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Comparative effectiveness of some novel fungicides and different biocontrol agents against two *Colletotrichum musae* isolates under laboratory condition

Abstract. *Colletotrichum musae*, is aggressive and devastating threat and causing huge losses in banana production globally. The use of various fungicides and as well as biocontrol agents can help to manage the crop. However, five different fungicides, namely., Antracol, Defeater plus, Ridomil gold, Kocide, and Topsin M at five different concentrations viz., 10 ppm, 100 ppm, 200 ppm, 500 ppm, and 1,000 ppm were used to check the growth inhibition of the two isolates (CM02 and CM11) of *C. musae* by food poison technique. The topsin M fungicide at all concentrations (10-1,000 ppm) was highly effective, which yielded 72-98% inhibition, followed by Ridomil gold 1,000 ppm caused 82.11% and 79% inhibition of both isolates. Ridomil gold at 500 ppm causes 75.13% and 69.44% mycelial growth inhibition of CM02 and CM11. In comparison, Kocide at 1,000 ppm caused more than 60% inhibition of both strains. However, we observed that, as the concentration decreased the mycelial growth of the pathogen increased. Furthermore, during the present investigation, three biocontrol agents, viz., *Trichoderma harzianum*, *Trichoderma polysporum* and *Paecilomyces variotii* were used for their antifungal activity against (CM02 and CM11) by dual assay test. *T. harzianum* proved highly effective biocontrol agents and cause (18.88% and 20.11%) mycelial inhibition of both strains, followed by *T. polysporum* (13.33% and 15%) and *P. variotii* (9.11% and 8.75%) on 3rd, 4th and 7th day of inoculation by dual assay test. In conclusion, among all tested fungicides, Topsin M was found highly effective against both strains of *Colletotrichum musae*. Therefore, biological control especially with *Trichoderma* species are promising method to control this pathogen quickly. Both isolates of *C. musae* showed high sensitivity against Trichoderma species on the third and fourth day of inoculation.
Key words: Banana, anthracnose, *Colletotrichum musae*, fungicides, biocontrol agents.

Introduction

Banana (*Musa spp*), members of the Musaceae family, are a major commercial fruit crop cultivated in many parts of the world [1]. Many species of big herbaceous blooming plants in the genus *Musa* yield an edible fruit known as a banana. The fruit may vary in size, colour, shape and firmness, but is frequently elongated and bent [2]. It has a starchy, juicy interior covered by thin skin that turns various colors when mature. It is the fourth-largest food crop consumed in the globe after wheat, rice and maize and also considers the fourth most valuable food after rice, meat, and milk [3, 4]. In Pakistan, banana fruit about 34.8 thousand hectares with annual production is 154.8 thousand tons, whereas Sindh province contributes 87% of the total banana production of the country [5]. Due to the absence of agrochemical and biocontrol applications and the extended time between harvesting and reaching the market, organic bananas are more prone to postharvest diseases, which can degrade their quality when compared to conventional banana crops [6]. Postharvest disease caused by fungal pathogens is most important and aggressive factor, which cause huge economically losses in the globe [7, 8]. Among them, anthracnose rot caused by *Colletotrichum (C.) musae* is a huge problem in bananas production, which affect the

fruit quality and commercial viability worldwide [9]. The fungus cause brown to black lesions appearing symptoms on banana, which are usually diamond-shaped. Symptoms of the disease appear when the banana is still green. Orange or salmon-colored rings consisting of fungal spores may pronounce in the latter stage around the lesions, and the lesion appears more sunken than orange-colored masses of spores [10]. Many postharvest disease including, anthracnose rot are managed through agrochemicals, which available in the market [11, 12]. Therefore, the continued application of these fungicides has led to the emergence of *C. musae* strains that have developed resistance to them [13]. Nowadays, consumers demand chemical-free fresh fruits and look for substitute approaches for managing diseases [9, 14].

Biological control has emerged as one of the most promising strategies for preventing and reducing postharvest losses. Especially when disease resistance or chemical control options are unavailable [15]. For the last few years, many researchers are showing interest in controlling plant disease through biological control [16]. Plant pathogen populations can be hidden from view by biological control, a method used to combat plant diseases [17]. Many *Trichoderma* species are valuable against plant diseases [18, 19]. Therefore, the present study was aimed to evaluate the five fungicides at five different doses and three biocontrol agents against two isolates of *C. musae* causing anthracnose rot disease in bananas.

Materials and methods

Collection, isolation, and identification of pathogens. To isolate the fungal pathogen, anthracnose-affected banana fruits were collected from different banana orchards in the district Matiari of Sindh province, Pakistan. Collected samples were kept in paper bags with location tags and carried out into the laboratory to isolate fungi associated with banana fruit. The sample was rinsed with tap water to cleanse the fruits from any adhering soil particles. The infected parts of the banana fruit skin having the disease were cut into small pieces (1 cm) with the help of a sterilized scalpers knife. They were surface sterilized for 1-2 minutes with 5% NaClO solution, rinsed three times with distilled sterilized water (DSW), and then put on PDA Petri plates amended with streptomycin sulfate and penicillin at 1 ml/L. Five pieces of banana fruit skin were placed in each Petri plate and incubated at 28°C for 3-4 days. The strains of *C. musae* were identified based on mycelial characters, color, size, and shape described by [20] and [21].

In vitro screening of fungicides against two isolates of C. musae. Five different fungicides viz., Antracol, Defeater plus, Ridomil gold, Kocide and Topsin M were used at five different doses viz., 10 ppm, 100 ppm, 200 ppm, 500 ppm and 1,000 ppm against two isolates (CM02 and CM11) of *C. musae* by food poisoned technique [22]. The details of fungicides, including brand names, active ingredients, chemical groups and distribution in Pakistan, are shown in (Table 1).

Table 1 – Details of different fungicides which are used in this experiment

Trade name	Active ingredients	Chemical group	Manufacturer/ Distributor in Pakistan
Antracol	Propineb	Dithiocarbamates	Bayer Crop Science
Defeater plus	Flumorph+fosetyl aluminium	Ethyl phosphate	Kanzo Ag Pharma
Ridomil gold	Mancozeb+mefenoxam	Dithiocarbamates& Acylalanines	Syngenta Pakistan Limited
Kocide	Copper hydroxyde	Inorganic	FMC Corporation
Topsin M	Thiophanate methyl	Thiophanates	Arysta Life Science Pakistan

Before pouring, the required concentrations were mixed in a PDA medium. Fungicide-free mediums were used as control. After PDA solidifying, a 5 mm disk of seven days old culture was placed to the centre of the Petri plate. Each treatment was depend on four replications and mean values were calculated. After inoculations, the petri plates were incubated at 27ºC for seven days. To evaluate the mycelial growth of the fungi, two perpendicular lines were drawn on the backside of the Petri plates and crossed in the centre of the plate. The colony growth (mm) was measured using a scale every 24 hours until the control plate

was filled in any treatment. The mycelial growth inhibition percentage was recorded through formula as given by [23]

$$PI = \frac{(R-S)}{R} \times 100,$$

where: PI – percent inhibition of fungal mycelial growth;

R – fungal mycelial growth in control plates;

S – fungal mycelial growth in treated plates.

Collection of fungal biocontrol agents. Three fungal biocontrol agents viz., two species of *Trichoderma* (*T. harzianum* and *T. polysporum*) and one *Paecilomyces variotii* were retrieved from the Department of Plant Protection, Sindh Agriculture University, Tando Jam, Pakistan to check the sensitivity of *Colletotrichum* isolates (CM02 and CM11) by dual assay method.

In vitro screening of biocontrol agents against *C. musae*. Three biocontrol agents, viz., *Trichoderma harzianum, Trichoderma polysporum*, and *Paecilomyces variotii*, were used for their antagonistic activity against *C. musae* isolates through a dual assay test [24].

A 5 mm agar disc was cut from 5-day-old pure cultures of fungal biocontrol agents were placed on one side on PDA-containing plates. On the opposite side, at the end of the same plate, a 5 mm agar disc of 7-day-old pure culture of each isolates of *C. musae* was also placed. Each biocontrol agent was tested separately in the same manner. The plates were incubated at (25 + 2^0C) for 3-4 days. Each treatment was divided into four replications with control and mean values recorded. One straight line was drawn at the center on the backside of the Petri plates. The colony growth (mm) was measured using a scale every 24 hours until the control plate was filled in any treatment. The mycelial growth inhibition percentage was recorded through a formula as given by [25]:

$$PI = \frac{(P1-P2)}{P1} \times 100,$$

where: percent inhibition of fungal mycelial growth;

P1 – Covered area by the fungal mycelial growth in control plate;

P2 – Covered area by the fungal mycelial growth in dual culture plate.

DNA extraction. For the extraction of DNA, a CTAB method developed by Doyle and Doyle (1987) was utilized with some adjustments from banana anthracnose causal agent *C. musae* [26]. To determine the DNA concentration and purity, the Li et al., (2006) method was used for performing a Nano-drop [27]. Furthermore, a 1% agarose gel was utilized to analyse the DNA concentration and purity by running the samples for 30 minutes.

PCR based detection. The Polymerase Chain Reaction (PCR) stands as a potent molecular technique, enabling the targeted amplification of distinct DNA sequences. Two primers, ITS1 and ITS4 were evaluate to amplify a specific sequence region [28]. The PCR reactions were conducted with a fixed amount of reagents, including 1.5 µl of each primer, 7 µl of master mix, and 0.5 µl of Platinum Taq-polymerase, in a total volume of 12.5 µl of reaction. An automated thermal cycler was employed to conduct the PCR amplification with a protocol consisting of an initial denaturation at 96°C for 9 min, followed by 40 cycles of denaturation at 96°C for 30 sec and annealing at 53°C for 1 min. The final extension was carried out at 72°C for 7 min. The amplified products were detected on a 1.5% agarose gel containing ethidium bromide [27]. This method finds extensive employment in molecular biology investigations, fundamentally transforming our capacity to scrutinize and manage DNA sequences with exceptional precision and sensitivity.

Characterization of the strains. The manufacturer's recommendations (Bio Product) were followed in sequencing the PCR-amplified products that were positive. A BioEdit v7.2 version software was used for the analysed to attained 16S rDNA sequences and (NCBI) blast tool was utilized for compared to those retrieved [29]. After that, the sequence was uploaded to MEGA-7 software and align with the help of ClustalW program. A phylogenetic tree was construct with the help of neighbor joining method with 1,000 bootstrap value and Tamura 3-parameter model. By employing this approach, we were able to discern connections among diverse sequences, yielding a profoundly enlightening and influential analysis that unveils novel insights into the intricate interplay among these vital genetic components.

Data analysis. The experiments were carried out Completely randomized design (CRD). The least significant difference test (LSD) was used to compare mean values at $p = 0.05$. The data was analyzed by Statistix 8.1 version computer software. The Graphpad prism 8 software was used for graphs.

Results and diacussion

Colonial appearance and morphological characters. The pathogen grown in isolation displayed a copious amount of white aerial mycelium lacking a distinct pattern. The colonies typically appear as fluffy and cottony. The mycelium was septate. The conidia are one-celled and slightly curved, with a tapering base and a rounded or slightly pointed tip and size 12-14 µm in length. Conidiophores structures are simple or branched.

Molecular characterization. In phylogenetic analysis, we included 09 closest sequences of *C. musae* revealed in the BLAST search along with representative sequences of other members of clade, namely *C. gloeosporiodes, C. siamense and C. fragariae*. In the ITS sequence analysis, our two sequences (OQ8917591 and OQ8917601) of *C. musae* was found to be 99.8% identical to the rest of the GenBank sequences of *C. musae* we used, except for Ok0415151 and MT3511141, which showed 99.2% and 99.5% sequence homology to our isolate, respectively. Moreover, our isolate showed only 98.7% and 98.8% sequence similarity with *C. gloeosporiodes* HM0158521 and *C. siamense* MZ0404911 and MT5974041, respectively. The other members of clade, such as *C. fragariae* MT5974041, was distantly related to our isolate, showing only 93.6 sequence homology (Figure 1).

In vitro sensitivity of CM02 and CM11 against different fungicides. Five fungicides at different doses were used to check growth inhibition of the two isolates of *C musae.* At all concentrations (10-1,000 ppm), Topsin M appeared highly effective, which yielded 85-98% inhibition, followed by Redomil gold 1,000 ppm caused 82.11% inhibition of CM02. Redomil gold at 200 and 500 ppm causes 75.13% and 69.44% inhibition of mycelial growth. In comparison, Kocide at 1,000 ppm caused 63.22% inhibition. While Antracol 10-1,000 ppm, Defeater plus 200-1,000 ppm, and Kocide 100-500 ppm showed more than 50% inhibition, followed by Redomil gold 100 ppm caused 43.11%, and Defeater plus 10-100 ppm reduced the 37.33%-40% mycelial growth of CM02, respectively (Figure 2).

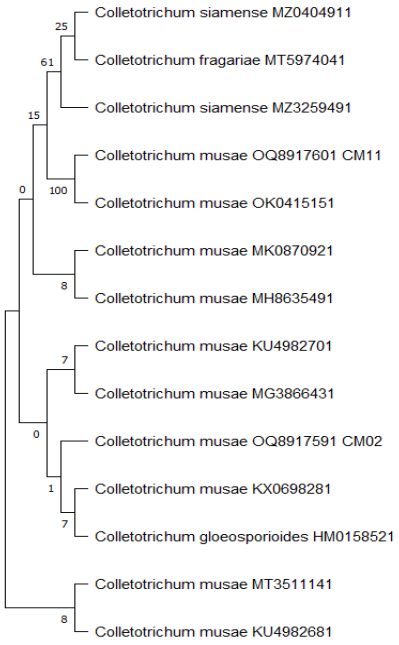

Figure 1 – Phylogenetic relationship of two sequence of *Colletotrichum musae* with NCBI BLAST search sequences, constructed with neighbor joining method based of partial sequence of ITS region

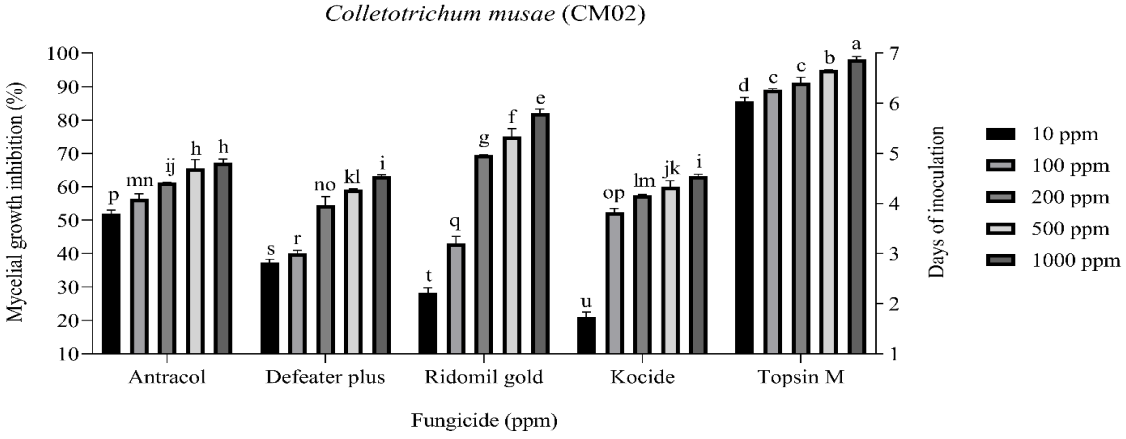

Figure 2 – Response of *Colletotrichum musae* strain (CM02) against five different fungicides in seven days of inoculation by food poison technique. The error bars and alphabetic letters show significant values of LSD (p>0.005) among each other

Against CM11, also Topsin M was found most effective which leading 72-95% mycelial growth inhibition at al dosses followed by Ridomil gold cause 79% and Antracol cause 73.22% mycelial growth inhibition at 1,000 ppm of tested patogen. In comparision, Kocide at 500-1,000 ppm, Ridomil gold 200-500 ppm, Antracol 200-500 ppm and Defaeter plus 500-1,000 ppm were showed moderately highly effective, leading more than 60% mycelial growth inhibition of CM11.

Therefore, Antracol 10-100 ppm, Defeater plus 200 ppm and Kocide 100-200 ppm showed moderately effective and cause 50-59% growth inhibition of CM11. However, Ridomil gold 10-100 ppm, Defeater plus 10-1,000 ppm and Kocide at 10 ppm were show least effective and cause 20-45% colony growth inhibition of tested fungus, respectively (Figure 3).

Antifungal activity of various fungal biocontrol agents. Three different fungal bio-control agents, including two species of *Trichoderma* (*T. harzianum* and *T. polysporum*) and one *Paecilomyces variotii* were used against two strains of *C. musae* by dual plate assay. Both *Trichoderma* species were found highly effective against (CM02 and CM11) and reached on the fungal mycelial growth in 3rd and 4th days. The *P. variotii* showed moderately effective and reached on the mycelial growth of the pathogen in 7th days. On 3rd day *T. harzianum* showed 18.88% inhibition of CM02 followed by 20.11% CM11 mycelial growth inhibition percentage of CM11. At 4th day of inoculation, *T. polysporum* cause 13.33% growth inhibition of CM02 followed by 15% CM11. The *P. variotii* growing slow and cause 9.11% of CM02 followed by 8.75% growth inhibition of CM11 on 7th day of inoculation (Table 2).

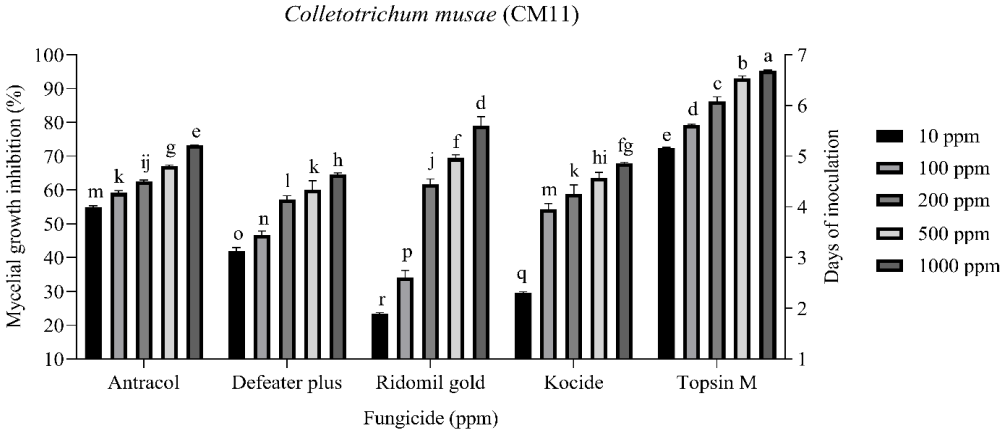

Figure 3 – Response of *Colletotrichum musae* strain (CM11) against five different fungicides in seven days of inoculation by food poison technique. The error bars and alphabetic letters show significant values of LSD (p>0.005) among each other.

Table 2 – Effect of three different fungal biocontrol agents against two isolates of *Colletotrichum musae* (CM02 and CM11) on mycelial growth by dual assay method. The + sign show the standard deviation and alphabetic letters show significant LSD (p>0.005) values among each other

Biocontrol agents	Inhibition pencentage (%) of *Colletotrichum musae* isolates					
	3rd day		4th day		7th day	
	CMO2	CM11	CMO2	CM11	CMO2	CM11
Trichoderma harzianum	18.88+1.462 a	20.11+2.588 a	-	-	-	-
	-	-	13.33+1.005 b	15+1.178 b	-	-
	-	-	-	-	9.11+2.007 c	8.75+1.558 c

C. musae is a devastating threat and causes considerable losses in banana crops worldwide. Using fungicides and biocontrol agents against *C. musae* is simple and can help manage the crop. Various agro-pesticides and fungicides are available in markets that control anthracnose rot disease of bananas but continuous application of these fungicides were hazards for human health and the environment [11-13].

However, during the present study, five different fungicides viz., Antracol (Propineb), Defeater plus (Flumorph + Fosetyl Aluminium), Ridomil gold (Mefenoxam + Mancozeb), Kocide (Copper Hydroxide), and Topsin M (Thiophanate-methyl) with five different concentrations viz., 10 ppm, 100 ppm, 200 ppm, 500 ppm, and 1,000 ppm were used against *C. musae*. All fungicides showed highly and moderately effective against both strains and cause significant mycelial growth inhibition. In addition, both isolates show highly sensitivity against Topsin M (Thiophanate-methyl) at all concentrations (10-1,000 ppm), leading 72-98% colony growth inhibition. In comparision, Ridomil gold show moderately effective and cause 61-82% growth inhibtion of both strains.

Similarly, Vieira et al. [30] reported that thiophanate methyl (Topsin M) is more effective against *C. musae*. In recent study, Mancozab and cupper oxychloride was found effective with 70% growth inhibition at high dosses against *Fusarium solani* causal pathogen of root rot disease in faba bean crop [31]. *Lasiodiplodia theobromae* causal agent of banana fruit rot disease was found most sensitive against four fungicides including Mancozab at 2,500 ppm [32]. The lowest doses (10-100 ppm) of two fungicides were found least effective which lead 21-43% growth inhibition followed by Kocide at 10 ppm cause 21.11% and 29.56% mycelial growth inhibition of CM02 and CM11. We observed that both strains rapidly grow on the lowest doses of various fungicides. Therefore, biological control is one of the most promising alternative methods of fungicides to control postharvest disease and reduce the economical losses, especially when pathogen show resistance against chemical fungicides [15, 33, 34].

Furthermore, three different biocontrol agents, namely, *Trichoderma harzianum*, *Trichoderma polysporum*, and *Paecilomyces variotii* were used for their antifungal activity against *C. musae* under laboratory conditions. Among biocontrol agents, *T. harzianum* was found highly effective and cause 18.88% and 20.11% mycelial growth inhibition percentage of CM02 and CM11 on 3rd day of inoculation followed by *T. polysporum* on 4th day cause 13.33% and 15% growth inhibition of CM02 and CM11. The *P. variotii* was found least effective. According to Tongsri et al. [35] *Trichoderma* spp. has been effective control against many phytopathogens including, *Phytopatora capsici* and *Colletotrichum gloeosporioides* [36], *Colletotrichum dematium* [37], *Lasiodiplodia theobromae* [38], *C. musae* [19, 39], *Fusarium oxysporum* [40], *Cladosporium spherospermum*, *Aspergillus niger* and *Fusarium oxysporum* [41] and *Fusarium solani* [42]. Out of 6 strains of *Trichoderma*, three strains were found highly effective against three pathogens viz., *Sclerotium rolfsii*, *Rhizoctonia solani* and *Fusarium solani*, leading 60-100% mycelial growth inhibition by dual assay test [18]. Our results indicated that, as compared to fungicides, the pathogen was quickly control through biocontrol agents especially *Trichoderma* species.

Conclusion

Among all tested fungicides, Topsin M was found highly effective at all concentrations (10-1,000 ppm) against *C. musae* isolates (CM02 and CM11), which led to 72-98% mycelial growth inhibition. After that, Ridomil gold was found moderately effective against both strains. However, we observed that, as the concentration decreased the mycelial growth of the pathogen increased. Biological control especially with Trichoderma species are promising method to control this pathogen quickly. Both isolates of *C. musae* showed high sensitivity against Trichoderma species on the third and fourth day of inoculation.

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Conflict of interest

All authors are aware of the article's content and declare no conflict of interest.

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