Antagonistic Effect and Plant Growth Hormone Produced by Endophyte *Bacillus amyloliquefaciens* LKM-UL Isolated from Cocoa Plant

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Abstract: In Malaysia black pod disease caused by *Phytophthora palmivora* is one of the serious problems causing yield loss in cocoa plantation. In this study, an endophytic bacterium living within tissues of healthy *Theobroma cacao* L. leaves was isolated and assessed its ability to inhibit the growth of *P. palmivora*. Based on molecular identification using 16S rDNA gene sequences analysis, this endophytic bacterium was identified as *Bacillus amyloliquefaciens* which was subsequently named as *Bacillus amyloliquefaciens* LKM-UL. Preliminary test using dual culture method showed *B. amyloliquefaciens* LKM-UL has strong ability to inhibit the growth of *P. palmivora*. *B. amyloliquefaciens* showed the highest antifungal activity after 24 h incubation in nutrient broth. Cells-free supernatant from *B. amyloliquefaciens* showed the highest antifungal activity of 30.6 mm with cell concentration of $Log_{10}10.63$ CFU/mL via agar disc diffusion method. *B. amyloliquefaciens* LKM-UL also produced plant growth hormone identified as 1-naphthalene acetic acid (NAA), tryptamine, 3-indole propionic acid (IPA), indole-3-butyric acid (IBA), gibberellic acid (GA), trans-zeatin and kinetin.

Key words: Antifungal activity, cocoa, endophyte, plant growth hormone.

1. Introduction

Cocoa (*Theobroma cacao*) was first introduced into Malaysia in 1950s to diversify the plantation agriculture after oil palm and rubber. Malaysia today is the fifth largest cocoa grinder in the world and grinding is expected to continue and expand further in future in between 350,000 to 400,000 ha [1]. However, high levels of yield loss to pests and disease is a major problem for world cocoa production in Africa, Brazil and Asia including Malaysia. One of the most threatening fungal pathogen that has significantly dropped the annual pod yield production in Malaysia is the black pod disease caused by *Phytophthora palmivora* which affects the cocoa pods [2].

In recent years, the use of chemical pesticides to control the disease have been substantial criticism, mainly due to their adverse effects on the environment, human health and other non-target organisms. The use of microbe-based biocontrol agents such as endophytic bacteria is preferable due to their capability to reside inside plants while producing bioactive substances (plant growth regulatory, antibacterial, antifungal,

antiviral and insecticidal) in enhancing the plant growth and controlling fungal pathogens [3]. The advantages of using endophytic bacteria to control the cocoa pathogen is because they have been adapting to colonization inside the cocoa plants and providing promising suppression of pathogen [4] without causing environmental contamination compared to fungicides and pesticides application [5].

Endophytes also have been used as inoculants to promote plant-growth. The ability of bacterial endophytes to promote plant-growth occurs as a result of direct mechanisms, either facilitates the acquisition of essential nutrients or modulates of level of hormones within a plant [6]. Modulation of hormone levels may cause PGPB (Plant Growth-Promoting Bacteria) synthesizing one or more of the phytohormones such as auxin, cytokinin and gibberellin [7], [8]. The indirect promotion of plant growth occurs when a PGPB decreases the damage to plants following infection with a phytopathogen by inhibiting the growth of pathogen.

In this study, endophytic bacteria from healthy cocoa leaves have been isolated and the antagonistic activities against cocoa fungal pathogen, specifically *P. palmivora* was evaluated. Subsequently, the selected endophyte was tested for its ability to produce plant-growth hormone.

2. Methodology

2.1. Isolation of Endophytic Bacteria

The isolations of endophytic bacteria were carried out using the protocol as described by Shimizu [9] with some modifications. Twigs and leaves were collected from healthy cocoa tree grown in Sabah, Malaysia. The surface-sterilized plant pieces were aseptically placed onto nutrient agar (NA) plate and incubated at 28^{o}C for 7 days. The colonies appeared around the plant tissues were restreaked onto fresh NA until pure culture was obtained.

2.2. Characterization and Identification of Endophytic Bacteria

The selected endophytic bacteria was identified by using morphological, biochemical and molecular procedures [10]. Gram staining was also performed to confirm the purity of the isolate. Molecular genetics identification of the bacterial isolate was performed by amplification of 16S rDNA gene using universal primers (forward [F]: 5-AGAGTTTGATCCTGGCTCAG-3, reverse [R]: 3-GGTTACCTTGTTACGACTT-5). Polymerase chain reaction (PCR) reaction was carried out and the PCR product was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). The bacterial 16S rDNA sequences obtained was then aligned with known 16S rDNA sequences in the GenBank database using the basic local alignment search tool (BLASTn) at the National Center for Biotechnology Information (NCBI).

2.3. Fungal Pathogen Culture P. palmivora

The cocoa pathogen, *P. palmivora* was obtained from Malaysia Cocoa Board Culture Collection. Stock cultures of fungal isolate was maintained on potato dextrose agar (PDA) and stored at 4^{o}C. The working culture was established by transferring the pathogen onto PDA and incubated for 7 days at 30^{o}C. This culture was used for subsequent experiment.

2.4. Antagonistic Activity of *B. amyloliquefaciens* LKM-UL against *P. palmivora*

The antagonistic activity was done via dual culture method [11]. The endophytic bacteria was streaked onto one side of PDA plate, while a 6 mm x 6 mm agar cube containing mycelia of cocoa pathogen from 7 days-old culture was placed close to the bacterial streak. The Petri dish was incubated at 30^{o}C for one week. The inhibition zones (mm) were recorded by measuring the clear distance between the edges of the fungal mycelium and the bacterial streak.

2.5. Determination the Growth of *B. amyloliquefaciens* LKM-UL and Antagonistic Activity

The bacterial isolate was inoculated in 100 mL nutrient broth (NB) in a 250 mL conical flask and incubated overnight at 28°C with agitation at 120 rpm. The culture was centrifuged at 4000 rpm for 30 min and the pellet was suspended in normal saline for preparation of standard inoculums [12]. Subsequently, 10% (v/v) of standard inoculums of bacterium was transferred into 100 mL NB and incubated at 28°C. The number of bacteria in the culture medium was enumerated using the spread plate method after 8, 12, 24, 36, and 48 hours of incubation and expressed as colony forming units per milliliter (CFU/mL).

For preparation of cell-free supernatant, 1.0 mL of culture was then centrifuged at 4,000 rpm for 30 min at 4°C. Sterile filter paper disc (6 mm) was impregnated with 100 µL of cell-free supernatants (after filtered through 0.2 µm filter) and placed onto 7 days old *P. palmivora* and incubated for 7 days at 30°C. The antifungal activity was determined by measuring the inhibition zone of mycelial growth of the pathogen around the filter paper disc.

2.6. Extraction of Plant Growth Hormones

The extraction of plant growth hormone was performed by method adapted from Miezah [13]. *B. amyloliquefaciens* was grown in 100 mL of nutrient broth in 500 mL conical flask and incubated at 28 ± 2°C with agitation of 120 rpm for 24 hours. The culture was centrifuged at 7,700 x g for 30 minutes. The cell-free supernatant was reduced to 50 mL by evaporation under vacuum and adjusted to pH 2.8 using 1 N HCl. The plant growth hormone from cell-free supernatant was extracted using ethyl acetate to produce ethyl acetate fraction and aqueous fraction. Ethyl acetate fraction was evaporated under vacuum and dissolved in absolute methanol for thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC) bioassay. Aqueous fraction was adjusted to pH 7.0 using 1 N NaOH and further extracted with water-saturated *n*-butanol. Fraction of *n*-butanol was separated and evaporated under vacuum and dissolved in absolute methanol for TLC and HPLC bioassay.

2.7. Detection Plant Growth Hormones Using Thin Layer Chromatography (TLC)

TLC chromatogram was run on 3 cm width x 10 cm length preparative silica gel plates (Gel silica $60GF_{254}$, Merck). The solvent system used were chloroform:ethyl acetate:formic acid (50:40:10, v/v) to separate indole compounds and gibberellins in ethyl acetate fractions. Another solvent *n*-butanol:acetic acid:water (12:3:5, v/v) was used to separate cytokinin in *n*-butanol fraction. One mL of fraction was spotted onto TLC plate, air-dried then subjected to the devolving solvent as mobile phase. The plates were then visualized under the UV light at wavelength 254 nm. Besides that, the plate was exposed to iodine vapour in a closed jar containing iodine crystals [14]. The relative fluidity (R_f) value of each separated spots were measured.

2.8. Identification of Plant Growth Hormones by High Pressure Liquid Chromatography (HPLC)

HPLC chromatogram was produced by injecting 10 µl of the filtered extracts onto a reverse-phase column (Nucleosil EC 300/4 100-10 C_{18}) using JASCO ChromPass Chromatography System (JAPAN), equipped with Photo Diode Array (MD 2010 plus) at 254 nm. Two solvent systems were used to separate hormones compounds. Solvent A was 85% of water:acetonitrile:acetic acid (85:15:1, v/v) and solvent B was 15% of 30% methanol in water, with flow rate of 1.0 mL/min. Retention times for peaks were compared to hormones standard; 3-indole acetic acid (IAA-Sigma), 1-naphthalene acetic acid (NAA-Sigma), indole-3-butyric acid (IBA-Sigma), 3- indole propionic acid (IPA-Aldrich), tryptamine (TRY-Aldrich), trans-zeatin (Sigma), kinetin (Sigma) and gibberellic acid (GA-Sigma).

3. Results and Discussion

Several endophytic bacteria that colonize inside plant tissues were successfully isolated from surface-sterilized of healthy cocoa twigs along with leaves. One of the isolate (designated as LKM-UL) showed strong inhibition distance of 32.2 mm against *P. palmivora*. The morphological and biochemical characterization showed the bacteria appeared as opaque colonies on the NA medium and Gram positive-rod. The isolate exhibited positive results for motility, catalase and Voges Proskauer tests but negative for methyl red.

The result of the 16S rDNA gene showed 100% similarity of isolate LKM-UL with *Bacillus amyloliquefaciens*, and thus designated as *Bacillus amyloliquefaciens* LKM-UL. The 16S rDNA sequence was submitted to GenBank with the accession number of KR560041.

The growth of *B. amyloliquefaciens* LKM-UL and the ability to inhibit cocoa pathogen from cells-free extract is showed in Fig. 1. Inhibitory activity was detected from the beginning of the cells growth, during exponential and stationary phases. The highest antagonistic activity was observed in the early stationary phase of bacterial growth obtained at 24 hours of incubation period. The optimum inhibition zone (30.6 mm) was concurrent with the optimum cells growth (Log_{10} 10.63 CFU/ mL). Further incubation period showed a decreased in the antagonistic ability and also the cells growth. The ability of *B. amyloliquefaciens* to reduce the mycelial growth of *P. palmivora* reveals certain substances produced by the bacterium that has fungistatic effect.

This finding is in accordance with previous reports, in which endophytic bacteria produced antifungal to protect plant from pathogens. Endophytic bacterium, *Bacillus subtilis* strain EDR4 showed inhibitory effect on stem rot caused by *Sclerotinia sclerotiorum* [15]. Meanwhile, *Bacillus amyloliquefaciens* isolated from tomatoes acquired longer cultivation periods of 48 hours to inhibit *Alternaria solani,* a pathogenic fungus which caused early blight in tomato [16]. The rapid growth of endophytic bacteria offered advantage in the race for space and nutrients against pathogenic fungi, before any antifungal activity was deployed [17].

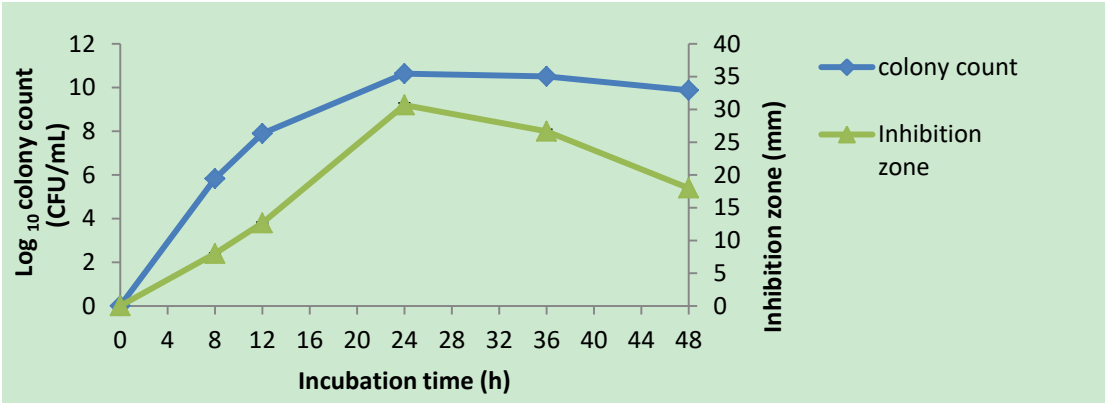

Fig. 1. The growth of *B. amyloliquefaciens* LKM-UL and inhibition zones of cells-free supernatant against cocoa pathogen.

Plant growth hormones extracted from *B. amyloliquefaciens* LKM-UL using ethyl acetate fraction on TLC plate showed three clear individual spots and several faded spots of indole (auxins) and gibberellins hormones (Fig 2a). The clear spots detected at *Rf* value of 0.3, 0.7 and 0.9. The faded spots were due to the type of solvent used for separation of indole compounds which damaged some hormones [18]. *Azospirillum brasilense* produced substance associated with auxin activity in cell-free supernatant which can be spotted by TLC apparatus [19]. Meanwhile, the *n*-butanol fraction revealed cytokinin spot detected at *Rf* value of 0.5, 0.7 and 0.9 (Fig. 2b). Cytokinin has been observed in several bacteria including *Rhizobium* sp., *Bacillus subtilis* BC1 and *Escherichia coli* K12 by TLC method showing *Rf* value of 0.65 [20].

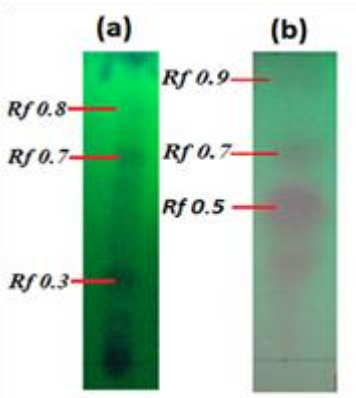

Fig. 2. TLC chromatogram of *B. amyloliquefaciens* LKM-UL from (a) Ethyl acetate fraction and (b) *n*-butanol fraction showing different R_f values.

Identification of indole (auxin) and gibberellins hormones from ethyl acetic fractions using HPLC revealed that, *B. amyloliquefaciens* LKM-UL produces NAA, TRY, IPA, IBA and GA (Fig 3a). Meanwhile, the cytokinin hormones from *n*-butanol fraction showed that the isolate produced trans-zeatin and kinetin (Fig 3b). Other study showed *Bacillus subtilis* LK14 isolated from *Moringa peregrine* plant produced auxin hormones that can improved the growth of *Solanum lycopersicum* [21]. Auxin hormones were known to play important role in cell elongation, division and enlargement [22] and GA could stimulated the shoot length, chlorophyll contents and increased biomass of rice plant [23]. Interestingly, *B. amyloliquefaciens* LKM-UL isolated from cocoa plant have the ability to produce multiple types of auxins (1-naphthalene acetic acid, tryptamine, 3-indole propionic acid and indole-3-butyric acid), gibberellic acid and cytokinins (trans-zeatin and kinetin). These growth-promoting hormones (auxin, cytokinins, gibberellic acid, and abscisic acid) either inhibit or potentiate stress-specific hormones in mediating the protection or susceptibility of the plant against the invading pathogen [24].

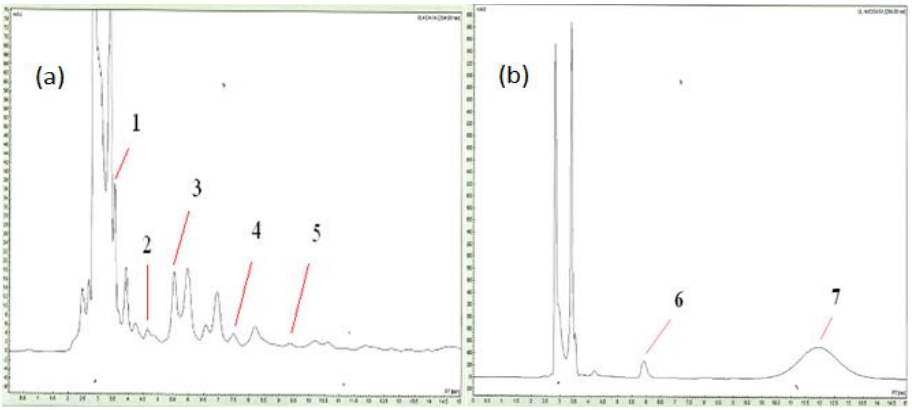

Fig. 3. HPLC chromatograms of *B. amyloliquefaciens* LKM-UL showed plant growth hormones from (a) ethyl acetate fraction and (b) n-butanol fraction; 1: NAA, 2: TRY, 3 IPA, 4: IBA, 5: GA, 6: Trans-zeatin and 7: Kinetin.

4. Conclusion

Endophytic bacteria have been reported to live in symbiosis within diverse plants but their known function is still limited. Since *B. amyloliquefaciens* LKM-UL exhibit antifungal activity against *P. palmivora* and also producing several growth hormones, therefore it can be developed as potential biocontrol and

biofertilizer for cocoa plant.

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