Research Paper :

Mode of action of the native potential antagonist, *Trichoderma fasciculatum* against *Colletotrichum gloeosporioides* causing mango anthracnose

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SUMMARY

For control of anthracnose of mango incited by *Colletotrichum gloeosporioides* Penz., native antagonistic microflora were used with different mechanisms. Under *in vitro* study, the four potential antagonists *viz.*, T_1, T_7, F_{11} and B_1 isolated from fructoplane showed the highest antagonistic activity in dual culture studies due to mycoparasitism and the efficacy of the four potential antagonists was confirmed in spread plate technique. Moreover, T_7 isolate was selected as the best fungicide compatible potential native antagonist among the fungicides evaluated in poison food technique. The effect of volatile and non-volatile metabolites produced by fructoplane isolate, *Trichoderma fasciculatum* (T_7) inhibited the mycelial growth and conidial germination over control on 3rd and 5th day of incubation, respectively through antibiosis. These findings indicated that the native potential antagonist T_7 which inhibited the growth of pathogen with different mechanisms combining with a compatible systemic fungicide Thiram at a lower concentration proved to be the best in integrated disease management of *Colletotrichum gloeosporioides*.

Key words : Mango, *Colletotrichum gloeosporioides*, Anthracnose, *T. fasciculatum* Mycoparasitism, Volatile and non-volatile compounds

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Mango (*Mangifera indica* L.) is considered as "king of fruits" grown throughout the tropics and subtropics worldwide. India is the world's largest producer of mango. *Colletotrichum gloeosporioides* causes anthracnose, which is the most important biological constraint to mango production in Southeast Asia resulting in 30-60% harvest losses (Dodd *et al.*, 1991). Though chemical control measures are effective, considering the cost of chemical pesticides and the environmental hazards involved, biological control is a viable strategy for sustainable disease management. The biologically active compounds from natural sources have always been of a great interest for scientists working on various diseases and understanding the mechanisms through which the biocontrol of plant diseases occurs is critical to the eventual improvement and wider use of biocontrol methods. The susceptibility of the pathogen to inhibitory metabolites of bioagents assumes greater relevance where the fungal pathogen is polyphasic, multicyclic and when all the different phases of life cycle of the pathogen are drastically affected by these antagonists. The present study dealts with the antagonistic effect of metabolites produced by the potential biocontrol agents against *Colletotrichum gloeosporioides*.

MATERIALS AND METHODS

Isolation, identification and pathogenicity:

The pathogen was isolated from infected mango fruits collected from mango orchards at Agricultural Research Station, Anantharajupeta, Kadapa (Dt), Andhra Pradesh by tissue segment method and purified by single spore isolation method (Rangaswami and Mahadevan, 1999). The isolates were identified by standard mycological keys (Barnett and Hunter, 1972) and maintained on Potato dextrose agar (PDA) for further studies. Wound inoculation method was used to test the pathogenicity on baneshan mango fruits (Bhuvanaeswari and Rao, 2001).

Screening of native potential bioagents by dual culture technique:

Serial dilution plate technique was used for the isolation of native antagonistic microflora from phylloplane and fructoplane of mango (Zenuchi, 2003). The antagonistic activity of microflora isolates against *C. gloeosporioides* was determined by dual culture technique under *in vitro* conditions (Bhuvaneswari and Rao, 2001).

Spread plate method:

1ml of conidial suspension of antagonistic fungi (10^4 conidia/ml) prepared using sterile

distilled water was poured into a Petriplate. To this 15ml molten and cooled PDA medium was added and gently rotated to mix the suspension uniformly and allowed to solidify. Then the test pathogen was inoculated at the centre of the Petriplate and incubated at 28±2^{0}C. Control plates without conidial suspension served as control. Three replications were maintained for each treatment and per cent reduction in growth of the 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

Effect of volatile metabolites of potential antagonist on C.gloeosporioides under in vitro:

Inverted plate technique as described by Uma Maheswari *et al*. (2002) was used to study the influence of volatile substances on the growth of *C.gloeosporioides*. 6mm disc of the fungal antagonist was placed at the centre of Petriplate containing sterilized PDA medium. In case of bacterial antagonist, 48 hour old culture was streaked at the centre of Petriplate containing nutrient agar (NA). The lid of each Petriplate was replaced by another bottom plate containing PDA medium inoculated with 6mm actively growing culture disc of *C.gloeosporioides* and sealed together with an adhesive tape. The lid of control plate, not inoculated with antagonist served as control. Three replications were maintained and the plates were incubated at 28±2^{0}C. Colony diameter of the pathogen was measured on 3rd day and 5th day after incubation. Per cent inhibition of growth of the test pathogen was calculated using the formula as described above.

Effect of non-volatile metabolites of native potential antagonist on radial growth of C.gloeosporioides under in vitro:

The effect of culture filterate of antagonist on *C.gloeosporioides* was studied following the method of Pant and Mukophadyay (2001). 6mm disc of the fungal antagonist was transferred into sterilized Potato dextrose broth (PDB) and kept in shaker@100 rpm at 28±2^{0}C for 15 days. The culture filterate was obtained by passing the broth culture successively through Whatmann no.1 filter paper and bacterial proof filter (0.2 µm, sartorius-single use filter). The culture filterate obtained was added to molten PDA to obtain concentration of 10% (v/v). The medium was poured into Petriplates (20ml/plate) and inoculated with 6mm disc of *C. gloeosporioides*. PDA plates inoculated with the pathogen but not amended with culture filterate served as check. The plates were incubated at 28±2^{0}C. The colony diameter of the pathogen was measured on 5th day of incubation and per cent inhibition of the growth of the isolates over the control was calculated using the formula as described above.

Effect of non-volatile metabolites on germination of conidia of C.gloeosporioides under in vitro:

The effect of non-volatile metabolites produced by antagonists on germination of conidia of *C. gloeosporioides* was studied by slide germination technique (Nene and Thapliyal, 1993). 0.2 ml of culture filterate of the antagonist was placed in cavity slide and allowed to dry. Same quantity of spore suspension of the pathogen (10^4 conidia/ml) was placed in the same cavity slide and incubated at 28±2^{0}C. Distilled water along with spores of pathogen and only medium served as the control. Three replications were maintained. The germination of conidia was recorded under microscope after 24 hours. A total of 100 conidia in a high power microscopic field were counted including germinated and non-germinated ones. The percentage of inhibition was calculated by the formula:

$$\text{Percentage of inhibition of the conidia} = \frac{\text{No. of spores germinated}}{\text{Total number of spores observed}} \times 100$$

Mycoparasitism:

Hyphal interactions between *C.gloeosporioides* and the antagonist were observed from dual culture. Mycelial mats were lifted gently from the zone of interaction from dual culture plates with the help of a needle and kept in a drop of cotton blue with lactophenol on a microscopic slide and observed under microscope (Pant and Mukhophadhyay, 2001).

Statistical analysis:

Completely Randomized Design (CRD) was used for radial growth, per cent disease incidence, poisoned food technique and dual cultural technique (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

The antagonistic effect of native microflora assessed revealed that all the test microbes inhibited the growth of *C.gloeosporioides* at varying degrees both in dual culture and spread plate method under *in vitro*. Among the antagonist mycoflora, the native *Trichoderma* isolate T_1 from fructoplane was superior with highest per cent of

inhibition (79.93%) followed by T_7 (71.38%) and F_{11} (71.00%) isolates (Table 1). Statistical analysis showed that there was significant difference between the per cent inhibition of T_1 and T_7 isolates and however there was no significant difference between the inhibition per cent of T_7 and F_{11} isolates.

Of all the nine bacterial isolates evaluated in dual culture studies, the isolate B_1 showed maximum inhibition of 63.09% followed by B_2 (52.33%) and B_9 isolate showed the minimum inhibition of 8.28%. The four potential antagonists *viz.*, T_1, T_7, F_{11} and B_1 that were made contact with the mycelia of test pathogen, the only fungal antagonist T_7 overgrew on the mycelium of the test pathogen whereas others just inhibited the growth of the pathogen. Microscopic examination of the zone of hyphal interactions between antagonist and pathogen revealed hyphal parasitism of antagonist on the test pathogen and production of specialized structures like haustoria which are responsible for arresting the growth of the test pathogen (Fig. 1). *Trichoderma* formed several loops coiling around the hyphae of pathogen and thick compact rope like structure followed by rupturing, twisting and leakage of hyphal protoplasm at a later phase of interactions. Sometimes the hyphae of *Trichoderma* produced haustoria like structures which entered the pathogen and disorganization of protoplasmic contents was also observed.

The efficacy of the above four potential antagonists were further confirmed by spread plate technique and all the four antagonists (T_1, T_7, F_{11} and B_1) successfully inhibited of the test pathogen (Table 2 and Fig. 2). The potential antagonistic fungal isolates *viz.*, T_1, T_7 and F_{11}

Table 1 : *In vitro* evaluation of the efficacy of antagonistic microflora against growth of *C.gloeosporioides* in dual culture technique

Antagonistic isolates	*Mycelial growth (mm)	Per cent inhibition over control
T_1	18.05	79.93
T_2	32.98	63.36
T_3	31.80	64.60
T_4	30.40	66.20
T_5	40.60	54.88
T_6	34.40	61.70
T_7	25.76	71.38
T_8	34.78	61.35
T_9	36.97	59.00
F_{10}	42.15	53.00
F_{11}	26.10	71.00
F_{12}	39.97	55.59
F_{13}	44.29	50.79
F_{14}	62.10	31.00
F_{15}	66.97	25.59
B_1	33.22	63.09
B_2	42.90	52.33
B_3	67.33	25.09
B_4	72.77	19.14
B_5	77.78	13.58
B_6	64.78	28.20
B_7	74.20	17.56
B_8	43.89	51.23
B_9	82.55	8.28
Control	90.00	---
T1SEm	1.3151	0.4369
C.D. (P=0.05)	3.7716	0.8921

*Mean of three replications; T – *Trichoderma* isolates; F – Fungal isolates; B- Bacterial isolates

Fig.1 : *In vitro* evaluation of the antagonistic activity of *Trichoderma* isolates - T_1 to T_9 ; Fungal isolates - F_{10} to F_{15} ; Bacterial isolates - B_1 to B_9 against *C.gloeosporioides* in dual culture technique

Table 2 : *In vitro* evaluation of the efficacy of antagonistic microflora against *C.gloeosporioides* in spread plate technique

Antagonistic isolates	Mycelial growth (mm)*	Per cent inhibition over control
T_1	0.00	100.00
T_7	0.00	100.00
F_{11}	0.00	100.00
B_1	0.00	100.00
Control	90.00	---

* Mean of three replications

Fig. 2 : *In vitro* evaluation of potential antagonists in spread plate technique. T_1 and T_7 – *Trichoderma* isolates, F_{11} - Fungal isolate, B_1 – Bacterial isolate

and bacterial isolate B_1 were further selected for fungicide compatibility studies as these suppressed the growth of test pathogen both in dual culture and spread plate techniques.

The compatibility of four antagonists with various fungicides at different concentrations evaluated showed different degrees of inhibition against *C.gloeosporioides* in poisoned food technique (data not shown/ communicated/in press). Mancozeb was 100% per cent compatible with both T_1 and T_7 antagonists whereas Thiram was compatible with only T_7 (76.44%) and inhibitory to T_1 (18.11%). Moreover, complete suppression of both T_1 and T_7 isolates was observed in case of Carbendazim, Propioconcazole and Prochloraz. The F_{11} bioagent was incompatible with all the fungicides except Mancozeb. Though the bioagent B_1 was compatible with most of the fungicides evaluated, it was inferior to T_1 and T_7 isolates in inhibiting the test pathogen. However, the antagonist T_7 was more compatible with the different fungicides particularly to Thiram when compared to T_1 isolate, which showed the highest per cent inhibition. Hence, T_7 was selected as the best fungicide compatible potential native antagonist and identified as *Trichoderma fasciculatum* at IARI, New Delhi (accession no. 6624). With the increasing interest in developing alternatives to chemical fungicides, production of *Trichoderma* as bio-protectant has become the focal point for research and development. The fungicides provide initial protection by weakening the pathogen and then the antagonists colonize the pathogen to suppress through antibiosis and mycoparasitism.

Some biocontrol agents secrete wide array of compounds with biological activity against other organisms, mostly products of secondary metabolism. These metabolites serve different functions, depending upon the ecological niche of the organisms. Some metabolites may be antibiotics to protect the BCA against antagonistic microorganisms or may prevent growth of saprophytic microbes on the host or suppress the growth of plant pathogens. The effect of volatile metabolites produced by *T.fascicualtum* (T_7) inhibited the mycelial growth up to the extent of 14.54% and 26.68% over control on 3rd and 5th day of incubation, respectively (Table 3 and Fig. 3). Further, the growth of the test pathogen after 5th day of incubation completely inhibited

Table 3 : *In vitro* evaluation of the efficacy of volatile metabolites produced by *Trichoderma fasciculatum* on the growth of *C.gloeosporioides* in inverted plate technique

	Group	Mycelial growth (mm)*	Per cent inhibition over control	SE	T value
3rd day	Treatment	33.89	14.54	0.1101	14.50**
	Control	39.66		0.333	
5th day	Treatment	44.44	26.68	0.1131	
	Control	60.70		0.9498	
Interaction	Treatment	33.89	---	0.1101	
	Control	44.44	---	0.1131	

* Mean of three replications ** Significant

Fig. 3 : Effect of volatile metabolites produced by *Trichoderma fasciculatum* on the growth of *C.gloeosporioides* in inverted plate technique

encircling by the hyphae of the antagonist. The effect of non-volatile metabolites in culture filtrate of *T.fasciculatum* showed 9.14% inhibition of test pathogen over control indicating the production of some inhibitory metabolites on the 5th of incubation (Table 4 and Fig 4). Understanding the mechanism(s) of action involved in biocontrol process is of primary importance for establishing the effectiveness of biocontrol agent will provide much insight into where and when the interaction occurs and the pathogen will be affected (Larkin *et al.*, 1990). The effect of non-volatile metabolites on germination of conidia of test pathogen revealed that the antagonist inhibited the conidial germination upto the extent of 9.60% (Table 5). The inhibitory volatile and non-volatile metabolites produced by *T. fasciculatum* at different stages inhibited the test pathogen which clearly indicates the potency to antagonize all the reproductive phases of the pathogen thus, effectively preventing its growth and proliferation. From the results obtained, it was presumed that the mechanisms involved in the inhibition of *C. gloeosporioides* by *T. fasciculatum* involved competition, production of inhibitory metabolites and mycoparasitism. The results are in agreement with the findings made by Adebanjo and Bankole (2004) who reported that *Trichoderma viride* coiled around the hyphae of *Colletotrichum truncatum* causing brown blotch of cowpea by penetrating into it. Similarly, Chakravarthy and Nagamani (2007) also reported that the inhibition of *Rhizoctonia solani* by *Trichoderma* spp. was due to competition for food and space, production of antibiotics and mycoparasitism.

Table 4 : *In vitro* evaluation of the efficacy of non-volatile metabolites produced by *Trichoderma fasciculatum* on the (5th Day) growth of *C. gloeosporioides*

Group		Mycelial growth (mm)*	Per cent inhibition over control	SE	T value
5th day	Treatment	52.24	9.14	0.1233	16.75**
	Control	57.50		0.2887	

* Mean of three replications ** Significant

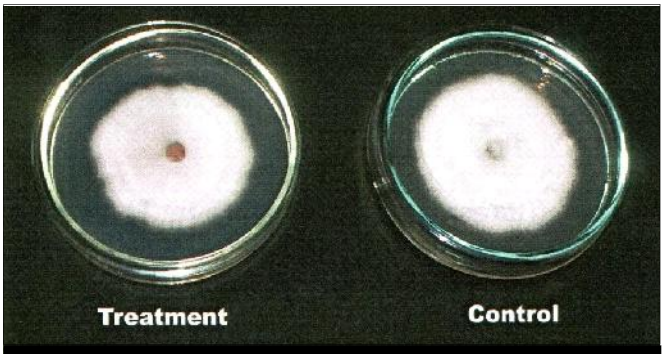

Fig. 4 : Effect of non-volatile metabolites produced by *Trichoderma fasciculatum* on the (5th Day) growth of *C.gloeosporioides*

Table 5 : *In vitro* evaluation of the efficacy of non-volatile metabolites produced by *Trichoderma fasciculatum* on germination of the conidia of *C.gloeosporioides*

Group	No. of conidia germinated*	Per cent inhibition over control	SE	T value
Treatment	56.50	9.60	0.3333	7.18**
Control	62.50		0.2887	

* Mean of three replications and counted 100 conidia per replication ** Significant

For sustainable crop production, the components involved in an integrated disease management should be eco-friendly, so that beneficial organism would be safe to use (Anahosur, 2001). Hence, future research is focused on the scientific investigation for bioactive components which were supposed to exhibit their activity in synergistic way or be activated by the presence of other substances or even some other chemical structures may be responsible for the proposed biological activity. Genetic and molecular analysis has demonstrated that production of various antifungal compounds is a primary mechanism of biocontrol for many strains, accounting for as much as 90 per cent of the disease-suppressing activity (Thomashow and Weller, 1995). Taking these in account, the quantities of volatile compounds in the vapour phase of the bioassay system will be measured by direct headspace sampling and GC analysis and also finding new natural, compounds of plant origin emphasize the importance to develop techniques for rapid identification of active structures, on the other side to obtain these structures selectively for the mass production and commercialization.

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