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ANTAGONISTIC POTENTIALS OF *TRICHODERMA* SPP. AGAINST FRUIT ROT OF CUSTARD APPLE CAUSED BY PHOMA LINGAM

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Abstract

Context: Custard-apple (*Annona squamosa* Linn.) is a popular tropical fruit and fruit-rot disease caused by *Phoma lingam* leads to considerable qualitative and quantitative damages to the fruit in the area under study.

Objective: Studies were conducted to investigate the antagonistic potential of five *Trichoderma* spp. namely, *T. harzianum, T. hamatum, T. lignorum, T. reesei* and *T. viride* against *in vitro* growth of the pathogen followed by field experiments.

Materials and Methods: Dual culture plate, closed petriplate and food poisoning technique were followed in order to ascertain the antagonistic potential of the five species of *Trichoderma*. Hyphal interaction between the pathogen and *T. viride* was studied by collecting mycelial samples from the interaction zone of dual culture plate and was processed for scanning electron microscope. Plants with infected fruits were sprayed with spore suspension of *T. viride* and two commercial bioformulations of *T. viride* viz. Trichofix and Trichoguard for three times at a dose of 0.1, 0.5 and 1.0% prepared in distilled water.

Results: All the *Trichoderma* species more or less effectively inhibited the growth of the pathogen through mycoparasitism, production of volatile and non-volatile metabolites. Scanning electron microscopic (SEM) micrographs revealed that hyphal interaction between *P. lingam* and *T. viride* leads to lysis of the pathogenic mycelium by the antagonist. Field experiments with spore suspension of *T. viride* and Trichofix and Trichoguard significantly reduced fruit rot incidence of custard-apple.

Conclusion: The results of the current study indicate that adoption of biocontrol based disease management programmes can be effectively utilized against similar fruit diseases.

Keywords: custard-apple, fruit rot, mycoparasitism, biological control, *Trichoderma, Annona squamosa*.

Introduction

Plants in their environment face potential deleterious organisms such as fungi, bacteria, viruses, nematodes, etc. and many of them are able to cause plant diseases resulting in loss of crop production worldwide. Custard-apple (*Annona squamosa* Linn.) belonging to the family Annonaceae is one of the best tropical fruits as it provides good carbohydrate nutrition with excellent sources of energy and minerals. The fruit pulp can be processed as frozen pulp, juice, jelly, sherbet and ice cream. Fruit rot disease of custard-apple caused by *Phoma* sp. is epiphytotic in the area that poses a serious threat to its production (Ojha *et al.* 2005). The affected plant showed blackening of the entire fruit, which gradually became hard and stony. The size of the fruits gets reduced and some shedded off from the branches.

While chemical control of plant diseases is usually expensive and may have a negative impact on the environment and on public health, the use of microorganisms to control plant pathogens, known as biological control, is accepted as a durable and eco- friendly alternative to plant disease management. Fungi have got maximum attention as antagonists probably because easy handling and identification compared to other

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microbes. In the past few years, *Trichoderma* spp. have received considerable attention as potential biocontrol agents for a number of plant pathogens.

Trichoderma- plant pathogen interaction suggests competition for nutrients and space (Chet 1987), inactivation of the enzymes produced by the pathogens (Ozbay and Newman 2004), modifying the environmental conditions (Benitez *et al.* 2004), secretion of antifungal metabolites (Dennis and Webster 1971a,b), mycoparasitism or hyperparasitism involving production of lytic enzymes (Elad *et al.* 1982, Lu *et al.* 2004), promotion of plant growth and plant defensive mechanisms leading to induced resistance in the host (Benitez *et al.* 2004, Ozbay and Newman 2004) and production of siderophores (Dutta *et al.* 2006) all of which play a significant role in the antagonism of other fungi.

Keeping these in view, the present study was undertaken to evaluate the efficacy of five *Trichoderma* spp. against a fruit rot pathogen *Phoma lingam* in terms of competition for nutrients, production of inhibitory volatile, non-volatile and soluble metabolites and hyphal interactions between the pathogen and the antagonists. Finally field trial was conducted with most effective antagonist to control the fruit rot incidence of custard-apple.

Materials and Methods

Fungal isolates: The pathogen was isolated from the infected custard-apple fruits and identified as *Phoma* sp. by Indian Agricultural Research Institute, New Delhi (ITCC No. 5864.04). The pathogen of the disease has now been found to be *Phoma lingam*. Five *Trichoderma* species were isolated from rhizosphere soil of different vegetable fields of Burdwan, West Bengal. All the organisms were maintained on potato dextrose agar (PDA) at 4°C.

Evaluation of antagonistic potential of the Trichoderma spp. through mycoparasitism: Dual culture plate technique was followed in order to ascertain the antagonistic potential of the five species of *Trichoderma*, *viz*. *T. viride, T. lignorum, T. reesei, T. harzianum* and *T. hamatum* in terms of competition and mycoparasitism. The pathogen as well as *Trichoderma* spp. were grown on PDA plates for a week at 28°C. One mycelial disc (5 mm diameter) each of the pathogen and the antagonist was placed on the surface of PDA plate at 5 cm apart. The inoculated plates were incubated at 28°C for 7 days and the inhibition of mycelial growth of the test pathogen was measured.

Evaluation of volatile compounds produced by Trichoderma spp.: In order to study the effect of volatile compounds produced by *Trichoderma* spp., PDA medium (15 ml) was poured both in the base and the lid of the petriplate. The medium was allowed to solidify. Then a mycelial disc (5 mm) of the pathogen was placed at the centre of the lid of the petriplate and the bottom of the petriplate was inoculated with a mycelial disc (5 mm) of the respective antagonist. The petriplate was sealed to one another and incubated at 28°C for 7 days. The percentage inhibition of growth of the pathogen was recorded as the difference in radial growth of the pathogen in the presence or absence of *Trichoderma*.

Evaluation of non-volatile compounds produced by Trichoderma spp.: To determine the effect of non-volatile compounds produced by *Trichoderma* spp. on growth of the pathogen, each of the respective antagonists was placed centrally on dialyser bag (Sigma) covered PDA plate. After 2 days of incubation at 28°C, the respective antagonist and the dialyser bag were removed. After that, a mycelial disc (5 mm) of *P. lingam* was placed centrally on the same PDA plate and incubated at 28°C for 7 days. Growth inhibition by the respective antagonist was recorded.

Effect of soluble metabolites of Trichoderma spp. on growth of the pathogen: Food poisoning technique was adopted to study the effect of soluble metabolites of *Trichoderma* spp. against the pathogen. Cultures of five *Trichoderma* spp. were grown in 250 ml conical flasks containing 10 ml Czapek's synthetic broth and

incubated at 28°C for 21 days. Culture filtrates were obtained by filtering through Whatman filter paper No. 1 and passed through a sterilized sintered glass filter (G-5) in order to remove the mycelial bits and spores of the antagonists. These cell free culture extracts were stored at 5°C for antagonistic studies.

Potato dextrose broth (150 ml) at pH 7.2 was taken in 250 ml conical flasks. Soluble metabolites of each antagonist at different doses (1, 2, 4, 6, 8 and 10 ml) were mixed separately with the broth. An inoculum disc (5 mm) of *P. lingam* was placed in the conical flask and incubated at 28°C for 7 days. Growth inhibition of the pathogen was recorded.

Hyphal interactions through scanning electron microscope: Hyphal interaction between the pathogen and the most potent antagonist *T. viride* was studied by scanning electron microscope (SEM). Mycelial samples from the interaction zone of 15 days old dual culture plate were cut by cork borer and were processed for SEM according to Pham *et al.* (2005). Sodium phosphate buffer (0.1M) was prepared by dissolving 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 800 ml of distilled water. The pH was adjusted to 7.4 by 1N HCl or 1N NaOH using a pH meter. The volumes were adjusted and sterilized by autoclaving. The buffer was stored at room temperature.

Glutaraldehyde solution (25%) was prepared from the available 70% solution and was stored in refrigerator over activated charcoal. For use, the clean solution was decanted from the top and was filtered through a filter paper. After that the mycelial samples were fixed in 2.5% glutaraldehyde buffered with 0.1M sodium phosphate buffer at pH 7.4 for one hour. The fixed samples were washed in distilled water three times at 15 minutes intervals. The clean samples were dehydrated through a graded series of ethanol *viz*. 30% alcohol (5-10 min), 50% alcohol (5-10 min), 70% alcohol (5-10 min), 80% alcohol (5-10 min), 95% alcohol (5-10 min) and 100% alcohol (10-15 min with three changes).

All specimens were mounted on aluminum SEM stubs using double-sided adhesive tape. After mounting the samples were transferred to IB2 ion coater vacuum evaporation unit. The specimens were placed about 10 cm from the evaporation source. The samples were coated at a pressure of 10^{-5} torr with approximately 100 Å thick layer of gold. The coated specimens were analyzed in scanning electron microscope (S530 Hitachi, Japan) at an anode potential of 20-25 KV.

Field experiment: Field experiment was conducted at the time of fruit development in the area under study i.e. during June-July, 2010. From *in vitro* experiment *T. viride* was found to be most effective antagonist and thus the isolate of *T. viride* and two commercial bioformulations of *T. viride* viz. Trichofix and Trichoguard was applied at a dose of 0.1, 0.5 and 1.0% prepared in distilled water.

The spore suspension of fungal antagonist was prepared in 100 ml distilled water using 7 days old actively growing culture of *T. viride* with the spore concentration of 10^8 cfu ml^{-1}. From this spore suspension different concentrations were prepared in distilled water. Three sprays were given for each of the treatments. First application was done prior to appearance of fruit and two subsequent applications were carried out at 10 days interval after the initiation of fruit.

Statistical analysis: There were five replications for each of the treatments. Data were analyzed statistically by one-way analysis of variance (ANOVA) and Duncan's multiple range tests using a statistical software package (SPSS 10.0 Inc.).

Results

Effect of Trichoderma spp. on growth of Phoma lingam through mycoparasitism in dual culture plate and production of volatile and non-volatile metabolites: It may be noted (Table 1) that five species of *Trichoderma* had been able to reduce the growth of the pathogen significantly through mycoparasitism in dual culture plate

and production of volatile and non-volatile metabolites. A comparison between the inhibition by volatile and non-volatile metabolites of *Trichoderma* isolates reflects that non-volatiles were able to check the growth of the test pathogen most efficiently than the volatile ones. Highly significant reduction in pathogenic growth was observed by *T. viride* (7.10 cm) and *T. harzianum* (6.64 cm) in dual culture plate.

Table 1. Effect of *Trichoderma* spp. on growth of *Phoma lingam* through mycoparasitism and production of volatile and non-volatile metabolites

Treatments	Growth inhibition of the pathogen through mycoparasitism		Growth inhibition of the pathogen through volatile metabolites		Growth inhibition of the pathogen through non-volatile metabolites	
	Radial growth of the pathogen (cm)	Radial growth of the antagonist (cm)*	Radial growth of the pathogen (cm)	Radial growth of the antagonist (cm)*	Radial growth of the pathogen (cm)	Radial growth of the antagonist (cm)*
T. hamatum	3.62	5.38b	4.28	4.72c	3.84	5.16 c
T. harzianum	2.36	6.64ab	3.44	5.56ab	2.82	6.18 b
T.lignorum	3.14	5.86b	4.08	4.92bc	3.26	5.74bc
T. reesei	3.96	5.04bc	4.40	4.60c	4.06	4.94c
T. viride	1.90	7.10a	2.94	6.06 a	2.43	6.57a
Control	9.00	0.00	9.00	0.00	9.00	0.00

*Means with the same letter are not significantly different according to Duncan's multiple range test at P < 0.05.

Table 2. Effect of soluble metabolites of *Trichoderma* spp. on growth of *P. lingam* following food poisoning technique in liquid medium

Treatments	Metabolite of *Trichoderma* spp. (ml)					
	1	2	4	6	8	10
T. hamatum	*141.36^{a}	120.98^{b}	92.00^{c}	67.68^{cd}	45.08^{d}	15.04^{e}
T. harzianum	136.98^{a}	116.86^{ab}	84.14^{bc}	59.22^{c}	23.86^{cd}	6.28^{d}
T.lignorum	140.40^{a}	121.06^{b}	96.18^{cd}	68.90^{d}	39.88^{de}	12.18^{e}
T. reesei	148.28^{a}	129.94^{bc}	105.32^{c}	82.18^{d}	49.94^{de}	29.04^{f}
T. viride	135.28^{a}	108.56^{ab}	74.06^{b}	40.16^{c}	9.12^{cd}	0.00^{d}
Control	156.00					

*Dry mycelial weight of the pathogen (mg); **Means with the same letter are not significantly different according to Duncan's multiple range test at P < 0.05.

Table 3. Effect of biocontrol treatments with *T. viride* isolate and two commercially available formulations of *T. viride* on development of fruit rot of cusard-apple

Treatments	Rate*	Total no. of fruits	No. of infected fruit	Percentage of disease	Percentage of reduction**
Control	-	25	25	100	-
Trichoderma viride	0.1	26	23	88.46	11.54^{d}
	0.5	22	15	68.18	31.82^{bc}
	1.0	22	06	27.27	72.73^{a}
Trichofix	0.1	23	18	78.26	21.74^{cd}
	0.5	25	12	48.00	52.00^{b}
	1.0	26	04	15.38	84.62^{a}
Trichoguard	0.1	21	15	71.43	28.57^{d}
	0.5	27	12	44.44	55.56^{c}
	1.0	24	02	8.33	91.67^{a}

*Rate = % application (w/v or v/v) **Means with the same letter are not significantly different according to Duncan's multiple range test at P < 0.05.

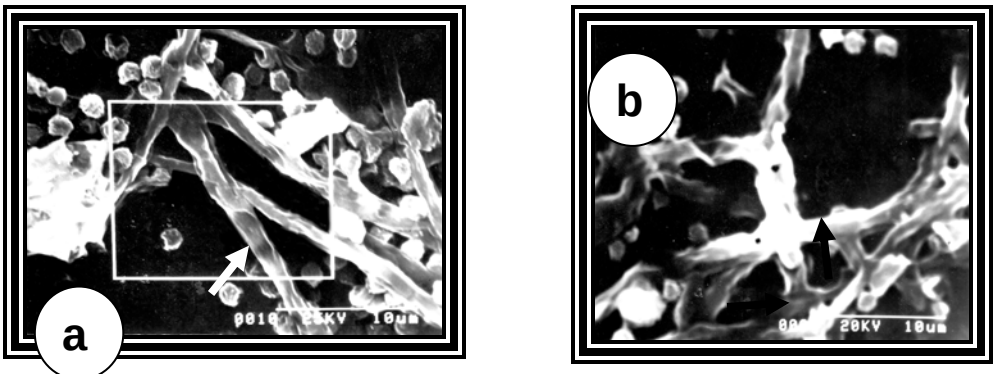

Fig 1. Scanning electron micrographs of hyphal interaction between the pathogen, *P. lingam* and the most potent antagonist, *Trichoderma viride* on 15 days old PDA dual culture plate. (a) Coiling of hypha of *T. viride* (→) around the hypha of *P. lingam*, (b) Formation of pore (→) on mycelium of the pathogen

Effect of soluble metabolites of Trichoderma spp. on growth of Phoma lingam: The soluble metabolites of *Trichoderma* spp were able to bring about significant reduction of radial growth of *P. lingam* and with the rise in the amount of growth metabolites of the antagonists, the biomass production of the pathogen gets retarded (Table 2). Growth of the pathogen was completely arrested in treatment with 10 ml metabolites of *T. viride*.

Hyphal interactions between T. viride and the pathogen: Scanning electron microscopic (SEM) studies of hyphal interaction between *T. viride* and the pathogen revealed that the hyphae of the antagonist underwent coiling around the hyphae of *P. lingam* (Figure 1a) followed by formation of pores on the hyphae of the pathogen (Figure 1b).

Field experiment: Plants sprayed with spore suspension of *T. viride* showed reduction in incidence of the disease up to 72.73% (Table 3). Among the two formulations of *T. viride*, Trichofix (1.0%) exhibited 84.62% reduction in the disease and Trichoguard was observed to be most effective as it at 1.0% rate resulted in 91.67% reduction in fruit rot disease of custard- apple. In all the cases effectiveness of the treatments was enhanced with increase in their doses.

Discussion

It is apparent from the results (Table 1) that *Trichoderma* spp. were able to retard the growth of *P. lingam* through competition for nutrients and space and by production of volatile and non-volatile substances. According to Benitez *et al.* (2004) *Trichoderma* has a superior capacity to take up and mobilize nutrients compared to other organisms. Papavizas (1985) stated that *Trichoderma* spp. are often very fast growing and rapidly colonize substrates, thus excluding pathogens. The efficient use of available nutrients is based on the ability of *Trichoderma* to obtain ATP from the metabolism of different sugars, such as those derived from polymers wide-spread in fungal environments – cellulose, glucan and chitin among others, all of them rendering glucose (Chet *et al.* 1997).

It may be suggested from the result that the variation in the potentiality of *Trichoderma* spp. to inhibit growth of the pathogen by the volatile and non-volatile substances indicates their differential ability in production of antibiotic principle. The results were also in accordance with the findings of Bruce *et al.* (2000) and Kucuk and Kivanc (2003) as they reported inhibitory effect of volatile and non-volatile compounds of *Trichoderma* origin on several plant pathogens. In the present study, a comparison between the inhibition by volatile and non-volatile metabolites of *Trichoderma* isolates reflects that non-volatiles were most efficient towards growth inhibition of *P. lingam*. Vey *et al.* (2001) suggested that most *Trichoderma* strains produce several volatile and non-volatile toxic metabolites such as harzianic acid, tricholin, peptaibols, 6-pentyl-α-pyrone, massoilactone, viridian, glioviridin, glisopenins, heptilidic acid that impede colonization by antagonizing microorganisms.

The result (Table 2) reveals that toxic metabolites, secreted in culture broth by five *Trichoderma* species, brought about significant reduction in vegetative growth of the pathogen and maximum inhibition was achieved with *T. viride*. Horvath *et al.* (1995) stated that *Trichoderma* isolates produce a wide range of soluble metabolites, which are inhibitory to other fungi and harzianolide is one of the important antifungal metabolites produced by *Trichoderma*. There is also strong evidence that the culture filtrates of *Trichoderma* spp. contain some kinds of antibiotics or enzymes which are involved in antifungal activity of the antagonists (Di Pierto *et al.* 1993).

Scanning electron micrograph clearly showed coiling of mycelium of *T. viride* around the hyphae of *P. lingam* and also formation of pore on hyphae of the pathogen. This is in concordance with other studies (Chet *et al.* 1981, Elad *et al.* 1982, Lu *et al.* 2004) which showed that *Trichoderma* coils around the hyphae of its host and then penetrates them by enzymatic digestion of the cell wall. Zaldivar *et al.* (2001) also recorded that

Trichoderma species producing antifungal substances which act as mycoparasite and lyse the pathogenic hyphae. So these SEM observations indicated that coiling of the antagonist around the pathogen is an early event preceding hyphal damage. Certain chemical stimulus of pathogenic fungi may attract the antagonist and induces a chemotropic response of the antagonist. The recognition between the pathogen and the antagonist is due to lectins and this is followed by interactions between hyphae of the pathogen and the antagonist. Thus, it seems likely that hyphal interactions between these fungi are crucial in the subsequent steps leading to growth inhibition of the pathogen.

The fruit rot incidence of custard-apple was significantly reduced in response to the treatments with spore suspension and two bioformulations of *T. viride* (Table 3). Thus the isolate which showed its potential role in suppressing the *in vitro* growth of the pathogen, was also found to exhibit promising effect in the field condition. These results are relatively consistent with earlier studies showing the effectiveness of formulation of biocontrol agents in reducing incidence of fungal diseases of plants (Boby and Bagyaraj 2003, Khan and Sinha 2005).

Therefore, the current study provides evidence that *T. viride* shows promising antagonistic effect on the pathogen through different modes of action like mycoparasitism, production of soluble metabolites and volatile and non-volatile compounds and also by hyphal interactions. In addition, field trial with *T. viride* and its two commercially available bioformulations was also found to be significantly effective to control the fruit rot disease of custard-apple.

Conclusion

In conclusion, the present study has focused on the possibility of managing other similar fruit diseases through proper screening of the bioagents followed by their field trial and moreover, this management strategy did not pose any risk to the environment and consumer health.

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