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Species diversity of *Trichoderma* in the soils of Manipur and their antagonistic activities against Apple rot pathogen *Penicillium expansum*

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The present investigation was undertaken to select the most potential isolates of *Trichoderma* spp. and to observe their antagonistic activities against Apple rot pathogen, *Penicillium expansum.* Fifty five *Trichoderma* isolates were obtained from 30 soil samples collected from various parts of Manipur which were identified as 6 species of the genus, *T. viride, T. koningii, T. hamatum, T. virens, T. harzianum,* and *T. longibrachiatum.* All the isolates were screened for their *in vitro* antagonistic activities against the pathogen. Out of the fifty five *Trichoderma* isolates, 10 highly antagonist *Trichodermà* spp. were selected and evaluated against the pathogen through production of volatile and non-volatile inhibitors. Maximum inhibition of mycelial growth of the pathogen was shown by the metabolites of *T. viride* isolate (TV19). Three fungicides, viz., captaf (captan), blitox (copper oxychloride) and derosal (carbendazim) were evaluated against the pathogen and *Trichderma* isolates. Captaf was highly suitable for integration with all the antagonists as it totally inhibited the mycelial growth of the pathogen but less inhibitory to the antagonists.

Key words : *Trichoderma,* species diversity, *Penicillium expansum,* fungicides

INTRODUCTION

Penicillium expansum Link. causes severe rots on Apple during postharvest storage and commercialization. (Scherm *et al.,* 2003; LIma *et al.,* 2003). The existing fungicides for postharvest disease control are freequently used to control rots on apple. However, long exposure and high dose of fungicide led to the onset of pathogen-resistant strains and risks for consumers' health. Therefore, biological fruit protection using a biocontrol agent that does not leave a toxic residue may be an effective alternative to chemical control. Wilson and Wisniewski (1994) and Lima *et al.* (1999) have reported that the use of antagonistic microorganism is effective in reducing the indidence of postharvest fungal pathogens of different fruits. *Trichoderma* species have long been reconized as biocontrol agent for the control of plant diseases (Papavizas 1985; Adams, 1990). They are becoming widely used in horticulture (Harman *et al.,* 2004). Mukhopadhyay (1987) has emphasized that biological control of plant pathogen can be successfully exploited in modern agriculture especially within the framework of integrated pest management system.

The purpose of this work is two fold: (1) to assess the species diversity of *Trichoderma* occurring in the soil of Manipur and to observe the antagonistic activities of the most promising isolates of *Trichoderma* species against *P. expansum,* and (2) to evaluate tolerance limit of *Trichoderma* isolates and *P. expansum* to different fungicides at various concentration.

MATERIALS AND METHODS

Isolation and maintenance of Trichoderma spp.

Soil samples were collected from different part of Manipur representing varied soil and habitat types. *Trichoderma* spp. were isolated using Soil dilution plate method (Waksman and Fred, 1922) on *Trichoderma* selective medium (Elad *et al.,* 1981). The suspected colonies of *Trichoderma* were isolated on Czapeks agar medium in pure form and identified on the basis of their morphological characters (Rifai, 1969). An identification number was assigned to each of the *Trichoderma* isolates and stored at 4°C for fruther use.

Isolation and maintenance and pathogenicity test of p. expansum

P. expansum was isolated from the naturally infected apple fruit (*Malus domestics* Borkh.) and stored at 4°C in a pure form on Czapeks agar medium. Pathogenicity was proved by inoculating injured healthy fruits with spores of *P. expansum* isolated from rotted fruits, collected during a survey of retail fruit markets. Five healthy fruits were surface sterilized and injured by a sterilized needle. With the help of inoculating needle the mycelia from the margin of growing pathogen culture was inoculated on the injured area. Fruits were then covered with perforated polythene bags and incubated at 25 ±1°C for 10 days. Typical symptoms of the rot were recorded after the incubation period.

Dual culture technique

Two mycelial discs (5 mm dia.) removed from the margins of actively growing colonies of the test pathogen and biocontrol agent were placed 5 cm away from each other on opposite side of 90 mm dia. Petriplate, containing about 20 ml of Czapeks agar medium. The paired cultures were incubated at 25 ±1°C for 5-7 days and then scored for degree of antagonism on a scale of class 1-5 (Bell *et al,* 1982) as below: Class 1: Biocontrol agent completely overgrew the pathogen and covered the entire medium surface (highly antagonistic); Class 2 : Biocontrol agent overgrew at least two-third of the medium surface (antagonistic); Class 3: Biocontrol agent and the pathogen each colonized approximately one half of the medium surface and neither organisms appeared to dominate the other (moderately antagonistic); Class 4 : The pathogen colonized at least two third of the medium surface and appeared to withstand enchroachments by the biocontrol agent (poor antagonist); and Class 5 : The pathogen completely overgrew the biocontrol agent and occupied the entire medium surface (non antagonist).

Hyphal interactions

From the zone of interaction between the antagonist and *P. expansum* in dual culture plate, the mycelial mats were gently lifted with a needle and put in a drop of cotton blue on a microscopic slide, spread with needle and observed under microscope for hyphal interaction.

In vitro evaluation of highly antagonistic Trichoderma spp. against P. expansum

Ten isolates of *Trichoderma* spp. which showed Class 1 type of antagonism were selected and evaluated in laboratory to screen out the most efficatious one, which inhibits growth of pathogen by producing volatile and non volatile substances following the techniques described by Dennis and Webster (1971 a,b). Czapeks agar medium was used for all the experiments.

Effect of volatile metabolites

The *Trichoderma* isolates were centrally inoculated by placing 5 mm discs taken from 3 days' old culture on the Petriplates containing agar medium and incubated at 25 ±1°C for 3 days. The top of each Petridish was replaced with bottom of the Petriplate containing agar medium and inoculated centrally with the pathogen. Plates with agar medium without *Trichoderma* spp. at the lower lid and plates inoculated with mycelial disc of the *P. expansum* on the upper lid were maintained as control. The pairs of each plates were sealed together with cellophane adhesive tape and incubated as mentioned. Colony diameter of the pathogen was recorded and the inhibition of mycelial growth was calculated.

Effect of non-volatile metabolites

The isolates of *Trichoderma* spp. were inoculated in 100 ml sterilized Czapeks broth in 250 ml conical flasks. Inoculated flasks were incubated at 25 ±1°C for 15 days. The culture was filterd through Millipore filter and culture filtrate was added to molten agar medium (at 40°C) to obtain 5,10 and 15% concentrations. The medium was poured aseptically into the Petriplates and inoculated after solidification with 5 mm disc of the pathogen. Control plates were maintained without amending with the culture filtrate. The inoculated plates were incubated at 25 ±1°C. Radial growth of pathogen was recorded and per cent inhibition was calculated.

Evaluation of fungicides

Three fungicides viz., carbendazim 50 WP (derosal), captan 50 WP (captaf) and copper oxychloride 50 WP (blitox) were tested at 5 different concentrations (50,100,200,300 and 400 µg/ml) against the pathogen and antagonists *in vitro* by using Poisoned food technique (Fisher, 1969). 5 mm mycelial discs of pathogen and antagonists removed from 3 days' old cultures were inoculated to Petriplates containing fungicide amended agar media. Control diameter was recorded after 72 hrs of inoculation at 25 ±1°C and per cent growth inhibition over control was calculated.

In all the experiments proper control sets and three replications were maintained. The per cent growth inhibition in all above experiments was calculated by the formula I = C-T/C×100 where I = per cent growth inhibition, C = colony diameter of the fungus in control and T = colony diameter of the fungus in treatment.

RESULTS AND DISCUSSION

Out of 30 soil samples examined during the study period, one or more colonies of *Trichoderma* could be isolated from 25 soil samples. Fifty five *Trichoderma* isolates could be obtained which were distributed into 6 species of the genus, *T. viride, T. koningii, T. hamatum, T. virens, T. longibrachiatum* and *T. harzianum. T. viride* respresented the largest number of isolates (22 isolates) followed by *T. hamatum* (11 isolates), *T. koningii* (8 isolates, *T. harzianum* (7 isolates, *T. virens* (4 isolates), and *T. longibrachiatum* (3 isolates) (Table 1). Thus colonies of 6 species of *Trichoderma* could be isolated from 83.33% of the soil samples examined. It showed that the genus is fairly distributed in the soils of Manipur. Domsch *et al.* (1980) stated that *Trichoderma* spp. is widely distributed all over the world and occur in nearly all types of soil expecially in those containing organic matter.

Table 1 : Distribution of *Trichoderma* spp. among different districts of Manipur

Trichoderma	Districts/Number of isolates									
species	IE	IW	BP	TB	CD	CCP	SP	TL	UK	Total
T. viride	2	7	1	5	–	1	1	1	4	22
T. virens	–	1	–	2	1	–	–	–	–	4
T. hamatum	2	3	–	2	1	–	2	–	1	11
T. koningii	–	3	–	1	–	–	1	1	2	8
T. harzianum	–	1	–	1	1	–	1	2	1	7
T. longibrachiatum	–	2	–	–	–	–	–	1	–	3
Total	4	17	1	11	3	1	5	5	8	55

IE=Imphal East; IW=Imphal West; BP=Bishnupur; TB=Thoubal; CD=Chandel; CCP=Churachandpur; SP=Senapati; TL=Tamenglong; UK=Ukhrul.

Table 2 : Distribution of *Trichoderma* isolates among different classes of antagonism against *P. expansum*

Trichoderma	Antagonism class/Number of isolates					Total
species	1	2	3	4	5	
T. viride	5	14	1	1	1	22
T. virens	–	3	–	1	–	4
T. hamatum	2	6	3	–	–	11
T. koningii	1	5	1	1	–	8
T. harzianum	1	6	–	–	–	7
T. longibrachiatum	1	2	–	–	–	3
Total	10	36	5	3	1	55

In dual culture method, 10 isolates, 5 belonging to *T. viride* (TV5, TV6, TV7, TV19 and TV20), 2 belonging to *T. hamatum* (TH8 and TH10) and 1 each belonging to *T. koningii* (TK3), *T. harzianum* (TH5) and *T. longibrachiatum* (TL1) had shown strong antagonism against *P. expansum* and were grouped under Class 1. The largest number of isolates (36 isolates) were grouped as antagonists (Class 2), 5 isolates as moderately antagonists (Class 3), 3 isolates as poor antagonists (Class 4) while 1 isolate do not show any antagonism and was grouped under Class 5 (Table 2). Among the species *T. viride* was most frequently isolated from the soils of Manipur. This species seems to be more

Table 3 : Effect of volatile metabolites of *Trichoderma* isolates on colony growth of *P. expansum*

Trichoderma species	Incubation period (hr)						
	24		48		72		Mean growth inhibition (%)
	Colony diameter (mm)	Inhibition (%)	Colony diameter (mm)	Inhibition (%)	Colony diameter (mm)	Inhibition (%)	
TL1	6.0	11.76	11.3	11.02	16.3	6.86	9.86
TV6	6.1	10.29	11.5	9.45	17.2	1.71	7.15
TK3	6.5	4.41	11.8	7.09	17.3	1.14	4.21
TH8	6.3	7.35	11.8	7.09	16.5	5.71	6.72
TH5	5.0	26.47	9.5	25.20	13.6	22.29	24.65
TV19	4.8	29.41	8.0	37.01	10.0	42.86	36.43
TV20	5.2	23.53	9.5	25.20	13.3	24.00	24.24
TH10	5.2	23.53	10.0	21.26	13.8	21.14	21.98
TV7	5.8	14.71	11.7	7.87	17.3	1.14	7.91
TV5	5.7	16.18	11.3	11.02	17.1	2.29	9.83
Control	6.8	–	12.7	–	17.5	–	0.0
SEm±	0.19	–	0.29	–	0.50	–	–
CD at 5%	0.58	–	1.82	–	2.30	–	–

Table 4 : Effect of non-volatile metabolites of *Trichoderma* isolates on *P. expansum*

Trichoderma isolates	*P. expansum*					
	5% connentration		10% concentration		15% concentration	
	Radial growth (mm)	Inhibition (%)	Radial Growth (mm)	Inhibition (%)	Radial Growth (mm)	Inhibition (%)
TL1	17.30	4.79	17.20	5.34	17.00	6.44
TV6	14.70	19.10	14.50	20.20	14.20	21.85
TK3	17.60	3.14	17.40	4.24	17.30	4.79
TH8	18.70	-2.92	18.50	-1.82	18.00	0.94
TH5	17.30	4.79	16.80	7.54	16.50	9.19
TV19	15.30	15.80	13.20	27.35	11.30	37.81
TV20	15.20	16.35	14.90	18.00	14.70	19.10
TV10	18.30	-0.72	18.20	-0.17	17.70	2.59
TV7	17.00	6.44	16.50	9.19	16.30	10.29
TV5	18.70	-2.92	18.50	-1.82	18.00	0.94
Control	18.17	–	18.17	–	18.17	–
SEm±	0:04	–	0.03	–	0.06	–
CD at 5%	0.52	–	0.35	–	0.35	–

Table 5 : Effect of different concentrations of fungicides on the radial growth of *P. expansum* and 10 highly antagonists *Trichoderma isolates*

		P. expansum		TL1		TV6		TK3		TH8		TH5		TV19		TV20		TH10		TV7		TV5	
Fungicide	Conc. µg/ml	Colony diameter (mm)	Inhib-tion (%)	Colony diameter (mm)	Inhib-tion (%)	Colony diameter (mm)	Inhib-tion (%)	Colony diameter (mm)	Inhib-tion (%)	Colony diameter (mm)	Inhib-tion (%)	Colony diameter (mm)	Inhib-tion (%)	Colony diameter (mm)	Inhib-tion (%)	Colony diameter (mm)	Inhib-tion (%)	Colony diameter (mm)	Inhib-tion (%)	Colony diameter (mm)	Inhib-tion (%)	Colony diameter (mm)	Inhib-tion (%)
	50	0	100	37.3	58.6	26.0	71.1	20.0	77.8	40.0	55.6	32.0	64.4	35.0	61.1	20.0	77.8	21.0	76.7	25.0	72.2	20.0	77.8
	100	0	100	35.5	60.6	19.0	78.9	17.5	80.6	35.0	61.1	29.0	67.8	11.3	87.4	19.0	78.9	14.0	84.4	20.0	77.8	15.0	83.3
Captaf	200	0	100	34.0	62.2	10.0	88.9	11.0	87.8	30.0	66.7	25.0	72.2	11.0	87.8	11.0	87.8	12.0	86.7	18.0	80.0	10.0	88.9
(Captan)	300	0	100	23.0	74.4	8.0	91.1	10.8	88.0	26.0	71.1	17.5	80.6	10.3	88.6	9.0	90.0	10.0	88,9	11.0	87.8	9.0	90.0
	400	0	100	21.0	76.7	6.6	92.7	8.3	90.8	20.0	77.8	10.0	88.9	7.3	91.9	7.0	92.2	8.2	90.9	9.0	90.0	7.5	91.7
	50	15.0	23.8	25.0	72.2	38.0	57.8	80.0	11.1	47.0	47.8	47.0	47.8	55.0	38.9	70.0	22.2	58.0	35.6	37.0	58.9	80.0	11.1
Blitox	100	14.3	27.4	22.0	75.6	32.0	64.4	71.0	21.1	40.0	55.6	40.0	55.6	50.0	44,4	65.0	27.8	52.0	42.2	35.0	61.1	70.0	22.2
(Copper	200	13.0	34.0	20.0	77.8	30.0	66.7	55.0	38.9	36.0	60.0	35.0	61.1	43.0	52.2	55.0	38.9	48.0	46.7	31.0	65.6	65.0	27.8
oxyhloride)	300	10.0	49.2	18.0	80.0	27.0	70.0	50.0	44.4	31.0	65.6	30.0	66.7	39.0	56.7	50.0	44.4	34,0	62.2	28.0	68.9	59.0	34.4
	400	8.7	55.8	16.0	82.2	20.0	77.8	40.0	55.6	25.0	72.2	26.0	71.1	35.0	61.1	46.0	48.9	31.0	65.6	24.0	73.3	50.0	44.4
Derosal	50	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
(Carbendazi	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
-m)	200	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100	-	100
	300	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
	400	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
Control	-	19.7	-	90	-	90	-	90	-	90	-	90	-	90	-	90	-	90	-	90	-	90	-
SEm±	-	0.08	-	0.24	-	0.16	-	0.15	-	0.33	-	0.24	-	0.14	-	0.08	-	0.21	-	0.06	-	0.08	-
CD at 5%	-	1.04	-	8.11	-	4.74	-	4.48	-	4.74	-	8.21	-	4.65	-	1.69	-	7.84	-	0.94	-	1.69	-

adapted to the soil and climatic conditions of the state. Rai and Upadhyay (1978) noted *T. viride* and *T. harzianum* to be the most abundant species when they examined 36 soil samples collected from different parts of India.

Microscopic examination of slides prepared from the zone of interaction between antagonist and pathogen showed no coiling or penetration of the pathogen hyphae. In dual culture experiment the pathogen colonies stopped their growth in Petriplates upon contact with the antagonists and hypae began to lie back while the antagonists continued its growth over the pathogen colongy. Similar result was reported by Bagwan (2003) in dual culture experiment containing *Trichoderma* spp. and *Penicillium* spp. Tabebordbar *et al.* (2008) reported the inhibition of the mycelial growth of *P. expansum* by 11 *Trichoderma* isolates in dual culture method. Competition for both nutrients and space is an important contributing factor in biologicl control (Baker and Cook, 1974). The *Trichoderma* spp. being the fast growers are good competitors for both nutrients and space. The result of the present study suggest that antagonistic activities of the *Trichoderma* isolates against *P. expansum* may be attributed to antibiosis and competition.

The results (Table 3) revealed that the highest growth inhibition through the production of volatile metabolites at all the three observation period (24, 48 and 72 hrs) was induced by the isolates TV19 followed by TH5 and TV20. The least growth inhibition (4.21%) was recorded with metabolite of TK3. Difference in inhibition per cent may be due to differences in the quantity and quality of the inhibitory volatile substances produced by antagonists. Culture filtrate of all *Trichoderma* isolates inhibited the growth of pathogen in varying degrees (Table 4). The highest growth inhibition (37.81%) was observed with TV19 at 15% filtrate concentration followed by that of TV6 and TV20. *T. hamatum* (TH8) and *T. viride* (TV5) were least effective.Tabebordbar *et al.* (2008) observed significant growth inhibition of the *P. expansum* by that of TV6 and TV20. *T.hamatum* (TH8) and *T. viride* (TV5) were least effective. Tabebordbar *et al.* (2008) observed significant growth inhibition of the *P. expansum* by volatile and non-volatile metabolites of *T. harzianum* and *T. viride*. Among these more inhibitory isolates with higher concentrations of their culture filtrate could induce higher growth inhibition. The *Trichoderma* spp. (*T. hamatum, T. viride* and *T. harzianum*) are known to produce volatile (6-pentyl-α-pyrone) and non-volatile (Trichodermin, Suzukacillim and Alamethicine) antibiotics (Reusser, 1967). In the present investigation also efficient *Trichoderma* spp. emitted intense coconut odour and secreted yellowish green metabolites in growth medium.

Among the tested fungicides captaf was found most effective (Table 5). It caused complete inhibition of the pathogen at 50 μg/ml. All bioagents showed tolerance to captaf and proved the least inhibitory. It inhibited the growth of TL1 ranging from 58.56 to 76.67% and TH8 55.56 to 77.78% indicating that these two bioagents was less inhibited by captaf as compared with the other *Trichoderma* isolates. Tolerance to captaf by *Trichoderma* spp. *in vitro* is in conformity with Sharma *et al.* (2001) and Khalko *et al.* (2006). Blitox at 400 μg/ml. inhibited 55.84% growth of the pathogen and biocontrol agents ranging from 44.44 to 82.22%. Thus blitox is also moderately inhibitory to the pathogen and less inhibitory to some of the antagonists. Derosal showed 100% growth inhibition of the pathogen as well as antagonists at all the concentration tested. Similarly Khalko *et al.* (2006) and Jha *et al.* (2008) reported 100% growth inhibition of *Trichoderma* spp. by carbendazim. Carbendazim should not be used for integration with the antagonists. Control of *Penicillium* decay of apple fruit is known as very difficult to achieve by biologicl means, due to the high competitiveness of the pathogen in the wound niche (Wilson and Wisniewski, 1989, 1994; Spotts *et al.,* 1999; Janisiewicz and Korstin, 2002). Integrated management practice may be an alternative and efficient method for the control of apple rot.

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