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Genetic Diversity of Endophytic *Bacillus* strains (101 and 201) Isolated from Native Neem (*Azadirachta indica* L.) Plants of West Malaysia

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ABSTRACT

Background: A large number of bioactive compounds are produced by neem tree (*Azadirachta indica*). The association between the neem tree and endophytes is not well explored in West Malaysia. Endophytes are the organisms that live inside the medicinal plants and produce bioactive compounds of medicinal importance. The present research work has been carried out to harness significant biocontrol activity showing endophytic bacterial strains.

Methods: Screening for bacterial endophytes was performed by using surface sterilisation method. Sterile explants were placed on nutrient agar plates and incubated at 37°C. Plates were observed for the presence of endophytes surrounding the explants. The bacterial endophytes were identified microscopically by Gram staining protocol and molecular characterization was performed by amplification of 16 S rRNA and sequencing.

Result: Twelve endophytic bacterial strains isolated in which, only two bacterial strains were found significant in terms of bio-control activity. Therefore, these two bacterial strains were selected for further study. The 16S rRNA amplification and phylogenetic tree construction of endophytic bacterial strains (101 and 201) confirmed that these bacterial strains are closest with *Bacillus cereus* and *Bacillus megaterium* strains.

Key words: 16S rRNA gene amplification, *Bacillus cereus, Bacillus megaterium,* Bacterial endophytes.

INTRODUCTION

Enhancement of agricultural crop yield, plant growth promotion and protection from plant pathogens are the major challenges all over the world. Use of chemical pesticides was one of the common practice for plant disease control in the past. These pesticides caused irreversible damage to the land and spoiled the balance of soil (Aktar *et al*. 2009).

To sort out this issue in long run and maintain healthier soil, there is an urgent need of bio-control based crop specific endophyte product (Noorzaid *et al*. 2019). This will be useful in coming future for sustainable agriculture.

Microorganisms that live inside the plants called as endophytes and this term was first introduced by De Bary in 1866. Endophytic associations are mutualistic symbiotic interactions, where both of the partners (plant and endophyte) help each other and grow together (Strobel *et al*. 2004). The endophytes colonize in various plant parts especially in roots of plant to protect the plant from plant pathogens. In return the plant provide nutrients for the survival and multiplication of the endophyte. These endophytes are also called as plant growth promoting bacteria (PGPBs) and are beneficial for the plants in drought, stress and other adverse conditions (Rosenblueth and Martinez-Romero, 2006; Bahgat *et al*. 2014).

A large number of significant plant growth promoting endophytic bacterial strains from diverse medicinal plants, were discovered by various researchers all over the world and *Bacillus* genus is one of them (Suneja *et al*. 2018; Hazarika *et al*. 2019; Dudeja *et al*. 2013). *B. cereus*, *B. subtilis*, *B. amyloliquefaciens*, *B. megaterium* and *B. pumilus* are well known examples of plant growth promoting endophytic bacteria (Lopes *et al*. 2018). These species of *Bacillus* not only support the plants in adverse conditions but also help in phosphorus and nitrogen solubilization so that these macro-elements will be available for plant development (Lopes *et al*. 2018; Rosenblueth and Martinez-Romero, 2006). In fact, the endospore forming potential of *Bacillus* genus is unique and outstanding, which help the plant to defend from plant pathogens in water scarcity (Ryan *et al*. 2008).

Neem (*Azadirachta indica* L.) is a well known medicinal tree all over the world from thousands of years. A large number of bioactive compounds produced by this tree and majority of them possesses antimicrobial properties. Endophytic association between neem plants of Indian

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region and microorganisms was thoroughly studied by various researchers (Singh *et al*. 2017; Tiwari and Thakur, 2014).

The recent study conducted by Kannan *et al*. (2018), reported the putative role of endophytic bacteria in salt tolerant polyembryonic mango accessions in Andaman and Nicobar islands. In West Malaysia, the bacterial endophytes isolated from *Neesia altissima* were found significantly antagonistic against human pathogens (Pratiwi *et al*. 2016). Therefore, this study was designed to harness novel endophytic bacterial strains from native neem plants of West Malaysia and to assess their biocontrol activity.

MATERIALS AND METHODS

Sample collection site and methods

Thirty neem plants, which are growing at unique environmental niches such as high altitude and different soil texture in (Shah Alam, Selangor, Penang, Port Dickson and Melaka) West Malaysia were chosen for this study. Six plants from each location have been selected and were collected by maintaining all aseptic conditions (Tiwari and Thakur, 2014; Noorzaid *et al*. 2019). The plant species were authenticated by botanist from University Putra Malaysia.

Isolation and identification

The isolation of endophytic bacteria was carried out according to the protocol given by Xu *et al.* (2020) and Ferreira *et al*. (2017). Explants (roots, leaves, nodes and internodes) of neem were used for the isolation of endophytic bacterial strains. Surface sterilization of explants was done thorough washing with tap water (15 min.), sterilized distilled water (5 min.), 70% Ethanol (3 min.), 4% Teepol (1 min.) followed by serial wash with sterile distilled water (1, 2 and 5 min.) and 0.1% $HgCl_2$ (1 min.) followed by serial wash with sterile distilled water (1, 2 and 5 min.) (Sharma *et al*. 2015; Singh *et al*. 2017).

Surface sterilized explants were transferred at nutrient agar media plates (Xu *et al*. 2020). After two days of incubation of plates at 37°C, the bacterial strains started growing on the nutrient agar medium from inoculated explants. These endophytic bacterial strains were carefully replaced to another set of nutrient agar medium for pure culture isolation and further bacterial strain identification (Tiwari and Thakur, 2014; Xu *et al*. 2020). The strains were examined for morphology *via* Gram staining. For biochemical identification of isolated bacterial strains, colony morphology, colony elevation, Gelatin and casein hydrolysis, H_2S production test, urease and catalase tests were performed as per the standard protocol (Singh *et al*. 2017; Tiwari and Thakur, 2014).

Measurement of biocontrol/antagonistic activity *In-vitro*

The biocontrol or antagonistic activity of all identified bacterial strains against phytopathogenic fungi was performed using the *in vitro* dual-culture assay. Mycelial discs with 8 mm diameter were cut from the target fungal colonies cultured on Czapek dox agar media plates for seven days and were placed on fresh Czapek dox agar media plate. A loopful colony (10^7 cells/colony forming units) was transferred to Luria broth from a previously grown pure cultures of bacterial strains (101 and 201). After 48 hours of incubation, 1 ml suspension containing active cells of 101 number bacterial strain was transferred to the Czapek dox agar media plates, in which 8 mm diameter fungal disc inoculated earlier, at the center of the plate. Control plates without bacterial strains were also prepared simultaneously. Plates were incubated at 28°C for 7-14 days and examined for the inhibition of fungal growth (Mardanova *et al*. 2017). The 28°C temperature is set to facilitate the growth of fungal pathogen not for the growth of bacterial strains, since the active bacterial cell suspension taken from previously incubated and grown pure culture on Luria broth. For the 201 number bacterial strain also same protocol was followed and for the calculation of growth inhibition percentage, these experiments were repeated three times (Mardanova *et al*. 2017).

The growth inhibition of the test fungus was measured by using the following formula:

$$\text{Growth inhibition (\%)} = [R\text{-}r / R \times 100]$$

R-represents the radial growth of target fungus in control. r-the radial growth of the target fungus with bacterial suspension.

Isolation of genomic DNA from bacterial strains

Genomic DNA from both endophytic bacterial strains (101 and 201) was isolated using DNA isolation kit (Qiagen® UK). Pure culture of bacterial strains on nutrient agar plates were transferred to nutrient broth and incubated at 37°C for 24 hours. Two milliliter of active bacterial culture from this broth were then used to isolate genomic DNA. The media supernatant was decanted and the pellet washed thrice with 1 ml of NaCl EDTA (30 mM NaCl, 2 mM EDTA, pH 8.0). The washed bacterial pellet was resuspended in 100 µL of NaCl-EDTA (30 mM NaCl, 2 mM EDTA, pH 8.0) and 100 µL of freshly prepared lysozyme solution (concentration 10 mg/ml in NaCl-EDTA) was added and mixed. The mixture was incubated at 37°C for 1 hour with intermittent shaking. To remove RNA, 4 µL of Rnase-A solution (Stock 10 mg / ml) was added to the mixture before incubation (Tiwari and Thakur, 2014).

Amplification and sequencing of 16S rRNA

PCR was carried out in a 50 µL reaction volume containing 50 ng of genomic DNA, 20 pmol of each primer, 1.25 units of Taq DNA polymerase, 200 µM of each dNTPs and 1X PCR buffer. PCR was carried out for 35 cycles in a thermal cycler (My cycler™ BIO-RAD USA) with the initial denaturation at 94°C for 3 minute, cyclic denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 2 minutes with a final extension of 7 minutes at 72°C (Qiagen® UK). Standard forward (5′-GTGCTAGTGTTAGAGGGTTTCCG-3′) and reverse (3′-TCCCAGGCGGAGTGCTTAATGCG-5′) primers used for

amplification reaction. The PCR product was checked by agarose gel electrophoresis, purified and was further subjected to DNA sequencing (Sekar and Prabavathy, 2014). The sequence data was analyzed using BlastN (BLASTN-OmicX FRANCE). Phylogenetic tree was constructed by using Mega7 software (Chen *et al*. 2013).

RESULTS AND DISCUSSION

A total of twelve (101 – 1201) endophytic bacterial strains isolated and identified on the basis of morphological, cultural and biochemical characteristics (Table 1).

Biocontrol/antagonistic activity

The ability to produce extracellular antifungal metabolites and volatile organic compounds (VOCs) against plant pathogens is known as antagonism. From dual culture assay, it was found that only two endophytic bacterial strains are showing significant biocontrol/antagonistic activity. These two bacterial strains (101 and 201) were significantly inhibiting the growth of phytopathogenic fungi *Colletotrichum coccodes* and *Fusarium avenaceum In-vitro* (Fig 1). Therefore, these two endophytic bacterial strains were selected for further study.

Phylogenetic tree

Genomic DNA from both bacterial strains (101 and 201) was isolated and amplified (Fig 2). The Phylogenetic tree was constructed using neighbor joining unrooted tree Method. The sequences of both strains were matched within NCBI data bank. These two bacterial (101 and 201) strains have shown 100% homology with the *Bacillus megaterium* and *Bacillus cereus* strains, when matched with BLAST results against NCBI 16S ribosomal RNA sequences (Fig 3 and 4). In this research, twelve endophytic bacterial strains were isolated from native neem plants of West Malaysia. Out of these twelve endophytic bacterial strains, only two bacterial strains were found significant in terms of bio-control activity. These bacterial strains identified as *Bacillus cereus* and *Bacillus megaterium* (101 and 201) through DNA sequencing and BLAST.

Wei *et al*. (2011) reported that endophytic *Bacillus cereus* strain MQ 23 is significant plant growth promoter in *Sophora alopecuroides* plant root nodules. This strain was found active for siderophore production, phosphate solubilization and nitrogen fixation by producing nitrogenase enzyme.

Study conducted by Yan *et al*. (2017) found that the endophytic *Bacillus cereus* (BCM2 and SZ5) and *Bacillus altitudinis* (CCM7) strains effectively controlled meloidogyne incognita on tomato plants through rapid rhizosphere occupation and repellent action. Chinnaswamy *et al*. (2018) also reported that the *Bacillus megaterium* strain isolated from *Medicago polymorpha* enhances growth, promotes nodulation using *Ensifer medicae* and alleviates salt stress in alfalfa plants.

In the recent study, similar results were obtained by Lopes *et al*. (2018) and found that the *B. subtilis*; *B. cereus*

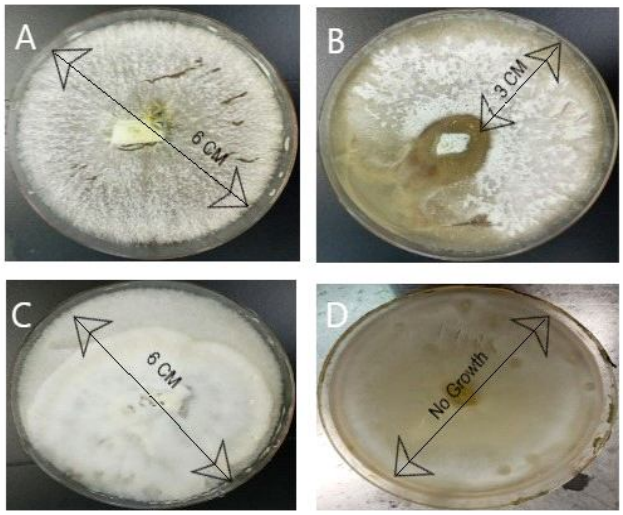


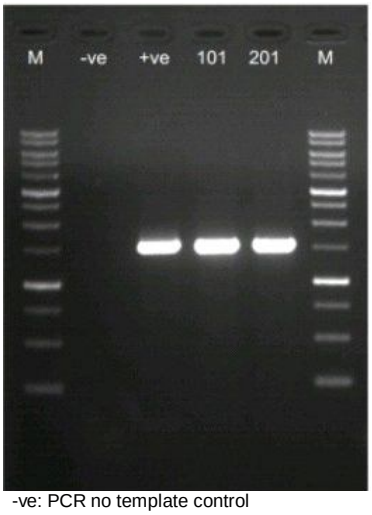
Fig 1: Biocontrol/antagonistic activity of bacterial strains (101 and 201) against fungal phytopathogens in dual culture assay on agar plate.

1A: Pure culture of *Colletotrichum coccodes* as positive control without bacterial strain - 101 suspension as positive control.

1B: Antagonism/Inhibition of fungal mycelial growth and conidial development shown by bacterial strain - 101 against fungal phyto-pathogen *Colletotrichum coccodes*. The growth inhibition is 50% according to the formula [Growth inhibition (%) = R-r/R × 100].

1C: Pure culture of *Fusarium avenaceum* as positive control without bacterial strain - 201 suspension as positive control.

1D: Antagonism/Inhibition of fungal mycelial growth and conidial development shown by bacterial strain - 201 against fungal phyto-pathogen *Fusarium avenaceum*. The growth inhibition is 100% according to the formula [Growth inhibition (%) = R-r/R × 100].



-ve: PCR no template control
+ve: Positive control

Fig 2: Agarose gel electrophoresis showing 16S rRNA amplified products (Left to right); Lane M - 1 Kb ladder; Negative control (-ve); Positive control (+ve); Endophytic bacterial strain - 101 (Amplicon size ~ 1.5 kb full length); Endophytic bacterial strain - 201 (Amplicon size ~ 1.5 kb full length); Lane M- 1 Kb ladder.

Table 1: Characteristic features of isolated endophytic bacterial strains from neem plants.

Description	101	201	301	401	501	601	701	801	901	1001	1101	1201
Location	Shah Alam	Petaling Jaya	Subang Jaya	Klang	Kajang	Kuala Selangor	Puchong	Penang	Port Dickson	Melaka	Sepang	Kuala Langat
Explant	Leaf	Root	Stem	Stem	Root	Leaf	Leaf	Stem	Stem	Root	Root	Root
Colony form	Convex	Convex	Wrinkled/ Variable	Wrinkled/ Variable	Wrinkled/ Variable	Smooth convex	Smooth convex	Convex	Convex	Convex	Convex	Convex
Colony elevation	Circular	Circular	umbonate	umbonate	umbonate	Irregular round to rough	Irregular round to rough	Raised mucila--genous	Raised mucila--genous	Raised mucila--genous	Round and slightly umbonate	Round and slightly umbonate
Colony pigmentation	-	-	-	-	-	Yellow	Yellow	-	-	-	-	-
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Short rod	Short rod
Gram's staining reaction	+	+	-	-	-	-	-	-	-	-	-	-
Cell arrangement	Single	Single	Single	Single	Single	Single	Single	Single	Single	Single	Single	Single
H_2S production	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-	-	-	-	-	+	+
Casein hydrolysis	+	+	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	-	-	-	-	-	-	-
Catalase production	+	+	-	-	-	-	-	-	-	-	-	-
Cellulase production	-	-	-	-	-	-	-	-	-	-	-	-
Urease production	-	-	-	-	-	-	-	-	-	-	-	-
Endospore formers	+	+	-	-	-	-	-	-	-	-	-	-

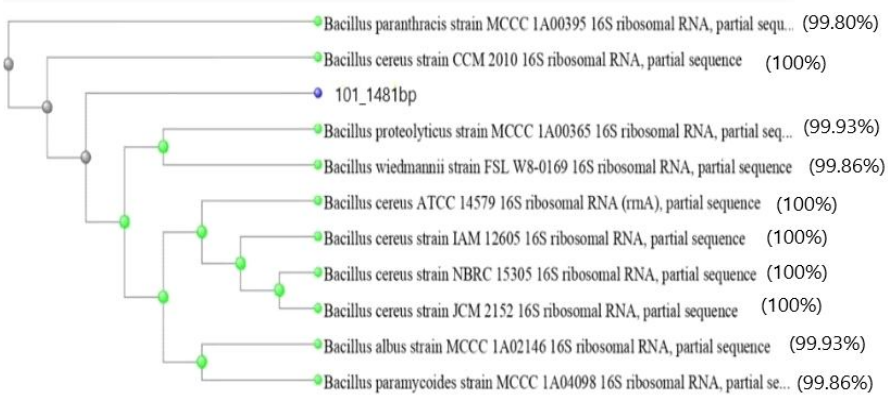


Fig 3: Phylogenetic tree of endophytic bacterial strain – 101 (1481 base pairs).

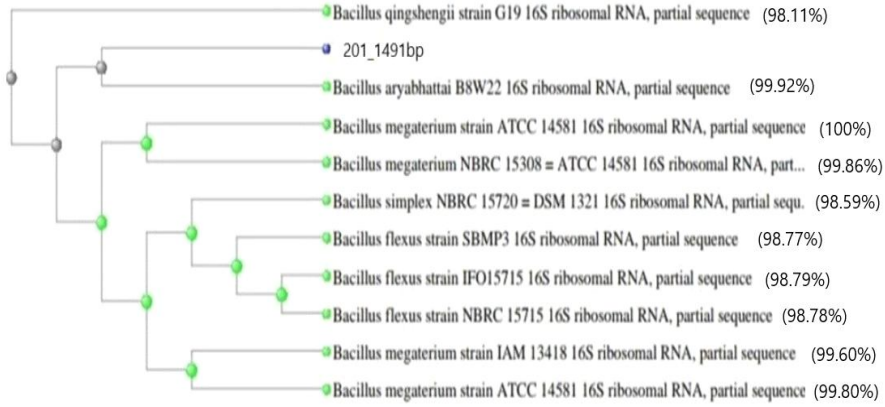


Fig 4: Phylogenetic tree of endophytic bacterial strain - 201 (1491 base pairs).

and *B. megaterium* strains are potentialistic source of bio-control by producing a large number of antimicrobials, such as polyketides, bacteriocins, non-ribosomally synthesized peptides and other secondary metabolites. Thus, the present research work unfolds the biocontrol activity of 101 and 201 endophytic bacterial strains (*B. cereus* and *B. megaterium*) from diverse neem plants and this will be particularly useful in coming future for selection of plant specific endophyte based product in various types of adverse conditions.

CONCLUSION

The endophytic mutualistic relationship between plant and bacteria is well known. In the present research, plant beneficial endophytic *Bacillus* strains *(B. cereus* - 101 and *B. megaterium* - 201) were isolated from neem plants in West Malaysia and have shown significant biocontrol activity against phytopathogens. Further research work is in progress, to purify these extracellular antifungal metabolites responsible for this biocontrol activity from these isolated strains.

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