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Isolation and identification of microorganisms from soil in a young oil palm plantation

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Abstract

Microorganisms in soil could be beneficial to the ecosystem or pathogenic to crop plants. Their distribution depends on the environmental habitat favoring each species. This study was intended to isolate and identify the types of microorganisms in soil habitat of a young oil palm plantation, and associate them to their role as being beneficial or pathogenic to the ecosystem. The isolation process was done by soil sampling and soil dilution technique. The bacterial population were identified through BIOLOG, whereas the fungal and actinomycetes were identified through conventional methods of microscopic observations of the isolates. Five of the isolates were bacterial species, identified as *Malikia spinosa*, *Bacillus humi*, *Bacillus cereus/thuringiensis*, *Bacillus pseudomycoides* and *Bacillus* sp. Other isolates were three fungi and two actinomycetes of *Mucor* sp., *Penicillium* sp. and *Aspergillus* sp., and *Streptomyces* spp. isolate B1 and C2, respectively. None of the isolated species were reported to be pathogenic on oil palm, but *Aspergillus* sp. could be pathogenic to other crop plants. *Bacillus cereus/thuringiensis* is commonly used commercially as biological control agent mainly for Lepidopterous insects.

Key words: Soil bacteria, soil fungi, soil actinomycete, oil palm soil.

Introduction

Soil provides diverse habitats for a wide range of organisms. It serves as a complex habitat of soil microorganisms, in which the populations are crucial for sustainable agriculture [1]. Soils are made up of different components of solid fractions including sand, silt, clay and organic matter, which provide a wide range of microhabitats for various type of microorganisms [2,3]. Major groups of microorganisms in soil include bacteria, fungi and actinomycetes, and their population varies according to the soil microhabitats.

Each fertile soil could contain high bacterial number, indicating that the microorganisms can be an important indicator of soil quality and ecologically important parameter [4-6]. Soil microorganisms are also crucial in primary production through decomposition of organic matter and nutrient cycling [7]. Soil microorganisms have essential roles in elemental cycles including carbon, nitrogen and sulfur cycle. These organisms also transform phosphorus, iron and potassium to more soluble form which assists the uptake of the compounds by plants [8].

Despite of the importance of microorganisms in soil processes, several soil-borne microbial species are known to cause plant diseases. In oil palm plantation, at certain stages of the crop growth, *Ganoderma boninense* is widely known to cause basal stem rot of oil palm in Malaysia [9]. *Fusarium oxysporum* f. sp. *elaeidis* has been reported to cause vascular wilt in African oil palm [10]. Other pathogenic soil-borne microorganisms include *Colletotrichum* spp. (antrachnose disease), *Phytophthora* spp. (root rot disease) and *Rhizoctonia solani* (damping-off disease) [11].

This study was aimed to isolate and identify the microorganisms present in soil of young oil palm plantation, and grouped them as beneficial or pathogenic to the ecosystem.

Materials and Methods

Soil samples: Soils were randomly sampled using auger from 0-15 cm layer in young oil palm area at University Putra Malaysia (UPM), Selangor, Malaysia. Soil composition was determined for organic carbon, analyzed by the Walkley-Black procedure: total N was extracted using micro-Kjeldahl method; total P was extracted using 6 M HCl; K, Mg and Ca were extracted by leaching the soil sample with 1 M ammonium acetate solution (pH 7.0), and K was determined using flame photometer, and Mg and Ca were determined using atomic absorption spectrophotometer. The composition was as follows: 1.94% C, 0.32% N; 219 ppm P, 104 ppm K, 119 ppm Ca and 32 ppm Mg, and were classified as sandy clay (40% clay, 10% silt and 50% sand); pH 4.1 ± 0.01.

Isolation of microbial colony: Microbial extracts were prepared from the soil samples by serial dilution in sterile distilled water. Ten g of soil was transferred into 90 mL sterile distilled water followed by serial dilutions to 10^{-5}. The dilutions were plated using the spread plate technique on the specific culture media accordingly: Potato Dextrose Agar (PDA) amended with 30 mg/L streptomycin sulphate for fungi; Nutrient Agar (NA) amended with 0.1 g/L cyclohexamide for bacteria; Actinomycete Isolation Agar (AIA) amended with 0.5 g/L cyclohexamide for actinomycete. Additions of streptomycin sulphate to PDA and cyclohexamide to NA and AIA were, respectively, for the suppression of bacterial and fungal growth. The plates were inverted and incubated at 25ºC in darkness. The plates were examined for microbial colonies within 24 hours to 10 days after incubation. Visible microbial colonies on the respective media were counted after 24 hours for bacteria, 5 days for actinomycetes and 7 days for fungi. The colonies were isolated by subculturing accordingly in the specific

media in order to obtain pure culture of single colony of the microbial species. The pure cultures were maintained in the respective media accordingly for identification purpose.

Identification of microbial isolates:

Bacteria: Bacterial identification was done using Biolog Gen III Technology, which needs no Gram staining, no pre-tests and no following tests. The technology is claimed to be much more simple and user-friendly compared to the previous Gen II Technology [12]. The Gen III Microplate™ test panel involves a standardized micromethod using 94 biochemical tests for profiling and identification of both Gram-positive and Gram-negative bacteria. This procedure requires sterile condition during bacterial inoculation steps. Two to three single colonies of unknown bacteria grown on NA for 24 hours were suspended in a special "gelling" Inoculation Fluid – A (IF-A) at the recommended cell density (90-98%) using the Biolog turbidometer. After a slight upside down conversion of the IF-A tube, the cell suspension was inoculated into the Gen III Microplate™ at 100 µL per well. The microplate was then placed in the Omnilog® incubator at 33ºC to allow the phenotypic fingerprint to form. The Omnilog® software identifies the bacterium from the phenotypic fingerprint in the Gen III Microplate™ by performing all reading and interpretation of results. ID results (microbial species) were obtained within 24 hours. Gram staining was conducted later, prior to viewing under light microscope for visual recording.

Actinomycetes: Classical approach was chosen in identifying the actinomycetes [13, 14]. The identification process was done macroscopically and microscopically based on morphological appearances. Macroscopic observations involved the examination of colonial appearance grown on AIA and Blood Agar (BA). Microscopic appearances were observed using light microscope upon Gram staining and acid fast staining using modified Kinyoun method. Biochemical tests were also conducted for confirmation of genus. The genus of actinomycete was determined based on the UK National Standard Method: Identification of Aerobic Actinomycetes [14] and Bergey's Manual of Determinative Bacteriology [13].

Fungi: The fungal identification involved morphological examinations of the fungal mycelium (radial growth, colour, and pigmentation) on PDA and Malt Extract Agar (MEA) [8, 15, 16]. The spores and other structures were examined under light microscope. The genus was identified based on "A Manual of Soil Fungi" [16] and "Compendium of Soil Fungi" [15].

Results

Microbial counts of total population of bacteria, actinomycetes and fungi showed that bacteria recorded the highest ($3.9x10^6$) colony-forming unit per gram of dry weight soil (CFU/g of dry weight soil) among the three microbial groups in the oil palm soil (Table 1). The colony count was not significantly different to that of the actinomycete population with a $3.8x10^6$ CFU/g of dry weight soil, but significantly higher than the fungal population count of $3.9x10^5$ CFU/g of dry weight soil.

Ten isolates were obtained from the isolation process, which consisted of three fungi, five bacteria, and two actinomycete strains. Specific identification of the isolates revealed that the fungal species were *Mucor* sp., *Penicillium* sp. and *Aspergillus* sp.; and actinomycete species were *Streptomyces* sp. isolate B1 and *Streptomyces* sp. isolate C2, as listed in Table 2. Bacterial species identified by BIOLOG (Table 3) were *Malikia spinosa*, *Bacillus humi*, *Bacillus cereus/thuringiensis*, *Bacillus pseudomycoides*, and *Bacillus* sp.

Table 1. General count of microorganisms in soil of young oil palm plantation.

Type of microorganisms	CFU/g of dry weight soil (mean ± SEM)
Bacteria	$3.9^{a} \pm 0.9 x10^6$
Actinomycetes	$3.8^{ab} \pm 1.4 x10^6$
Fungi	$3.9^{b} \pm 0.7 x10^5$
LSD	$3.5 x10^6$

Note: Values in the same column followed by a similar letter are not significantly different by LSD ($P<0.05$).

Table 2. Specific identification of fungal and actinomycete isolates from oil palm soil.

Microbial group	Microbial isolate
Fungi	*Mucor* sp.
	Penicillium sp.
	Aspergillus sp.
Actinomycete	*Streptomyces* sp. isolate B1
	Streptomyces sp. isolate C2

Table 3. BIOLOG identification of bacterial isolates from oil palm soil.

Bacterial ID	Probability Index (%)	Similarity Index
Malikia spinosa	96.8	0.7
Bacillus humi	64.5	0.5
Bacillus cereus/thuringiensis	86.1	0.6
Bacillus pseudomycoides	76.2	0.5
Bacillus sp.	-	0.2

Mucor sp. (Fig. 1) was a fast growing organism, which was observed to be cottony white turning grayish on PDA with radial growth of 8 cm within 3 days. The sporangiophores could grow up to more than 10 mm high and unbranched. The sporangia would turn from yellowish to dark brown after several days with ellipsoidal and smooth-walled spores.

The vegetative hyphae of *Penicillium* sp. were observed as septate and branched (Fig. 2). The spores were formed in chains from verticillate phialides, and the conidiophores branched to form a brush-like head structure. The conidial areas appeared on PDA were yellow-green and reverse side were yellow with some red spots. The radial growth was between 2 and 3 cm within 7 days.

The vegetative mycelium of *Aspergillus* sp. consisted of branching hyphae (Fig. 3). The conidiophores were nonseptate and smooth with blackish brown conidial heads. The conidial areas appeared on PDA were blackish brown, and reverse side were yellowish white. The radial growth was between 2 and 5 cm within 7 days.

Streptomyces spp. produced extensively branched vegetative hyphae which did not fragment easily. Both isolates, B1 (Fig. 4) and C2 (Fig. 5) grew well on AIA, and the colonies were small, discrete, and creamy white, similar to the reverse-side. Later, aerial mycelia developed which appeared powdery. On BA, the colonies appeared glisten. The colonies adhered to both agar, and could be hardly removed by loop. The isolates were not acid fast when stained using modified Kinyoun method, catalase-positive and reduced nitrate to nitrite. They also produced strong earthy smell.

Malikia spinosa was only Gram-negative among the bacterial

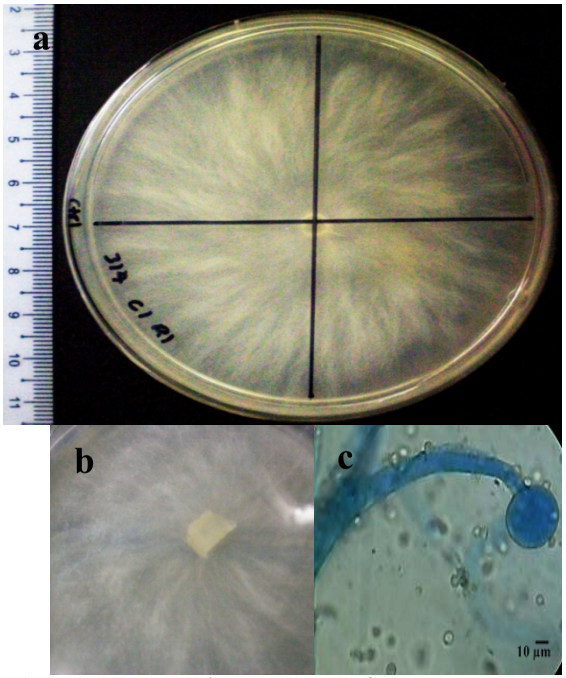

Figure 1. Macroscopic appearances of *Mucor* sp. on PDA (**a** and **b**), and *Mucor* sp. (**c**) under 1000x magnification.

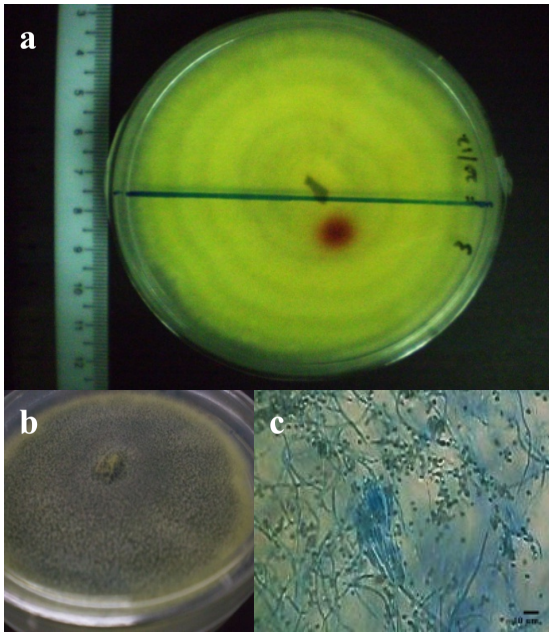

Figure 2. Macroscopic appearances of *Penicillium* sp. on PDA (**a** and **b**) and *Penicillium* sp. (**c**) under 1000x magnification.

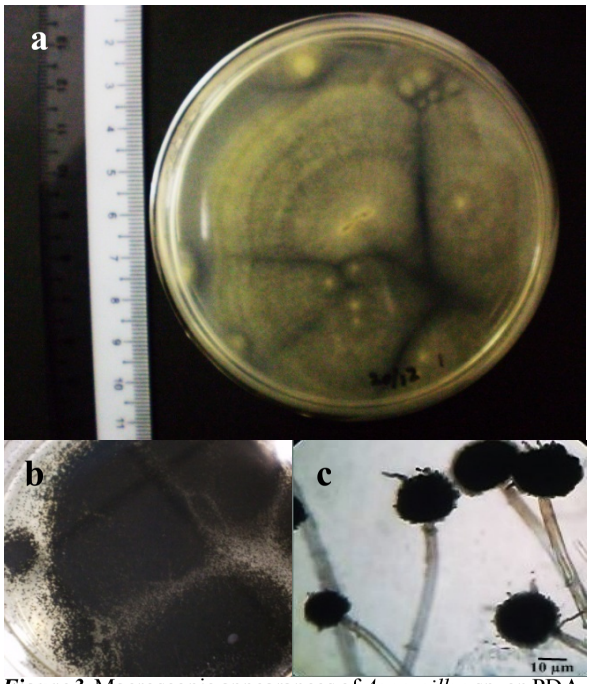

Figure 3. Macroscopic appearances of *Aspergillus* sp. on PDA (**a** and **b**) and *Aspergillus* sp. (**c**) under 400x magnification.

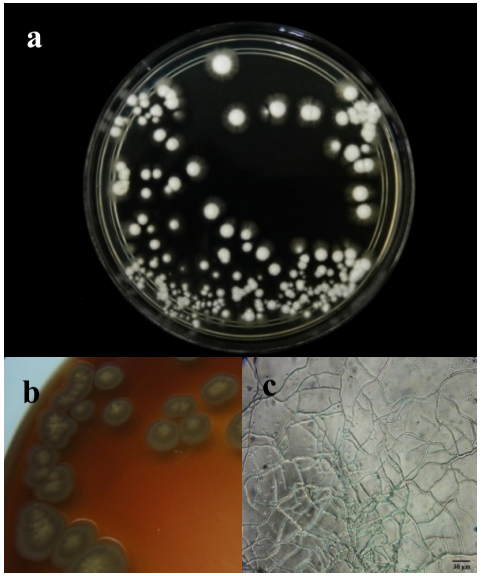

Figure 4. Macroscopic appearances of *Streptomyces* sp. isolate B1 on AIA (**a**) and BA (**b**), and *Streptomyces* sp. isolate B1 (**c**) under 1000x magnification.

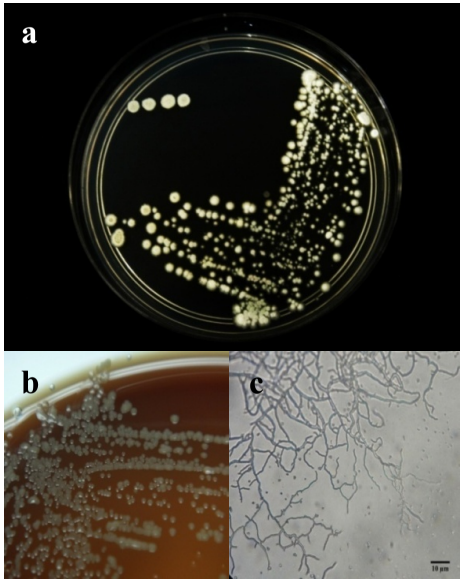

Figure 5. Macroscopic appearances of *Streptomyces* sp. isolate C2 on AIA (**a**), and BA (**b**), and *Streptomyces* sp. isolate C2 (**c**) under 1000x magnification.

isolates. The cells were straight rods with rounded ends which occurred singly, in pairs or in short chains. The colonies on NA appeared to be circular, raised, smooth, creamy white and translucent. After few days, the colonies appeared mucoid.

Bacillus humi was observed to be a rod shaped Gram-positive organism. The cells were slightly curved with rounded end, occurred singly or in pairs. On NA, the colonies were convex and appeared to be glossy and white with watery texture.

Bacillus cereus/thuringiensis appeared Gram-positive, and when grown on NA, the colonies were white, large, raised and dull surface, irregular margins and opaque. The cells were rod shaped and occurred singly or in pairs.

Bacillus pseudomycoides was also observed as Gram-positive. The appearance of colonies on NA was whitish cream and opaque. Microscopic observation showed that the cells were rod shaped and occurred in short chains.

Another species, only identified as *Bacillus* sp. appeared to be convex, white and opaque on NA. The cells were stained Gram- p ositive, and rod shaped.

Discussion

Fungi, bacteria and actinomycetes were recorded to be present in soils from young oil palm plantation. Bacteria and actinomycetes recorded significantly higher population counts compared with that of the fungi. Bacteria, however, were observed to have slightly higher CFU/g of dry weight soil than actinomycetes. Bacteria were reported to replicate rapidly in favourable conditions [17]. There was indication in this study that actinomycetes had slower growth rate compared with bacteria. This could be explained by the visible and distinct colonies of actinomycetes that could only be seen after 5 to 10 days, compared with bacterial colonies which normally appeared after 12 to 24 hours. Fungi recorded the lowest CFU/g of dry weight soil compared with bacteria and actinomycetes. Despite of the low population count, the presence of fungi in the soil of young oil palm plantation remains important as they can still dominate the soil through their extensive mycelia and reproductive spores, which ensures the survival of most fungal species [18].

The fungal species identified were all considered as common soil fungi. *Mucor* sp. can be regarded as one of the commonest soil fungi [15]. Certain *Mucor* spp. are beneficial, capable of producing large quantities of gamma-linolenic acid (GLA) which is effective in curing certain human diseases [19].

Penicillium species occurred with particular frequency in soil dilution plate technique; differences in soil washing number will not alter the number of colonies [15]. *Penicillium* sp. is a fungus of major importance due to its ability to produce penicillin, used as antibiotics to control human pathogens [20]. *Penicillium* sp. and *Aspergillus* sp. are strong phosphate solubilizing microorganisms in soil, which convert phosphate for plant uptake [21]. *Aspergillus* sp., unlike *Penicillium* sp., is found predominantly in warmer regions [15]. The species are known to cause serious infections in humans and animals by producing aflatoxin [22]. In agriculture, certain *Aspergillus* spp. were recorded as plant pathogens by producing mycotoxins [23].

Streptomyces spp., which represented the actinomycete group, were considered non-pathogenic. *Streptomyces* spp. are known as antibiotic producers, particularly *Streptomyces griseus*, which is widely recognized to produce streptomycin, essential antibiotic in medicine [8]. *Streptomyces* has been observed to produce antibiotic depending on the growth phase [24]. Candicidin (anti-fungal antibiotic) was produced after vegetative growth in liquid-grown culture of *S. griseus*, while *Streptomyces antibioticus* grown on agar surface only produces oleandomycin at the end of the vegetative growth state [25].

The BIOLOG Gen III Identification System was not able to differentiate between *B. cereus* and *B. thuringiensis*. These species are closely related, and can only be distinguished through molecular methods such as polymerase chain reaction combined with a restriction endonuclease [26]. Many literatures have reported *B. cereus* to be responsible of minor food-borne illnesses, causing nausea, vomiting and diarrhea [27, 28]. This opportunistic pathogen has endospore which can survive and stimulate bacterial growth, thus producing enterotoxin. This enterotoxin can survive high temperatures and wide range of pH, which finally will cause poisoning in humans [29]. *B. thuringiensis* is also known to produce toxins, which are termed Cry proteins which specifically targets insects including Hymenoptera, Diptera, Lepidoptera and others[30].

B. pseudomycoides was also reported to be closely related to *B. cereus,* but can easily be separated through fatty acid composition [31]. It was even proposed as a new species, after previous studies showed that this species was genetically distinct but phenotypically similar to *B. mycoides* [31]. Therefore, no previous studies were found on the pathogenicity of this species. *B. mycoides,* a non-pathogenic bacterium, which has distinctive rhizoidal colony formations, was reported to induce systemic resistance in sugar bit, thus acting as biological control agent [32, 33].

Little is known of *B. humi* and *M. spinosa*, as both species were proposed in 2005, and therefore, no report was found on the pathogenicity of both species. *B. humi* was first isolated in agricultural research area in Netherlands [34], whereas, *M. spinosa* has been misclassified as *Pseudomonas spinosa* and was proposed to the new genus *Malikia* in 2005 [35].

Conclusions

Fungi, bacteria and actinomycetes made up the major groups of microorganisms present in soil of young oil palm plantation from UPM. Ten microbial isolates were obtained from the soil, consisting of three fungi, two actinomycetes and five bacteria. The fungal isolates were identified as *Mucor* sp., *Penicillium* sp. and *Aspergillus* sp. Two actinomycete isolates were identified as *Streptomyces* spp. isolate B1 and C2. The bacterial isolates were identified as *M. spinosa*, *B. humi*, *B. cereus/thuringiensis*, *B. pseudomycoides* and *Bacillus* sp. No previous report was found on the pathogenicity of all the identified isolates towards oil palm. However, *Aspergillus* sp. could be pathogenic to other plant crops.

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