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Full Length Research Paper

Inhibitory influence of biocontrol agents, plant oils and an inorganic salt on *Mycosphaerella fijiensis* and *Cordana musae*, the causal pathogen of black sigatoka and leaf spot of banana

Dionisio G. Alvindia

Philippine Center for Postharvest Research and Mechanization (PhilMech), Science City of Muñoz, Nueva Ecija 3120, Philippines. E-mail: dgalvindia@yahoo.com. Tel: 63-44-4560213. Fax: 63-44-4560110.

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The search for alternatives to chemical treatments to control the causal-pathogens of black sigatoka and Cordana leaf spot in banana was studied *in vitro*. We examined the effect of biocontrol agents, plant oils and an inorganic salt, on the mycelium growth of *Mycosphaerella fijiensis* and *Cordana musae* for 10 weeks. *Trichoderma harzianum* DGA01 and *Bacillus amyloliquefaciens* DG14 inhibited the development of *M. fijiensis* throughout the duration of observation while Serenade ASO (*B. subtilis* 1.34% EC) gave total control for six weeks. Piper betel oil (2.5% EC), garlic oil (15% EC), capsicum oil (20% EC) were not successful in killing dormant mycelium but do cause significant delay in development of *M. fijiensis* by 37, 9, and 43%, respectively. Potassium bicarbonate (14% EC) restricted the growth of *M. fijiensis* by 43%. Meanwhile, *T. harzianum* DGA01, *B. amyloliquefaciens* DG14, and Serenade ASO sustained total control of mycelium development of *C. musae* in 10 weeks while piper betel oil only limited the mycelium growth by 60%, garlic oil and capsicum oil by 20% each and potassium bicarbonate by 28%.

Key words: Banana, biocontrol agent, inorganic salt, plant oil, black sigatoka, Cordana leaf spot.

INTRODUCTION

Black sigatoka leaf spot disease (BSLSD) caused by *Mycosphaerella fijiensis* is considered the most damaging and costly disease of banana (Jacome and Schuh, 1992). The control of BSLSD accounts for 27% of the total production cost (Stover, 1980). By attacking the leaves, the fungus disrupts the process of photosynthesis that feeds the growing bunch. Infected plants have fewer leaves, which lead to fewer and smaller fruits, a delayed harvest and lower quality fruit (Ramsey et al., 1990). BSLSD reduce banana green life which is the time between harvest and natural fruit ripening initiation via the synthesis of endogenous ethylene (Chillet et al., 2009).

Cordana leaf spot (CLS) caused by *Cordana musae* is known to attack and to cause great losses to banana production (Tangonan, 1999). The pathogen causes oval lesions of 5-7 cm or more in length, brown with a bright yellow border. There is progressive drying of the leaves beginning with the oldest as in Sigatoka (Morton, 1987). Necrotic portions of leaves have concentric zonations, which are most noticeable on the upper leaf surface. Lesions may eventually encompass entire leaf margins and large portions of the leaf lamina (http://www.ipmcenters.org/cropprofiles/docs/prplantainbanana.html).

Commercial plantations in the Philippines are maintaining periodic agrochemical spray program for the control of banana leaf diseases, and have been criticized on the grounds of environmental and human health concern. With this, an ordinance was passed in Davao City, Mindanao Island banning the aerial spray for banana leaf diseases control. Banana growers claimed that such regulation make the plant vulnerable to leaf diseases resulting to loss in jobs and bankruptcy. The situation encouraged banana industry and scientists to look for alternative methods of leaf disease control. Searching for

sustainable alternatives to synthetic chemicals obviously is the best long-term goal for leaf disease control, especially for smallholding farmers who cannot afford the costly synthetic chemical spray.

The current trends in search for alternatives to agricultural pesticides include the increased use of screening processes to identify materials with potential for control of disease-causing pathogens. As a preliminary stage, to provide additional tools for disease management alternatives with different mechanisms of action than chemical pesticides, the present study reported the *in vitro* efficacy of biological control agents, plant extracts, and a generally regarded as safe (GRAS) compound against *M. fijiensis* and *C. musae*. Serenade ASO is commercially available biological control agent based on *Bacillus subtilis* QST 713 and claimed to control ranges of fungal and bacterial plant pathogens (http://www.basf.com/group/corporate/en/brand/SERENA DE). We included *Trichoderma harzianum* DGA01 and *Bacillus amyloliquefaciens* DGA14, isolated from banana fructosphere and effective against crown rot-causing pathogens (Alvindia and Natsuaki, 2008, 2009). Plant extracts and/or distilled oil from piper betel, capsicum and garlic are reported having antimicrobial and antioxidant activities against ranges of microbial pathogens (Erturk, 2006; Irkin and Korukluoglu, 2009; Portz et al., 2008; Suppakul et al., 2006). Meanwhile, potassium bicarbonate is a registered pesticide for the control of sigatoka disease in Florida, USA (http://edis.ifas.ufl.edu/pdffiles/HS/HS17700.pdf).

MATERIALS AND METHODS

Recognition of disease symptoms

The first symptoms of BSLSD disease are tiny, chlorotic spots that appear on the bottom (abaxial) surface of the 3rd or 4th open leaf. The spots grow into thin brown streaks that are limited by leaf veins. The color of the streaks becomes darker, sometimes with a purple tinge, and visible on the top (adaxial) surface. The lesions then enlarge, becoming fusiform or elliptical, and when the disease severity is high, large areas of the leaf may become blackened and water-soaked. On the necrotic tissue, numerous, tiny, black, globose fruiting bodies (pseudothecia) containing sac-like structures (asci) filled with ascospores will emerge from the underside of the leaf. Black spots contain asci, or sacs, each containing eight spores can be examined under the microscope (Marin et al., 2003).

CLS caused by *C. musae* are pale brown, oval patches, ranging from one to several mm; lesions are surrounded by bright yellow halo. Necrotic portions of leaves have concentric zonations, which are most noticeable on the upper leaf surface. Lesions may eventually encompass entire leaf margins and large portions of the leaf lamina. There is progressive drying of the leaves beginning with the oldest as in Sigatoka (Morton, 1987).

Collection of specimen

Dwarf Cavendish banana (*Musa* AAA) leaves showing disease symptom of BSLSD or CLS were collected from a banana orchard in Quezon, Nueva Ecija, and 130 km north of Manila, Philippines. Bananas are grown in this area without agro-chemical application during production. Proper cultural practices are maintained in the farm such as de-leafing, mat sanitation, weeding, and sanitation. Collected specimens were placed in zip locked plastic bags and transported to the laboratory of the Food Protection Division, Philippine Center for Postharvest Development and Mechanization (PhilMech), Science City of Muñoz, Nueva Ecija, 150 km N of Manila.

Isolation of disease-causing pathogen

Banana leaves infected with BSLSD or CLS were washed in running tap water for 1 min, air dried before wiping with sterile tissue paper with 95% ethanol. Sections were cut at 2-3 mm from the margins of young actively growing lesions. The underside of a cut leaf piece was fixed with a double-sided adhesive tape, attached to the underside of 90 mm Petri dishes, and inverted over 2% water agar. Plate was incubated at 25°C for 12-24 h. Under a compound microscope, a germinating ascospore (Figures 1 and 2) was isolated using sterile loop-needle in a test tube slanted with potato dextrose agar (PDA) + 5% malt-extract (ME). The pure culture was stored at 25°C.

Pathogenicity test for the isolated fungi

One-month-old pure culture of *M. fijiensis* or *C. musae* grown in PDA plate was flooded with sterile distilled water. The colony was lightly scraped with sterile spatula to separate the fungus from the agar. The resulting suspension was transferred to a beaker and gently stirred using a spatula for the fungal vegetative parts to break into smaller units enough to be dispensed by a hand sprayer. The suspension was further diluted with sterile water and sprayed on the newly banana opened leaves. Banana leaves were covered with transparent plastic bags to provide favorable condition for disease development. Three leaves were used for each test fungus. Leaf was inspected after two months for symptoms resembling those of BSLSD or CLS. The experiment was repeated twice.

The test materials

Chemrez Technologies, Inc., a company in the Philippines engaged in developing environment-friendly innovations in plant protection, provided the materials for testing such as Serenade ASO 1.34%, piper betel oil (PBO) 2.5% EC, capsicum oil (CO) 20% EC, garlic oil (GO) 15% EC, and potassium bicarbonate (PB) 14% EC. The concentrations for the test materials were pre-determined by the company. On the other hand, *B. amyloliquefaciens* DGA14 and *T. harzianum* DGA01 earlier isolated from banana fructoplane were effective biocontrol agents for controlling banana crown rot-causing pathogens (Alvindia and Natsuaki, 2008, 2009).

Preparation of cell suspension

A loopful of *B. amyloliquefaciens* DGA14 was streaked on NA plates at four right angle directions, flaming the loop after each directional streak. After 24 h incubation at 25°C, a single colony was picked up with a sterile wire loop and grown in 250 mL Erlenmeyer flasks containing 100 mL nutrient broth (NB) + 5% ME. After 48 h shake incubation (rotary shaker, 70 rpm) at 28°C, cells were harvested by centrifugation for 25 min at 13,000 rpm. The resulting pellet was dissolved in 10 mL of sterile ¼ concentration Ringer's solution (Daigo, Tokyo, Japan). Cell concentration was determined with a bacteria counter (SLGC, Saitama, Japan), and a

Figure 1. Germinating ascospores of *Mycosphaerella fijiensis* in 2% water agar as seen under compound microscope.

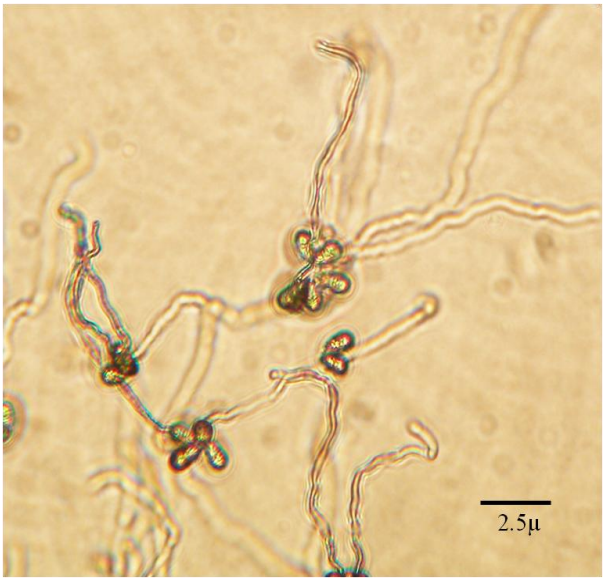

Figure 2. Germinating conidia of *Cordana musae* in 2% water agar as seen under compound microscope.

dilution was made to give cell concentrations of 10^8 cfu/ml.

Also from a stock culture, a mycelial disks of *T. harzianum* DGA01 was inoculated in a 250 ml Erlenmeyer flask containing 100 ml NB +5% ME. The flask was incubated on a rotary shaker (70 rpm) at 25°C for 15 days. The mycelial growth was separated using sterile gauze, and the filtrate was examined under a compound microscope using a hemacytometer (Bright-Line, Hausser Scientific, Horsham, PA, USA) for spore concentration. Spore suspension was adjusted to 1 x 10^6 cfu/ml in a Ringer's solution. Fungicide (Mancozeb, 2 g/l) and untreated control (distiiled water) were provided as control checks.

Screening for efficacy of the test materials

Mycelial discs (4 mm) were taken using sterile cork borer from a two-month-old colony of *M. fijiensis* or *C. musae* in PDA + 5% ME. Mycelial discs were dipped in a test material for 15 min and plated at 3-equidistant points into fresh PDA + 5% ME. The untreated control plate contained mycelial discs of a pathogen without dipping in a test material. This was repeated with other test materials. Plates were incubated for 10 weeks at 25°C. Mycelium growth was recorded weekly for 10 weeks by measuring colony diameter using a caliper, and the efficacy of the treatment (ET) percentage was obtained using the formula: (*Cdc*-*Cdt*)/*Cdc* x 100 = ET; wherein *Cdc* is the colony diameter (mm) of the control, *Cdt* is the colony diameter of the treatment. The experiment was repeated twice with three replications per treatment.

Statistical analyses

All of the comparisons of means were subjected to analysis of variance and the significant differences among treatments were determined with a least significant difference separation test using Microsoft Corp. STATISTICA® Ver. 6.

RESULTS

B. amyloliquefaciens DGA14 and *T. harzianum* DGA01 controlled colony growth of *M. fijiensis* throughout the period of observation. Serenade ASO was effective inhibiting colony growth of *M. fijiensis* for six weeks. Thereafter, the mycelium growth reached 10 mm in diameter and remained in constant size until the end of observation (Figure 3). On the other hand, a progressive colony growth of *M. fijiensis* was noted 2 weeks after treatment with CO, GO, PBO, and PB. The colony diameter of *M. fijiensis* after 10 weeks of treatment with CO, GO, PBO, and PB was 30, 32, 22, and 20 mm, respectively. The colony diameter of treated- and untreated control reached 35 mm in 10 weeks.

B. amyloliquefaciens DGA14, *T. harzianum* DGA01, and Serenade ASO controlled the mycelium development of *C. musae* throughout the period of observation (Figure 4). On the contrary, mycelium development of *C. musae* was noted two weeks after treatment with plant oils and salt. The colony size gradually increased reaching a final diameter of 20 mm for those treated with CO and GO, 10 mm with PBO, and 18 mm for PB. The colony diameter of untreated control was 25 mm while no growth was recorded on fungicide-treated mycelium disc.

B. amyloliquefaciens DGA14 and *T. harzianum* DGA01 were the best treatments with 100% effectiveness in controlling the mycelium growth of *M. fijiensis* after 10 weeks, followed by Serenade with 71%, PB by 43%, PBO by 37%, CO by 14%, GO by 9%. Mancozeb had no effect in reducing the colony growth of *M. fijiensis* (Table 1.) Meanwhile, the treatments with 100% control over the mycelium development of *C. musae* were *B.*

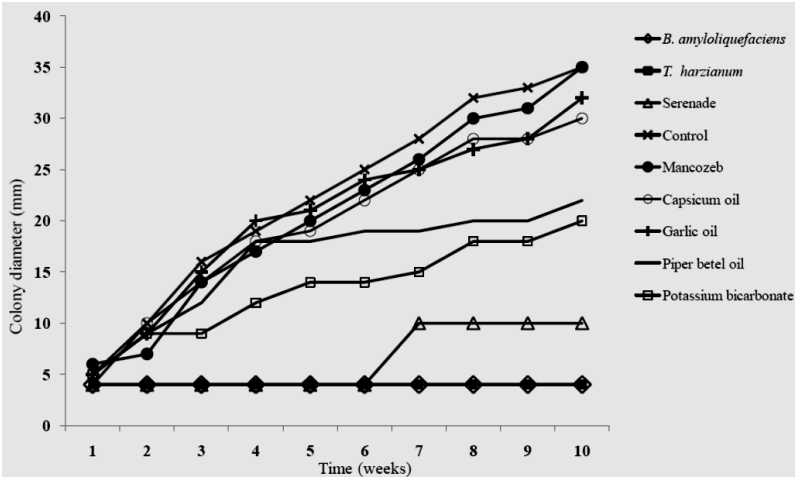

Figure 3. Progress in colony development of *Mycosphaerella fijiensis* for10 weeks with various treatments.

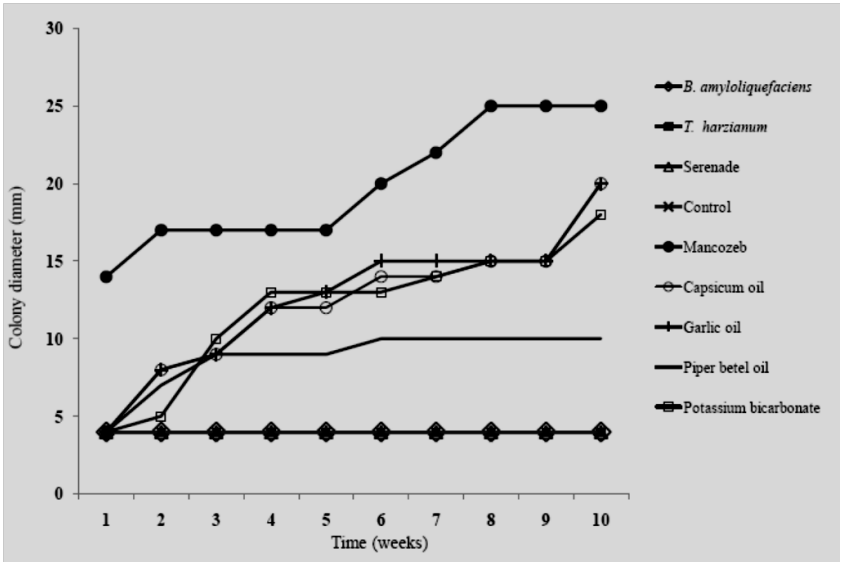

Figure 4. Progress in colony development of *Cordana musae* for10 weeks with various treatments.

amyloliquefaciens DGA14, *T*. *harzianum* DGA01, Serenade ASO, and Mancozeb followed by PBO with 60%, PB by 28%, and CO and GO by 20%. The isolates of *M*. *fijiensis* and *C*. *musae* were both pathogenic to banana plant.

DISCUSSION

Little attention and few researches on developing alternatives to chemical control for important leaf diseases of banana such as BSLSD and CLS are done at present due to the presence of effective chemicals. The cost of research and development may be added to the reasons for limited interest and financial support to find alternative methods. Nevertheless, an ordinance prohibiting chemicals aerial spray in banana plantations in Davao City, Mindanao Island, Philippines compelled us in exploring alternatives for leaf disease control. This study as an initial step, investigated non-chemical alternatives in controlling the growth of CLS- and BSLSD-causing pathogens. We gathered data needed for further research in developing sustainable and environment-friendly innovations in plant protection for controlling BSLSD and CLS.

The results show effectiveness of *T. harzianum* DGA01

Table 1. Colony diameter and reduction of colony diameter (%) in pathogens 10 weeks after treatment.

Treatment	*Mycosphaerella fijiensis*		*Cordana musae*	
	Colony diameter (mm)	Efficacy of treatment (%)	Colony diameter (mm)	Efficacy of treatment (%)
B. amyloliquefaciens	4 a	100	4 a	100
T. harzianum	4 a	100	4 a	100
Serenade	10 b	71	4 a	100
Capsicum oil	30 d	14	20 c	20
Garlic oil	32 de	9	20 c	20
Piper betel oil	22 c	37	10 b	60
Potassium bicarbonate	20 c	43	18 c	28
Mancozeb	35 e	0	4 a	100
Untreated control	35 e		25 d	

*Values with the same letter in a column are not significantly different according to LSD test (P ≤ 0.01). Colonies measuring 4 mm had zero growth as the size of the mycelial plug initially inoculated onto agar plate was 4 mm. Efficacy of treatment (ET) was obtained using the formula: (*Cdc-Cdt*)/Cdc x 100 = ET; wherein *Cdc* is the colony diameter (mm) of the control, *Cdt* is the colony diameter of the treatment.

and *B. amyloliquefaciens* DGA14 in controlling *C*. *musae* and *M*. *fijiensis in vitro*. The striking feature of *T*. *harzianum* DGA01 was the rapid growth, which overgrew the 90-mm agar plate within 48 hrs (Alvindia and Hirooka, 2011) of dual incubation with a pathogen. Also, *T*. *harzianum* DGA01 is a necrotrophic fungus that inflicts control over the growth of the target fungus by direct parasitism and production of volatiles (Alvindia and Natsuaki, 2008). For *B*. *amyloliquefaciens* DGA14, antibiosis perhaps is the mechanism involved in controlling the development of *C*. *musae* and *M*. *fijiensis*. The pinkish cottony mycelium plug of *M*. *fijiensis* or cottony white mycelium plug of *C*. *musae* turned black after 7 days of dual incubation with *B*. *amyloliquefaciens* DGA14. The antimicrobial substances produced by *B*. *amyloliquefaciens* DGA14 (Alvindia and Natsuaki, 2009) could had been reacted to mycelial discs of *C*. *musae* or *M*. *fijiensis*. It was reported that *B. amyloliquefaciens* produce surfactin, iturin A, fengycin A and fengycin B. These are cyclic lipopeptides composed of seven (surfactin and iturin A) or 10 α-amino acids (fengycins) linked to a β-amino (iturins) or β-hydroxy (surfactins and fengycins) fatty acid which may vary from C-13 to C-16 for surfactins, from C-14 to C-17 for iturins and from C-14 to C-18 for fengycins (Arguelles-Arias, 2009; Chen et al., 2007; Hiradate et al., 2002). *B*. *amyloliquefaciens* strains producing iturin A have been used as biocontrol agents to suppress fungal plant pathogens (Yoshida et al., 2001; Yu et al., 2002). Meanwhile, Serenade ASO, a known broad spectrum, preventative biofungicide for the control of many important plant diseases, did not sustain the inhibitory effect on *M*. *fijiensis* but do cause a significant delay in mycelium growth by 71%. Remarkably, Serenade ASO totally inhibited the growth of *C*. *musae*. Comparably, our biocontrol agents *T*. *harzianum* DGA01 and *B*. *amyloliquefaciens* DGA14, were effective in controlling *M. fijiensis* than previously reported such as *Serratia marcescens*, *S. entomophyla* and *Bacillus* spp. with 40% level of control under laboratory conditions (Riveros et al., 2002).

The efficacy of plant oils and salt is affected by type of pathogens to be treated. For instance, plant oils controlled the growth of *C*. *musae* by 20-60% as compared with 9-37% in *M*. *fijiensis*. Similarly, PB recorded a 43% reduction in mycelium growth of *M. fijiensis* while 28% in *C*. *musae.* Meanwhile, Mancozeb had no effect on the growth of *M. fijiensis* indicating resistance of the pathogen to fungicide treatments. In general, the non-chemical alternatives we tested have better inhibitory influence in *C*. *musae* than in *M. fijiensis*. The limitation of our study was inadequate bio-screening to determine the optimum effective doses of plant oils and salt against the target pathogens. Such information is very useful during practical application in the field. Increasing the concentration of plant extract, as in pure *Jatropha curcas* fruit and seed extracts, exhibited higher level of control against CLS of banana *(*Dela and Tangonan, 2008).

Our present study demonstrated the efficacy of various non-chemical alternatives in controlling leaf spot-causal pathogens of banana *in vitro*. Though this study has encouraging result however, the next step should be testing the efficacy of materials in the field. Interaction of the biocontrol agents with causal-pathogens in the phyllosphere needs to be elucidated to understand their activities under natural conditions. In a same manner, the ability of biocontrol agents to efficiently colonize surfaces of banana leaves as prerequisite for effective biocontrol, should be done. Likewise, the phytotoxic effect of plant oils and salt on banana plant must be determined.

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