Microbial Control of *Mycosphaerella fijiensis* Morelet A Notable Pathogen of Bananas and Plantains

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Abstract: The ability of one bacterium and two fungi to inhibit the mycelial growth of *Mycosphaerella fijiensis* was determined in this study. The culture filtrates of the three microorganisms (*Bacillus subtilis*, *Trichoderma asperellum* and *Trichoderma longibrachiatum*) at different concentration were evaluated in – vitro against mycelia growth of *M. fijiensis* on two culture media using spread plate and mycelia dry weight method. Generally, the culture filtrates of the three microorganisms had significant effect on the mycelial growth of *M. fijiensis*. Culture filtrates of *Bacillus subtilis* inhibited mycelial growth of *M. fijiensis* at concentration of 1.5%, 2.5% and 5%. *Trichoderma asperellum* and *Trichoderma longibrachiatum* culture filtrates were observed to be less effective at concentration of 1% and 1.5% but became effective on mycelial growth of *M. fijiensis* at concentration of 2.5% and 5%. When spread plate method was used, culture filtrates of *Trichoderma asperellum* was observed to be highly effective than that of the other microorganisms. When mycelial dry weight method was used, culture filtrates of *Bacillus subtilis* exhibited high antagonistic effect to mycelial growth of *M. fijiensis*. Malt extract agar and malt extract broth significantly ($P < 0.05$) supported the antagonistic activity of microbial agents culture filtrates on mycelial growth of *M. fijiensis* compared to potato dextrose agar and potato dextrose broth. Effective screening of microbial biocontrol of *Mycosphaerella fijiensis* can contribute to reduction in chemical pesticides usage that has been reportedly linked to environmental pollution and threat to public health. [Nature and Science 2010;8(10):299-305]. (ISSN: 1545-0740).

Key words: microbial agents; *Mycosphaerella fijiensis*; culture filtrates; antagonistic activity.

1. Introduction

Black sigatoka, caused by *Mycosphaerella fijiensis* Morelet (teleomorph of *Pseudocercospora fijiensis* (Morelet) Deighton), is regarded as the most damaging and economically important leaf disease of bananas and plantains worldwide (Carlier *et al*., 2000). Black sigatoka disease attacks plantains and bananas in several countries of central and south America, Africa and Asia (Anon, 1982). The fungus reduces the green leaf area and impedes photosynthesis, which is needed for fruit filling (Marin *et al*., 2003). The greatest loss occurs as a result of premature ripening of fruits in the field and during transportation and storage (Marin and Romero, 1992).

In 1992, the cost to control this fungus in Honduras alone was estimated at US$1,000,000 compared to an annual income of US$100million reported by banana industry (FHIA, 1991). Mobambo (1993) reported minimum yield loss of 33%, and greater losses may occur if control measures are either not adopted or fail (Craenen and Ortiz, 1998; Marin *et al*., 2003).

Black sigatoka is controlled with frequent applications of fungicides and cultural practices, such as the removal of affected leaves, and adequate spacing of plants and efficient drainage within plantation. These control methods are either require high levels of expensive imputs or have a high labour requirements, which adds to the cost burden to the grower (Ploetz, 2001). Effective chemical control methods are available but are not used by most small scale producers who can not afford to purchase equipment and chemicals. Murhorpadhyay and Mukherjee (1996) listed the major problems associated with use and overuse of chemical fungicides to include, residue problems, health and environmental hazards and the development of fungicides resistance in the pathogens. Generally, biological control of black sigatoka is extremely challenging because of the polycyclic nature of the disease, the presence of plants of all ages, and the

unfurling of highly susceptible leaves every 6 - 12 days. Biological control of *M. fijiensis* has received little attention because of the availability of highly effective fungicides as well as the limited interest and financial support to find alternative methods of control. However, the development of strains of *M. fijiensis* less sensitive or resistant to systematic fungicides and the increasing demand for environmentally safe control measures has increased the interest in finding alternatives for the control of black sigatoka over the past decade (Marin *et al*., 2003). One of the alternatives is the use of microbial agents which demands to be evaluated in – vitro in the laboratory before field application. Therefore, the main objective of this study was to evaluate (in – vitro) the crude culture filtrate of microbial agents at different concentration as an antagonist to mycelial growth of *M. fijiensis*.

2. Materials and Methods

2.1 Innoculum Preparation

Dried and naturally infected leaf samples at various stages of disease development were collected at different location in University of Ibadan, Nigeria and International Institute of Tropical Agriculture (IITA), Ibadan in Nigeria and were transferred to the laboratory in paper bags. Infected leaves samples, noted for abundant sporulation lesions were cut into 2cm x 2cm. These were stapled to 9cm diameter filter paper, immersed in sterile water for 5 minutes and then placed in the lid of petri dish with the leaf sections directly above 3% water agar composition which was then incubated for 24hours for the release of ascospores as described by Ayodele (1997). The ascospores forcibly discharged on the agar surface after 1hour. Single germinating ascospores were transferred onto V8 juice agar composition after 24hours. Plates were incubated at temperature of 27^{o}C at 12 hours light and 12 hours darkness. Identification of *Mycosphaerella fijiensis* was done according to the description of Meredith and Lawrence (1969) adapted by Natural (1990) based on the structure of the conidia.

Prior to antagonistic activity experiment, the inoculum of the mycelia was quantified. Scraped fragments of *M. fijiensis* were grounded with already sterilised mortar and pestle containing sterile water. Finely grounded mixture was filtered with well-folded cheesecloth into another sterile beaker. The counting of the mycelial fragments using a haemocytometer was re-adjusted with sterile water to concentration of 1.006 x10^{6} mycelial fragments/ml following the method of Twizeyimana *et al.* (2007).

2.2 Growth of *M. fijiensis* on Different Culture Media

M. fijiensis with average mycelia diameter (1.1-1.2mm) were subcultured onto separate plates of potato dextrose agar (PDA) and malt extract agar (MEA). The average growth diameter was measured after every six days for eigthteen days. The experiment was done in four replicates.

2.3 Extraction of Culture Filtrates of Microbial Agents

Five millilitres mycellia disc carrying the confluent growth of 7 days old culture of *Trichoderma spp* (*Trichoderma asperellum*, NGT – 161 and *Trichoderma longibrachitum*, NGT – 167) were aseptically inoculated into separate 125ml conical flask containing 50ml of sterile potato dextrose broth (PDB) and were incubated on shaker incubator at temperature of 28^{o}C and 150 rpm for 7 days. They were then collected after the required period of incubation. Filtration was carried out using whatman No1 filter paper to separate the mycelia mat and the culture filtrate while the culture filtrate of *Bacillus subtilis* was obtained by growing the *Bacillus* in flask containing 100ml of nutrient broth and was then incubated using shaker incubator for 48 hours at room temperature. After incubation, the cell free – extracts was obtained by centrifugation at 4500rpm for 40 minutes, followed by vacuum filtration on 0.22 um membranes. Extracts were kept at 4^{o}C in sterile flasks.

2.4 Antagonistic Activity of Microbial Agents using Spread Plate Method

One milliliter of 1.006 x10^{6} mycelial fragments/ml of *M. fijiensis* suspended in sterile distilled water was pipetted into MacCartney bottles containing 10ml of different concentration (1%, 1.5%, 2.5% and 5%) of each treatment sample and into 10ml of sterile distilled water as control. There were three replicates per treatment. The suspension were agitated in a vortex mixer and incubated for one hour at room temperature. From each suspension including the control, 0.1ml was pipetted into potato dextrose agar (PDA), malt extract agar (MEA) prepared plates and spread with sterile

glass rod, as described by Ayodele (1997). The plates were incubated at temperature of 27°C and periodically observed after each 24 hours for numbers of colonies formed by the survived mycelia of *M. fijiensis* in various concentrations of microbial agents' cultured plates. Percentage of mean growth was calculated as follows;

Mean growth (%) = $N_c - N_t / N_c \times 100$

Where ; N_c = Numbers of mycelial growth of *M. fijiensis* in the control

N_t = Numbers of mycelial growth of *M. fijiensis* in the treatment

2.5 Antagonistic Activity of Microbial Agents using Mycelia Dry Weight Method

The mycelial growth measurement was determined using liquid media. Potato dextrose broth (PDB) and malt extract broth (MEB) were dissolved separately into 1000ml of sterile distilled water in media bottles according to manufacturer's specifications. They were dispensed at varying volumes into 250ml conical flasks and covered with aluminium foil, sterilized by autoclaving at temperature of 121°C and pressure of 1.2kg/cm^2 for 15 minutes. The media were allowed to cool down to room temperature. Extracts of microbial agents were aseptically pipetted into each flask to give different percentage concentration as shown below. Aseptically, one milliliter (1ml) of 1.006 x 10^6 mycelial fragments/ml of *M. fijiensis* was pipetted onto the surface of already prepared plates of potato dextrose agar (PDA) and spread with glass rod. This was incubated for 7 days at temperature of 27°C. After the incubation period the pathogen was cut with sterilized 5mm cork borer carrying confluent growth of *M. fijiensis* and inoculated into each flask. The flasks were incubated using shaker incubator at room temperature for 14 days at 250rpm. On the 14th day, the mycelial mats of the fungal isolate were harvested by filtering on a pre - weighed filter paper after which the filter paper with the mycelia mat were then oven dried at 80°C until constant weight was maintained. The weight of the mycelia was determined by subtracting the weight of the filter paper from the total weight of filter paper plus mycelial. There were three replicates for each treatment. Percentage mean growth was calculated as follows;

Mean growth (%) = $W_c - W_t / W_c \times 100$

Wheres;

W_c = Weigth of mycelial growth of *M. fijiensis* in the control.

W_t = Weigth of mycelial growth of *M. Fijiensis* in the treatment

Potato dextrose	Malt extract	Microbial agents
100ml (Control)	100ml (Control)	0.0%
99.0ml	99.0ml	1.0%
98.5ml	98.5ml	1.5%
97.5ml	97.5ml	2.5%
95.0ml	95.0ml	5.0%

2.6 Data collection and statistical analysis

Data for antagonistic activity using spread plate method were obtained after the 5th day, while the data for antagonistic activity for mycelial dry weigth method were obtained after 14 days. The data collected were analysed using the analysis of variance procedures of SAS (version 9.1, of 2008, SAS Institute, Cary, NC) for factorial, and Randomised Complete Designs (CRD). The least significant different (LSD) test at 0.05 level of significant was used to compare treatment means for each parameter.

3. Results

3.1 Evaluation of *M. fijiensis* Growth on PDA and MEA

There was significant difference at $P<0.05$ level of probability in the average mycelial growth of *M. fijiensis* grown on PDA and MEA from day 12 to 18 (table 1). It was observed that the fungus does not grow fast.

Table 1: Growth of *M. fijiensis* on potato dextrose agar and malt extract agar

	Day1	Day6	Day12	Day18
Media	**Mycelial mean growth**			
PDA	1.1±0.1^a	2.4±0.5^a	4.2±1.2^{ab}	6.3±1.6^{ab}
MEA	1.1±0.1^a	1.9±0.1^a	3.4±0.7^b	4.6±0.9^b

Data are presented as means for four replicates, followed by standard deviation within replicates.Values followed by the same letters are not significantly different at P=0.05. PDA ; Potato dextrose agar, MEA; Malt extract agar.

3.2 Effect of Microbial Agents on Mycelial Growth of *M. fijiensis* Cultured on PDA and MEA

All the microbial agents at concentration of 1%, 1.5%, 2.5% and 5% has significant effect on the mycelial growth of *M. fijiensis* compared to the control at P<0.05 level of probability. *Trichoderma asperellum* inhibited the mycelial growth of *M. fijiensis* at concentration of 1.5%, 2.5% and 5% irrespective of the media and moderately effective at 1%, 1.5% and 2.5% concentration on PDA (Table 2). Observation revealed that *Trichoderma longibrachiatum* significantly inhibited the mycelial growth of *M. fijiensis* at 1.5%, 2.5% and 5% on MEA compared to PDA (Table 2). The culture filtrate of *Bacillus subtilis* at different concentration showed significant effect at P<0.05 level of probability on mycelial growth with the exception of 1% concentration that shows little or no effect on the pathogen (Table 2). Anova uncovered in Table 3 that all the culture filtrates from the microbial agents were significantly different in their antagonistic activity on mycelial growth of *M. fijiensis.*

Table 2: Effect of Microbial Agents on Mycelial Growth of *M. fijiensis* Cultured on Potato Dextrose Agar and Malt Extract Agar

		Concentration of culture filtrate (%)				
		0%(Control)	1.0 %	1.5%	2.5%	5.0 %
Microbial agents	**Media**	**Mycelia mean growth (%)**				
Trich. asp.	PDA	100.0 ± 0.0	45.3± 0.0	28.3±0.5	24.5±0.3	15.6±0.4
	MEA	100.0 ± 0.0	19.7±0.8	10.6±0.2	7.4±0.2	4.2±0.1
Trich. long.	PDA	100.0 ± 0.0	74.2±4.0	36.7±1.9	31.1±1.1	20.0±0.3
	MEA	100.0 ± 0.0	26.1±1.2	20.5±0.8	10.0±0.4	6.1±0.1
B. sub.	PDA	100.0 ± 0.0	60.2±0.9	50.9±1.2	37.6±0.7	15.7±0.4
	MEA	100.0 ± 0.0	46.2±1.6	30.6±2.5	17.4±0.6	2.9±0.2

Data are presented as means for three replicates, followed by standard deviation within replicates. Trich. asp= *Trichoderma aperellum, Trich*. long= *Trichodema longibrachiatum,* B.sub.=*Bacillus subtilis*.

Table 3: Comparism of microbial agents effectiveness on mycelial growth of *M. fijiensis* cultured on potato dextrose agar and malt extract agar

Treatments	**Mean growth**
Trichoderma asperellum	19.38[a]
Trichoderma longibrachiatum	28.10[b]
Bacillus subtilis	32.68[c]

Data are presented as means for three replicates. Means with the same letter are not significantly different according to Duncan's multiple range tests (P=0.05).

3.3 Effect of Microbial Agents on Mycelial Growth of *M. fijiensis* Cultured in PDB and MEB

Mycelia dry weigth method was used to ascertain the in - vitro effect of microbial agents on the mycelial growth of *M. fijiensis* in liquid culture media. All the microbial agents has significant effect on the pathogen (*M. fijiensis*), but were observed to be significantly different and more effective than each other at P<0.05 level of probability. Observation in Table 4 revealed that *Trichoderma asperellum* significantly inhibited mycelial growth of *M. fijiensis* at higher concentration in the liquid media most especially in malt extract broth, and moderately at lower concentration in potato dextrose broth. *Trichoderma longibrachiatum* at higher concentration exhibited strong inhibition on the mycelial growth of *M. fijiensis* in potato dextrose broth and malt extract broth but showed moderate significant effect at lower concentration (Table 4). Appreciable inhibition was recorded for *Bacillus subtilis* at concentration of 1%, 1.5% and 2.5% but became significant at concentration of 5% on the mycelial growth of *M. fijiensis* in malt extract broth and potato dextrose broth (Table 4). Anova in Table 5 justified that *Bacillus subtilis* was more effective in liquid media compared to other microbial agents.

Table 4: Effects of microbial agents on mycelial Growth of *M. fijiensis* Cultured in Potato DextroBroth and Malt Extract Broth.

		Concentration of culture filtrate (%)				
		0%(Control)	1.0 %	1.5%	2.5%	5.0 %
		Mycelia mean growth (%)				
Microntsbial age	**Media**					
Trich. asp.	PDB	100.0 ± 0.0	95.6±0.4	82.4±0.2	67.1±0.2	45.9±0.2
	MEB	100.0 ± 0.0	50.5±0.3	35.3±0.2	9.6±0.3	5.3±0.3
Trich. long.	PDB	100.0 ± 0.0	64.5±0.2	62.1±0.3	42.4±0.2	37.7±0.2
	MEB	100.0 ± 0.0	73.7±0.5	57.9±0.1	36.0±0.3	29.1±0.2
B. sub.	PDB	100.0 ± 0.0	48.5±0.1	32.0±0.2	30.0±0.2	17.4±0.1
	MEB	100.0 ± 0.0	41.6±0.2	37.4±0.1	37.6±0.3	16.6±0.1

Data are presented as means for three replicates, followed by standard deviation within replicates. Trich. asp= *Trichoderma aperellum, Trich*. long= *Trichodema longibrachiatum,* B.sub.=*Bacillus subtilis.*

Table 5: Comparism of microbial agents effectiveness on mycelial growth of *M. fijiensis* cultured on potato dextrose broth and malt extract broth

Treatments	**Mean growth**
Trichoderma asperellum	32.64[a]
Trichoderma longibrachiatum	48.97[b]
Bacillus subtilis	50.42[c]

Data are presented as means for three replicates. Means with the same letter are not significantly different according to Duncan's multiple range tests (P=0.05).

4. Discussion

This research work discussed how spread plate and mycelial dry weigth method can be used to evaluate antagonistic activity of microbial agents for the control of slow growing *Mycosphaerella fijiensis*. Mycelial growth of *M. fijiensis* was studied on PDA and MEA prior introduction of microbial agents. The fungus grew on both PDA and MEA in normal incubator of 12 hours light / 12 hours darkness at temperature of 27^{o}C. This was in accordance with the report of Ayodele (1997) that PDA and MEA are among the media that supported the mycelial growth of *Mycosphaerella fijiensis.*

Many research studies have reported the potential value of *Trichoderma species* for control of plant diseases. Adekunle (1998) reported that *Trichoderma species* possess toxic substances, which they release in plant to inhibit fungal germination in - vitro and in organic substrate in the field. This was observed in the inhibitory activity exhibited by culture filtrates of *Trichoderma asperellum* and *Trichoderma longibrachiatum* on the mycelial growth of *M. fijiensis*.

Based on our results, the in – vitro antagonistic activities of these culture filtrates on the mycelial growth of *M. fijiensis* agreed with the reported work of Odebode (2006) that *Trichoderma species* are capable of producing a range of metabolites, which have antifungal activities in liquid cultures. The inhibition of mycelial growth of *M. fijiensis* by culture filtrates of *Trichoderma species* in this study could be due to antibiotics or specific enzymes. Papavivas (1985) reported that enzymes such as exo – and endo – glucanases, cellobiase and chitinase were noted for various cell wall degradation. This study has therefore demonstrated possible presence of antifungal substances in the culture filtrate of *Trichoderma asperellum*, NG - T161 and *Trichoderma longibrachiatum*, NG - T167 against the mycelial growth of *M. fijiensis*.

Observation from analysis of variance in this research study revealed that *Trichoderma asperellum* had more significant effect on the mycelial growth of *M. fijiensis* compared to that of *Trichoderma longibrachiatum* irrespective of the media and antagonistic method used. This agreed with the report of Odebode, (2006) that different *Trichoderma species* do produces different antifungal substances to inhibit growth of plant pathogens.

Antagonistic activity of *Bacillus subtilis* in this study agreed with the reported work of Marin *et al.*

(2003). The culture filtrate of *Bacillus subtilis* showed significant effect at P<0.05 level of probability on mycelial growth of *M. fijiensis* in both antagonistic methods used. The culture filtrate of *Bacillus subtilis* at 5% concentration in PDA and MEA significantly suppressed the growth of the pathogen while also at low concentration of 1% in MEB and PDB showed significant effect on the pathogen which agreed with the report of Riveros *et al*. (2003) that culture filtrates of bacteria GBC02 and SE/P02 at 0.1ppm concentration stopped the physical growth of *M. fijiensis* after five days incubation in comparison to control. Comparing the antagonistic activity of *Bacillus subtilis* on different culture media with respect to the antagonistic methods used, it was observed that, there was no difference in their in – vitro effect on the mycelial growth of *M. fijiensis.* This suggested that, the same metabolites were released by *Bacillus subtilis* into the culture media which agreed with the reported work of Young *et al*. (1994) that at least five antibiotics were produced by *B. subtilis*, namely, subtilin, bacitracin, bacillin, subtenolin and bacilomycin. These substances might be responsible for the inhibition observed in this study.

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