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

Vithiya Yoganathan, Kartikeya Tiwari

**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 16S 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 phylogenetetic 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
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 polycrystalline 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% HgCl₂ (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, H₂S 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 (10² 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:

\[
\text{Growth inhibition} = \frac{[R_r \times 100]}{R}
\]

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 minutes, 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'-GTGCTAGTGTTAGGTTCCCG-3') and reverse (3'-TCCCCAGGCGAGTGCTTAATGGG-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 SZS) 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...
### 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 | Root | Leaf | Leaf | Stem | Stem | Root | Root | Root | Root |
| Colony form               | Convex | Convex | Wrinkled/Variable | Wrinkled/Variable | Smooth | Smooth | Convex | Convex | Convex | Convex | Convex | Convex |
| Colony elevation          | Circular | Circular | umbonate | umbonate | umbonate | umbonate | Irregular | Irregular | Raised | Raised | Raised | Round and slightly raised |
| Colony pigmentation       | - | - | - | - | - | - | - | - | - | - | - | - |
| Cell shape                | Rod | Rod | Rod | Rod | Rod | Rod | Rod | Rod | Rod | Rod | Short rod | Short rod |
| Grant's staining reaction | + | + | + | + | + | + | + | + | + | + | + | + |
| Cell arrangement          | Single | Single | Single | Single | Single | Single | Single | Single | Single | Single | Single | Single |
| H₂S production           | - | - | - | - | - | - | - | - | - | - | + | + |
| Gelatin hydrolysis        | - | - | - | - | - | - | - | - | - | - | - | - |
| Casein hydrolysis         | + | + | + | + | + | + | + | + | + | + | + | + |
| Starch hydrolysis         | + | + | + | + | + | + | + | + | + | + | + | + |
| Catalase production       | + | + | + | + | + | + | + | + | + | + | + | + |
| Cellulase production      | - | - | - | - | - | - | - | - | - | - | - | - |
| Urease production         | - | - | - | - | - | - | - | - | - | - | - | - |
| Endospore formers         | + | + | + | + | + | + | + | + | + | + | + | + |
In this regards you may please reply of the reviewer comments mail.

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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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