Screening potential microbes against whitefly 
(*Bemisia tabaci* (Gennadius)), the most important 
est of cassava (*Manihot esculenta* Crantz)

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Abstract

The study was conducted to isolate potential bacteria and fungi from soil, cow dung and to study their pathogenicity on the most important pest of cassava, cassava whitefly (*Bemisia tabaci*). Entomopathogenic microbes were isolated from rhizosphere soil and cowdung, and their pathogenicity was tested against *Bemisia tabaci*. Microbes showing promising results were subjected to DNA extraction and PCR study; subsequently sequenced for isolate identification. The Basic Local Alignment Search Tool (BLAST) analysis of sequences identified the bacterial isolates as *Bacillus cereus* and *B. pumilus*, and fungus as *Beauveria bassiana*, *Metarhizium anisopliae* and *Penicillium citrinum*.

Keywords: *Bemisia tabaci*, cassava, cassava mosaic disease, microbes

1. Introduction

Cassava, *Manihot esculenta* Crantz, is an important crop and its tubers are used as staple or subsidiary diet in developing countries. Though it is drought tolerant and highly adaptable to grow in marginal soil, cassava is vulnerable to pests and diseases, causing heavy yield losses. *B. tabaci*, the most important pest of cassava generally seen on the underside of the leaves. Adult is approximately 1.5 mm long and its body is covered with white waxy coating. Female lays eggs on the underside of the plant leaf. After an incubation period of 12 days, the eggs will hatch out into nymphs. Nymph sucks and reaches adult stage the sap and within approximately six weeks they grow. Their life span is about one to two months. Whitely, *B. tabaci* have been causing yield loss past few centuries. Even though these insects, attacking cassava may vary both morphometrically (Harish et al., 2016) [11] and genetically (Harish et al., 2019) [10], they are well-known vectors of cassava mosaic disease (CMD). Indian cassava mosaic virus (ICMV) is associated with CMD in India (Hong et al., 1993) [12]. Indian cassava mosaic virus (ICMV) was the first CMV to be recorded from South Asia (Malathi et al., 1985) [19], followed by Sri Lankan cassava mosaic virus (SLCMV) several years later (Saunders et al., 2013) [24]. Although SLCMV was initially reported from Sri Lanka, it was subsequently shown to occur also in Southern India, together with ICMV (Jose et al., 2011; Patil et al., 2005) [21]. The earliest reports of CMD in India noted that the disease was restricted to the cassava-growing regions of southern India: primarily Kerala and Tamil Nadu, and to a lesser extent Karnataka and Andhra Pradesh (Malathi et al., 1985). More recently, CMD was also reported from Sri Lanka (Austin, 1986) [22]. The endosymbionts of whitely may be the reason for making it a successful vector (Harish et al., 2019) [10]. Many studies are being conducted to control whitely. Pesticides were generally considered for vector control until the late 1950s. However, the rapid increase in use of organic insecticides during the 1940s and 1950s aroused public concern about their safety (Casida and Quistad, 1998) [15]. The consequences followed due to extensive pesticide use paved the way for using natural enemies for pest management. Among several groups of biocontrol agents for whiteflies and other sap-sucking insects, entomopathogenic fungi possess the unique ability to infect their host directly through the integument. Moreover, they play a role in the natural mortality of whitely populations (Lacey et al., 1996) [16]. Among entomopathogenic bacteria, *Bacillus* spp., especially *Bacillus thuringiensis*, have been used extensively for control of insect pests in crops (Lacey et al., 2000) [17]. Because of the ill effects caused by chemical insecticides, adoption of ecofriendly strategies should always be given a priority in pest management. The aim of the study was to manage the
main threat in cassava production, *B. tabaci* using potential microbes.

2. Materials and Methods

2.1 Materials
Rhizosphere soil was collected from five tuber crop plants, cassava, sweet potato, *Amorphophallus*, *Colocasia* and *Dioscorea* from different locations of Thiruvananthapuram, Kerala. Five plants were selected for soil collection for each crop and soil collected for a particular crop were pooled together and brought to lab in sterile polythene bags. The soil samples and cow dung collected from different locations were dried in shade in order to remove the excess moisture content. Whitefly adults were collected from cassava fields of ICAR-CTCRI, Kerala using aspirator. Nymphal and pupal stages of whitefly were also collected from cassava leaves.
Culture media used for the study were nutrient agar medium for bacteria; rose bengal agar and potato dextrose agar for fungi. For DNA Extraction and PCR, various materials used were liquid nitrogen, phenol: chloroform, proteinase k, RNase, ice cold isopropanol, 70% ethanol, TE buffer, dNTPs, Taq polymerase, forward and reverse primers (ITS1 and ITS4 for fungi; 16SF and 16SR for bacteria), Taq buffer and SDS.

2.2 Methods
After pooling soil samples collected from each crop separately, one gram each of soil and cow dung were weighed and serial dilution was performed until $10^{-6}$ concentration. Among various dilutions $10^{-6}$ of the sample was used for spread plating in nutrient agar plates, whereas $10^{-4}$ dilution was used in rose bengal agar plates and potato dextrose agar plates. Fungal spores were extracted using tween 20 media and spore counting was done using hemocytometer. Number of spores obtained by counting the spores under hemocytometer is calculated using the formula- Number of spores per μl = average cell count per four corner square x $10^4$.

Whiteflies were collected using aspirator and were transferred to large Petriplates containing surface sterilized cassava leaves by dipping them in 70% ethanol for 20 seconds. Ten whiteflies each of nymphs and adults were used for each fungal sample per Petriplate with three replications. The spores extracted previously were transferred to small sprayers and mist of spores was sprayed on the whiteflies. For bacterial bioassay, bacterial suspensions were prepared and ten ml each of sterilized nutrient broths were prepared in test tubes. The bacterial suspensions were transferred to small sprayers and were sprayed on both sides of surface sterilized cassava leaves. The whiteflies (nymphs and adults) were transferred to Petriplates for feeding on the sprayed leaves. After incubation the plates were observed for fungal and bacterial growth. Surface sterilization was done on whiteflies with 70% ethanol for five seconds. The fungal and bacterial colonies were selected, when grown from the body surface of treated and surface sterilized whiteflies and when mortality happened compared to control treatments after placing on PDA and nutrient agar plates respectively. DNA was isolated from the microbes using standardized DNA extraction protocol (DNeasy blood and tissue kit (Qiagen®)) and PCR was carried out in Biorad thermal cycler with the thermal cycle programme of 94 °C for 2 minutes initial denaturation, 94 °C for 30 seconds final denaturation, 51.7 °C for 1 minute annealing, extension 72 °C for one minute 30 seconds and final extension of 72 °C for 8 minutes. The amplified products were resolved on 1.2 % agarose gel. The DNA bands of 600bp and 1200bp for fungi and bacteria respectively were visualized using gel documentation system and 20 μl of the PCR product were sent for sequencing (Agrogenome, Ernakulam). DNA ladder of 1kb plus (Thermo Fisher Scientific, USA) was used for determining the size of the amplicon. The 16S rRNA and Internal Transcribed Spacer (ITS) sequences were analyzed using NCBI - BLAST for the identification of microorganisms.

3. Results and Discussion
From rhizosphere soil total of 14 bacteria, 7 fungi and from cow dung 3 bacteria were isolated. The isolated colonies were separated based on their morphology. From sample one (S1) – *Amorphophallus* (rhizosphere soil), four bacterial colonies and two fungal colonies were isolated. Three bacterial colonies and one fungal colony were isolated from sample two (S2) – *Dioscorea* (rhizosphere soil). From sample three (S3) which was cassava (rhizosphere soil) one bacterial colony and four fungal colonies were isolated. In case of sample 4 (S4) – *Colocasia* (rhizosphere soil), 6 bacterial colonies were isolated. One fungal colony could isolate from *Colocasia* (rhizosphere soil). From sample 5 (S5) which was sweet potato (rhizosphere soil), no bacterial colony and only two fungal colony could be isolated. From cow dung sample, three bacterial colonies were isolated. Out of nine fungal samples isolated, samples S5, S2 and S4 did not give sufficient spores for examining their pathogenicity to whitefly, whereas samples S1 C1, S1 C2, S1 C3, S1 C4 and S2 C1 (C is colony number) provided appreciable spore counts. According to Jackson et al. (2013) [14] the ideal samples for fungal bioassay should contain spore concentration of minimum $10^6$ per ml. In this study also only those samples which had spore concentration of $10^6$ and more were used (Table 1).

![Fig 1: Adult pair of Bemisia tabaci](Image)

Table 1: Number of fungal spores obtained from samples

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Number of spores per μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>$1.4x10^5$</td>
</tr>
<tr>
<td>S1 C1</td>
<td>$2.5x10^7$</td>
</tr>
<tr>
<td>S2</td>
<td>$6x10^4$</td>
</tr>
<tr>
<td>S2 C1</td>
<td>$9.1x10^4$</td>
</tr>
<tr>
<td>S2 C2</td>
<td>$1.27x10^5$</td>
</tr>
<tr>
<td>S2 C3</td>
<td>$1x10^6$</td>
</tr>
<tr>
<td>S2 C4</td>
<td>$2.85x10^7$</td>
</tr>
<tr>
<td>S3</td>
<td>$7x10^4$</td>
</tr>
<tr>
<td>S3 C1</td>
<td>$1.6x10^8$</td>
</tr>
</tbody>
</table>
After 5 days of incubation, the plates with samples $S_1C_1$, $S_3C_1$ and $S_5C_1$ showed mortality of whitefly in bioassay. The mortality was observed in 5th day, as it takes about 5 days for the fungus to grow and fully enter the whitefly. When the whitefly was viewed under microscope after lactophenol cotton blue staining, growth of fungi on whitefly was observed. Surface sterilized whiteflies (using 70% ethanol) were placed on PDA plates (which showed mortality after spraying fungal spore suspension), resulted in the growth of fungi on the surface of insects after four days of incubation. Thus the samples are expected to be pathogens of whitefly. Figure 4 shows bioassay technique and figure 5 shows the microscopic view of fungus grown on whitefly. In case of bacteria, the colonies $S_1C_1$, $S_2C_1$, and $S_6C_1$ showed mortality in whitefly when leaves dipped in the bacterial suspensions fed to nymphs and adults of *Bemisia* (Figure 6 & 7). Serial dilutions of the extracts from dead and surface sterilized whiteflies were plated on nutrient agar plates and resulted in the growth of promising bacteria.

For the identification of bacterial and fungal isolates, DNA extraction, PCR and sequencing were done. Among the fungal isolates, sample 3 (S3), which was isolated from cassava rhizosphere, colony 1($C_1$), colony 2 ($C_2$) and colony 3 ($C_3$) were used for DNA extraction. The quantity of DNA was 338.65 ng/μl, 440.65 ng/μl and 315.15 ng/μl respectively. Quantity of DNA for $S_1C_1$ was 353.08 ng/μl and for $S_5C_1$, 362.12 ng/μl. For bacteria, sample 1, which was isolated from *Amorphophallus* rhizosphere, only colony number 4 ($S_1C_4$) was used for DNA extraction and the quantity of extracted
DNA was 56.87 ng/μl. From sample 4, which was isolated from Colocasia rhizosphere, only colony number 2 (S4C1) was used for DNA extraction and the quantity of DNA obtained was 29.88 ng/μl. From sample 6, which is cow dung, only colony number 3 (S6C1) was used for DNA extraction and the quantity of DNA obtained was 12.41 ng/μl. PCR analysis was carried out for fungi and bacteria and Figure 8 and 9 shows these isolates respectively.

**Figure 8:** PCR analysis of fungal isolates using ITS primers (L: 1kb plus marker, lane 1- S1C1, lane 2- S2C1, and Lane 4- S3C1)

**Figure 9:** PCR analysis of bacterial isolates using 16s primers (L: 1kb plus marker, lane 1- S1C4, lane 2- S6C3 and lane 3- S2C1)

Based on the sequencing results, the organisms were identified using NCBI-BLAST (Table 2). The BLAST results for fungal samples S3C1, S4C1 and S5C1 showed the organisms are Penicillium citrinum, B. bassiana and M. anisopliae respectively. For bacterial samples, the organisms identified as B. cereus (S5C4 & S6C1) and B. pumilus (S3C1). The order of effectiveness (mortality of nymphs and adults in bioassay) of fungi and bacteria were B. bassiana (80.5) > M. anisopliae (77.2) > Penicillium citrinum (68.7) and B. cereus (65.5) > B. pumilus (61.0) respectively. The corrected mortality percent are 70.8, 67.5, 59.0 and 55.8, 51.3 for fungi and bacteria respectively (Table 3).

According to Mascarín et al. (2018) [20] fungal entomopathogens are very good option against arthropod pests. Entomopathogenic deuteromycete fungi of the genera Beauveria and Paecilomyces have been recognized as important biocontrol agents of Aleurodoid pests of field and greenhouse crops for more than 20 years (Wright et al., 1998) [20]. Based on the study conducted by Dara (2017) [6] B. bassiana is compatible with many chemical fungicides. Previous studies conducted by Wright and his co-workers (1998) [20] proved that B. bassiana are highly pathogenic to B. argentifolii nymphs. The entomopathogenic fungus B. bassiana produced higher mortality to the first instars and adults of the silver leaf whitefly (Negasi et al., 1998) [22] and 52-98 % mortality to Bemisia at concentrations of 1-4 x 10^6 conidia mL^-1 (Eyal et al., 1994) [9]. Many researchers emphasized the role of B. bassiana and M. anisopliae as potent entomopathogens (Barreto et al., 2004 [3]; Imoulan and Elmezziane, 2014 [13]; Annuaykanjanasin et al., 2013 [1]) in this study also Beauveria showed mortality of whitefly, which proved it to be an effective biocontrol agent for Bemisia. De Faria and Waight (2007) [7] identified 171 fungal-based products used as biocontrol agents since the 1960s, most of them based on B. bassiana, Beauveria bronniartii, M. anisopliae, and Isaria fumosorosea. The other entomopathogenic fungus proved to be effective against white fly in the present study is Penicillium citrinum and it was sample S5C1 obtained from cassava rhizosphere soil. Similar study conducted using the fungus by Makeiton et al. (2014) [18] also gave satisfying result on mosquito larvae mortality. The first bacterial entomopathogen to be discovered was B. thuringiensis by Ishiwata in Japan (1901). Since its discovery a little over a century ago (Beegle et al., 1992) [4], a number of other entomopathogenic bacterial species have been identified. Reviews suggest that members of genus Bacillus

![Table 2: Fungal and bacterial isolates identified using NCBI-Blast](image)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Organism identified</th>
<th>NCBI Accession No.</th>
<th>Mortality Percent</th>
<th>Corrected Mortality Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1C1</td>
<td>Penicillium citrinum</td>
<td>MN647586</td>
<td>68.7</td>
<td>59.0</td>
</tr>
<tr>
<td>S1C2</td>
<td>Beauveria bassiana</td>
<td>MN647585</td>
<td>80.5</td>
<td>70.8</td>
</tr>
<tr>
<td>S3C1</td>
<td>Metarhizium anisopliae</td>
<td>MN647130</td>
<td>77.2</td>
<td>67.5</td>
</tr>
<tr>
<td>S4C1</td>
<td>Bacillus cereus</td>
<td>MN647516</td>
<td>65.5</td>
<td>55.8</td>
</tr>
<tr>
<td>S5C1</td>
<td>Bacillus cereus</td>
<td>MN647519</td>
<td>65.3</td>
<td>55.6</td>
</tr>
<tr>
<td>S6C1</td>
<td>Bacillus pumilus</td>
<td>MN649215</td>
<td>61.0</td>
<td>51.3</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9.7</td>
</tr>
</tbody>
</table>

- Wraight and his co-workers (1998)
- Previous studies conducted by Wright and his co-workers (1998)
- Based on the study conducted by Dara (2017)
- B. bassiana is compatible with many chemical fungicides.
- Recent study conducted using the fungus by Makeiton et al. (2014) also gave satisfying result on mosquito larvae mortality.
are ubiquitous in nature (Turnbull et al., 1990) [25]. Many surveys of soil bacteria have identified strains of *Streptomyces* and *Bacillus* as potential biocontrol agents (Emmert and Handelsman, 1999) [8]. All the entomopathogenic bacterial isolates used in this study belongs to the genus *Bacillus* which are natural agents for biological control of invertebrate pests and are the bases of many biological commercial insecticides (Molina et al., 2010) [21]. Spores of Gram-positive bacteria offer a scope for commercial product formulations, because of its robustness and durability (Emmert and Handelsman, 1999) [8]. Increased intensity in research on the Gram-positive bacteria associated with plants will provide the potential for a suite of products that may vary in their biological target while sharing a unique formulation (Emmert and Handelsman, 1999) [8]. Studies by Emmert and Handelsman (1999) [8], states that interaction of *B. cereus* with the host plant revealed some promising avenues for improving biocontrol. The other promising bacterium identified in the study (sample S9C, isolated from cow dung) was *B. pumilus*. Identification of a novel strain of *B. pumilus*, that is highly toxic to *Ceratitis capitata* larvae was already reported (Molina et al., 2010) [21].

4. Conclusion

The fungal isolates *Penicillium citrinum*, *B. bassiana*, *M. anisopliae* and the bacterial isolates *B. cereus* and *B. pumilus* isolated from rhizosphere soil of different tuber crop plants and cow dung, were found to give encouraging results for the control of nymphs and adults of cassava whitefly, *B. tabaci* in bioassay study and further studies may be conducted to know their effectiveness in field conditions. As an ecofriendly option and as a viable, sustainable pest management strategy use of these microbial insecticides should be encouraged against the notorious pest; whitefly and their effectiveness can be tested against other major sucking pests also.

5. References


23. Patil BL, Rajasubramanium S, Bagchi C, Dasgupta I. Both Indian cassava mosaic virus and Sri Lankan cassava mosaic virus are found in India and exhibit high variability as assessed by PCR-RFLP. Archives of Virology. 2005; 150(2):389-397.

