

EFFICACY OF SOIL BACTERIA IN CONTROLLING SUGARCANE SMUT DISEASE FUNGI

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Abstract

Some sugarcane varieties which have been identified previously as resistant to sugarcane smut disease have shown some disease incidence in commercial plantations. Therefore, in addition to breeding for resistance to the disease, there is a necessity to control the disease in an eco-friendly manner. This study investigates the efficacy of soil bacteria in controlling sugarcane smut disease fungi in a laboratory. Seven soil samples collected from different locations of the Sugarcane Research Institute, Sri Lanka were used to isolate bacteria in Nutrient Agar medium. They were named and the antagonism was detected through dual culture technique on the Potato Dextrose Agar medium. Bacterial isolates were identified based on morphological and bio-chemical characteristics. Molecular identification was done using DNA sequence analysis. A total of twenty eight bacteria were isolated. Four isolates were found to have antagonistic ability against sugarcane smut fungi. The highest inhibition rate of 77.87 % was shown by the bacterial isolate BCB 017. The isolates BCB 010, BCB 021 and BCB 022 showed relatively high inhibition rates of 61.11 %, 60.31 % and 43.52 % respectively. DNA sequence analysis revealed that the bacterium with highest inhibition rate was *Bacillus subtilis*. This result could be used in the development of an integrated smut disease management program.

Keywords: Bacterial isolates, *Bacillus subtilis*, Bio control, Sugarcane smut disease, *Ustilago scitaminea*

INTRODUCTION

Among the biotic constraints for increasing sugarcane production in Sri Lanka, sugarcane smut disease caused by Basidiomycetes fungi has been a significant factor responsible for serious crop losses in plantations (Sydow, 1924; Thokoane *et al.*, 2011). The development and severity of this disease depends on the environmental conditions and the resistance of the sugarcane varieties (Ramesh Sundar *et al.*, 2012). Genetically, sugarcane varietal reaction to smut disease has been identified as polygenic, and therefore, varietal resistance is the most appropriate method in the management of this disease in plantations. As such, the development of resistant varieties to this disease has been one of the major objectives in sugarcane breeding programs in almost all sugarcane-producing countries including Sri Lanka. Although, all sugarcane varieties recommended and released by the Sugarcane Research Institute (SRI), Sri Lanka are tolerant to this disease, the existing plantations have been infected with the disease at varying levels. For example, the sugarcane varieties, SL 83 06, SL 96 128 and SL 96 328 which have been identified previously as resistant to sugarcane smut disease have shown some disease incidence in commercial plantations. Disease surveys carried out by SRI, Sri Lanka have shown that in Sevanagala plantations, the sugarcane variety SL 83 06, one of the best varieties recommended and released by SRI, has recorded smut disease incidence of nearly 33% in some fields. At Pelwatte, the same variety has recorded disease incidence of 28% in some fields.

This collapse of the resistance of varieties could be due to gradual varietal degeneration, development and build up

of new strains of pathogens, etc. Thus, in addition to breeding for resistance against sugarcane smut disease, implementation of an effective disease management program has become essential to maintain the sugarcane plantations free of the disease. The management practices adopted so far included hot water treatment of seed setts at 54^o C for 50 minutes followed by treatment with fungicide at 0.5 ml/l for 5 minutes and rouging out of infected clumps to minimise crop losses due to the disease. SRI, Sri Lanka has been studying to develop an integrated disease management program with the emphasis on introducing bio-control agents and the minimum use of fungicide to minimise the adverse effects on the environment. This paper presents the results of an attempt made to develop a method to control the smut pathogen in an environment benign manner by making use of bio-control agents to be included in an integrated smut disease management program.

MATERIALS AND METHODS

Isolation and purification of soil micro-organisms

Soil samples were collected from different locations of the Sugarcane Research Institute, UdaWalawe, Sri Lanka for isolation of micro-organisms. One gram (1g) of soil sample was diluted in 9 ml of sterilised distilled water and thoroughly shaken to get a soil solution of 10⁻¹ dilution. One millilitre (1ml) of the above solution was again aseptically transferred to 9ml of sterilised distilled water to form 10⁻² dilution. Similarly 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, and 10⁻⁷ serials were made for each soil sample. A volume of 0.1ml of each dilution was aseptically transferred and

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spread on Petri plates with Nutrient Agar (NA) in three replicates and labelled. The plates were incubated at $\pm 28^{\circ}\text{C}$ temperature until appearance of the colonies. The bacterial isolates were characterised morphologically in terms of their size, shape, colour and margin. A loop full of colonies from each Nutrient Agar plate was streaked on respective media and incubated at $\pm 28^{\circ}\text{C}$ temperature. Isolation and streaking were repeated on the same media until pure cultures were obtained from each selected bacterial colony. The bacterial isolates were identified on the basis of morphological characteristics, and they were named as BCB 001, BCB 002, etc.

***In-vitro* screening of bacteria isolates against smut pathogen**

The isolated bacteria were tested *in-vitro* against smut pathogen using dual culture method (Morton and Stroube, 1955). Smut mycelia disc of 5mm diameter was placed in the centre of a Potato Dextrose Agar plate, and the purified bacteria were inoculated in a circle of 35 mm radius around the smut mycelium disc. This was replicated thrice. One control set was also maintained without inoculating the bacterial isolates.

All plates were incubated at $\pm 28^{\circ}\text{C}$ temperature, and the colony diameter of the smut pathogen in each plate was recorded for every 24 hours regularly up to 10 days of inoculation. The percentage of mycelial growth inhibition was calculated against the control according to Mishra *et al* (2011). Where,

$$\text{Growth of bacteria in treatment} - \text{Growth of the bacteria in control} \times 100$$

Growth of bacteria in control

Microscopic and Bio-chemical characterisation of effective bacterial isolates

Gram staining test was done to observe morphology and bio-chemical tests, namely, Starch Hydrolysis Test, Gelatin hydrolysis test, Catalase Test, Fermentation Test and Cellulose Test were done on each effective bacterial isolates for bio-chemical characterisation.

DNA isolation of bacteria

Total DNA was extracted from 0.1 g of pure bacterial isolates which showed the highest inhibition, BCB 017 using Promega extraction kit according to manufacturer's protocol.

PCR amplification of bacteria

The DNA sample isolated from the superior bacteria was subjected to polymerase chain reaction (PCR) using two pairs of universal primers which were designed to the 16S rRNA region of bacteria. The sequences of the primers, synthesised by Macrogen (Korea), were

AGAGTTTGATCMTGGCTCAG (27 F) and TACCAGGGTATCTAATCC (800 R) for one PCR test and CCAGCAGCCGCGGTAATACG (518 F) and TACGGYTACCTTGTTACGACTT (1492 R) for the other. Amplification reactions were performed in 50 μl reaction volumes containing primers 10 μmol each, template (total DNA) 5 μl , dNTPs 2 mM each, 5 μl of 10 x PCR buffer and Taq DNA polymerase 0.5 U. Amplification was performed with an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds annealing at 55°C for 1 minute and extension at 72°C for 1 minute. Immediately after thermal cycling, a final extension at 72°C for 10 minutes was done and the sample was cooled and kept at 4°C until use. The PCR amplicon of the bacterial isolate was recovered and sequenced.

RESULTS AND DISCUSSION

Isolation of antagonistic bacteria from soil

Twenty eight bacterial species were isolated from the soil samples collected from various locations. The results revealed that four bacterial isolates were able to show the antagonistic activity *in-vitro* on the control of smut pathogen while other twenty four were not effective. The four positive isolates were BCB 010, BCB 017, BCB 021 and BCB 022. Therefore, these four isolates were subjected to characterisation and further evaluation.

Morphological characterisation

The morphological characterisation showed that there was a slight variation among all bacterial isolates. Most of the bacterial isolates have round margins with smooth surface. Some of the isolates have round margins with wavy surface and others have irregular margins with smooth surface. One isolate showed filamentous margin with branching surface.

The colour of the colonies showed a wide variation ranging from pure white to yellow and orange. The results are summarised in Table 1.

Antagonistic activity of the identified bacteria against *Ustilago scitaminea*

The results revealed that four (04) bacterial isolates were positive on the control of smut pathogen while the other twenty four bacterial isolate were not effective as the smut pathogen had grown on the whole Petri plate by covering the bacterial colony after few days of inoculation.

The growth rate of the smut pathogen against each positive bacteria isolate up to 10 days are summarised in Table 2, and the antagonistic activity of the bacterial isolate BCB 017 after 10 days is shown in Fig. 1.

Table 1. Morphological characteristics of the isolated bacteria

Bacterial Isolate	Configura-tion	Margin	Elevation	Colour
				Yellowish
BCB 001	Round	Smooth	Convex	Orange
BCB 002	Round	Smooth	Flat	Yellow
BCB 003	Irregular	Smooth	Flat	Orange
BCB 004	Round	Smooth	Convex	White
BCB 005	Round	Lobate	Convex	Yellow
	Round with radiant margin			Yellowish orange
BCB 006	Round	Branching	Convex	orange
BCB 007	Round	Smooth	Convex	Cream
BCB 008	Round	Lobate	Convex	Cream
BCB 009	Round	Smooth	Convex	White
BCB 010	Round	Smooth	Convex	Off white
BCB 011	Round	Smooth	Umbonate	Off white
BCB 012	Irregular	Wavy	Flat	Yellow
BCB 013	Round	Smooth	Raised	Off white
BCB 014	Round	Smooth	Flat	Yellow
BCB 015	Round	Smooth	Convex	White
				Yellowish brown
BCB 016	Round	Wavy	Flat	brown
BCB 017	Round	undulate	Flat	Off white
BCB 018	Filamentous	Branching	Flat	White
BCB 019	Round	Smooth	Convex	Cream
BCB 020	Round	Wavy	Flat	Yellow
BCB 021	Round	Smooth	Convex	Off white
BCB 022	Round	Smooth	Convex	Pure white
BCB 023	Round	Lobate	Flat	Cream
BCB 024	Irregular	Smooth	Convex	Yellow
				Whitish yellow
BCB 025	Round	Smooth	Flat	yellow
BCB 026	L- form	Smooth	Sumbonate	Cream
BCB 027	Irregular	Lobate	Flat	Cream
BCB 028	Irregular	Lobate	Umbonate	Off white

Table 2. The growth rate of the smut pathogen against four bacterial isolates.

Bacterial isolate	Growth rate of smut pathogen against bacterial isolates (mm/day)				
	Days after inoculation				
	2	4	6	8	10
BCB 010	0.20	0.37	0.47	0.47	0.47
BCB 017	0.16	0.60	0.70	1.00	1.27
BCB 021	0.20	0.43	0.60	0.60	0.70
BCB 022	0.20	0.37	0.43	0.43	0.47

The results revealed that the smut pathogen has shown different growth rates against different bacterial isolates. In the isolate BCB 010, there was no difference in the growth rate after 6 days of inoculation. In other isolates the growth rates of the pathogen have varied with time. The inhibition percentage of each antagonistic bacterium on smut pathogen up to 10 days after inoculation is shown in Fig. 2.

In bacterial cultures BCB 010 and BCB 017, the inhibition

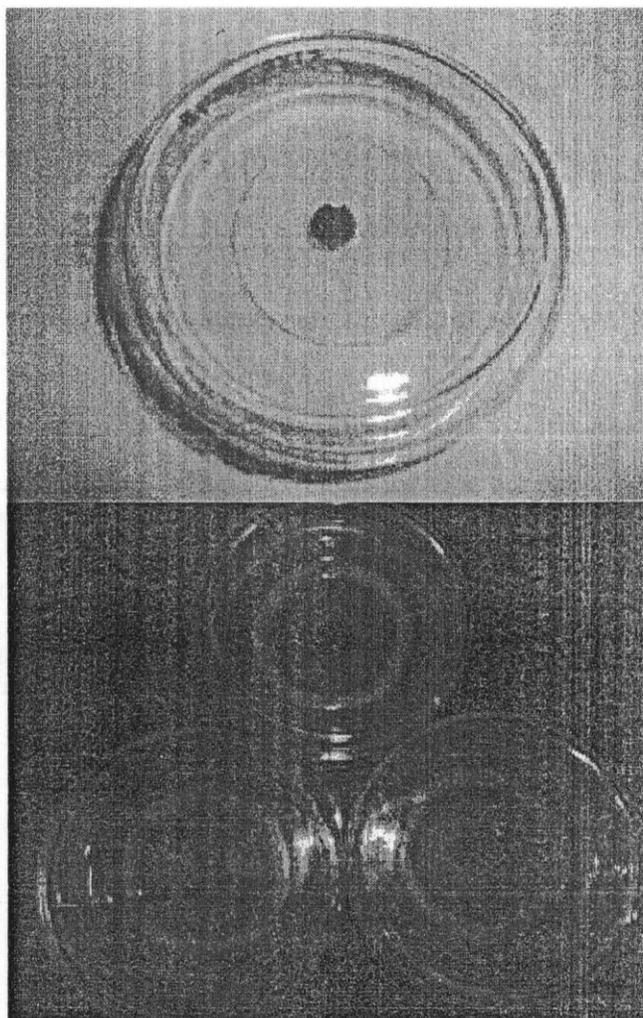


Fig 1. Antagonistic activity of BCB 017 bacterial isolate after 10 days

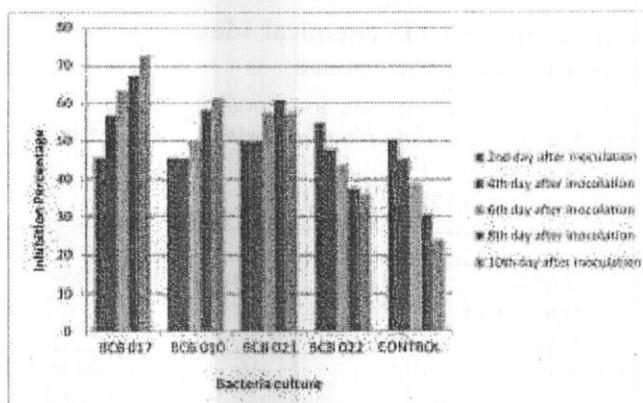


Fig 2. Inhibition percentage of isolated bacteria against smut pathogen

percentage gradually increased up to 10 days and in bacterium culture BCB 021 inhibition percentage decreased after 8 days of incubation. The bacterium culture BCB 022 has behaved differently from the others and its inhibition percentage decreased with time.

Furthermore, the behaviour of each bacterium on smut pathogen was different. In the culture BCB 017, inhibition of the pathogen increased after 2 days of incubation and in bacteria culture BCB 022, it was after 3 days of incubation. In other two cultures, the increase in inhibition occurred after 4 days of incubation. The culture BCB 017 has shown the highest inhibition rate after 10 days of inoculation. This is graphically illustrated in Fig. 3.

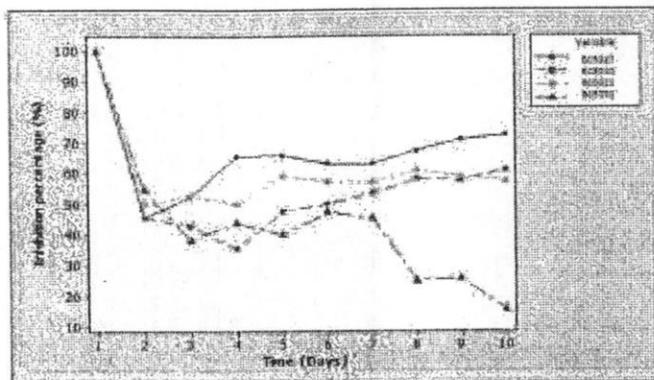


Fig. 3. Behaviour of different bacteria isolates with time

The inhibition percentage of different bacteria isolates is summarised in Table 3.

Table 3. The inhibition percentage of the bacteria isolates against *Ustilago scitaminea*

Bacteria Isolate	Inhibition percentage
BCB 010	61.11
BCB 017	77.87
BCB 021	60.31
BCB 022	43.52
Control	0.00

The microscopic and bio-chemical identification of the effective bacterial isolates

The microscopic studies of the four positive isolates revealed that two isolates out of four bacterial isolates were

gram negative rod shape and the other two isolates were gram positive rod shape. The results are summarised in Table 4.

Table 4. The microscopic results of the four positive bacterial isolates

Bacterial Isolate	Gram +ve/-ve	Cell shape
SCSB 010	Positive	Rod shape
SCSB 017	Positive	Rod shape
SCSB 021	Negative	Rod shape
SCSB 022	Negative	Rod shape

The results of the bio-chemical tests carried on the bacterial isolates which have shown antagonistic ability are summarised in Table 5.

Molecular characterisation

The universal primers used in PCR to amplify bacterial DNA, resulted in an expected size of about 700 bp to 900 bp (Fig. 4). Basic local alignment search tool (BLAST) analysis of the sequence data revealed that the BCB 017 has identity of 99 % with *Bacillus subtilis* (Accession no. KJ 143749.1).

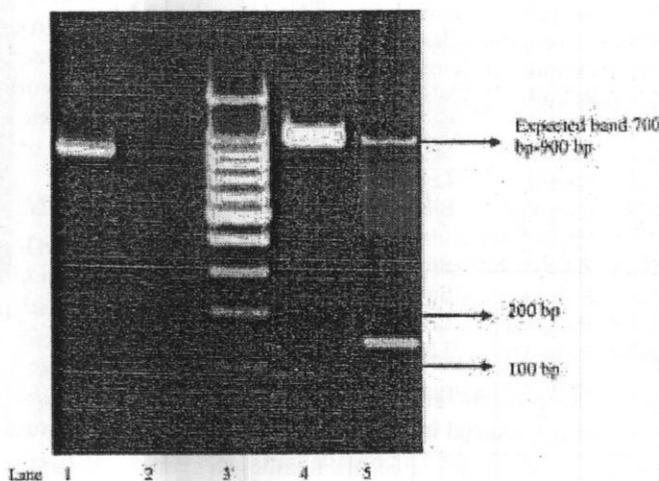


Fig. 4. PCR products resulted from amplification with universal primers

Table 5. The results of biochemical tests

Bacterial isolate	Bio chemical test								
	Starch Hydrolysis Test	Gelatin Hydrolysis Test	Cellulose Test	Catalase Test	Fermentation Test				
					Glucose	Lactose	Sucrose	Maltose	Peptone
BCB 10	-Ve	-Ve	-Ve	Medium bubbling	Yellowish orange	Yellow	Yellowish Green	Yellowish Green	Yellow
BCB 17	+Ve	+Ve	-Ve	Medium bubbling	Purple colour	Purple colour	Purple colour	Purple colour	Purple colour
BCB 21	-Ve	+Ve	-Ve	Medium bubbling	Yellowish orange	Yellowish orange	Yellowish Green	Yellowish Green	Yellow
BCB 22	-Ve	-Ve	-Ve	Immediate bubbling	Orange Gas	Yellowish orange	Yellowish orange Gas	Yellow	Yellow

Note: -Ve = Negative; +Ve = Positive

Lanes: 1-Bacteria sample with 27F & 800R primers; 2-Bacteria water PCR with 27F & 800R primers 3- 100 bp ladder, 4 - Bacteria Sample with 518F & 1492R primers, 5-Bacteria water PCR with 518F & 1492R primers.

Bacillus subtilis has been found antagonistic to different pathogens in different crops by various workers in the world (Bais *et al*, 2003, Ashwini *et al*, 2014). But there are no previous reports of *Bacillus subtilis* the control of smut pathogen. Therefore, this is the first report of *Bacillus subtilis* on the control of *Ustilago scitaminea* in the world.

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