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Biological Control of *Pythium* Damping-off of Cauliflower by *Trichoderma harzianum.*

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ABSTRACT

Out of eight antagonists, one isolate of *Trichoderma harzianum* exhibited fastest growth rate and strong antagonism against *Pythium apanidermatum in vitro*. The prinicpal mechanism of antagonism was direct parasitization of *Trichoderma* by coiling around the hyphae of *Pythium* leading to digestion of protoplasmic contents and lysis. Occasional presence of *Trichoderma* in the lumen of *Pythium* hyphae was also recorded. Soil application of wheat bran saw dust (WBSD) preparation of *Trichoderma* gave 32.4 to 77.0 percent control of damping-off under glass house conditions. Seed coating with *Trichoderma* (1.6×10^{10} spores/ml) resulted in 31% control of the disease. WBSD preparation of *T. harzianum*, either live or killed, promoted the growth and vigour of cauliflower seedlings. Apron at 5 and Thiram at 100 µg/ml proved better and completely inhibited the radial growth of *P. ahpanidermatum in vitro*. None of the fungicides was found inhibitory to *T. harzianum* except Thiram. Seed treatement with Apron, Fytolan, SAN 506 F and Thiram gave 15.7 to 85.8% disease control. Integration of fungicidal seed treatment with soil application of *T. harzianum* resulted in 66.4% to 90% control of damping-off. Colony forming units of *Trichoderma* and *Pythium* monitored on selective media revealed that the population of *Trichoderma* remained almost stable where it was applied at higher rates but declined with time in lower rates of application. On the other hand, the population of *Pythium* declined in *Trichoderma* amended soil.

Key words: Biocontrol, damping-off, cauliflower, *Pythium, Trichoderma*

Damping-off, caused by *Pythium aphanidermatum* (Edson) Fitz. is a serious threat to cauliflower (*Brassica oleracea* var. *botrytis* L.) seedlings. The disease is so serious and rapid that if high temperature and high moisture prevail at the juvenile stage, 80-90 per cent seedlings may be killed within 48 h (Mukhopadhyay, A.N. Unpublished). Only a few reports have appeared in literature on the chemical control of this dreaded disease (Desai *et al.*, 1972, 1974; Sandhu and Gill, 1982).

During the last decade, *Trichoderma* species have shown tremendous potential of controlling several plant diseases especially the soil borne ones. Papavizas (1985) has published an exhaustive review on the ecology, biology and biocontrol potential of *Trichoderma* and *Gliocladium*. Though, there is no published report on the use of *Trichoderma. in bio-integrated control of Pythium* in cauliflower, it has been successfully tried for the management of several pathogens, *viz.*, *Sclerotium rolfsii* in sugarbeet (Upadhyay and Mukhopadhyay, 1986); *P. aphanidermatum* in cucumber (Lumsden *et al.*, 1982), peas, cucurbits, tomatoes and peppers (Sivan *et al.*, 1984), in sugarbeet (Mukhopadhyay and Chandra, 1986), and in tobacco (Mukhopadhyay *et al.*, 1986).

In the present investigation, we report mycoparasitism and biocontrol potential of *T. harzianum* either alone or in conjunction with fungicidal seed treatment to control *Pythium* damping-off of cauliflower. Preliminary reports on this work have already been published (Mukherjee and Upadhyay, 1988; Mukherjee *et al.*, 1989).

MATERIALS AND METHODS

Eight antagonist isolates (three of *Trichoderma harzianum* Rifai, IMI nos. 304056, 304057; 304058; two of *Trichoderma viride* Pers: Fr., IMI nos. 304054, 304060; two of *Trichoderma koningii* Oud., IMI nos. 304055, 304059; one of *Gliocladium virens*, Miller, Giddens, Foster, IMI no. 304061) were screened for antagonism *in vitro* against *P. aphanidermatum* on potato dextrose agar (PDA) by dual culture technique described by Morton and Stroube (1955). For selection of antagonist, linear growth rate of each antagonist isolate was also recorded.

Hyphal interaction between *P. aphanidermatum* and the most potent antagonist, *T. harzianum*-2 (IMI no. 304057) was studied by taking

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small mycelial fragments from the zone of interaction of the two fungi in dual culture. These were put on glass slide, stained with cotton blue in lactophenol, teased out with the help of needles and observed under Olympus microscope at a magnification of x 600 and x 1500.

Four fungicides, Apron 35 SD (metalaxyl), Fytolan 50 WP (copper oxychloride) Thiram 75 WP (tetramethyl thiuram disulphide) and an experimental compound SAN 506 F 50 WP (mixture of copper oxychloride and Oxadixyl, 4:1) were assayed against *P. aphanidermatum* and *T. harzianum* by using poisoned food technique (Grover and Moore, 1961). The concentrations (a.i.) evaluated were 5,10 and 15 µg/ml in case of Apron and 50, 100 and 150 µg/ml in remaining fungicides.

The antagonist *T. harzianum* was mass cultured on sterilized wheat bran saw dust tap water mixture (WBSD) in the proportion of 3:1:3.5 w/w/v contained in autoclavable polypropylene bags. This is a modification of the wheat bran saw dust medium developed by Elad *et al.* (1980). A quantity of 60 g WBSD per bag (18 x 13 cm) was found optimum for the growth of the antagonist. The bags were inoculated by making two slits on one side with a sterilized blade and then introducing four discs (11 mm) of *T. harzianum* cut from the edge of a 3-day-old plate culture. The slits were than sealed with adhesive tape and incubated at 28 ± 1°c for 10 days.

The pathogen was mass cultured by inoculating 6 mm mycelial discs in 250 ml Erlenmeyer flasks containing 75 g sterilized sorghum grains presoaked overnight in 2 percent sucrose solution. The flasks were incubated at 28 ± 1°c for 10 days. For artificial inoculation, 30 g culture of *P. aphanidermatum* was blended in 250 ml sterile water and 25 ml of this suspension was drenched in each pot and mixed with the surface soil to a depth of 3 cm.

A glasshouse experiment was conducted to study the biocontrol efficacy of *T. harzianum*. Plastic pots of 2 kg capacity, non-autoclaved sandy loam soil and the cauliflower variety 'Pant Shubhra' were used throughout the investigations. Thirty seeds per pot were sown at a depth of 1 cm. The average temperature of the glasshouse during the experiment ranged from 25°c to 33°c. Pot soil duly infested with *Pythium* was amended with 10-day-old WBSD preparation of *T. harzianum* at four different rates *viz.*, 5,10,20 and 30 g/pot mixed thoroughly with upper 3 cm soil. After 7 days, sowing was done with untreated seeds or seeds coated with *T. harzianum* spore suspension (1.6 x 10^{10} spores/ml). For seed coating, the seeds were soaked in *Trichoderma* spore suspension (1.6 x 10^{10} spores/ml) prepared in sterile water for 6h and then air dried before sowing. For treating one g seed, 0.2 ml of spore suspension was used. Observation on seedling mortality was recorded 20 days after sowing.

Ten-day-old WBSD preparation of *T. harzianum* live or autoclaved, *Trichoderma*-coated seeds (1.6 x 10^{10} spores/ml) and double sterilized WBSD were evaluated to assess their effect on growth and vigour of cauliflower seedlings in pot soil where no pathogen was added. The preparations were mixed with upper 3 cm soil at the rate of 20 g/pot, 7 days before sowing. After emergence, 15 plants were maintained in each pot. Seedlings were uprooted 20 days after sowing to record the height, fresh weight and dry weight.

In a Chemical control experiment, seeds were treated with Apron @ 8 g/kg seed, Fytolan, SAN 506F and Thiram @ 5 g/kg seed by dry seed dressing. Thirty seeds in each pot were sown after 1 day of *Pythium* infestation. Observation on seedling mortality was recorded 20 days after sowing.

All the fungicides evaluated as seed treatment for the control of damping-off were tried in conjunction with soil application of 20 g WBSD preparation of *T. harzianum* per 2 Kg pot to study the effect on disease control.

From the biocontrol experiment, soil samples upto a depth of 3 cm were taken 1,15 and 30 days after inoculation of *T. harzianum*. The CFU of *Trichoderma* were enumerated by dilution plate technique (Johnson, 1957) on *Trichoderma* selective medium (TSM) developed by Elad *et al.* (1981), except that Dexon was replaced by 15 µg/ml of Apron 35 SD. The soil samples from different treatments were serially diluted to get a 10^{-3} dilution. One ml of this suspension was poured on the solidified TSM and spread evenly by horizontal shaking. The plates were incubated for 72 h and observations on CFU were recorded with the aid of a colony counter. CFU of *Pythium* were enumerated by sprinkling 100 mg of soil per plate

on solidified *Pythium*-selective medium (PSM) developed by Peethambaran (1975). The plates were incubated at 28 ± 1°c for 72 h.

RESULTS AND DISCUSSION

All the antagonists (*T. harzianum, T. viride, T. koningii* and *G. virens*) overgrew the colony of *Pythium* in dual culture. *G. virens* took minimum time (68 h) to completely over-grow the *Pythium* colony. However, by that time, *T. harzianum*- attained a linear growth of 86.6 mm, statistically equal to that of *G. virens* (Table 1). In monoculture, *T. harzianum*-2 and 3 attained 90 mm growth in 56 h, while *G. virens* attained a linear growth of 75 mm exhibiting the slowest growth rate. *T. harzianum*-2 in terms of both antagonism and growth rate proved to be superior to all and therefore, selected for further studies.

Table 1. Linear growth of antagonist isolates in dual and monoculture

Isolates	Linear growth (mm)	
	Dual culture (68h)	Mono culture (56h)
T.harzianum-1	83.30^{b}	78.33^{c}
T.harzianum-2	85.60^{ab}	90.00^{a}
T.harzianum-3	82.00^{b}	90.00^{a}
T.viride -1	83.33^{b}	84.33^{b}
T. viride-2	83.30^{b}	82.00^{b}
T.koningii 1	83.00^{c}	84.00^{b}
T. koningii -2	77.66^{a}	83.33^{b}
G.virens	90.00^{a}	75.00^{d}

In vertical columns means followed by similar letters are not different statistically (P = 0.05) by Duncans multiple range test.

Microscopic examination of the hyphal interaction between the antagonist and the pathogen reveled that, initially, the hypha of *T. harzianum* ran parallel and got adpressed to the hypha of *P. aphanidermatum*. Thereafter, contact was established and, in most cases, the hypha of *Trichoderma* coiled around the *Pythium* hypha. The hyphal coiling was sparse or intense. At the point of contact, sometimes, knob-like structures were produced by *Trichoderma* hypha which seemed to be penetrating the *Pythium* hyphae. Irregular septation in *Pythium* hypha as a result of contact with the antagonist was also a common feature. The hyphae of *Trichoderma* were occasionally found in the lumen of *Pythium* hyphae revealing direct penetration. The ultimate result of all acts of mycoparasitism was partial to complete digestion of protoplasmic contents and lysis of *Pythium* hyphae. The results indicate that *T. harzianum* is a destructive mycoparasite on *P. aphanidermatum*. There are reports regarding coiling of *Trichoderma* around *Pythium* hyphae (Bell *et al.*, 1982; Hadar *et al.*, 1984; Mukhopadhyay *et al.*, 1986) and direct penetration (Fajola and Alasoadura, 1975). Formation of septa in *Pythium* hypha may be attributed to an attempt to prevent the draining-out of the cell contents due to lysis (Mukhopadhyay *et al.*, 1986).

Apron at 5,10 and 15 µg/ml and Thiram at 100 and 150 µg/ml showed complete inhibition of linear growth of *P. aphanidermatum in vitro* (Table 2). Thiram at the lowest concentration tested (50 µg/ml) gave 75.9 percent growth inhibition. Fytolan and SAN 506 F proved to be inferior to Apron or Thiram and caused 82.6 and 72.2% inhibition in linear growth of *Pythium* at 150µg/ml. No adverse effect on radial growth of *T. harzianum* was noted in Apron, Fytolan and SAN 506 F upto 15, 100 and 150µg/ml concentrations respectively. However, Thiram 50, 100 and 150µg/ml concentrations could inhibit the growth by 35.2, 74.1 and 81.5 per cent respectively.

Table 2. Effect of fungicides on the linear growth of *P aphanidermatum* on PDA incubated at 28 ± 1° for 36 hours

Fungicides	Concentration(µ g/ml)	Linear growth (mm)	Inhibition (%)
Apron	5	0.00^{a}	100.00
	10	0.00^{a}	100.00
	15	0.00^{a}	100.00
Fytolan	50	90.00^{g}	0.00
	100	76.67^{f}	14.81
	150	15.67^{b}	82.58
SAN 506F	50	71.67^{e}	20.36
	100	35.00^{d}	61.10
	150	25.00^{c}	72.21
Thiram	50	21.67^{c}	75.92
	100	0.00^{a}	100.00
	150	0.00^{a}	100.00
Check		90.00^{g}	

Means followed by similar letters are not different statistically (P = 0.05) by Duncans multiple range test.

Soil application of different rates of *T. harzianum* resluted in 32.4 to 77 per cent disease control, the minimum being in 5 g and maximum in 20 g/pot (Table 4). Coating of seeds with spore suspension of *Trichoderma* gave 31 per cent dis-

Table 3. Effect of *Trichoderma harzianum* (TH) on the growth and vigour of cauliflower seedlings

Treatments	Rate (g/pot)	Height (cm)	Increase in height (%)	Fresh weight (mg)	Increase in fresh weight (%)	Dry weight (mg)
TH	20	64.14[b]	11.32	640.00[b]	60.00[b]	99.99
TH (autoclaved)	20	72.42[a]	25.68	772.50[a]	82.50[a]	174.99
WBSD (autoclaved)	20	60.87[bc]	5.64	515.00[cd]	40.00[c]	33.33
TH (Seed coating)	1.6×10^{10} spores/ml	62.39[b]	7.69	560.00[bc]	40.00[c]	33.33
Check		57.42[c]		447.50[d]	30.00[c]	

In vertical columns, means followed by similar letters are not different statistically (P = 0.05) by Duncans multiple range test

Table 4. Biological control of *Pythium* damping-off of cauliflower by *T. harzianum* (TH) under glass house condition

Rate of TH application(g/pot)	Seedling mortality (%)	Disease control (%)
5	45.86	32.42[c]
10	29.35	56.75[b]
20	15.59	77.02[a]
30	19.25	71.62[a]
Seed coating (1.6×10^{10} spores/ml)	46.78	31.07[c]
Check	67.88	

Means followed by similar letters are not different statistically (P = 0.05) by Duncans multiple range test.

ease control. Significant variations in disease control were not observed between *Trichoderma* seed coating and 5 g/pot of soil application, as also in 20 g and 30 g/pot of soil application of *Trichoderma.*

Soil application of both autoclaved and live WBSD culture of *T. harzianum* significantly increased the height, fresh weight and dry weight of cauliflower seedlings, while double- autoclaved WBSD or *Trichoderma* seed-coating failed to do so (Table 3). Autoclaved culture of *T.harzianum* increased the height, fresh weight and dry weight of seedlings by 25.7, 72.6 and 175 per cent respectively, and proved superior to the effect of live cultures of *T. harzianum* in respect to growth promotion. This observation leads to the presumption that fermented organic matter may be playing an important role in growth promotion in addition to other factors.

Seed treatment with all the four fungicides gave significant disease control over check. Seed-treatment with Apron resulted in maximum disease control (85.7%). This observation is supported by its high degree of lethality to *P. aphanidermatum in vitro*. The disease control recorded with SAN 506 F and Thiram was 54.3 and 52.9 percent respectively (Table 5).

Table 5. Chemical control of *Pythium* damping-off in Cauliflower under glass house condition

Fungicides	Seed treatment rate (g/kg)	Seedling mortality (%)	Disease control (%)
Apron	8	9.16	85.72[a]
Fytolan	5	55.87	15.70[c]
SAN 506 F	5	29.35	54.28[b]
Thiram	5	30.27	52.28[b]
Check		64.21	

Means followed by similar letters are not different statistically

The integration of 20 g WBSD preparation of *T. harzianum* with fungicidal seed treatment significantly reduced the seedling mortality and gave 66.4 to 90 per cent control of the disease (Table 6). Intergration of *Trichoderma* with Apron or Thiram

Table 6 Integration of *T harzianum* (TH) and fungicidal seed treatment to control *Pythium* damping-off of cauliflower under glass house condition

Treatments	Seedling mortality (%)	Disease** control (%)
TH* + Apron 0.8 %	7.26	89.99[a]
TH + Fytolan 0.5 %	19.87	72.63[b]
TH +SAN506 F 0.5 %	24.38	66.44[b]
TH* + Thiram 0.5%	9.96	86.27[a]
TH	23.48	67.67[b]
Check	71.16	

* TH @ 20g
** Means followed by similar letters are not different statistically (P = 0.05) by Duncans multiple range test.

seed-treatment gave 90 per cent and 86.3 per cent disease control respectively. Disease control achieved with *T. harzianum* alone or in combination with SAN 506 F or Fytolan did not differ significantly among the different treatments. Integration of biological and chemical control seems to be a very promising way of controlling pathogens with a minimal interference with biological equilibrium (Papavizas, 1973). In this study, integration of biological and chemical control proved beneficial and improved the degree of disease control.

In a biological control programme by the introduction of an antagonist, monitoring the population of the antagonist as well as the pathogen is very important (Mukhopadhyay, 1987). This may be helpful in assessing the persistence of the antagonist and the pathogen in soil. The results on CFU of *Trichoderma* per g soil at different time intervals revealed that all the treatments had significant higher population of *Trichoderma* as compared to check (Fig. 1). At all the three observations, there was no significant difference in CFU

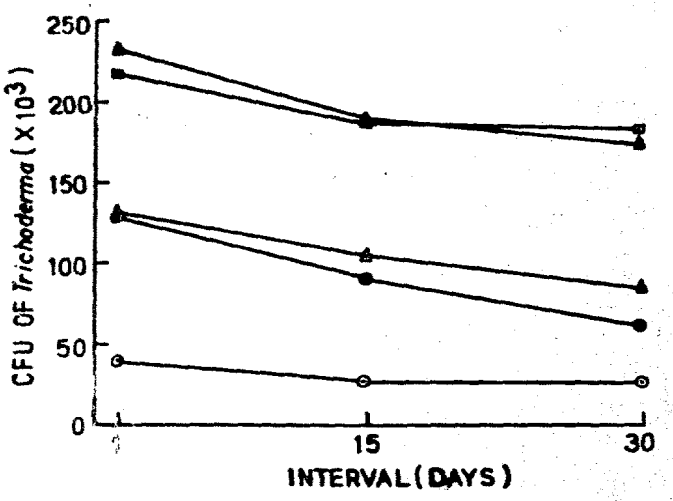

Fig. 1. Colony forming units (CFU) of *Trichoderma* per g soil from pots inoculated with WBSD preparation of *T. harzianum* @ 5g (●), 10g (Δ), 20g (▲) and 30g (□) per pot and check (○).

between *T. harzianum* 5 g/pot and 10 g/pot. The same was true for *T. harzianum* 20 g/pot and 30 g/pot. With time, a decline in *Trichoderma* population was observed in all the treatments, though, the decline was sharper where it was applied at lower rates, i.e., 5 and 10 g/pot, but the population was almost stable at higher rates of application, i.e., 20 and 30 g/pot. Where *Trichoderma* was applied @ 20 g/pot, the initial count on CFU was 217.5 x 10^3/g which declined to 186.2 x 10^3/g by the 16th day and to 182 x 10^3/g by the 30th day. Whereas, in check, the count on CFU 38.5 x 10^3/g initially, which declined to 26.7 x 10^3/g and 26 x 10^3/g by the 15th and 30th days respectively. The stability of *T. harzianum* populations at higher rates of application may be due to the higher saprophytic ability of the introduced antagonist at higher population density.

The CFU of *Pythium* sharply declined in *Trichoderma*-amended soil upto 15 days (Fig. 2).

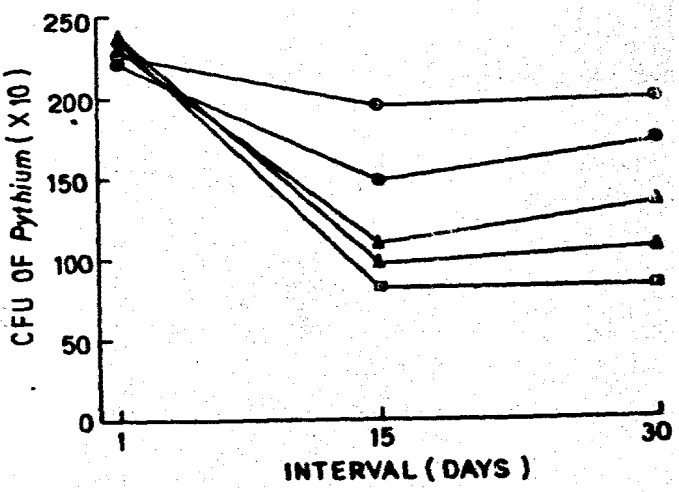

Fig. 2. Colony forming units (CFU) of *Pythium* per g soil inoculated with WBSD preparation of *T. harzianum* @ 5g (●), 10g (Δ), 20g (▲) and 30g (□) per pot and check (○).

On 15th day, the CFU was significantly less in all the *Trichoderma* amended soils. However, there was no significant difference in CFU of *Pythium* among *T.harzianum* 5 g and 10 g/pot, *T. harzianum* 10 g and 20 g/pot, *T. harzianum* 20 g and 30 g/pot. The CFU of *Pythium* was maximum in check (1940/g soil) and minimum in soil amended with *T. harzianum* at 30 g/pot (815/g soil). On 30th day, there was no significant differenc in CFU between check and soil amended with *T. harzianum* 5 g/pot. This was also true in case of *T. harzianum* 20 g and 30 g/pot. The CFU of *Pythium* in check was 1975/g soil on 30th day, whereas, it was 830/g in soil amended with *T. harzianum* @ 30g/pot. The decline in CFU of *Pythium* in *Trichoderma*-amended soil appears to be due to the direct antagonistic effect of *Trichoderma* on *Pythium*.

The results of the present investigation suggested high degree of biocontrol potential of *T. harzianum* against caluliflower damping-off caused by *P. aphanidermatum*. However, further investigations are needed to develop a suitable technology for application of *Trichoderma* in soil

and especially on seed for successful and economic control of this dreaded disease under field conditions.

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