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Endophytic bacteria *Bacillus aerius*, as a potential biocontrol agent against bacterial leaf spot of betel vine

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Abstract
Bacterial leaf spot is one of the major threats in betel vine production as it can cause economic loss. Concern on the environment and human health has led an attempt to replace existing methods of chemical control and avoid extensive use of bactericides by using endophytic bacteria. The present study was conducted to isolate and characterize of endophytic bacteria *Bacillus aerius* isolated from betel vine that has potential as antagonistic bacteria against *Xanthomonas axonopodis* pv. *betlicola*, the causal agent of bacterial leaf spot of betel vine. *Bacillus aerius* were successfully isolated from leaves of betel vine and showed strong antagonistic activity indicated against *X. a.* pv. *betlicola* on nutrient agar plate. Molecular identification successfully identified the antagonistic endophytic bacteria as *Bacillus aerius*. The findings in this study revealed the biocontrol abilities of isolated endophytes as an excellent option to be used by agriculture sectors to have a sustainable environment.

Keywords: Endophytic, potential, bacterial, betel vine, *Bacillus aerius*

Introduction
Betel vine (*Piper betle* L.) is an economically and medicinally important cash crop in the world. It belongs to the family Piperaceae and the most probable place of origin of betel is central Malaysia (Chattopadhyay and Maity, 1990) [3]. It is valued both as a mild stimulant and for its medicinal properties. Betel vine is extremely prone to diseases, pests and various types of natural disasters (Sayeeduzzaman, 1988) [10]. The disease is a major barrier to the cultivation of betel vine, since the microclimate created in the orchards is highly conducive for the growth of pathogens. Major diseases of betel vine are foot rot or leaf rot caused by *Phytophthora parasitica* var. *piperina*, Anthracnose caused by *Colletotrichum piperis* and Bacterial leaf blight (BLB) caused by *Xanthomonas axonopodis* pv. *betlicola*, among them bacterial leaf spot is the most destructive disease which decreases the production as well as the quality of betel vine to a great extent.

Now a day, biological control has become a predominant component in integrated disease management (IDM) and organic farming. Recently, the use of endophytes has become an outstanding approach to plant protection. Endophyte, by definition, resides in the tissues beneath the epidermal cell layers and causes no apparent harm to the host (Stone *et al*., 2000) [12]. They are ubiquitous, colonize all plants and have been isolated from almost all plants examined (Nair and Padmavathy, 2014) [8].

Therefore present study aimed to isolate and identify endophytic bacteria *Bacillus aerius* from betel vine, to evaluate their effect as biocontrol agents against bacterial leaf spot caused by *Xanthomonas axonopodis* pv. *betlicola*.

Materials and Methods
Isolation of endophytic bacteria *Bacillus aerius*
Bacillus aerius was isolated from leaf samples of betel vine. The modified isolation method of Doley and Jha (2015) [4] was used. Leaf samples were surface-sterilized with 70 percent ethanol for 1 minute and then with 2 percent sodium hypochlorite for 2 minutes, then rinsed three times with sterilized double distilled water (SDW) and dried in laminar airflow. A 100µl aliquot from the final wash was inoculated on nutrient agar media by the pour plate method to confirm sterility (Pandey *et al.*, 2015) [9]. Outer tissues of the collected leaf samples were removed and inner tissues were cut into small pieces with a sterile scalpel and sets of four segments were evenly placed in each Petri dish containing nutrient agar.

The plates were incubated at 28 ± 2 °C for 72 hr for observing the colonies development and isolated colonies were picked up and streaked again on fresh nutrient agar plates and incubated. Finally pure cultures were transferred on NA slants and stored for further studies in a refrigerator at 4 °C.

Antagonistic activity assay against *X. a.* pv. *betlicola*

To evaluate the antagonistic activity of *Bacillus aerius* against *X. a.* pv. *betlicola* dual culture technique was followed. The two lines of antagonistic bacteria *Bacillus aerius* were streaked first and then the pathogen was streaked in between two streaks of *Bacillus aerius* parallelly. Plates streaked with only *Xanthomonas* were used as controls and then incubated at 32 °C for 48 hours. The visual observation of inhibition of *X. a*. pv. *betlicola* by the antagonistic bacteria were recorded according to Kumar *et al*., (2020) [6].

Antagonistic Bacteria *Bacillus aerius* identification by Using Molecular Method

Bacillus aerius grown in nutrient broth and incubated at 32 °C for 48 hours. The total genomic DNA of endophyte was extracted using (Cetyl Trimethyl Ammonium Bromide) method (Murray and Thompson, 1980) [7] with some modifications. The DNA was amplified by Mastercycler® Nexus Gradient machine for polymerase chain reaction amplification. The gene fragments of antagonistic endophyte were sequenced using universal primers pair BVF (5'-GGGGAGCGAACAGGATTAGA-3') and BVR (5′ GTAAGGTTCTTCGCGTTGCT -3′) that was synthesized by Chromous Biotech Ltd., Bangalore. The 50 µl of PCR reaction mixture contained 10x assay buffer with 15mM $MgCl_2$ (5 µl), dNTPs (4 µl), Forward primer (2.5 µl), Reverse primer (2.5 µl), genomic DNA (5 µl), EX Tag HS (0.25 µl) and nucleus free water (30.75 µl). Amplifications of the gene were performed for 30 cycles in Eppendorf gradient thermal cyclers programmed (Kathleen 2006). The reaction will proceed as follows: 5 minutes at 95 °C (Denature), 1 minute at 95 °C (Denature), followed by 60 °C for 1 minute (Anneal) and 1 minute at 72 °C (Elongation).

The PCR product was finally preceded for Gel Electrophoresis, 1.2% agarose gel was used by preparing 1.2 g agarose powder with 100 ml 1X TBE buffer then was stained with 2.5 µl DNA Gel Stain. Gel was visualized by using Bio RadTM gel documentation system under UV light to observe the DNA band, 100 bp of ladder acted as a marker. The PCR product was sequenced using forward and reverse primers at Chromous Biotech Ltd., Bangalore. Homology search was done by using the BLAST algorithm available at http://www.ncbi.nlm.nih.gov.

Results

In vitro* evaluation of *Bacillus aerius* against *X. a.* pv. *betlicola

Bacillus aerius was tested against *X. a.* pv. *betlicola* to know the antagonistic activity through dual culture technique under *in vitro* condition. The results are depicted in Plate 1 indicated that bacterial endophyte *Bacillus aerius* showed strong/high inhibition activity against *X. a.* pv. *betlicola.*

Molecular characterization of *Bacillus aerius*

Bacillus aerius genomic DNA was isolated by following CTAB method and subjected to PCR using species specific primers of *Bacillus aerius*. *Bacillus aerius* were amplified with amplicon size of 210bp and it confirmed that isolated endophytic bacterial isolate were *Bacillus aerius* (Plate 2). For further confirmation amplified PCR products were subjected for sequencing by using forward and reverse primers and out sourced for sequencing to Chromous Biotech Ltd., Bangalore. Homology search was done by using BLAST algorithm available at http://www.ncbi.nlm.nih.gov.

Based on sequence similarity matrix, LBBaMa-2 was identified as *Bacillus aerius* (*B. aerius*) which shared 98.99 percent identity with the *Bacillus aerius* strain PC3.

Discussion

Endophytic microbes are microorganisms that live in the intercellular spaces in plants tissues for most if not all of their life cycles with no pathogenic effects on their hosts (Azevedo *et al*., 2002) [2]. Endophytes are sheltered from environmental stress and microbial competition and they seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, stems, roots and seeds of various plant species (Kobayashi and Palumbo, 2000) [5]. *Bacillus aerius* was isolated from apparently healthy betel vine plants. Similarly, bacterial endophytes were isolated from betel vine by Singh *et al*. (2017) [11] where they isolated 71 endophytic bacteria from leaf, stem and root of four different varieties (Mahoba, kalkatia, desawari and wild type). Aravind *et al*. (2009) [1] isolated 74 endophytic bacteria from root and stem tissues of black pepper (*Piper nigrum* L.).

Further, the endophytic bacteria *B. aerius* were identified with species specific primers and based on sequence similarity matrix, *B. aerius* which shared 98.99 percent identity with the *Bacillus aerius* strain PC3. Similar endophyte identification was done by Kumar *et al*. (2020) [6] identification was done based on the BLASTn analysis of nucleotide sequences of 16S rRNA gene sequence, endophytic bacterial isolates were identified as *Bacillus cereus*, *B. pumilus*, *B. subtilis*, *B. velezensis*, *B. thermophilus*, *B. xiamenensis*, *B. stratosphericus* which belong to phylum Firmicutes. Some isolates belong to phylum Protobacteria includes; *Klebsiella pneumoniae*, *Enterobacter cloacae*, *E. tabaci*, *E. asburiae*, *Xanthomonas sacchari and Leclercia adecarboxylata*.

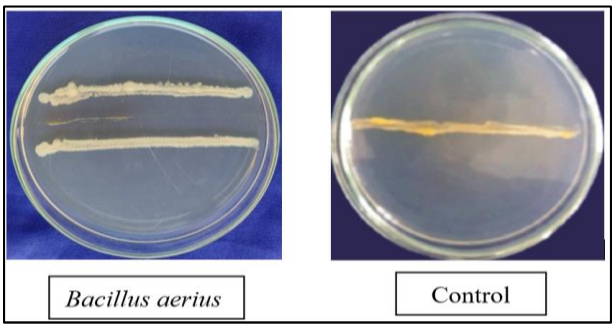

Plate 1: Antagonistic activity of *Bacillus aerius* against *X. a.* pv. *betlicola*

Plate 2: Gel image showing amplicon of *Bacillus aerius*
M- Marker (100 bp), Ba- *Bacillus aerius*, C-Control (Sterile water)

Conclusion

Present study showed the potential of *B. aerius* endophyte for bacterial leaf spot disease reduction. Further research is needed for the identification of secondary metabolites production from *Bacillus aerius* and its effectiveness as biocontrol agent against other important pathogens of betel vine.

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