design for the field trial was completely randomized, with fifty replicates for each treatment, randomly distributed over the plot. Microbial treatments were applied once a month for six months consecutively.

2.2. Preparation of microbial treatments:

Two treatments were tested in this trial. These were:

TR1; Living Soil Microbes® manufactured by One Good Earth (M) Sdn Bhd

TR2; Agriorganica® manufactured by Organica Biotech Sdn Bhd

All products were commercially-produced in Malaysia as BCAs and are suited to local environmental conditions. Each product was applied following the standard operation procedure (SOP) of the product.

a. Treatment 1 (TR 1):

This treatment contained a combination of *Bacillus* spp. and *Trichoderma* spp. TR 1 was prepared by mixing 800 mL of product with clean tap water to give a final volume of 20 L. The mixture was sprayed around the palms, with spraying radius of 2-3 m from the base using a high pressure (350 kPa) motorized knapsack sprayer with a walking speed of 50 m min^{-1}, and a nozzle output of 1.5 L min^{-1}. One palm was sprayed approximately with 800 mL of TR1.

b. Treatment 2 (TR 2):

TR2 was prepared by mixing 265 mL of the product with 130 g of humic acids in tap water, to give a final volume of 20 L. The mixture was sprayed around the palms as described above.

2.3. Soil sampling:

A total of 10 soil samples were randomly collected from the study site at an oil palm plantation located in Sapi Nangoh, Sandakan, Sabah. Soil samples from the top soil layer (0-15cm) were collected using an auger. The humidity of soil samples were maintained and transferred immediately to laboratory of Universiti Malaysia Sabah for further analysis.

2.4. Microbial counts:

Ten grams of each soil sample were added with sterile distilled water until the final volume was 100mL and stirred for 30min until all soil samples were homogenized in water. After homogenization, this solution was decimally diluted (10^{-1} to 10^{-5}). From the serial diluted solutions, 0.1 ml of the soil mixture was taken from every dilution which were then spread onto the petri dish which contained different media.

2.5. Culture media:

The media selected were among the basic media used in isolation of different types of soil microorganisms. Nutrient Agar (NA) was used to isolate bacteria and cultures were incubated at 37°C for 24 hours. Potato Dextrose Agar (PDA) with 0.1% streptomycin for prevention of bacteria growth was used to isolate fungi. The cultures were incubated at 27°C for 72 hours. Meanwhile, Malt Extract Agar (MEA) was used to isolate yeasts from soil samples. Cultures were incubated at 27°C for 48 hours. Three replicates were prepared for each medium and dilution. The developed colonies on plates were counted and the average number of colonies per three plates was determined. The number of total colony forming unit (CFU) per gram of soil was calculated. Individual colonies which varied in colour and shape were further picked and isolated on new NA, PDA and MEA, while actinomycetes were isolated on Oat Meal Agar (OMA). All microbes isolated were identified using Biolog identification technique.

2.6. Biolog Identification:

Microbial identification for bacteria, fungi and yeast were done using Biolog technique in different microplates, which were GEN III microplate, FF microplate and YT microplate respectively. Colonies were picked up with sterile cotton swab and suspended in Inoculation Fluid (15mL). Suspension was prepared at appropriate inoculums density using turbidimeter depending on the Inoculation Fluid (IF) used and types of microbes. Using a sterile micropipette, 100 µL of the suspension was dispensed into each well of the microplate. The conditions of incubation for the microplates were depending on the category of microorganism which ranging 26°-37°C for 24 to 72 hours. After incubated, the plates were placed in the reader for reading.

2.7. Molecular Identification:

Unidentified microbes from the Biolog's technique were identified using PCR method based on homology sequence analysis. DNA extraction for prokaryotes and eukaryotes microorganisms were using the DNeasy® Blood &Tissue Kit (Qiagen) and DNeasy® Plant Mini Kit (Qiagen) respectively, following their standard protocol. PCR protocol used are as described by Boyle *et al*. (2008) with general primers, Eub 518 (5'-ATTACCGCGGCTGCTGG-3') and Eub 338 (5'-ACTCCTACGGGAGGCAGCAG-3) for prokaryotes and ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and 5.8s (5'-GCGTGCGTTCTTCATCG-3') for eukaryotes. Each

PCR tube contains 15 µL of MM for a 25 µL PCR and 10 µL of sample DNA were added to respective PCR tube. Amplification was performed in a conventional thermocycler (Eppendorf Mastercycler). The reaction began with an initial 94ºC denaturation for 3 min, followed by 30 cycles of annealing, 96ºC for 15 s, 60ºC for 1.5 min, 72ºC for 2 min. A final extension step at 72ºC for 5 min allowed all amplicons to be fully extended. Cloning and DNA sequencing were done at First BASE Laboratories Sdn Bhd in Selangor, Malaysia. The sequence was trimmed for a better BLAST search in the NCBI gene bank. BLAST search was conducted to obtain sequences for closest matches in the NCBI GenBank database.

Results:

3.1. Soil Colony Forming Unit (CFU):

Population density of microorganisms in all soil samples collected before application of treatments is shown in Table 1. Based on the results, bacterial population from oil palm soil was ranging from 10^2 to 10^6 cfu g^{-1} of soil. Meanwhile, the fungal population on PDA was lower compares to bacteria with 10^2 cfu g^{-1} of soil. No growth was observed on PDA for soil samples collected from TR 1.1, TR 1.3, TR 1.5, TR 2.1, TR 2.4 and TR 2.5. All soil showed the presence of yeasts with CFU between 10^2 to 10^6 cfu g^{-1} of soil.

Table 1: Types of microorganism collected from oil palm plantation soil of Sapi Nangoh, Sandakan, Sabah before microbial treatments. The microorganism isolated were divided into groups based on their morphological (in CFU g^{-1} of soil sample weight).

Sample	Growth medium		
	NA (for bacteria)	PDA (for fungi)	MEA (for yeast)
TR 1.1	3.0×10^6	-	1.5×10^3
TR 1.2	7.2×10^4	-	4.7×10^4
TR 1.3	1.7×10^3	1.0×10^2	6.3×10^2
TR 1.4	1.6×10^3	1.0×10^2	2.0×10^6
TR 1.5	7.0×10^2	-	1.5×10^4
TR 2.1	4.6×10^3	-	6.4×10^4
TR 2.2	3.8×10^3	3.0×10^2	7.4×10^3
TR 2.3	3.2×10^3	1.0×10^2	4.7×10^3
TR 2.4	5.7×10^3	-	4.7×10^4
TR 2.5	6.7×10^5	-	7.2×10^4

NA denotes Nutrient Agar; PDA: Potato Dextrose Agar; MEA: Malt Extract Agar; S: Sample; TR 1: Living Soil Microbes; TR 2: AgriOrganica

The population density of microorganisms from soil samples collected after the application of microbial treatments for six months consecutively is shown in Table 2. Based on the results, bacterial population grown on NA was ranging from 10^4 to 10^6. The population of bacteria remained the same before and after the microbial treatments. However, fungal population in the soil samples showed an increase in comparison prior to treatments, which was between 10^2 to 10^4 cfu g^{-1} of soil. Meanwhile yeast population growth on MEA was between 10^4 to 10^6 cfu g^{-1} of soil.

3.2. Microbes Identification:

Identification using Biolog technique showed the bacterial population in Sapi Nangoh oil palm plantation soil before microbial treatments was predominated with *Bacillus* sp. such as *B. thuringiensis, B. pumilus, B. humi, B. lichenformis, B. albus, B. pseudomycoides* and *B. amyloliquefaciens* (Table 3). Some actinomycetes such as *Cornyebacterium* sp.were also observed from the soil samples. Various genera of yeasts identified from the soils were *Rhodotorula* sp., *Guillermondella* sp., *Filobasidiella* sp., *Fellomyces* sp., *Sporidiobolus* sp., *Bulleromyces* sp., *and Cryptococcus* sp. Meanwhile, fungi species identified from the soil were *P. solitum Westling, P. neoechinulatum, C. herbarum, F. tricinctum* and *F. kiliense.* Other species are as shown in the Table 3.

Table 2: Microorganism collected from oil palm plantation soil of Sapi Nangoh, Sandakan, Sabah after TR 1 and TR 2 treatments. The microorganism isolated were divided into groups based on their morphological (in CFU g^{-1} of soil sample weight).

Sample	Growth medium		
	NA (for bacteria)	PDA (for fungi)	MEA (for yeast)
TR 1.1	5.6×10^4	9.0×10^2	2.5×10^4
TR 1.2	2.5×10^6	1.5×10^4	4.4×10^4
TR 1.3	4.6×10^6	6.5×10^2	2.7×10^4
TR 1.4	1.9×10^4	1.1×10^4	8.0×10^4
TR 1.5	1.7×10^6	2.0×10^2	1.5×10^6
TR 2.1	2.9×10^4	1.1×10^3	1.6×10^5
TR 2.2	2.1×10^6	2.1×10^3	4.1×10^4
TR 2.3	2.2×10^4	2.0×10^2	2.3×10^5
TR 2.4	1.8×10^5	1.1×10^3	4.4×10^5
TR 2.5	1.1×10^4	1.0×10^2	2.9×10^6

NA denotes Nutrient Agar; PDA: Potato Dextrose Agar; MEA: Malt Extract Agar; S: Sample; TR 1: Living Soil Microbes; TR 2: AgriOrganica

Table 3: Identity of microorganisms isolated from Sapi Nangoh Oil Palm Plantation soil before application of microbial products based on Biolog technique.

Type of micro-organism	Genus	Species
Bacteria	*Bacillus*	*thuringiensis*
		cereus
		pumilus
		humi
		lichenformis
		albus
		pseudomycoides
		amyloliquefaciens
	Arthobacter	*woluwensis*
		globiformis
	Kytococous	*sedentarius*
	Ralstonia	*picketii*
Actino-mycete	*Corynebacterium*	*boris*
		mycetoides
	Brevibacterium	*epidermis*
	Rhodococcus	*fascians*
Yeast	*Rhodotorula*	*minuta*
		graminis
		pustula
	Guilliermondella	*selenospora*
	Filobasidiella	*neoformans bacillisporus*
	Fellomyces	*fuzhouensis*
	Sporidiobolus	*johnsonii*
	Bulleromyces	*albus*
	Cryptococcus	*albidus*
Fungi	*Penicillium*	*solitum Westling*
		neoechinulatum
	Aspergillus	
	Cladosporium	*herbarum*
	Fusarium	*tricinctum*
	Acremonium	*kiliense*

After application of microbial products, bacterial population in Sapi Nangoh oil palm plantation soil was still predominated with *Bacillus* sp. with some additional of other *Bacillus* sp. such as *B. subtilis, B. amylocoides, B. spherius, B. atrophaeus* and *B. coagulans* (Table 4). New genuses were also recorded such as *Arthobacter* sp., *Ralstonia* sp., *Enterobacter* sp., *Microbacterium* sp., *Burkholderia* sp., *Chromabacterium* sp., *Staphylococcus* sp., *Lactobacillus* sp., *Pseudomonas* sp.and *Pantoea* sp. Appearanceof new actinomycetes from the genus of *Corynebacterium* sp which was *C. accolens* and *B. parabrevis* from the genus of *Brevibacterium* was also recorded. Meanwhile yeast populations in the soil samples were predominated with *Candida* sp. but some other new genuses were also recorded, which were *Zygosaccharomyces*, *Yarrowia*, *Metshnikowia*, and *Pichia.* After the microbial treatments, several species of *Penicilium* were recorded such as *P. paxilii, P. chrysogenum, P. pinophilum* and *P. steckii zaleski.* Some new genuses were also recorded such as *Trichoderma, Aspergillus, Neosertoya* and *Paecilomyces.* Species belonging to cosmopolitan genera of *Penicillium* and *Trichoderma* were the most abundant in the fungal community. The dominant species identified after the treatments are mainly the cellulose-degrading fungi belonging. *T. harzianum* and *T. viride*. Several microbes which were unable to be identified using biolog were further identified using on homology sequence analysis. Several species such as *Streptomyces* sp. JAR6, *S. parvulus* strain CFA-9, *P. pinophilum* strain SGE7S, *T. harzianum* strain ITEM 908, *T. aureoviride* strain T77 and *A. cellulolyticus* Y-94 were identified using molecular technique.

Discussion:

In recent years, some studies have reported the microbial population in different environments such as in rice plantation and botanic garden [22,7] but none many have reported the microbial diversity of soil in the oil palm plantation. In oil palm plantation soils, the microbial community has typically been exposed to a long term and frequent soil disturbance. Agricultural activities, such as uses of pesticides and fertilizers in oil palm plantation have significant implication for the microorganism present in the soil [21]. Land use intensification has decreases the soil quality. The resistance and resilience of a community to disturbance decrease due to fewer organisms can adapt to the disturbance. The species richness diminished in disturbed soil in respect to undisturbed forest soil [4]. The decrease in diversity observed in the disturbed agricultural soil was mainly due to the fall in species richness.

The number of microbial population from this study varied in term of population size among bacteria, fungi and yeast. The population of bacteria in all samples collected after the treatments was varied from 10^3 to 10^6 bacteria/g of soil. Normal

estimation of number of bacteria in soil depth of 0-15cm based on plate counts usually range from 10^5 to 10^8 bacteria/g of soil as due to the high moisture content, which, along with surrounding humidity that enhances the microorganism cycles in the soil [14]. However, no major changes in CFU for bacterial population was observed after the microbial treatments which could probably due to the low nutrient sources available resulted from heavy application of chemical inputs history in the plantation [21,18]. Meanwhile, a significant increment up to 10^4 on the fungal CFU population was observed after the application of microbial treatments. Mohaptra [16] reported the population density of fungi usually ranges from 10^5 to 10^6 per gram of soil in undisturbed soil. The population of fungal has increased after the microbial treatments although not reached the normal range as described by Mohaptra [16]. Introduction of BCAs treatment had probably increased the fungi species richness and therefore might explain the slight increase in fungi population. Oil palm mono-cultivation has a great impact to the bacterial and fungal population in soil, but had only low impact on yeast quantity, which could probably explained the yeast population remained similar throughout the study period [26].

The result in this study showed that there is a great change in microbial diversity after the application of treatments. Microorganisms in the soil might be in a dormant state or inhibited by the effects of adverse environment. However, the introduction of biocontrol agents into the soil may important in shifting the resident microbes to an active state [13]. From Table 3 and 4, *Bacillus* spp. remains the major bacteria genus in the soil and the species richness increased at the end of the project. Their increment was also in accordance with the presence of new beneficial species such as *Pseudomonas* spp., *Burkholderia* sp., *Lactobacillus* sp., *Enterobacter* spp. and *Streptomyces* sp. These species are well known for their ability in decomposing organic matter, promoting plant growth and suppress soil borne pathogens [6,10]. This observation suggested that antibiotic-producing BCA such as *Bacillus* spp., could alter the microbial community structure in microhabitats to which it is applied. Meanwhile, from Table 4, the shift in the composition of fungal community were characterized with the presence of *Trichoderma* spp., *Paecilomyces* sp., *Neosartoya* spp., *Colletotrichum* sp., and as well as a detectable increase in *Penicillium*. Absence of some fungi detected before the treatment application also observed, supporting the prediction that *Trichoderma* spp. is an aggressive microorganism capable in mycoparasitism which had limited the growth of other fungi [8]. The changes in fungal community after the treatments, might aid in decomposing plant residues, releasing nutrients for plant growth and antagonizing the pathogenic species on the crop studied. The shift in yeast community was also recorded with the appearance of new species such as *Candida* spp., *Zygosaccharomyces* spp., *Yarrowia* spp., *Metshnikowia* spp., *Pichia* spp., *Hypopichia* spp. and *Kluveromyces* spp. Although their function in soil ecosystem remains unclear [3], a wide range of yeast strain are known to secrete proteinous toxins which kills susceptible yeast and fungi [12]. Detection of increment in yeast diversity in the soil community could probably give positive expression of their involvement in suppressing pathogenic fungi in soil.

This study showed that application of BCAs onto the soil could affect the resident microbial community. Detection of beneficial microbes in the soil after the treatments may contribute in the activation of antagonistic activities in the soil ecosystem against plant pathogens. However, microbial plate counts may represent only a small proportion of the soil microbe population, since many soil microbes can exist viable but at non culturable state and others may not be cultured easily [19].

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Conclusion:

The total microbial population and diversity in the oil palm plantation was poor in comparison to other undisturbed soils. This report provides a novel understanding on the microbial diversity and population in oil palm plantation. Although there was no major change in soil microbial population after the application of microbial treatments, positive changes in the composition of the soil microbes were observed. This shows introduction of these BCAs into soil had enhanced the growth of some other beneficial microorganisms.

Table 4: Identity of microorganisms isolated from Sapi Nangoh Oil Palm Plantation soil after application of TR1 and TR2 based on Biolog and PCR/sequence homology technique.

Type of micro-oganism	Genus	Species
Bacteria	*Bacillus*	*thuringiensis*
		cereus
		lichenformis
		pumilus
		amyloliquefaciens
		subtilis *
		amylocoides *

		spherius *
		atrophaeus *
		coagulans *
	Arthobacter	*globiformis*
		illicis * *woluwensis* *
	Ralstonia	*picketii*
	Enterobacter	*aerogenes* *
		brevis *
	Micro-bacterium	*terregenes* *
	Burkholderia	*caryophylli*
	Chroma-bacterium	*violacearum*
	Staphylococcus	*succinus* *
	Lactobacillus	*casei* *
	Pseudomonas	*fluorescens* *
	Pantoea	*dispersa* *
Actino-mycete	*Coryne-bacterium*	*accolens* *
	Brevibacterium	*epidermis*
		parabrevis *
	Rhodococcus	*fascians* *erythropolis* *
	Streptomyces	sp JAR6 *
		parvulus strain CFA-9 *
Yeast	*Candida*	*rugosa* *
		krusei *
		incommunis *
		zeylanoides *
	Rhodotorula	*minuta* *pustula*
		aurantiaca *
	Zygosaccharomyces	*bailli* *
	Yarrowia	*lipolytica* *
	Metshnikowia	*pulcherrima* *
	Pichia	*anomala* *
	Hypopichia	*burtonii* *
	Kluveromyces	*delphensis* *
	Cryptococcus	*curvatus* *
Fungi	*Penicilium*	*paxilii* * *chrysogenum* * *pinophilum* strain SGE7S* *steckii zaleski* * *simplicissimum* * *ochrochloron* *
	Trichorderma	*viride* * *harzianum* strain ITEM 908* *hamatum* * *aureoviride* strain T77*
	Aspergillus	*nidulans* * *alliaceus* *
	Cladosporium	*herbarum*
	Colletotrichum	*gloeosporioides* *
	Acremonium	*charticola* * *cellulolyticus* Y-94 *
	Neosertoya	*fischeri* *
	Paecilomyces	*lilacinus* *

* denotes new species

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