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IN-VITRO STUDY ON THE EFFECT OF ENDOGEIC EARTHWORM ON BLO DISEASE BACTERIUM (BDB) IN BANANA- A PRELIMINARY OBSERVATION

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ABSTRACT

Blood disease is a wilt disease that greatly affects the banana cultivation. Besides chemicals, the utilization of natural soil-biological entity may provide an alternative in searching for effective and environmental friendly approach against the disease. Endogeic earthworms are known as soil-engineers that modify soil properties that may favor or suppress different microbial populations. In the study, the effect of endogeic earthworm, Pontoscolex corethrurus on blood disease bacterium (BDB) was evaluated through in-vitro study. Earthworm casts, mucus and worm-worked soil collected were inoculated onto tetrazolium chloride (TZC) medium seeded with BDB at different concentrations. Molecular identification using 16S rRNA primers was then performed to identify the microbial isolates that inhibited BDB. Phylogenetic tree was constructed to determine the similarity among the isolates with Pseudomonas sp. sequences deposited in the GenBank. The results showed that bacterial colonies from earthworm mucus inhibited BDB at 10^9 and 10^5 cell/ml. However, no inhibition was observed at higher BDB concentration (10^7 cell/ml). Similarly, earthworm cast and worm-worked soil did not show inhibition towards BDB. Bacterial isolates obtained from earthworm mucus were classified into the genus Pseudomonas. The study suggested the potential role of P. corethrurus in enhancing the growth of beneficial microorganism in remediating blood disease.

Keywords: Banana, Blood disease bacterium (BDB), Cast, Mucus, *Pontoscolex corethrurus*, *Pseudomonas Sp.*

Contribution/ Originality

This study is one of very few studies which have investigated the effect of endogeic earthworm on banana blood disease. Earthworm mucus promoted the growth of beneficial microorganisms that inhibited blood disease bacterium (BDB), suggesting the potential role of endogeic earthworms as biological control for bacterial wilt diseases.

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1. INTRODUCTION

Blood disease is the bacterial wilt in banana that is caused by blood disease bacterium (BDB). This disease is a major problem to the banana cultivation due to its high virulence and ability to infect most banana cultivars [1]. In the field, blood disease can be transmitted through flower visiting insects, contaminated soil environment and planting equipment. The leaves of infected plants become yellow and wilted. Dark red discoloration is observed when the inner fruit pulp and pseudostem are cut open [2]. It is recommended that a fallow period of up to a year is needed for plantations that are infected with blood disease [3]. Blood disease has caused low production of banana for five consecutive years in Indonesia [4]. Besides very few resistant varieties, the control procedures are economically and ecologically costly [5]. The occurrence of blood disease in Malaysia was previously reported by Kogeethavani, et al. [6]. This possesses a great challenge to the banana cultivation in the country in view of the great impact experienced by Indonesia for the past decades. Therefore, the search for a viable, preferably nature-friendly approach is highly stipulated.

Endogeic earthworms are soil-burrowers that have intimate relationship with plant root system. Previous studies have documented that the feeding, casting and burrowing activities of the earthworms could alter soil physical and chemical environment [7] as well as increasing soil microbial population and plant biomass [8]. Earthworms are also found to secrete antimicrobial substances in response to pathogen infection [9, 10]. A number of studies have reported the role of earthworms in decreasing Fusarium biomass in soil [11] reduce the severity of fungal root disease in wheat [12] and nematode infection in banana [13]. These interactions reduce and/or suppress the populations of pathogen by altering the environment that is favorable for the growth of various microbes, which exhibit antagonism interaction with the pathogens. Plants would also gain benefits from the improved soil conditions that allow them to be more resistant to disease and pathogen infections.

However, knowledge on such interactions is limited and there is a lack of information on how these beneficial soil fauna influence the population of other soil-borne pathogens, such as BDB. Therefore, the present study aims to determine the effect of *Pontoscolex corethrurus* (a common endogeic earthworm) on BDB. The in-vitro study evaluates the presence of this earthworm and its secretions (mucus and cast) towards BDB.

2. MATERIAL AND METHODS

2.1. Earthworm Mucus and Cast Collection

P. corethrurus were sampled via digging and hand-sorting from a field adjacent to the Biology Department, Universiti Putra Malaysia. *P. corethrurus* is the dominant species in the area and can be easily differentiated from other earthworm species. Earthworms sampled were acclimatized in the native soils (kept in darkness, at room temperature, 26°C) for a week. All earthworms were

adults with clitellum, with average fresh weight of 1.0-1.4g. They were then transferred into containers with 300g of soils (5 adults per container, 4 replicates) and maintained for 3 weeks in the dark at room temperature. The control was containers with similar soils with the absence of earthworm.

After 3 weeks, earthworms were removed from the containers. Mucus collection was performed following the method proposed by Oleynik and Byzov [10] with some modifications. Fresh earthworm mucus and cast were obtained by inoculating the earthworms in sterile petri dishes with moist filter papers (5 earthworms per dish). Earthworms were bathed in sterilized distilled water for 3 times and blotted dry before placing into petri dishes. They were allowed to empty their guts on a moist filter paper in the dark for 6 hours, at room temperature (26°C). In the meantime, worm-worked soil (WWS) and control soil (CS) were collected and placed into sterilized micro centrifuge tubes.

After 6 hours, earthworms were removed from the petri dish and earthworm cast were carefully collected and placed into sterilized micro centrifuge tubes. Earthworm mucus was obtained from the outer body of the earthworms using micropipette. The mucus obtained was slimy and colorless. The mucus was then centrifuge at 8000 rpm for 5 minutes to remove any soil particles that were present. The supernatant was transferred into a clean microcentrifuge tube and vortex to mix well. Approximate 1ml of the earthworm mucus was filtered through a 0.2µm sterilized filter to obtain earthworm mucus that was free from microbes. Both unsterilized (EM) and sterilized earthworm mucus (CM) were used in the inhibition study.

2.2. Inhibition Study

BDB from the stock culture was revived on casamino peptone glucose (CPG) medium prior to the inhibition study. The culture was then transferred into sterilized distilled water and the concentration of the inoculums were adjusted to 10^3, 10^5 and 10^7 cell per milliliter (cell/ml) using Bio-Rad Smart Spec Plus spectrophotometer with sterilized distilled water (as positive control). The inoculum was then poured into lukewarm tetrazolium chloride (TZC) medium and mixed well before pipetting into petri dishes (10ml per dish).

Five drops of EM (20µl each) were carefully pipetted onto the solidified TZC medium. The same procedures were repeated with CM and sterilized distilled water (as controls) to evaluate the effect of earthworm mucus (both unsterilized and sterilized) on BDB. Meanwhile, approximately 0.5mg of earthworm cast (EC) and worm-worked soil (WWS) were put onto the medium with a sterilized spatula. The controls were similar soil without earthworm inoculation (CS), with 4 replicates for each treatment. The petri dishes were incubated at room temperature (26 °C). They were observed at 24 hours intervals for 7 days for the formation of visible inhibition zones. The length of the visible inhibition zones was measured, recorded and photographed. The inhibition experiment was repeated twice.

2.3. Molecular Identification

Pure cultures of microbial isolates that showed inhibition on BDB were obtained by streaking on TZC medium. A total of 16 bacterial isolates were obtained from EM. The antimicrobial property of each of these isolates was evaluated by streaking on TZC medium seeded with BDB. Isolates that showed inhibition were then selected for molecular identification using 16S rRNA universal primers. There were a total of 9 bacterial isolates that showed inhibition towards BDB, and therefore they were selected for molecular identification.

Molecular identification was performed on the bacterial isolates following the procedures described by Sim, et al. [14] with some modifications. The bacteria were cultured on CPG medium for 48 hours and sent for DNA extraction, PCR and sequenced at First BASE Laboratories Sdn. Bhd, Selangor, Malaysia.

Table-1. DNA sequences obtained from NCBI for the construction of phylogenetic tree

DNA Sequences obtained from GenBank	NCBI accession number
Pseudomonas sp. S61 16S ribosomal RNA gene, partial sequence	KT025916.1
Pseudomonas putida strain SKPf11 16S ribosomal RNA gene, partial sequence	KR492889.1
Pseudomonas monteilii strain LZU-9 16S ribosomal RNA gene, partial sequence	KP056325.1
Pseudomonas putida gene for 16S rRNA, partial sequence, strain: KF751	AB110608.1
Pseudomonas sp. NXUSASOPD003 16S ribosomal RNA gene, partial sequence	KP165021.1
Pseudomonas sp. PVR-YHB-5-2 16S ribosomal RNA gene, partial sequence	KP986957.1
Pseudomonas sp. INBio_4042AY 16S ribosomal RNA gene, partial sequence	KM242486.1
Pseudomonas sp. TL14(2) 16S ribosomal RNA gene, partial sequence	KF444360.1

Source: Accession numbers of DNA sequences obtained from National Centre for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/

For the PCR, 16S rRNA primer pair 357F (5' CCT ACG GGA GGC AGC AG 3') and 926R (5' CCG TCA ATT CMT TTR AGT 3') was selected for the amplification [14]. Each of the reaction contained 1.0 µl of DNA, 1 µl of the forward and reverse primer (10 µM), 12.5 µl of PCR Master Mix and 9.5 µl of nuclease free water. The reaction cycle consisted of initial denaturation at 95 °C for 1 minute, followed by 30 cycles of denaturation at 95 °C for 15 seconds, annealing at 46 °C for 15 seconds and extension at 72 °C for 10 seconds. Amplified DNA was subjected to gel electrophoresis and viewed under UV illumination for documentation. Samples with visible DNA bands were then purified and sequenced by the same laboratory. The sequenced fragments obtained were aligned with Bioedit software before BLAST with the DNA sequences deposited in the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/).
Maximum likelihood phylogenetic tree was constructed using MEGA v.6.0 program, where 1000 bootstrap replicates were performed. DNA sequences from the GenBank were obtained to

construct the phylogenetic tree (Table 1). *Escherichia coli* strain 761/12 (accession number: HG917880.1) was used as the out-group.

3. RESULTS

3.1. Effect of Earthworm Mucus, Cast and Worm-Worked Soil on BDB

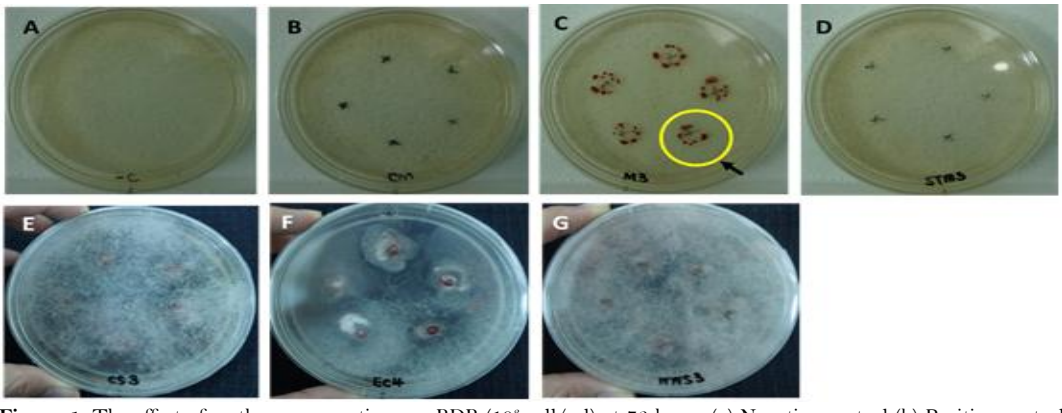

Figure-1. The effect of earthworm secretions on BDB (10^3 cell/ml) at 72 hours (a) Negative control (b) Positive control (sterilized distilled water) (c) Earthworm mucus (EM) (d) Sterilized earthworm mucus (CM) (e) Soil without earthworm treatment (CS) (f) Earthworm cast (EC) (g) Worm-worked soil (WWS)
Source: Photos of culture plates captured with Canon DS126151 camera in the laboratory

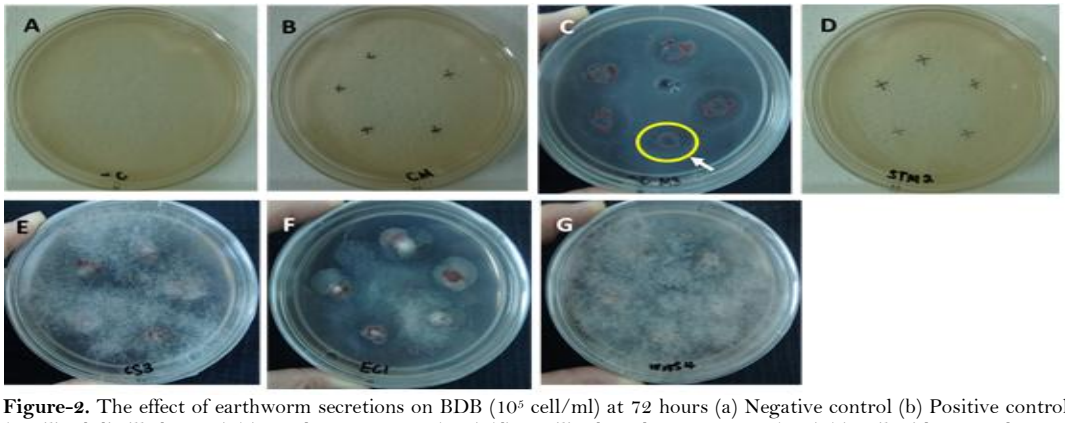

Figure-2. The effect of earthworm secretions on BDB (10^5 cell/ml) at 72 hours (a) Negative control (b) Positive control (sterilized distilled water) (c) Earthworm mucus (EM) (d) Sterilized earthworm mucus (CM) (e) Soil without earthworm treatment (CS) (f) Earthworm cast (EC) (g) Worm-worked soil (WWS)
Source: Photos of culture plates captured with Canon DS126151 camera in the laboratory

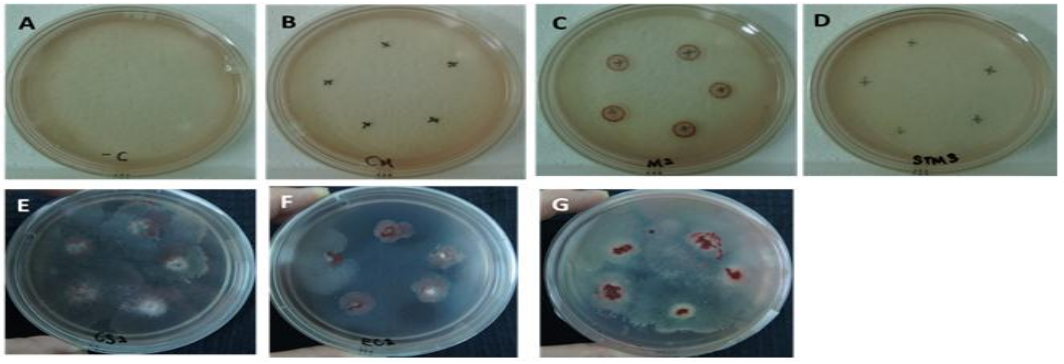

Figure-3. The effect of earthworm secretions on BDB (10^7 cell/ml) at 72 hours (a) Negative control (b) Positive control (sterilized distilled water) (c) Earthworm mucus (EM) (d) Sterilized earthworm mucus (CM) (e) Soil without earthworm treatment (CS) (f) Earthworm cast (EC) (g) Worm-worked soil (WWS)
Source: Photos of culture plates captured with Canon DS126151 camera in the laboratory

Observations on TZC medium seeded with BDB for a period of 7 days showed that earthworm mucus (EM) inhibited the growth of BDB at 10^3 and 10^5 cell/ml, with the formation of visible inhibition zones at 72 hours (Figure 1c and 2c). However, no inhibition was observed on BDB at 10^7 cell/ml (Figure 3c). Interestingly, sterilized earthworm mucus (CM) did not show any inhibition of BDB at 10^3, 10^5 and 10^7 cell/ml (Figures 1d, 2d and 3d). Bacterial colonies were observed around EM whereas both bacterial and fungal colonies grew around soil without earthworm treatment (CS), earthworm cast (EC) and worm-worked soil (WWS). However, there were no inhibition zone observed on BDB at 10^3, 10^5 and 10^7 cell/ml respectively (Figures 1e, f and g; 2e, f and g; 3e, f and g).

The length of inhibition zones that surrounded EM were measured and recorded for a period of 7 days (Figure 4). There was no inhibition zone observed around EM on Day 1 and 2 for BDB at 10^3, 10^5 and 10^7 cell/ml. However, the EM treatment on BDB at 10^3 cell/ml produced inhibition zones at Day 3. The zone increased from Day 4 to 6 and remained constant after Day 6. Meanwhile, for EM treatment on BDB at 10^5 cell/ml, the inhibition zone appeared on Day 3 did not increase in length thereafter. The length decreased from Day 4 to 5 and remained constant after Day 5. There was no inhibition zone observed on mucus treatment on BDB at 10^7 cell/ml.

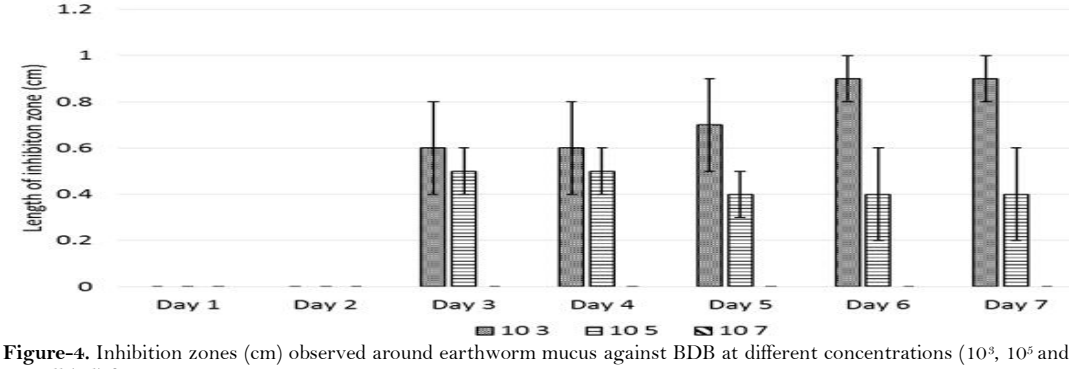

Figure-4. Inhibition zones (cm) observed around earthworm mucus against BDB at different concentrations (10^3, 10^5 and 10^7 cell/ml) from Day 1 to 7
Source: Figure constructed from the average length of inhibition zones formed on the seeded BDB culture medium

3.2. Molecular Identification

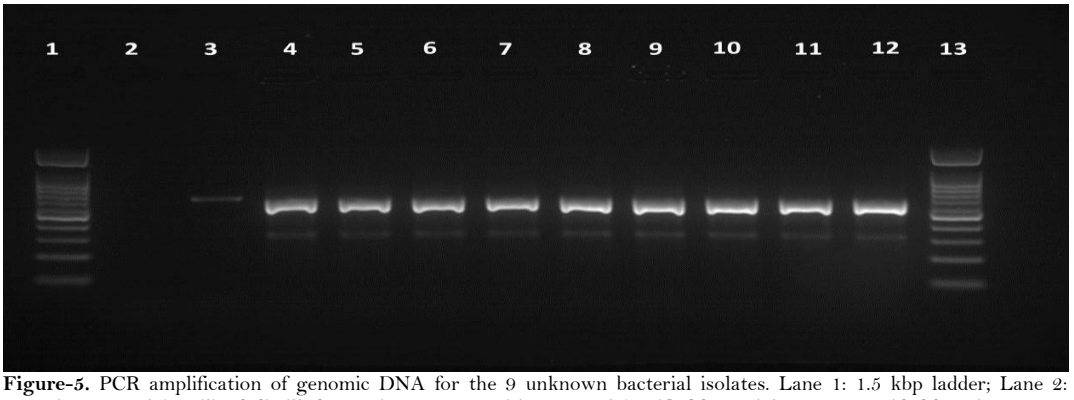

Figure-5. PCR amplification of genomic DNA for the 9 unknown bacterial isolates. Lane 1: 1.5 kbp ladder; Lane 2: Negative control (sterilized distilled water); Lane 3: Positive control (purified bacterial gDNA provided by First BASE Laboratories Sdn. Bhd.); Lane 4: Isolate M1C; Lane 5: Isolate M1D; Lane 6: Isolate M2B; Lane 7: Isolate M2C; Lane 8: Isolate M2E1; Lane 9: Isolate M2E2; Lane 10: Isolate M3C; Lane 11: Isolate M3D; Lane 12: Isolate M3E and Lane 13: 1.5 kbp ladder

Source: Image of gel electrophoresis documented by First BASE Laboratories Sdn. Bhd, Selangor, Malaysia

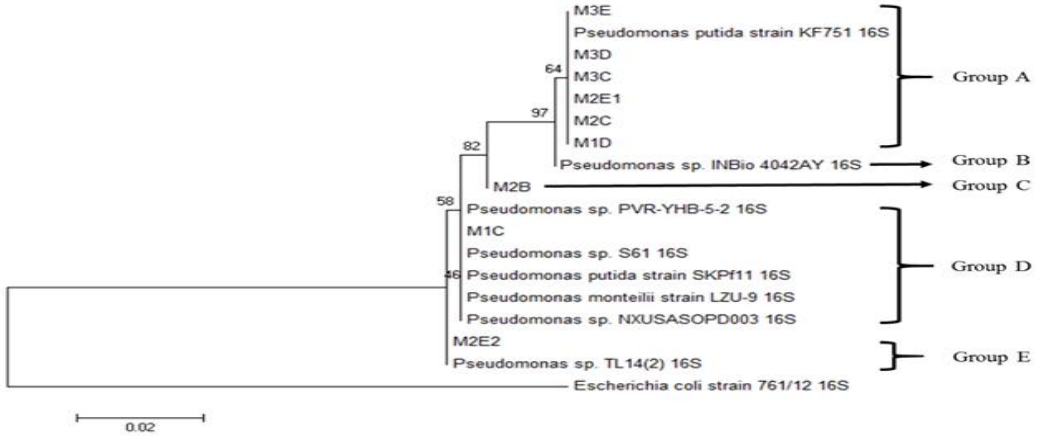

Figure-6. Phylogenetic tree built using Tamura-Nei method with 1000 bootstrap replicates. The maximum likelihood bootstrap values that are more than 50% are shown above the branches

Source: Phylogenetic tree constructed using MEGA v.6.0 program

DNA isolated from the 9 bacterial isolates were amplified with the universal primer (Figure 5) and compared with the sequences deposited at GenBank. The sequence homology from the BLAST search at NCBI suggested that all the 9 bacterial isolates have high percentage of similarity (100%) with the bacteria from the genus *Pseudomonas*. Phylogenetic tree suggested that all the bacterial isolates are grouped into the genus *Pseudomonas* (Figure 6), with 6 of them are clustered into Group A together with *Pseudomonas putida* (bootstrap value of 64%). Meanwhile, isolate M2B is grouped differently from Group A and B with bootstrap value of 82%. Isolate M1C is clustered differently from Group A, B and C with bootstrap value of 58% whereas isolate M2E2 is clustered into Group E with bootstrap value of 46%.

4. DISCUSSION

In the *in-vitro* study, mucus secreted by *P. corethrurus* inhibited the growth of BDB at 10^3 and 10^5 cell/ml. Inhibition zones were observed to form around the mucus at 72 hours and the inhibition zone remained for up to 7 days (168 hours). However, no inhibition zone was observed when the mucus was sterilized (CM). The observations suggested that the microbes in earthworm mucus were responsible in suppressing BDB, as negative result was shown in the sterilized mucus counterpart. From the sequence homology from BLAST search and phylogenetic tree, the bacteria obtained were grouped into the genus *Pseudomonas*. Pseudomonads have been widely studied due to their extensive distribution in soil and ability to colonize root rhizospheres [15]. They are also found to produce various metabolites and enzymes that mediate plant growth and involve in disease suppressions [16]. Pseudomonads isolated from the mucus of *P. corethrurus* may lead to antagonism effect or secretion of chemicals that suppressed BDB. Bityutskii, et al. [17] reported that earthworm mucus promoted higher organic residues mineralization and soil microbial populations. This was mainly due to the presence of various dissolved and available amino acids, sugars, ammonium and maltose in earthworm mucus that favored the growth and development of soil microbes. This serves as the priming effect in promoting higher microbial community that resulted in competition for various resources [17] hence creating a dilution effect and reduce the pathogenic effects of BDB towards the host plant.

The inhibitory effect of *Pseudomonas* sp. was not observed when similar amount of earthworm mucus (20µl) was inoculated onto TZC medium seeded with higher density of BDB (10^7 cell/ml). This was probably due to the density of *Pseudomonas* sp. was not sufficient in inhibiting the growth of BDB at this relatively high concentration. It could also be the effect of *Pseudomonas* sp. was masked by the high density of BDB, resulting in no inhibition observed. Nevertheless, the study provides a promising alternative in disease control, besides the use of resistant cultivars and chemicals. In soil with bacterial wilt disease incidences, the bacterial density are normally difficult to be detected and can be as low as $10^2 - 10^4$ cfu/g [2]. Introduction of endogeic earthworms or inducing their population in the soil could therefore, be useful as a mode of prevention or bioremediation for blood disease. It is useful in limiting the density of pathogen below the threshold level, which prevents the occurrence of the disease.

Soil fumigation is one of the effective treatments in controlling bacterial wilt diseases [18]. However, it is costly and possess threat to the environment [19]. The present study suggested the potential role of *P. corethrurus* in suppressing BDB. It also indicated that plants may indirectly gain protection from pathogen in soils rich with endogeic earthworms. The wall of earthworm burrows (drilospheres) that are rich in mucus will play an essential role in disease suppression. As earthworms burrow in the soil, they push soil aside, ingest and egest soil, incorporate litter and fine particles as well as secrete mucus to aid their movement in the burrows [20, 21]. These burrow walls create a conducive environment for a diversity of microbes and maintain a balance soil microbial community. Such interaction will benefit the root rhizosphere and reduce the detrimental effect of various soil-borne pathogens towards the host plant. Therefore, biological

suppression is naturally established when the pathogen is direct or indirectly suppressed by the native microbial community [22].

Interestingly, there was no inhibition observed for both earthworm cast (EC) and worm-worked soil (WWS) on BDB at 10^3, 10^5 and 10^7 cell/ml. This indicated that the microbial populations colonizing earthworm cast and worm-worked soil were different from the microbes found in mucus. This was probably due to the presence of different composition or density of microbial populations in earthworm cast and worm-worked soil when the soil passed through earthworm gut. Several studies had reported that earthworms selectively include certain microbes in their diet [23, 24] and alter soil physical, chemical and biological properties through their ingestion and egestion [25, 26]. Therefore, it promoted the growth and development of different microbial populations upon gut passage. Previous studies had documented higher microbial populations in soil and drilospheres enriched with earthworm mucus compared to surrounding soil [21, 27, 28]. However, the results vary depending on the earthworm species, and the extent to which microbial population that is associated to this alteration is not known. Therefore, more study should be conducted to further identify the microbial community that are present or influence by earthworm and its secretion. This is important to gain more in-depth understanding on the mechanisms and microbial compositional changes driven by earthworms in improving soil and plant health.

5. CONCLUSION

Pseudomonas sp. found in the mucus of *P. corethrurus* inhibited the growth of BDB at 10^3 and 10^5 cell/ml. The presence of *P. corethrurus* may contribute to the formation of natural suppressive soil that is able to reduce the severity or disease incidence, despite of the presence of BDB in soil. Though it is fairly premature, but the present study recorded promising result of utilizing earthworm in remediating blood disease in banana. The presence of a rich diversity of soil microbial population creates a dilution effect that reduce the dominancy of certain microbes, including potential pathogens to the host plant. Nevertheless, such hypothesis needs further studies to verify the role of *P. corethrurus* in remediating bacterial wilt disease.

6. ACKNOWLEDGEMENT

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