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Endophytic bacteria with antagonistic activities against pathogenic fungal *Oncobacidium theobromae* of cocoa

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Vascular streak dieback disease caused by pathogenic fungal *Oncobasidium theobromae* known as one of the serious issue causing great decline in Malaysia cocoa plantations. The objectives of this study were to determine antagonistic activity and growth rates of four endophytic bacteria against cocoa pathogen, to determine the potential of the endophyte as biological control against pathogen on cocoa seedlings and to identify active compounds produced by the selected endophyte. Four selected endophytic bacteria previously isolated within tissues of healthy *Theobroma cacao* L. designated as *Bacillus amyloliquefaciens* LKM-UL, *Pantoea agglomerans* LKM-PA, *Bacillus pumilus* LKM-PD and *Bacillus subtilis* LKM-BL were assessed for their abilities to inhibit the growth *O. theobromae.* Preliminary tests via dual culture method showed endophyte *B. subtilis* LKM-BL has the strongest antagonistic activity to inhibit the growth of *O. theobromae* followed by *B. amyloliquefaciens* LKM-UL, *P. agglomerans* LKM-PD and *B. subtilis* LKM-PA. The cell-free supernatant at 24 hours incubation from LKM-BL produced the highest antifungal activity compared to others. *B. subtilis* LKM-BL with strongest antagonistic activity was selected as biological control on cocoa seedlings. *B. subtilis* LKM-BL significantly protected cocoa seedlings against *O. theobromae* and the protective value of this treatment was 87.9% (at 30 days inoculation) and produced growth-promoting effects on cocoa seedlings compared to non-inoculation. Identification of antifungal compounds revealed two suggested compounds identified as Macrolactin A and Macrolactin M. These findings demonstrated that *B. subtilis* LKM-BL can be the most promising candidate to be exploited as biological control agent against cocoa fungal disease.

Keywords: Endophyte, bacteria, cocoa, antagonistic, *Oncobasidium theobromae*

INTRODUCTION

The profitable and quality beans of *Theobroma cacao* L. are used in money-making chocolate industry, thus making the plants as one of the leading crops in Malaysia. Unfortunately, the crop industry suffers from damaging fungal pathogen that causes the vascular-streak dieback (VSD) disease and widely spread by basidiomycete of *Oncobasidium theobromae* (Samuels et al., 2012). The fungus grows entirely inside the vascular vessels through the cuticle above the leaf veins and spreads internally to all branches and mostly destroys the whole tree if it extends to the trunk (Guest and Keane, 2007).

Controlling the disease by spraying chemical fungicide can be costly, as well as can increase exposure to environmental contaminations and not completely effective in high rainfall areas (Agbeniyi et al., 2014). Endophytic bacteria are now a center of attention because of the demand to reduce the use of chemicals, especially in concerns about uphold of environmental protection (Vale et al., 2010). The awareness of agriculture and food contaminations by chemical residue in gaining productivity by shielding plants from pathogens using chemical-based management that are more environmental friendly(Paul et al., 2013).

Endophytic bacteria capable to colonize the internal tissues of healthy plants and protect the plants by producing bioactive substances are suitable to be adapted as biological control agent (Lin et al., 2013). The advantages of using endophytic bacteria to control the cocoa pathogen *O*. *theobromae* is that they had been found to adapt rather well colonizing inside of cocoa plants, therefore, they could provide promising suppression of VSD without causing environmental contamination (Paul et al., 2013). The mechanism is like a lethal shield to protect the plant from getting invaded by the cocoa pathogen *O*. *theobromae*.

Endophytic bacteria, as previously reported, have the capability to produce antifungal substances that can be used in controlling pathogens of tomato (Yi et al., 2015), rapeseed (Chen et al., 2014), poplar (Ren et al., 2013), cucumber (Cao et al., 2012), corn (Petatan-Sagahon et al., 2011), banana (Fu et al., 2010), and many other plants. Antifungal activities from many endophytic bacterial isolates against a wide range of phytopathogens like *Rhizoctonia* sp., *Sclerotium* sp., *Colletotricum* sp., *Phytophthora* sp., *Fusarium* sp., *Macrophomina* sp. and *Alternaria* sp. had been studied (Jasim et al., 2016). Among the various bacterial genera, the genus *Bacillus* has already been known to produce antifungal bioactive compounds but the identities of the compounds vary.

The aims of this study were to determine the antagonistic activities and growth rates of four selected endophytic bacteria against cocoa pathogen, specifically *O. theobromae*. Subsequently, the endophyte with the highest antagonistic activity were selected to determine its potential to be developed as biological control agent on cocoa seedlings and subsequently used for the identification of antifungal compounds it produces. Since studies on endophytic bacteria isolated from cocoa plant are very limited, the study is significant and will be developed as locally produced biological control agent for cocoa plantation specifically in Malaysia.

MATERIALS AND METHODS

Endophytic bacteria cultures

Four selected endophytic bacterial *Bacillus amyloliquefaciens* LKM-UL*, Pantoea agglomerans* LKM-PA, *Bacillus pumilus* LKM-PD and *Bacillus subtilis* LKM-BL designated as LKM-UL, LKM-PA, LKM-PD and LKM-BL respectively were previously isolated from internal tissue of healthy cocoa plant were obtained from Malaysia Cocoa Board Culture Collection. Stock cultures of the endophytic isolates were stored at -80^{o}C in nutrient broth with 15% glycerol (Shin et al., 2007). The working cultures were established by a streaking technique from the stock cultures onto freshly prepared nutrient agar (NA) in Petri dishes and incubated for 24 h at 28^{o}C.

Fungal pathogen culture

The cocoa pathogen, *O*. *theobromae* was obtained from Malaysia Cocoa Board Culture Collection. Stock culture 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 freshly prepared PDA and incubated for 7 days at 30^{o}C. This culture was used for subsequent experiment.

Antagonistic activity of endophytic bacteria against *O. theobromae*

The antagonistic activity was done via dual culture method (Shin et al., 2007). 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 dishes were incubated at 30^{o}C for one week. The inhibition zone (mm) was recorded by measuring the clear distance between the edges of the fungal mycelium and the bacterial streak.

Determination of endophytic bacterial growth and antagonistic activity

The bacterial isolates were inoculated in 100 mL nutrient broth (NB) in a 250 mL conical flask and incubated at 28^{o}C, 120 rpm for 24 hours. The culture was centrifuged at 4000 rpm for 30 min and the pellets were suspended in normal saline for preparation of standard inoculums (Ainon et

al., 2010). Subsequently, 10% (v/v) of standard inoculums (10^8 $CFUmL^{-1}$) of each bacterium was transferred into 100 mL NB and incubated at 28°C, 120 rpm for 24 hours. The growth was monitored by measuring the absorbance at 550 nm (A_{550}) using a UV-spectrophotometer after 4, 8, 12, 16, 24, 28, 32, 36, 40, 44 and 48 hours of incubation.

For preparation of cell-free supernatant, one mL of each culture at different growth time interval was then centrifuged at 4000 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). About 100 µL of mycelia suspension culture of *O*. *theobromae* was spread on PDA plate and paper disc with cell-free supernatants was placed at the centre of the plate 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.

Evaluation of selected endophytic bacteria for biological control against *O*. *theobromae* on cocoa seedlings

The potential endophytic bacterium with the highest antifungal activity was used as biological agent on cocoa seedlings. The healthy cocoa seed were washed in running tap water for 5 min. Seeds were surface sterilized for 2 min in 70% ethanol, 10 min in 3.5% sodium hypochlorite, washed 3 times in sterile water and germinated on sterile moist towels. The rooted seeds were transplanted into plastic pots filled with autoclaved soil in greenhouse. Two months after transplantation, the seedlings were inoculated at a dose of 100 mL suspension culture of selected endophytic bacteria (10^8 $CFUmL^{-1}$) by directly spraying to the leaves. After 24 hours the seedlings treated with endophyte and untreated were challenge-inoculated with 100 mL mycelium suspension of *O*. *theobromae* by applying with brush to both surfaces of each leaves. Seedlings without endophyte and pathogen were sprayed with sterile distilled water as control. The seedlings were maintained by suitable watering. The seedlings were assayed for disease 2, 4, 6, 8, 10, 15 and 30 days after the inoculation with three replicates. The disease was scored by the percentage of seedlings with any lesions symptom that developed on the leaves. Protective value as a degree of suppression was calculated by the following formula:

Protective value = (1 – percentage of endophytic bacteria treatment/percentage of non-endophytic bacteria as control) x 100 (Tsuda et al., 2001).

Identification of antifungal bioactive compounds from selected endophytic bacteria

The selected endophytic bacterium with the highest antifungal activity was used to identify antifungal bioactive compounds. About 10% (v/v) of standard inoculums (10^8 $CFUmL^{-1}$) of bacterium was transferred into 100 mL NB and incubated at 28°C with agitation at 120 rpm for 24 h. After 24 hours incubation, the culture was centrifuged at 10,000 rpm for 20 min to get cell-free supernatant. The cell-free supernatant was freeze-dried (Labconco, USA) and 1 g of the dried sample was mixed with 1 mL of methanol. The sample was fractionated via Thin Layer Chromatography (TLC). The TLC analysis of cell-free supernatant was performed on silica gel plate (pre-coated $60GF_{254}$, Merck) and chloroform: methanol (8: 2) (v/v) was used as a mobile phase. The separated spots on the TLC chromatograms were visualized under UV_{254} nm and the R_f value of each separated spots was measured. The separated spots were removed by scraping individually and dissolved in 1 mL methanol and centrifuging at 4000 rpm for 30 min. The samples were tested for antagonistic activity against pathogens using paper disc as described above.

Samples with positive results were analyzed via High Performance Liquid Chromatography (HPLC) (Eksigent,NanoLC) using two mobile phase solvents; solvent A (H_2O containing 1% formic acid, 5 mm ammonium formate) and solvent B (Acetonitrile containing 1% formic acid, 5 mm ammonium formate) at a flowrate of 8 µL/min with gradient: 10-95% B for 20 min and 95-10% B for 10 min. Then, the purified fraction was analyzed through direct infusion to the ion source Liquid Chromatography Mass Spectrometer (LCMS) (AB Sciex Triple TOF 4600). The mass spectrometer was operated with a Duo Spray source and an Electrospray Ionization (ESI) probe. The compounds were analysed based on their MS/MS fragmentation spectra data using Eksigent Software v. 4.1 and were identified by using the ChemSpider database.

Statistical analysis

All experiments were conducted in triplicate and the data are expressed as mean ± standard deviation after analyzed via one-way ANOVA using the SPSS 13.0. The statistical significance was set at a confidence level of $p< 0.05$.

RESULTS AND DISCUSSION

Antagonistic activity of endophytic bacteria against *O. theobromae*

The antagonistic activities of four selected endophytic bacteria via dual culture method against cocoa pathogen *O. theobromae* showed that endophytes capable to produced antifungal activity to inhibit the growth of plant pathogen. Endophytic bacteria LKM-BL produced the highest inhibition distance of 39.2 ± 0.4 mm and had been significantly different $p < 0.05$ compared to LKM-UL, LKM-PD and LKM-PA (30.4 ± 0.8 mm,27.8 ± 1.6 mm, and 17.6 ± 0.8 mm respectively) (Table 1).

Table 1: Antagonistic activities of selected endophytic bacteria against cocoa pathogen *O. theobromae*

Endophytic bacteria	Inhibition zones (diameter, mm)*
LKM-UL	30.4 ± 0.8^{c**}
LKM-PA	17.6 ± 0.8^{a}
LKM-PD	27.8 ± 1.6^{b}
LKM-BL	39.2 ± 0.4^{d}

*Antifungal activities in triplicate with the mean and standard deviations. **The number followed by different letters in the column showed significantly different results based on Duncan test at $p< 0.05$.

A dual culture method is widely used as one of *in vitro* test for preliminary screening of biological control agents (Desai et al., 2002). The method allows the biological agent and pathogen to interact directly and degree of inhibition is recorded by observing the inhibition zone produced. Various results have revealed the potential of endophytic bacteria to inhibit the growth of plant pathogenic fungi for examples, Abla et al., (2015) demonstrated that endophytic bacteria isolated from *Mentha rotundifolia* L. plant showed antagonistic activity using dual culture method. Meanwhile, Paul et al., (2013) studied that *Bacillus tequilensis* (CNU082075), *Burkholderia cepacia* (CNU082111), *Pseudomonas aeruginosa* (CNU082137 and CNU082142) isolated from healthy tissues of leaves, stems and roots of chili pepper plants resulted antifungal activity against *Botrytis cinerea* pathogen. Also endophytic bacteria isolated from cucumber plants revealed antifungal activity against plant pathogen *Fusarium oxysporum* using dual culture method (Ozaktan et al., 2015).

Determination the growth of endophytic bacteria and antagonistic activity

The growth of four selected endophytic bacteria and the ability to inhibit cocoa pathogen from cells-free extract was showed in Figure 1. The results revealed that antagonistic activities against cocoa pathogen increases as the number of endophytic cells increases. Inhibitory activities were detected from the beginning of the cells growth, during exponential and the highest at early stationary phases (24 h) and further incubation (36 and 48 h) showed decrease in antagonistic capability due to cells growth entering death phases. Time consumed from initial cells growth to exponential phases, stationary phases till the death phases depending on several factors, such as bacterial species and the growth conditions (Lloren et al., 2010). According to Li et al., (2011), increasing the number of cells growth correlated with the production of bioactive compounds. This indicated that the antifungal compounds are produced throughout the growth of the endophytes, where the antifungal mainly generated within the cells and secreted into the supernatant (Wang and Liang 2014). Studied by Azizah at et al., (2015) showed that isolates *B. amyloliquefaciens* SAHA 12.07 and *Serratia marcescens* KAHN 15.12 produced highest active compounds in the stationary phase at 36-72 hours of growth. However, Yuan et al., (2014) reported that *B. amyloliquefaciens* NJN-6 produced the highest antifungal compound known as iturin A at 44 h incubation and decreased after that. Interestingly, endophytic bacteria from this study gave the highest antagonistic activity against cocoa pathogen and slightly at shorter time of 24 h compared to those isolates. The highest antagonistic from cells-free extract was isolate LKM-BL (18.3 ± 1.3 mm) followed by LKM-UL (15.3 ±0.8 mm), LKM-PD (13.3 ± 1.3mm) and LKM-PA (10.5 ± 0.9 mm). Endophytic bacterium LKM-PA exhibited the lowest cells growth based on absorbance value of 1.75 ± 0.01 at 24 h of cultivation. The results also showed that endophytic bacterium LKM-BL and LKM-UL obtained the highest cells growth of 1.88 ± 0.01 and 1.85 ± 0.01 respectively absorbance value at 24 h of cultivation. On the other hand, the growth of cells for endophytic bacterium LKM-PD slightly reduced to 1.84 ± 0.01.

Table 2: Antagonistic activities of endophytic bacteria *B. subtilis* LKM-BL against cocoa pathogen *O. theobromae*

Treatment	Percentage (%) of seedlings with lesions symptom at different days (d).							Protective value at 30 days (%)*	Heightat 30 days (cm)
	2d	4d	6d	8d	10d	15d	30d		
Endophyte + pathogen	0	1.7±1.5^{a**}	2.8±3.5^{a}	5.1±5.8^{a}	10.3±11.9^{a}	11.4±11.9^{a}	11.4±11.9^{a}	87.9	64.4±1.8^{a}
Non-endophyte + Pathogen	0	18.3±3.8^{b}	44.6±10.2^{b}	80.6±13.4^{b}	90.3±11.9^{b}	94.3±7.8^{b}	94.3±7.8^{b}	-	40.8±1.3^{c}
Control	-	-	-	-	-	-	-	-	46.4±2.7^{b}

*Protective value = (1 – percentage of endophytic bacteria treatment/percentage of non-endophytic bacteria as control) x 100
**The number followed by different letters in the column showed significantly different results based on Duncan test at $p< 0.05$.

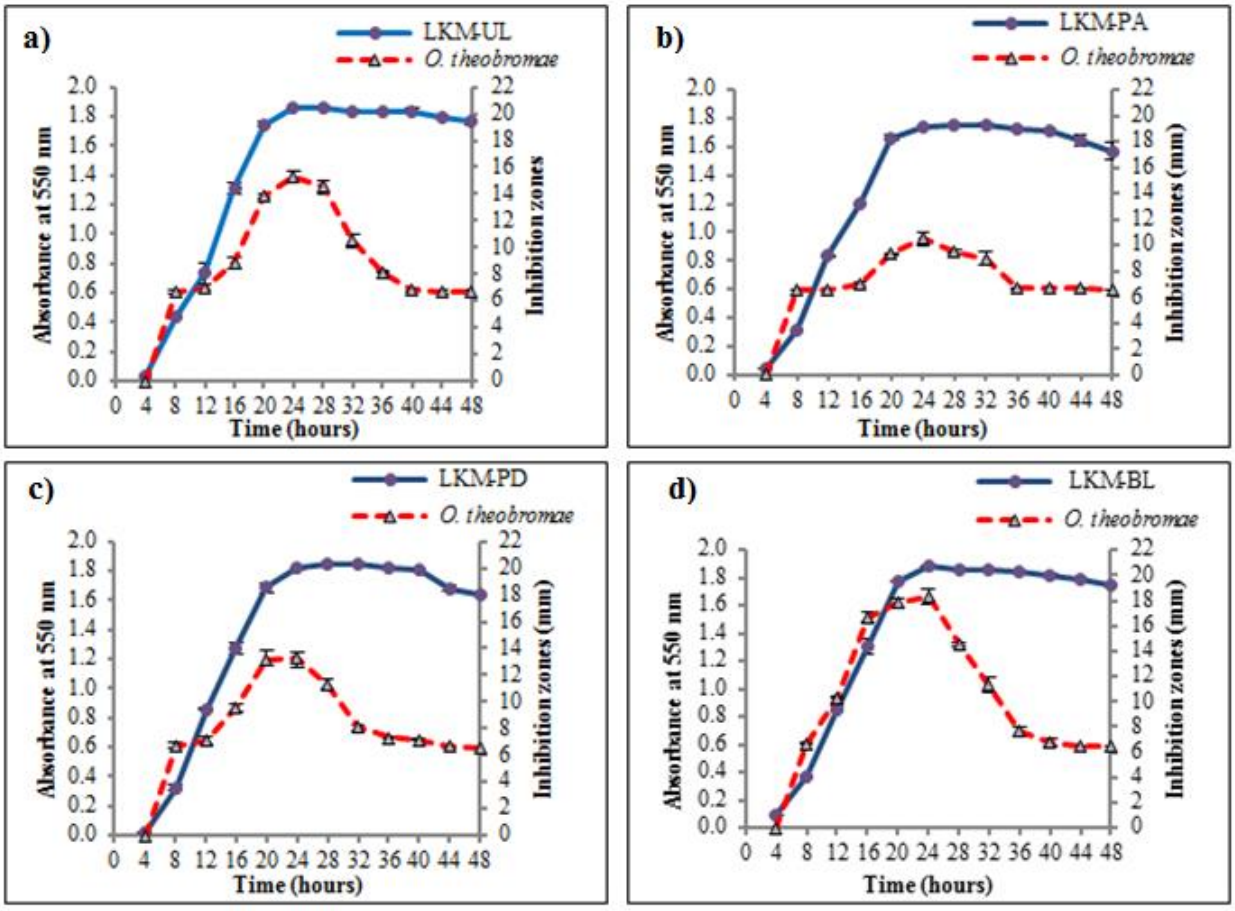

Figure 1. The growth curve and antagonistic activity of endophytic bacteria against *O. theobromae* a) LKM-UL, b) LKM-PA, c) LKM-PD and d) LKM-BL.

Evaluation of selected endophytic bacteria for biological control against *O. theobromae* on cocoa seedlings

Endophytic bacteria LKM-BL with the highest antagonistic activity was selected for evaluation as biological control against *O*. *theobromae* on cocoa seedlings. The protection activity against disease discovered at 4 days inoculation till the end of the experiment (30 days) compared to non-inoculation. LKM-BL significantly protected cocoa seedlings against *O. theobromae* and the protective value of this treatment was 87.9% (at 30 days inoculation) compared to non-inoculation. Meanwhile, the cocoa seedlings treated with this endophyte was the tallest (64.4 ± 1.8 cm) and significantly different $p < 0.05$ compared than with infected seeds (40.8 ± 1.3 cm) and control (46.4 ± 2.7 cm) after 30 days inoculation.

According to Tsuda et al., (2001), endophyte *Enterobacter cloacae* SM10 gave 44.4% of protection value from *Fusarium oxysporum*. Meanwhile, *B. subtilis* isolated from mulberry (*Morusalba* L.) plants showed high protection value of 70.1% (sterile soil) and 68.7% (nonsterile soil) against *Ralstonia solanacearum* (Ji et al., 2008). Endophytic bacteria LKM-BL had been demonstrated to be an endophyte that lives inside plants tissues and effective to control pathogenic fungal. *Bacillus* species are among the most common endophytic bacteria that many reports describing the ability to suppress several important plant pathogens and have been used as a biocontrol agent to protect crops against plant pathogens and provide an alternative to chemical fungicides (Ding et al., 2017). Meanwhile, (Kefi et al., 2015) have reported the capability of *Bacillus* strains isolated from tomato plants displaying protection against a phytopathogenic fungus to protect from destructive gray mold disease causing by *Botrytis cinerea*. However this species of the genus has not been reported for their

capability to protect against pathogenic fungal *O. theobromae* of cocoa. In fact, the isolate produced growth-promoting effects on cocoa seedlings. Similar result also revealed from Ji et al., (2008) studied, where the mulberry seedlings with the treatment of endophyte were all taller than the disease controls.

Identification of antifungal bioactive compounds

Endophytic bacteria LKM-BL was selected for identification of antifungal compounds against cocoa fungal disease. Thin layer chromatography (TLC) plate analysis resulted one spot with R_f value of 0.8. Similar R_f value was also reported by Chalasani et al., (2015) that obtained from antimicrobial compound produced by *B. subtilis* URID 12.1 which were able to inhibit the growth of *Staphylococcus aureus*. The separated antifungal spot whenpurified with HPLC and identified with LCMS revealed two compounds identified as Macrolactin A with molecular formula $C_{24}H_{34}O_5$ and molecular mass of 402.2 Da (Figure 2) and Macrolactin M with molecular formula $C_{25}H_{36}O_5$ and molecular mass of 416.2 Da (Figure 3).

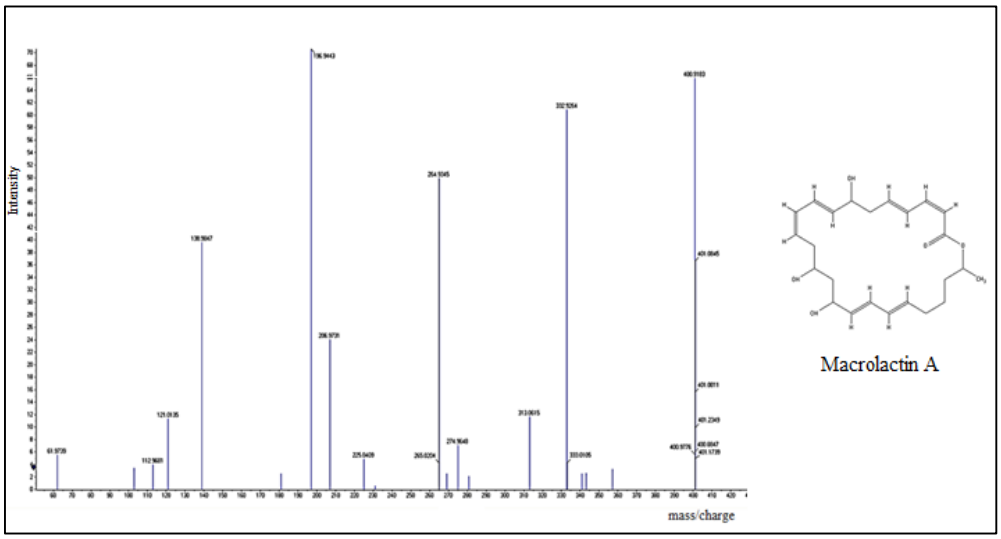

Figure 2. MS/MS spectrum and structure of antifungal bioactive compound Macrolactin A detected from endophytic bacteria LKM-BL.

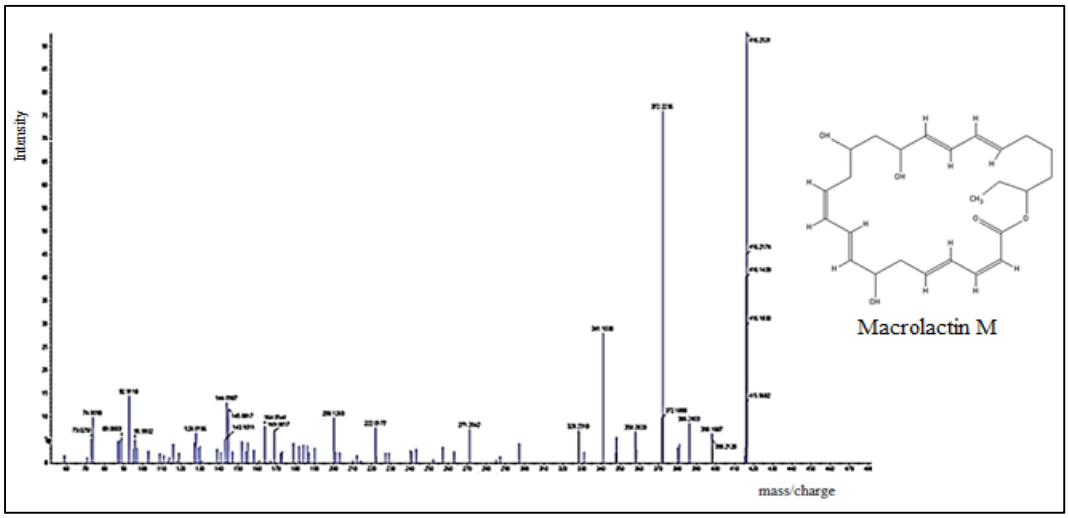

Figure 3. MS/MS spectrum and structure of antifungal bioactive compound Macrolactin M detected from endophytic bacteria LKM-BL.

Macrolactins revealed a wide range of active compounds such as antimicrobials, antiviral and anticancer (Mondol et al., 2013). Similar reports on macrolactin A was from *B. amyloliquefaciens* NJN-6 strain that produced antifungal activity against soil borne plant pathogen *Fusarium oxysporum* (Yuan et al., 2012) and from *B. subtilis* B5 that demonstrated antifungal activities against tea pathogenic fungi *Pestalotiopsis theae* and *Colletotrichum gloeosporioides* (Li et al., 2016). Meanwhile, macrolactin M produced by *Bacillus* sp. PP19-H3 which was isolated from marine macroalga of *Schizymenia dubyi* showed antibacterial activityagainst *Staphylococcus aureus* (Nagao et al., 2001).The position of hydroxyl group or keto group in macrolactone ring affected antimicrobial activity. According to Nagao et al., (2001), hydroxyl group placed at C-15 of macrolactin compounds was suggested that play an important role in the antibacterial activity, but there are no reports about the mechanism of action of this group compounds till now. The results from this studied, showed that endophytic bacteria *B. subtilis* LKM-BL capable to produced two antifungal compounds Macrolactin A and Macrolactin M to protect cocoa seedlings from *O. theobromae* pathogen.

CONCLUSION

Four selected endophytic bacteria LKM-UL, LKM-PA, LKM-PD and LKM-BL via dual culture method against cocoa pathogen *O. theobromae* showed that endophytes capable to produced antifungal activity to inhibit the growth of cocoa plant pathogen. All selected endophytes revealed inhibitory activities from the beginning of the cells growth, during exponential and the highest at early stationary phases (24 h) and further incubation (36 and 48 h) showed decrease in antagonistic capability. Endophytic bacteria *B. subtilis* LKM-BL showed highest antagonistic activity by dual culture method and cells-free extract at 24h growth against pathogen was selected for biological control on cocoa seedlings. The strain significantly protected cocoa seedlings against *O. theobromae* and produced growth-promoting effects on cocoa seedlings. It also produced bioactive compounds of macrolactin A and macrolactin B which exhibits a strong antifungal activity against cocoa pathogen, *O.theobromae*. Thus, *B. subtilis* LKM-BL can be the most promising candidate to be exploited as biological control agent against cocoa fungal disease in Malaysia cocoa plantations.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

IZ, EERS, AH and WSA designed and performed the experiments and also wrote the manuscript. IZ performed the analysis and reviewed the manuscript. All authors read and approved the final version.

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