

COMPATIBILITY OF NATIVE POTENTIAL BIOAGENTS WITH DIFFERENT FUNGICIDES AGAINST *COLLETOTRICHUM GLOEOSPORIOIDES* PENZ. CAUSING MANGO ANTHRACNOSE

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ABSTRACT

Managing the anthracnose through chemicals alone is not satisfactory in view of the development of resistance, environmental concerns and cost benefit ratio. Some reports are available regarding the resistance of *C. gloeosporioides* to fungicides in mango and as well as in other crops. Keeping this in view, ninety six native potential antagonists were isolated from phylloplane and leaf endophytes during the roving survey conducted in different regions of Andhra Pradesh. Of the 96 antagonists screened by dual culture technique, four fungal and six bacterial antagonists identified as fungicidal tolerant potential bioagents. Further these antagonists were evaluated for their compatibility with commonly used four systemic fungicides viz., carbendazim (50 ppm), thiophanate-methyl (50 ppm), propiconazole (25 ppm), hexaconazole (25 ppm) and two non-systemic fungicides viz., mancozeb (1000 ppm) and copper oxychloride (1000 ppm) to manage the disease through an integrated approach by combining bioagents and economic use of chemicals.

INTRODUCTION

Mango (*Mangifera indica* L.) is one the world's most important and esteemed fruits and described by some as the "king of all fruits". India is the world's largest producer and Andhra Pradesh ranks first in production (45.89 lakh tonnes). Because of diverse production conditions and the vast area grown, mango suffers from a number of diseases, some of them taking heavy toll on the crop and representing limiting factors for production and productivity. Anthracnose caused by *Colletotrichum gloeosporioides* Penz., is the most destructive disease of mango causing substantial yield losses which can reach 60% or higher during the heavy rainy season (Ann *et al.*, 1997). The indiscriminate use of different fungicides causes potential threat to human health, increase in pathogen resistance, mutation and cause environmental hazards. Due to extensive cultivation of mango, this pathogen has emerged as a major constraint in its successful cultivation. Although biocontrol is rarely used for foliar diseases, numerous organisms capable of antagonizing fruit and leaf pathogens have been reported in recent years. Formerly, less attention was paid to biocontrol of foliar microflora was known to consist of relatively a few organisms whose populations fluctuate dramatically according to environmental conditions. The rapid growth of foliage provides better opportunities for pathogen growth rather than organisms in the phyllosphere (Fry, 1982). However, it is presumed that the survival ability of endophytes may be better when compared to phylloplane microflora. Therefore, in the

present study an attempt has also been made to isolate fungicide tolerant antagonists against *C. gloeosporioides* from leaf endophytes. We opted for using a combination of biocontrol agents against foliar pathogen *C. gloeosporioides* from phylloplane and leaf endophytes with specific fungicides that will enhance the efficacy and duration of active disease control which is crucial for an effective disease management. Information is available on the compatibility of biocontrol agents with chemicals (Gupta and Sharma, 2004; Veena *et al.*, 2006). The efficiency of the biocontrol agent could further be improved when it was applied with the recommended fungicide, used at a lower concentration (Korsten *et al.*, 1992; Silimela and Korsten, 2001). Fungicide resistance problems, concerns regarding pesticide residues and revocation of registration of certain widely used fungicides have led to increased activity in the development of bioagents against plant pathogens. It is therefore, aimed to identify the compatible potential bioagents with commonly used systemic and non-systemic fungicides for the eco-friendly management of anthracnose fungus *Colletotrichum gloeosporioides* obtained from different regions of Andhra Pradesh.

MATERIALS AND METHODS

Isolation and screening of potential bioagents

Roving survey was conducted for the collection of healthy and diseased leaf samples to isolate the potential antagonists and pathogen respectively from major mango growing regions

of Rayalaseema, Telangana, Godavari and Krishna districts of Andhra Pradesh, India. Pathogen isolates were collected in order to identify the virulent isolate by conducting pathogenicity test on baneshan mango variety. The highly virulent isolate will be used for screening of potential bioagents.

Isolation from phylloplane

Isolation of native biocontrol agents was made from washings of healthy mango leaves (Zenichi *et al.*, 2003). Leaves were washed with sterile distilled water and serial dilution is prepared. One ml of this suspension was transferred to sterile petriplates containing solidified potato dextrose agar (PDA) and nutrient agar (NA) for the isolation of fungi and bacteria respectively. The plates were incubated at $28 \pm 2^\circ\text{C}$ for the development of colonies.

Isolation of leaf endophytes

Five grams of healthy leaves were surface sterilized for five minutes with 70 per cent ethanol and homogenized in 20 ml of sterilized phosphate buffer using a mortar and pestle. Appropriate dilutions of these suspensions were plated on PDA and one fourth of nutrient agar. The plates were incubated for 72 hrs at $28 \pm 2^\circ\text{C}$ (Kishore *et al.*, 2005a). In each sample, single colonies of predominant strains with distinct morphologies and well separated from other were subcultured and used for further studies.

Identification of native potential bioagents by dual culture technique

Dual culture technique was used to identify the potential antagonistic isolates of microflora obtained from phylloplane and leaf endophytes. The antagonistic activity of microflora isolates against *C. gloeosporioides* was determined by dual culture technique under *in vitro* condition (Bhuvanawari and Rao, 2001).

In case of fungi, mycelial discs of 6 mm diameter from seven day old cultures of both fungal antagonist and the test pathogen were placed at equidistant on sterile petriplates containing PDA medium. Whereas in bacteria, one day old cultures were streaked on opposite side of the pathogen on NA medium. The petriplates were then incubated at $28 \pm 2^\circ\text{C}$. Three replications were maintained in each treatment. Suitable controls were kept without antagonist. Growth of the pathogen was measured at 7th day after inoculation of antagonist. Inhibition percentage of mycelial growth of test pathogen was calculated by the formula:

$$I = \frac{C - T}{C} \times 100$$

Where,

I = Per cent reduction in growth of test pathogen

C = Radial growth (mm) in control

T = Radial growth (mm) in treatment

Compatibility of native potential antagonists with different fungicides by poisoned food technique

Fungicides that were found effective viz., four systemic fungicides viz., carbendazim (50 ppm), thiophanate-methyl (50 ppm), propiconazole (25 ppm), hexaconazole (25 ppm) and two non-systemic fungicides viz., mancozeb (1000 ppm) and copper oxychloride (1000 ppm) were evaluated for

fungicidal resistance / sensitivity (Sampath *et al.*, 2007) against *C. gloeosporioides* for their compatibility with potential antagonists by poisoned food technique for fungal isolates (Nene and Thapliyal, 1993). Hundred milliliters of double strength PDA was mixed with 100 mL of double concentrated fungicidal solution so as to get required final concentration of the fungicides. Twenty ml of poisoned media was plated in 9 cm sterile petriplates. A 5 mm mycelial disc of seven days old culture was inoculated at the centre of the petriplates and incubated at $28 \pm 2^\circ\text{C}$ for ten days. A control was maintained without fungicide. Three replications were maintained for each treatment. Per cent reduction in radial growth over control was calculated by using the following formula:

$$I = \frac{C - T}{C} \times 100$$

Where,

I = Per cent reduction in growth of test pathogen

C = Radial growth (mm) in control

T = Radial growth (mm) in treatment

Based on radial growth, the isolates were classified as highly sensitive, sensitive, moderately resistant, resistant and highly resistant.

Spectrophotometric method

This method was followed to study the compatibility of potential bacterial isolates with commonly used fungicides against *C. gloeosporioides*. Five hundred microliters of antagonistic bacterial cultures grown in Nutrient Broth (NB) for over night at $28 \pm 2^\circ\text{C}$ and 180 rpm were added to 50 mL of NB in 250 mL flasks containing different fungicides. Inoculated flasks were incubated at $28 \pm 2^\circ\text{C}$ and 180 rpm. Bacterial growth was determined by measuring optical density (OD) at 600 nm after 24 hours of incubation. Each treatment consisted of three flasks as individual replications. The nutrient broth without bacteria served as control.

Statistical analysis

Wherever necessary the data was statistically analyzed (Gomez and Gomez, 1984). Completely Randomized Design (CRD) was used for radial growth, per cent disease incidence, poisoned food technique and dual cultural technique. Two-way CRD was used for spectrophotometric method.

RESULTS

During the survey, thirty six isolates of *C. gloeosporioides* were collected and subjected to pathogenic variability on Baneshan mango grafts and classified as highly virulent, moderately virulent and less virulent. The results revealed that isolate Cg23 was found to be highly virulent and the same was used to test against the antagonistic activity of microflora isolated from phylloplane and leaf endophytes (Data not shown).

A total of 96 antagonistic microflora were isolated from phylloplane and leaf endophytic regions of healthy mango leaves against *Colletotrichum gloeosporioides*. The bacterial antagonists isolated from phylloplane were designated as PB₁ to PB₂₈ and endophytes as EB₁ to EB₃₉, whereas, fungal antagonists from phylloplane were named as T₁ to T₂₉. The

Table 1: Compatibility of potential fungal antagonists with different fungicides in poisoned food technique

Fungicides	Concentration	Mycelial growth (mm)				Percent compatibility			
		T3	T17	T23	T26	T3	T17	T23	T26
Carbendazim	50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Thiophanate-methyl	50	12.00	10.00	38.50	13.55	13.33	11.11	42.78	15.06
Propiconazole	25	15.50	11.00	0.00	0.00	17.22	12.22	0.00	0.00
Hexaconazole	25	12.50	32.50	13.30	11.10	13.89	36.11	14.78	12.33
Mancozeb	1000	86.00	85.00	88.50	88.50	95.37	94.44	98.33	98.33
Copper oxychloride	1000	56.00	31.50	69.50	24.55	62.22	35.00	77.22	27.28
Control	---	90.00	90.00	90.00	90.00	---	---	---	---

*Mean of three replications

	CD (5%)	SEM
Concentrations	1.5896	1.3574
Fungicides	0.5789	1.9546
Interaction	0.2398	0.5879

fungal antagonists T₃, T₁₇, T₂₃ and T₂₆ identified as *Trichoderma* spp. were found to be superior compared to other isolates in inhibiting the growth of *C. gloeosporioides*. In case of thirty nine endophytic bacterial antagonists EB₇, EB₉, EB₃₅ and EB₃₉ completely inhibited (100%) the growth of the pathogen and among the twenty eight phylloplane bacterial isolates tested, complete inhibition (100%) of the pathogen was recorded by PB₁₈ and PB₂₈ antagonists (Data not shown).

The potential fungal and bacterial antagonistic isolates viz., T₃, T₁₇, T₂₃, T₂₆, EB₇, EB₉, EB₃₅, EB₃₉, PB₁₈ and PB₂₈ were selected for fungicidal compatibility studies against highly virulent isolate Cg23. The compatibility of the potential fungal antagonistic isolates with different fungicides tested by poisoned food technique revealed the complete inhibition of T₃, T₁₇, T₂₃ and T₂₆ isolates was observed with carbendazim (50 ppm), T₂₃ and T₂₆ in the case of propiconazole (25 ppm). Mancozeb (1000 ppm) was found to be least effective about 94.44 to 98.33 per cent compatibility with all the fungal antagonists (Table 1).

Spectrophotometric method was used to test the compatibility of the above six potential bacterial isolates. These results revealed that PB₁₈ was more compatible with thiophanate-methyl (96.07%) at 50 ppm followed by mancozeb, carbendazim, copper oxychloride and propiconazole. The compatibility was less (16.09%) with hexaconazole at 25 ppm compared to other fungicides. Higher compatibility of PB₂₈ was recorded with propiconazole (88.05%) at 25 ppm

followed by copper oxychloride, carbendazim, thiophanate-methyl and mancozeb and the least compatible with hexaconazole (01.07%) at 25 ppm. All the isolates were significantly differing with each other statistically (Table 2).

The endophytic bacterial isolate EB₇ was highly compatible with the fungicide, copper oxychloride (98.07%) at 1000 ppm followed by thiophanate-methyl, carbendazim, mancozeb and propiconazole. The least compatibility (31.12%) was recorded in case of hexaconazole at 25 ppm. EB₉ was highly compatible with the fungicide hexaconazole at 25 ppm (85.89%) followed by thiophanate-methyl, carbendazim, mancozeb and copper oxychloride and the least compatibility (07.29%) was recorded in case of propiconazole at 25 ppm concentrations.

The bacterial antagonist EB₃₅ was found to be more compatible with thiophanate-methyl (97.02%) at 50 ppm followed by copper oxychloride, carbendazim, mancozeb and propiconazole. Hexaconazole was found less compatible with EB₃₅. Highest fungicidal compatibility was recorded with mancozeb (87.02%) in EB₃₉ at 1000 ppm. This isolate was compatible with all fungicides but the compatibility was least with hexaconazole (10.03%).

The mean compatibility of bacterial antagonists with different fungicides was highest in EB₇ followed by EB₃₅, PB₁₈, PB₂₈, EB₃₉ and EB₉. Among the fungicides, mancozeb was found to highly compatible with all the six bacterial antagonists followed by thiophanate-methyl, carbendazim, copper oxychloride and propiconazole whereas hexaconazole at 25 ppm exhibited less compatibility with all the isolates.

DISCUSSION

Table 2: Compatibility of potential bacterial antagonists with different fungicides by spectrophotometry

Fungicides	Optical density at 600nm				Percent compatibility							
	PB18	PB 28	EB 7	EB 9	EB35	EB39	PB18	PB 28	EB 7	EB 9	EB35	EB39
Carbendazim(50 ppm)	0.903	0.619	0.815	0.736	0.923	0.828	87.03	60.03	78.29	71.59	89.00	72.00
Thiophanate-methyl(50 ppm)	1.000	0.605	0.955	0.835	1.008	0.721	96.07	59.00	91.73	81.22	97.02	62.07
Propiconazole(25 ppm)	0.335	0.908	0.645	0.075	0.667	0.426	32.04	88.05	61.95	07.29	64.03	37.01
Hexaconazole(25 ppm)	0.174	0.017	0.324	0.883	0.038	0.118	16.09	01.07	31.12	85.89	03.07	10.03
Mancozeb(1000 ppm)	0.969	0.577	0.696	0.623	0.866	1.003	93.07	56.02	66.85	60.60	83.05	87.02
Copper oxychloride (1000 ppm)	0.612	0.733	1.021	0.497	1.002	0.708	59.02	71.04	98.07	48.34	96.06	61.06
Control	1.035	1.027	1.041	1.028	1.038	1.151	---	---	---	---	---	---
Mean	0.666	0.576	1.041	1.028	0.751	0.639	---	---	---	---	---	---

*Mean of three replications

	CD (5%)	SEM
Concentrations	1.2544	1.9874
Fungicides	1.5860	1.9786
Interaction	1.3680	1.2879

The term "anthracnose" literally means "like coal" and was first used by Fabre and Dunal to describe a disease of grapes in which blackening of a tissue was a characteristic feature. Anthracnose caused by *Colletotrichum gloeosporioides* is an

important disease of mango and prevalent in tropical regions of the world. *C. gloeosporioides* can cause fruit set reduction and quiescent infection results in post harvest losses (Dodd *et al.*, 1991; Donkin and Oosthuysen, 1996). The genus *Colletotrichum* includes more than 900 species, of which 100 species have been recorded in India (Wijesekara and Agarwal, 2006). *C. gloeosporioides* produces conidia on lesions on leaves, twigs, panicles, and mummified fruit. Conidia can be rain-splashed to other leaves or flowers and cause secondary infections; thus the disease is polycyclic in these organs. Usually, mango is infected by the pathogen during rainy season and survives in infected leaf debris during off season and serve as a primary source. Under favourable conditions, the pathogen spreads from leaves to inflorescence stalk, flowers and fruits by water borne conidia (Fitzell and Peak, 1984).

During the early phase of fungicidal usage (1950s and 1960s) the benefits of fungicides became increasingly apparent and resulted in significant increase in yield. Development of resistance to these fungicides was not a problem even after many years of their regular use (Russell, 1999). Unfortunately, due to the site-specific action, many brought with them the problem of resistance development soon after their introduction and the experience were sometimes painful as resistance could lead to total loss of effective control of the target pathogen (Davidse *et al.*, 1981). Due to the development of resistance in pathogen and the adverse effects of synthetic chemical residues on human health (Lichtenberg and Zilberman, 1987) and on environment (Weaver *et al.*, 1990) used in plant disease management has diverted the plant pathologist to intensify world-wide research efforts to develop alternative control strategies with little or no adverse effect on environment. Hence, search for effective, simple, inexpensive and nonhazardous methods of managing plant pathogens globally is gaining attention. Nonchemical and eco-friendly fungal and bacterial bioagents have been found to provide an answer to the nondiscriminatory and broad spectrum fungicides.

The use of biological control methods to reduce disease incidence caused by plant pathogens is continually being developed and is being used in a variety of crops (Soytong *et al.*, 2001). The efficiency of the biocontrol agent could further be improved when it was applied with the recommended fungicide, used at a lower concentration (Korsten *et al.*, 1992; Silimela and Korsten, 2001). The incorporation of eco-friendly biocontrol agents for the control of *C. gloeosporioides* as a part of integrated disease management will help in managing the disease as well as in delaying the development of tolerance/resistance to fungicides. In the present study, the native potential fungicidal tolerant biocontrol agents were identified from phylloplane and leaf endophytes against *Colletotrichum gloeosporioides*. Fungal antagonists, T₃, T₁₇, T₂₃ and T₂₆ which were identified as *Trichoderma* spp. in inhibiting the growth of *C. gloeosporioides*. Uses of *Trichoderma* has long been known as effective antagonists against wide range of plant pathogens with capability of attacking through mycoparasitism. The results evident that fungicidal tolerant potential antagonists from leaf endophytes viz., EB₇, EB₉, EB₃₅ and EB₃₉ that play beneficial role has been recorded. Our study also revealed that the leaf endophytes that act as a potential source of metabolites against foliar pathogen *C.*

gloeosporioides. The effectiveness of native potential antagonists from phylloplane and leaf endophytes warrants a further exploration of other products as alternatives to the fungicides.

Thus, these fungal and bacterial biocontrol agents which would benefit the industry where use of biological products to replace or supplement chemical use is extremely important. It is therefore clear that standardization of material preparation for fungicidal tolerant bioagents are urgently required. This approach might presumably become good and effective for integrated disease management strategies. Research is in progress to evaluate the viability and efficacy of some of the potential biocontrol agents identified during the present study. The main emphasis will be given to endophytes to test on a large scale under field conditions keeps the feasibility for commercialization. Simultaneously, molecular characterization of fungicidal tolerant potential biocontrol agents will be carried out to develop SCAR markers. These nucleic acid based fungal and bacterial biocontrol markers will be highly useful for mapping the distribution of biocontrol populations in production environments.

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