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Screening potential microbes against whitefly (*Bemisia tabaci* (Gennadius)), the most important pest 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. Whitefly, *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 [15]; Patil *et al*., 2005) [23]. 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) [2]. The endosymbionts of whitefly may be the reason for making it a successful vector (Harish *et al.,* 2019) [10]. Many studies are being conducted to control whitefly.
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) [5]. 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 whitefly populations (Lacey *et al*., 1996) [16]. Among entomopathogenic bacteria, *Bacillus* sp., 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.

Fig 1: Adult pair of *Bemisia tabaci*

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 (Agrigenome, 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 (S_1) – *Amorphophallus* (rhizosphere soil), four bacterial colonies and two fungal colonies were isolated. Three bacterial colonies and one fungal colony were isolated from sample two (S_2) – *Dioscorea* (rhizosphere soil). From sample three (S_3) which was cassava (rhizosphere soil) one bacterial colony and four fungal colonies were isolated. In case of sample 4 (S_4) – *Colocasia* (rhizosphere soil), 6 bacterial colonies were isolated. One fungal colony could isolate from *Colocasia* (rhizosphere soil). From sample 5 (S_5) 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 S_1, S_2 and S_5 did not give sufficient spores for examining their pathogenicity to whitefly, whereas samples S_1 C_1, S_3C_1, S_3C_2, S_3C_3, S_3C_4 and S_5C_1 (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).

Table 1: Number of fungal spores obtained from samples

Isolate	Number of spores per µl
S_1	$1.4x10^4$
S_1C_1	$2.5x10^6$
S_2	$6x\ 10^5$
S_3C_1	$9.1x\ 10^7$
S_3C_2	$1.27x10^6$
S_3C_3	$1x10^6$
S_3C_4	$2.85x10^7$
S_5	$7x10^4$
S_5C_1	$1.6x10^6$

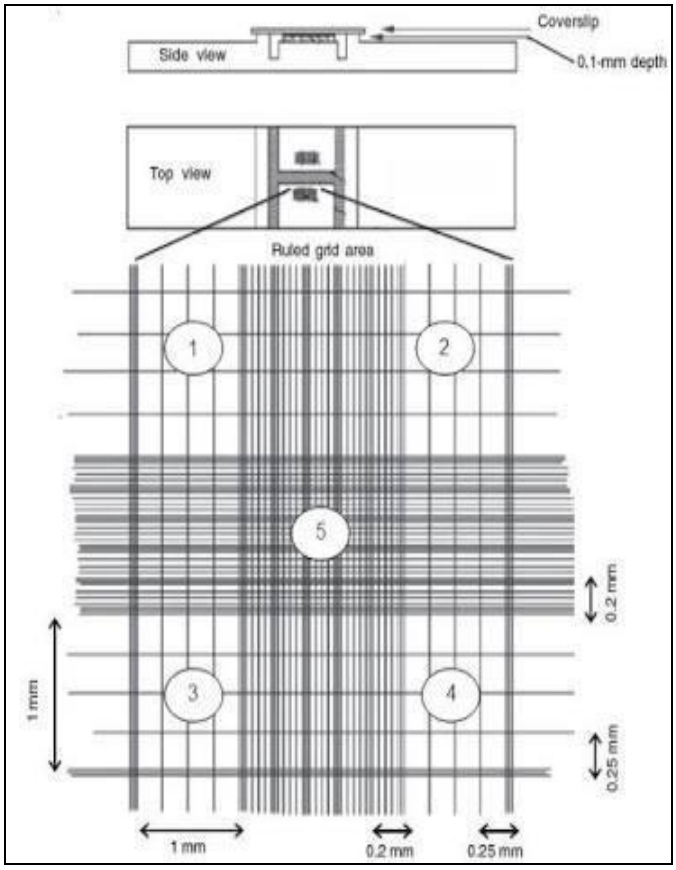

Fig 2: Haemocytometer

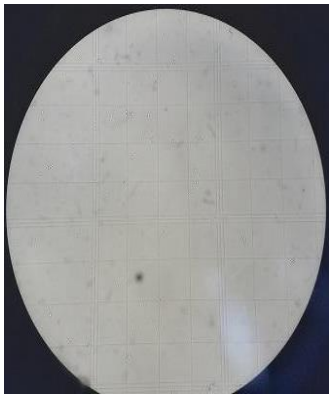

Fig 3: Spores as seen under Haemocytometer

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_4, S_2C_1, and S_6C_3 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.

Fig 4: Bioassay of white fl**y**

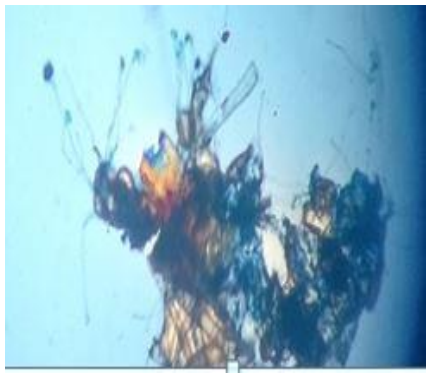

Fig 5: Microscopic view of fungus grown on whitefly

Fig 6: Bacterial bioassay in whitefly nymphs

Fig 7: Bacterial bioassay in whitefly adult

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 (S_1C_2) 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 (S_6C_3) was used for DNA extraction and the quantity of DNA obtained was 9.29 ng/µl. From sample 2, which was isolated from *Dioscorea* rhizosphere, only colony number 1 (S_2C_1) was used for DNA extraction and the quantity obtained was 12.41 ng/µl. PCR analysis was carried out for fungi and bacteria and Figure 8 and 9 shows these isolates respectively.

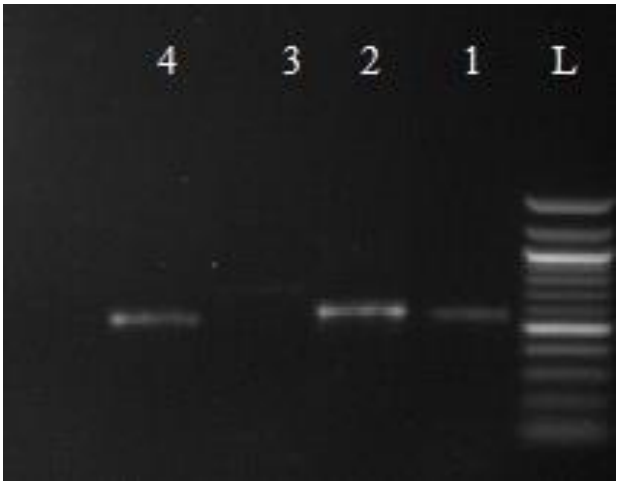

Fig 8: PCR analysis of fungal isolates using ITS primers (L: 1kb plus marker, lane 1- S_3C_1, lane 2- S_1C_1, and Lane 4- S_5C_1)

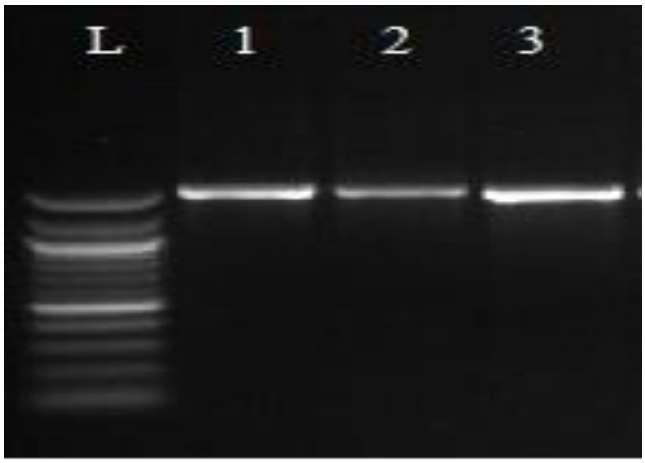

Fig 9: PCR analysis of bacterial isolates using 16s primers (L: 1kb plus marker, lane 1- S1C4, lane 2- S6C3 and lane 3- S2C1)

Table 2: Fungal and bacterial isolates identified using NCBI-Blast

Isolate	Organism identified	Similarity Percent	NCBI Accession No. (Reference sequence)
S_3C_1	*Penicillium citrinum*	100.0	MH793858.1
S_1C_1	*Beauveria bassiana*	97.7	MK049981.1
S_5C_1	*Metarhizium anisopliae*	99.8	MG825184.1
S_1C_4	*Bacillus cereus*	100.0	KX355774.1
S_2C_1	*Bacillus cereus*	99.9	MG651594.1
S_6C_3	*Bacillus pumilus*	99.9	KU844051.1

Table 3: Effectiveness of fungal and bacterial isolates in whitefly

Isolate	Organism identified	NCBI Accession No.	Mortality Percent	Corrected Mortality Percent
S_3C_1	*Penicillium citrinum*	MN647586	68.7	59.0
S_1C_1	*Beauveria bassiana*	MN647585	80.5	70.8
S_5C_1	*Metarhizium anisopliae*	MN647130	77.2	67.5
S_1C_4	*Bacillus cereus*	MN647516	65.5	55.8
S_2C_1	*Bacillus cereus*	MN647519	65.3	55.6
S_6C_3	*Bacillus pumilus*	MN649215	61.0	51.3
Control	-	-	9.7	

Based on the sequencing results the organisms were identified using NCBI- BLAST (Table 2). The BLAST results for fungal samples S_3C_1, S_1C_1 and S_5C_1 shown the organisms are *Penicillium citrinum*, *B. bassiana* and *M. anisopliae* respectively. For bacterial samples the organisms identified as *B. cereus* (S_1C_4 & S_2C_1) and *B. pumilus* (S_6C_3). 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 Mascarin *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 Aleyrodid pests of field and greenhouse crops for more than 20 years (Wraight *et al*., 1998) [26]. Based on the study conducted by Dara (2017) [6] *B. bassiana* is compatible with many chemical fungicides. Previous studies conducted by Wraight and his co-workers (1998) [26] 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 Elmeziane, 2014 [13]; Amnuaykanjanasin *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 Wraight (2007) [7] identified 171 fungal-based products used as biocontrol agents since the 1960s, most of them based on *B. bassiana*, *Beauveria brongniartii*, *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 S_3C_1 obtained from cassava rhizosphere soil. Similar study conducted using the fungus by Maketon *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*

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 S_6C_3 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.

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