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ISOLATION, SCREENING AND *IN VITRO* EVALUATION OF BACTERIAL ANTAGONISTS FROM SPENT MUSHROOM SUBSTRATE AGAINST *COLLETOTRICHUM MUSAE*

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ABSTRACT

Colletotrichum musae* is a major pathogen in banana fruit which causes heavy losses at the postharvest stage. As a viable alternative to harmful chemical usage and physical control measures, the potential of using bacterial antagonist against *C. musae* was evaluated in this study. Out of a total of 92 bacterial isolates derived from dilution plates of aqueous extracts of spent mushroom substrate (SMS), 43 isolates were initially selected for screening for antagonistic activity against the *C. musae* isolate CMK01. Screening of the selected bacterial isolates revealed that 39 of the tested isolates significantly inhibited fungal growth *in vitro* compared to the control (P <0.05). The diffusible substances secreted by the five selected isolates showed 100% inhibition of colony growth, while (13-58%) inhibition was observed with volatile compounds. Light microscopic observations revealed deformation and blackening of *C. musae* hyphal tips in the colony margins facing the antagonists. Four of the selected five bacterial isolates were identified with 100% precision as *Pseudomonas aeruginosa* based on partial 16S rDNA sequences. These promising antagonists have the potential to be developed as effective biocontrol agents against *C. musae.

Key words: *Colletotrichum musae,* bacterial antagonism, spent mushroom substrate, *Pseudomonas aeruginosa*

INTRODUCTION

The phytopathogenic fungus *Colletotrichum musae* is responsible for highly destructive anthracnose fruit rot in many Sri Lankan cultivars of banana that cause high postharvest losses (Perera *et al.* 1999). In Sri Lanka, of the total banana production, about 20-80% (Illeperuma and Jayathunge 2001) accounts for post harvest losses mainly due to diseases that could be prevented by proper handling and effective disease control. So far, various physical and chemicals treatments such as Calcium chloride and weak acids (Perera and Karunarathna 2001, 2002) plus highly toxic synthetic fungicides are been utilized to control the problem however, the anthracnose is still considered to be a major problem in harvested banana. With developing concerns on food safety and environmental issues, extensive research on selection of antagonists against post harvest fruit diseases is becoming increasingly popular. Biocontrol using antagonists involves the use of naturally occurring nonpathogenic microorganisms that are capable of reducing the activity of plant pathogens thereby suppressing the disease. The postharvest stage provides particular advantages to employ biological control as the controlled conditions in store houses can provide a unique environment ideal for biological systems.

The disease suppressive properties of composting materials are known for many decades and much scientific evidence have revealed favourable properties of composts for the management of plant diseases (Hoitink and Fahy 1986, Hoitink *et al.* 1997). Due to the unique chemical constitution present in spent mushroom substrate (SMS), it is inhabited by many microorganisms such as actenomycetes, bacteria and fungi, those which not only play a role in its further decomposition, but also exert antagonism to the normal pathogens surviving and multiplying in the ecosystem. Much scientific literature such as Yohalem *et al.* (1994), Yohalem *et al.* (1996) and Gea *et al.* (2011) discussed the potential utilization of aqueous extracts of SMS in controlling foliar diseases. Even though enumeration of SMS inhabiting microorganisms provide solid evidence for dis-

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ease suppressive and curative properties of SMS that emphasizes the effectiveness of direct utilization of mushroom compost or their aqueous extracts in agriculture, modern technology can be applied to improve this valuable source of antagonism much further in order to develop a commercially viable, highly efficient and environmentally sound biological control strategy.

The objective of the present study is to screen the presence of potential bacterial antagonists in spent oyster mushroom substrate against *Colletotrichum musae* and to evaluate their antifungal properties under *in vitro* conditions.

METHODOLOGY

Isolation of the pathogen

Colletotrichum musae (CMK01) was isolated from an infected fruit of the local cultivar "*seeni kehel*" (genotype ABB) collected in Matara district, Sri Lanka. The fruit tissue was swabbed with 70% ethanol and a small infected tissue was aseptically transferred to potato dextrose agar (PDA) medium and subsequently monoconidial cultures were prepared from sporulating cultures. The Pathogen was identified using morphology of the asexual conidia produced on PDA plates. The cultures were maintained at room temperature (26-±2^{o} C) with 12 h light and dark cycles.

Isolation of bacteria from spent mushroom substrate

Four months old discarded oyster mushroom cultivation media (saw dust of mango wood with 10% rice bran, 0.2% Magnesium Sulphate and 2% Calcium Oxide) was used to isolate antagonists for this study. Hundred grams of the spent mushroom substrate (SMS) was suspended in 100 ml of sterile distilled water and the aqueous suspension was filtered through four layers of sterilized cheesecloth. The filtrate was centrifuged at 3000 rpm (DAMON) for 10 minutes. The supernatant was collected and subjected to serial dilution procedure using sterile distilled water. Two hundred microlitre aliquots of the suspensions of 10^{-4} and 10^{-6} dilution levels were evenly spread on PDA media using a sterile bent glass rod. The plates were incubated at 28^{o} C for 24 h to get single bacterial colonies. Well discriminating individual bacterial colonies were then transferred to fresh PDA media and were maintained until further used

Screening for potential antagonists

Initially, all the isolated bacterial isolates from SMS were screened for antagonistic activity against C. *musae* isolate CMK01 by dual culturing on PDA plates: A small agar plug (5mm^2) containing the pathogen was placed in the middle of the plate while four bacterial isolates were spotted near the periphery of the plate so that they were equidistant from the pathogen. The plates were sealed and incubated at room temperature for one week. The bacterial isolates that showed visible fungal inhibition were selected for the second round of screening on dual cultures (Sariah 1994) where, each selected bacterial isolate was smeared at one side of the PDA plate while an agar plug (5 mm^2) of the test fungus was placed at the opposite end of the plate. The inverted plates were incubated at 28^{o} C. The treatments were arranged in completely randomized design with three replicates for each selected isolates and control of axenic CMK01culture. Three and five days after incubation, radius of each of fungal colony was recorded. Agar plugs obtained from the middle of the inhibition zones were cultured on fresh PDA plates in order to assess the viability of the fungus in the interaction zone at seven days after culturing. The percent inhibition of radial growth (PIRG) of the fungus was calculated for using the following formula:

$$PIRG = R_1\text{-}R_2/R_1 \times 100$$

Where, R_1 = Average radius of the *C. musae* colony in the control plate; R_2 = Average radius of the *C. musae* colony subjected to antagonism.

The results were subjected to the ANOVA procedure and the mean value of each interaction was compared against a control using the DUNNET'S test at P=0.05 value using SAS 9.1.3 statistical software.

Five highly efficient antagonists were selected for further testing based on the above results.

Production of diffusible and volatile antifungal substance

For these assays five antagonists namely, B01, B04, B09, B34 and B39 were selected based on the results of the above screening experiment.

The effect of diffusible substances secreted by the antagonists was evaluated using the cellophane overlay technique (Nourozian *et al.* 2006). Cellophane membranes (gift wrap material) of 9 mm diameter were boiled in water for 5 min and autoclaved placing the cellophane membrane in between two paper towels. A sterile cellophane membrane was placed on the PDA medium in each petri plate and air dried under the laminar flow cabinet for 15 minutes. A 24 h old culture of bacterial isolate was suspended in a very small amount of SDW and the suspension was smeared over the entire surface of the cellophane. For the control experiment, SDW was applied on the cellophane membrane. The plates were incubated at 28^{o}C for 48 hours after which the cellophane membrane smeared with bacterial culture was removed. A small disc of the *C. musae* isolate (CMK01) was placed in the centre of the plate. The plates were incubated at 28^{o}C for 5 days and radius of the fungal colony was measured in each plate. The treatments were arranged in completely randomized design with 5 replicates. The results were expressed as percent inhibition of radial growth compared to the control.

The effect of volatile substances was evaluated according to the agar strip removal method as described by Choi *et al.* (2006). An agar strip (1 cm wide) was removed from the centre of a PDA plate and the plate was air dried under sterile conditions to remove excess moisture on the cut surfaces. Each bacterial isolate was streaked on the centre of one half of the PDA plate while an agar disc of the *C. musae* isolate (CMK01) was placed in the centre of the other part of the PDA plate. The culture plates were sealed and incubated at 28^{o} C for 5 days. The treatments were arranged in completely randomized design with 5 replicates. The results were expressed as means of percent inhibition of growth of *C. musae* in the presence and absence of bacterial isolate.

Effect of antagonism on mycelial growth and morphology

Seven Days after establishment on dual cultures, microscopic slides were prepared mounting the leading edges of the *C. musae* mycelia that growing towards the bacterial antogonist. A control mount was also prepared from the seven-day old contro, axenic *C. musae* (CMK01) culture. The mounts were observed under the binocular microscope (x 40) to detect any alterations in mycelia growth and morphology.

Molecular identification of antagonistic bacteria

The five antagonists selected for above screening were identified using partial 16S rDNA sequencing analysis at the microbial identification facility of CABI international organization in the United Kingdom.

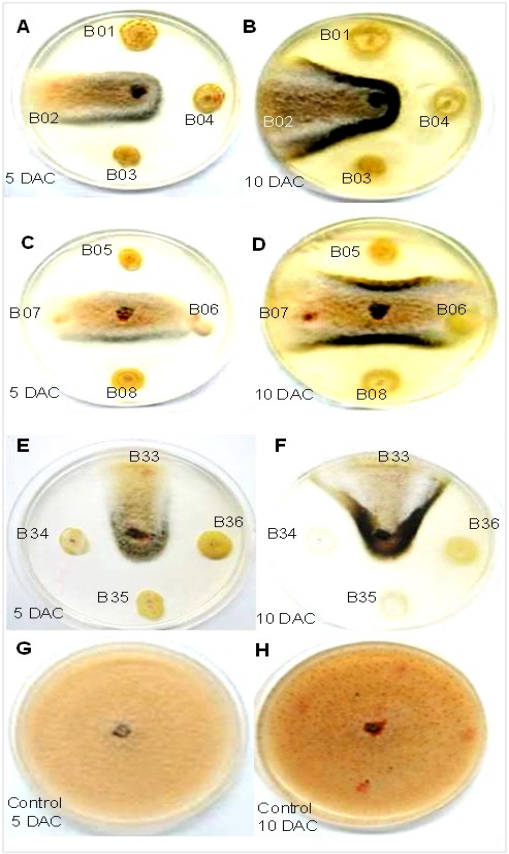
RESULTS

Screening for potential antagonists

The *C. musae* isolate (CMK01) used for this study showed an average growth rate of 9 mm per day at 26-±2^{o}C and 12 h light/dark. The slightly pink mycelium was very compact and grew profusely on PDA medium. *C. musae*

produced salmon pink colour spore masses as early as 7 days after culturing on PDA media at 26-28^{o} C temperature. The fungus was confirmed as *C. musae* based on straight, cylindrical orange cinnamon colour conidia that were obtuse at the apex and truncate at the base (Bailey *et al.* 1992).

Figure 1. Co-culturing and initial *in vitro* screening of potentially antagonistic bacteria against *Colletotrichum musae* at 5 and 17 days after culturing (DAC). Centre of each plate (and the control plate) shows the fungus and the surrounding colonies with labels (isolate numbers) indicate 23 of the bacterial isolates used for the initial screening. Figures A, C and E: Clear inhibition zones were observed at 5 DAC except in isolates B02, B06, B07 and B33. Figures B, D and F: At 10 DAC, colony margins of the inhibited fungus became dark and dense indicating growth termination.



Initially, 92 morphologically different bacterial isolates were obtained from spent mushroom substrate and co cultured with *C. musae* to test antagonistic activity. From which, 43 isolates were selected for further screening based on formation of a clearly visible inhibition zone between the bacterial and fungal colony by five days after culturing (DAC) (Figure 1).

The second round of screening of the selected 43 potential isolates on dual cultures with the *C. musae* isolate revealed that by 3DAC, 39 of the tested isolates significantly inhibited fungal growth *in vitro* compared to the control ($P < 0.05$) (Table 1). Seven of these positive isolates namely B01, B04, B05, B09, B34, B39 and B40 showed more than 60% inhibition. The maximum inhibition value of 80% was exhibited by the isolate B09.

Five days after co culturing, the level of inhibition increased in 38 isolates of which, 21 showed more than 70% inhibition with a maximum value of 78.5% for the isolates B39 and B40 (Table 1).

Table 1. Mean Inhibition of radial growth of *Colletotrichum musae* isolate (CMK01) by antagonistic bacteria extracted from spent oyster mushroom substrate.

	Inhibition of radial growth (%)			Inhibition of radial growth (%)	
Isolate Number	3DAC**	5DAC**	Isolate Number	3DAC**	5DAC**
B01	63.67	78.15	B23	45.67	67.41
B02	02.67$^{\#}$	00.74$^{\#}$	B24	50.00	69.63
B03	49.33	65.93	B25	52.00	48.89
B04	61.67	77.04	B26	50.67	65.04
B05	61.00	75.56	B27	52.00	56.30
B06	19.67	39.63	B28	51.67	66.37
B07	17.33	31.11	B29	48.00	67.41
B08	50.00	69.63	B30	55.67	72.59
B09	80.33	73.33	B31	51.33	68.52
B10	58.00	74.81	B32	55.67	71.48
B11	50.67	69.63	B33	0.00$^{\#}$	05.19$^{\#}$
B12	57.00	73.33	B34	64.00	78.52
B13	51.33	70.37	B35	50.00	00.71
B14	56.67	73.70	B36	56.33	70.00
B15	52.00	70.37	B37	12.33	40.00
B16	45.33	67.41	B38	58.67	73.33
B17	56.33	73.33	B39	64.67	78.52
B18	49.67	71.85	B40	63.00	77.04
B19	46.00	64.81	B41	51.67	70.37
B20	53.67	72.96	B42	32.00	51.11
B21	01.33$^{\#}$	00.89$^{\#}$	B43	03.33$^{\#}$	00.74$^{\#}$
B22	39.33	63.70	Control	00.00	00.00

Means with the symbol $^{\#}$ are not significantly different from the control plate at $P<0.05$ according to Dunnett's test. ***** Mean percentage inhibition of radial growth. ****** Days after culturing.

No mycelia growth was observed even seven days after incubation, when PDA plugs from the middle of the interaction zone was re-cultured on fresh PDA plates.

Production of diffusible and volatile antifungal substance

Experiment on diffusible substances revealed 100% inhibition of fungal growth on the third day after culturing (Table 2, Figure 2A). Fungal growth was not observed in those plates until 10 DAC.

Table 2. Mean growth inhibition of *Colletotrichum musae* isolate CMK01 by volatile and diffusible compounds produced by antagonistic bacteria.

Isolate number	Inhibition of radial growth (%) at 3DAC[b]	
	Volatile substances	Diffusible substances
B01	13.33*	100.00
B04	19.26*	100.00
B09	05.19	100.00
B34	58.52*	100.00
B39	36.30*	100.00
Control	00.00	000.00

Means with the symbol * are significantly different from the control plate at P<0.05 according to Dunnett's test.
[a] Mean percentage inhibition of radial growth. [b] Days after culturing.

In the experiment on volatile antifungal substances, significant (P<0.05) growth inhibition was observed in isolates B01, B04, B34 and B39 compared to the control colony (mean diameter 2.7 cm) by 3 DAC (Table 2, Figure 2B).

Effect of bacterial isolates on C. musae mycelial growth and morphology

Light microscopic observations revealed that hyphal tips at the leading edge of the colony, that faced the bacterial smear was thick and deformed as opposed to smooth normal hyphae in the control colony (Figure 3). These deformation lead to total inhibition of fungal growth and blackening of the colony margins that faced the antagonists (Figures 1B, 1D and 1F).

Molecular identification of antagonistic bacteria

According to the partial 16S rDNA sequence analysis, B01, B04, B34 and B39 isolates gave 100% matches to the species *Pseudomonas aeruginosa* when subjected to BLAST search. B09 was not able to identify since the sequencing reaction failed.

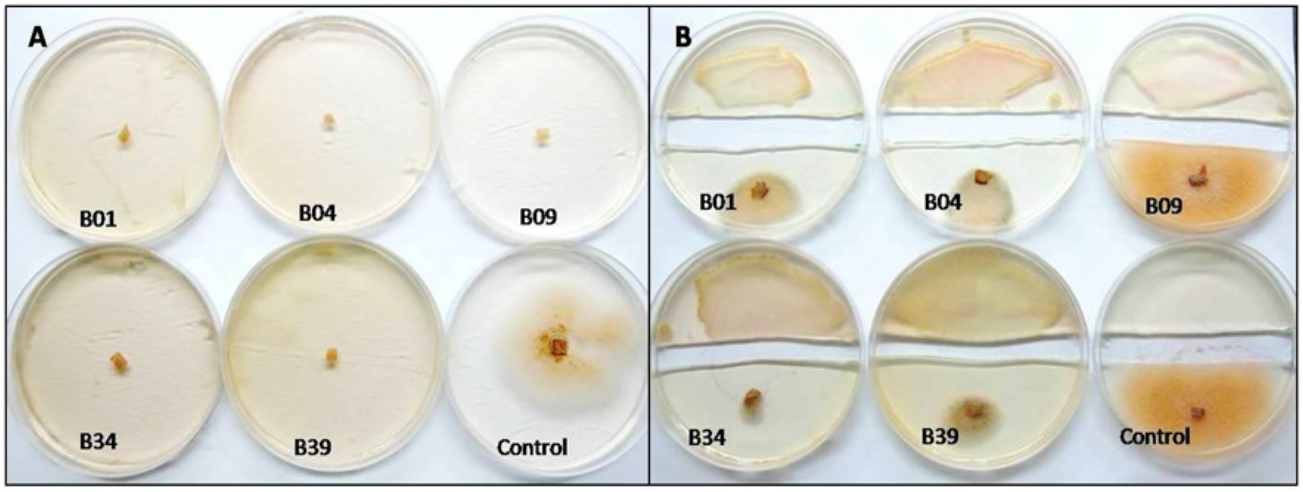


Figure 2: In-vitro screening for diffusible (A) and volatile (B) antifungal compounds produced by antagonistic bacteria against *Colletotrichum musae* isolate CMK01 three days after culturing.

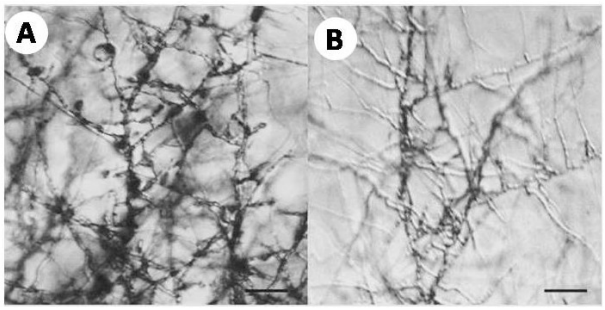


Figure 3: Light micrographs showing the effect of bacterial antagonism on hyphal growth (A) of *Colletotrichum musae* isolate CMK01 compared to a control mycelium (B). Bars = 20 µm

DISCUSSION

Results of this study clearly showed the potential of using antagonistic bacteria from spent mushroom substrate to control *C. musae* infecting banana.

There are various bacterial species, fungal and actinomycete groups that occur in the spent mushroom substrate are known to have antagonistic features. Ranathunge *et al.* (2012) reported isolation of sixteen different bacterial antagonists from aqueous extracts of SMS against *C. gloeosporioides* in papaya. Many different antagonistic bacterial species have been reported to inhabit SMS that include *Bacillus licheniformis, B. subtilis, Paenibacillus macerans, Pseudomonas aeruginosa* and *Streptomyces albidoflavus* (Romaine and Holcomb 2000, Viji *et al.* 2003, Phae *et al.* 1990).

In this study, four of the most promising bacterial isolates were identified as *Pseudomonas aeruginosa* using partial 16S rDNA sequencing analysis which gave numerous matches up to 100% to this species. *Pseudomonas aeruginosa* is a very common bacterium in the environment. There are numerous citations in published literature regarding the inhibitory properties displayed by this species towards *Colletotrichum.* For example, Rahman *et al.* (2007) reported occurrence of antagonistic *Pseudomonas aeruginosa* strains from the fructosphere of papaya against *C. gloeosporioides*. Masyahit *et al.* (2009) also reported the antagonistic association between *Pseudomonas aeruginosa* and *C. gloeosporioides* in dragon fruit. There are many other reports on the efficacy of *Pseudomonas aeruginosa* as a potential biocontrol agent for various phytopathogenic fungi (Siddiqui and Shaukat 2002, Mansoor *et al.* 2007, Prakob *et al.* 2009, Deshwal 2012, Maiti *et al.* 2012).

The results of the present study demonstrated that the antagonistic isolates produce antifungal compounds *in vitro* that inhibited mycelia growth of *C. musae*. Death and blackening of hyphae at the colony margins could be due to the toxic effect of antifungal substances which interfere with normal growth and multiplication. *Pseudomonas* strains are known to produce numerous compounds which are responsible for disease control. These inhibitory compounds are siderophores, HCN, degradative extracellular enzymes such as chitinase, protease, cellulose, β-1,3 glucanase and antibiotics such as pyrrolnitrin, pyoluteorin, phenazine (Dowling and O'gara, 1994, Choi *et al.*., 2006, Deshwal *et al.*, 2011, Maiti, *et al.*. 2012). Similar observations have been reported with respect to *Bacillus* spp. antagonistic to *Colletotrichum* spp. (Sariah, 1994; Rahman *et al*, 2007).

The present study revealed that *P. aeruginosa* is capable of producing volatile substances and diffusible substances with antifungal properties that significantly inhibited the mycelia growth of *C. musae* on PDA. Production of volatile antimicrobials by *P. aeruginosa* has also been reported in numerous occasions (Baligh *et al.* 1996, Conway *et al.* 1989, Rahman *et al.* 2007, Maiti, *et al.* 2012). This approach offers an efficient, economical and environmentally sound means of suppressing the disease. However, further studies are required to evaluate the *in vivo* efficacy of the antagonists as well as to determine the time of application and the most suitable form in which the antagonists can be applied on fruits.

CONCLUSION

In this study, *P. aeruginosa* isolates showed potential agonistic activity against *C. musae* causing anthracnose fruit rot of banana.

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