Full Length Research Paper

Evaluation of disease control and plant growth promotion potential of biocontrol agents on *Pisum sativum* and comparison of their activity with popular chemical control agent - carbendazim

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The superiority of chemicals over biocontrol agents in terms of effective and quick disease control was confirmed in this research but the ill effects of chemicals on human health and environment are a major set back to application of chemical pesticides in the long run. In the present work, biocontrol agents showed significant control on *Fusarium* wilt disease of pea but the results were not consistent indicating that repeated and well planned application schedule was necessary to obtain desired results. In the greenhouse pot trial, biopesticides; *Trichoderma* and *Pseudomonas* showed 50% disease control on an average compared to carbendazim with about 83% result. On the contrary, deleterious effect of carbendazim on non target micro-organisms was also proved in another experiment of this research.

Key words: Biocontrol, pesticide, toxicity.

INTRODUCTION

Are pesticides a necessary evil? Quick and ensured effectiveness of chemical pesticide on pathogen gives it an edge over biopesticide. Yet, the health hazards of chemical pesticides and their ill effects on environment prove to be a major set back to the unrestricted use of these chemicals; leaving us in a dilemma. Attempts to use chemicals and biopesticides in combination and to gradually minimize use of chemicals are necessary keeping in consideration environment protection for future.

The present research discusses the need of gradually shifting to biological control methods from total dependence on chemical methods. Biocontrol agents may take some time to establish in soil before their significant effects are seen on disease control and crop yield. In certain cases, several application schedules over a period of time may be required to obtain desired effects. This is a limitation of biological methods but keeping in consideration human and animal health and ecology one has to give a thought to the problems that may arise from excessive use of chemical pesticides.

Forcelini et al. (2001) and Deising et al. (2008) focused specifically on alarming increase of fungicide resistance in plant pathogens of a number of crops which has become an issue of concern in modern agricultural practices in several countries.

Biological control methods involving use of natural antagonists of plant pathogens have been suggested as a safe alternative to chemical methods by several workers. Haggag and Amin (2001) found *Trichoderma* effective in controlling *Fusarium* root rot and nematode disease complex in sunflower. *Trichoderma* also enhanced seed germination (Mukhtar, 2008). On applying *Trichoderma* and *Pseudomonas* in combination to control *Fusarium* infection on chickpea better efficacies were recorded than using them singly (Khan et al., 2004). These biocontrol agents can also be formulated by simple methods. Workers have used various agents for formulation of *Trichoderma* such as peat, vermiculite, Koalin, bentonite, lignite, molasses, cellulose granules, diatomaceous earth, wheat bran, charcoal (Singh, 2003; Lewis et al, 1998; Cozzi and Gasoni, 1997; Prasad and Rangeshwaran, 2000; Sarode et al., 1998). Thus, culturing and maintaining biocontrol agents is inexpensive and uncomplicated. Biological pest control agents are relatively safer than chemical pest control agents, yet, their market value is weak and needs a boost up. This may be possible by educating and spreading awareness in farmers regarding hazards of excessive chemical application in fields.

Table 1. Randomized design of experimental setup.

Treatment set no.	Abbreviation	Treatments
1	F+T	*Fusarium* + *Trichoderma*
2	F+P	*Fusarium* + *Pseudomonas*
3	F+T+P	*Fusarium* + *Trichoderma* + *Pseudomonas*
4	F+CARB	*Fusarium* + Carbendazim (chemical pesticide)
5	T	*Trichoderma*
6	P	*Pseudomonas*
7*	F	*Fusarium*
8	T MED	Cow Dung-Wheat bran-Sand medium used for *Trichoderma* culture
9	P MED	Pea seeds soaked in uninoculated *Pseudomonas* selective medium
10	F MED	Wheat bran-Sand medium used for *Fusarium* culture
11*	No treatment	

*All comparisons were made with Treatment 7 and 11.

MATERIALS AND METHODS

Sampling, *in vitro* screening and identification of potential biocontrol agents were performed as a separate research and have not been detailed here. Potential biocontrol agents (*Trichoderma* and *Pseudomonas*) isolated from rhizosphere and rhizoplane of various plants were screened for antagonistic activity on pathogenic *Fusarium oxysporum* [NFCCI-2195] isolated from root of wilt afflicted pea plant. The agents showing best antagonistic activity (strain of *T. harzianum* which was later accessioned as *Trichoderma atroviride* [NFCCI-2063] after molecular identification by ARI, Pune (India) and *Pseudomonas* sp. P5 which was tentatively identified and coded by the author) were selected for further experiment. Modifications of published methods have been used as per availability of resources and environmental conditions.

Formulation of biocontrol agents

Cow dung, sand and wheat bran (3:1:1) were used for mass culture as well as formulation of *Trichoderma* in transparent polythene bags placed under blue light reflection. *Trichoderma* culture was stored below 20°C. *Pseudomonas* was cultured in *Pseudomonas* selective medium (HiMedium, M120) for 48 h and 20% (v/v) glycerol was added. The preparation was stored at about 10°C (Islam et al., 2006; Manikandan et al., 2010).

Preliminary test (*in vitro*) of interaction between host, pathogen, and biocontrol agents in combination

The compatibility of strains of *Trichoderma* and *Pseudomonas* used in this experiment were tested previously in a separate experiment not detailed here.

Pea seeds of Arkel variety obtained from Jabalpur (India) were soaked for three hours in *Pseudomonas* culture suspension (10^9 cfu of *Pseudomonas* per ml of *Pseudomonas* selective broth medium) diluted to 10^4 cfu/ml with sterilized distilled water.

A suspension mix was prepared with about 10^7 cfu/ml of pathogen and about 10^7 cfu/ml of *Trichoderma*. 0.5 ml of the suspension mix was spread on Water Agar medium and treated seeds were placed on the medium. Observations were taken after every 24 h. Infection of *F. oxysporum* on pea seeds was studied and evaluated by referring to methods mentioned by Ondrej et al. (2008) and Gunawardena (2005).

Greenhouse pot trial

The experiment was performed in earthen pots of 900 g capacity with dimensions of $13 \times 13 \times 5$ cm^3. Eleven sets of treatments were prepared in triplicates. Loam soil, obtained from agricultural field of Jabalpur, was autoclaved for 30 min twice on alternate days. Appropriate quantity of soil as per capacity of pots was mixed with test agents according to treatment chart mentioned Table 1. Surface disinfected (HgCl$_2$ 0.05%, 20 s.) and thoroughly washed healthy seeds of pea (*Pisum sativum*) were used in the experiment. The experiment was done in February 2010 applying completely randomized design of experimental setup shown in Table 1 (Singh et al., 2002; Nawar, 2007).

10% v/v of biopesticide (*Trichoderma*) and pathogen (*Fusarium*) at about 10^7 cfu/g medium were mixed in soil (that is, 100 g of mixture contained 10 g *Trichoderma* formulation, 10 g *Fusarium* formulation and 80 g soil) two days before sowing seeds whereas *Pseudomonas* was applied as seed treatment by soaking pea seeds in *Pseudomonas* suspension as mentioned in preliminary test. 2 g/100 ml of Carbendazim 50 WP was also used as seed treatment by soaking pea seeds for 2 h in the preparation (Ibiam et al., 2008). Seeds were sown in the pots maintaining appropriate distance. Water was supplied after intervals of 24 to 48 h as required. Readings were taken after about one month.

Parameters recorded to study growth enhancement included seed germination, fresh weight, dry weight and length of shoot and root, whereas, number of wilted and unwilted plants per pot was recorded to study disease control. The results were statistically analysed by ANOVA (software - Excel Data Analysis ToolPak) at α = 0.05.

(i) Calculation of wilt% per set:

(Number of wilted seedlings / Total number of seedlings) × 100

(ii) Calculation of disease control % per set:

[(wilt% in set 7- wilt% in set A) / wilt% in Set 7] × 100

*Set A represents all the sets except Set 7

Garden trial

In this experiment, bioagents were tested only for their effect on

plant growth and not on disease control. *Trichoderma* formulation of about 10^7 cfu/g viable density was mixed with top soil (7 to10 cm depth) in a garden plot of about 3×3 sq.ft. containing loam soil. Pea seeds treated with *Pseudomonas* as in pot trial were sown in the garden plot. Irrigation was provided at intervals of 24 to 48 h as required.

A control plot was also maintained. No treatment was given to soil or seed in this plot. The control plot was adjacent to the experimental plot. Care was taken to avoid flow of irrigation water from one plot to the other by building up a barrier between the two plots. Growth parameters were recorded and compared as in pot trial.

Effect of chemical pesticides on pathogen: *Fusarium*; biocontrol agent: *Trichoderma*; pea root symbiont: *Rhizobium* and general microbial flora of soil

Poison plate technique was employed to test the effect of carbendazim on micro-organisms. To prepare medium plate incorporated with 0.05 mg/ml of pesticide, carbendazim was dispensed in sterilized distilled water at concentrations of 0.5 mg/ml. 1 ml of this preparation was homogeneously mixed with 9 ml of medium and poured on 90 mm petri plate. Similarly, media plates with 0.25 and 1.25 mg/ml of pesticide concentrations were prepared (Rathore and Misra, 1988; Dhingra and Sinclair, 1995).

Pathogen and biocontrol agent were separately point inoculated in three sets of PDA (Potato Dextrose Agar) medium incorporated with three different concentrations of carbendazim (0.05, 0.25, 1.25 mg/ml).

Similarly, the general microbial flora of soil was subjected to this test. 1 g rhizosphere soil was suspended in 99 ml of sterilized distilled water. 0.1 ml of this suspension was spread on pesticide incorporated PDA medium set and NA (Nutrient Agar) medium set as mentioned previously.

Rhizobium were isolated from root nodules of pea in Yeast Manitol Agar medium and subcultured in Yeast Manitol broth without Congo red and then 0.1 ml suspension of this culture (10^{-1} dilution) was spread on pesticide incorporated NA medium plate set for each concentration. The response of the tested organisms was recorded and the experimental sets were compared with control sets.

RESULTS AND DISCUSSION

Formulation of biocontrol agents

Selected strain of *Trichoderma* (*T. atroviride* NFCCI-2063) and *Pseudomonas* sp. P5 were formulated for use in experiment and further storage. The basis of *Trichoderma* formulation was to use a preparation which could readily support growth and sporulation of *Trichoderma*. Cow dung, sand and wheat bran (3:1:1) were used for mass culture as well as formulation. These are materials which are easily available to farmers and are inexpensive. Such, that farmers can prepare it at their own production units on small scale.

Within 10 days *Trichoderma* spread and proliferated throughout the medium. In later stages heavy sporulation was achieved. Abundant green clumps of conidia were observed. *Trichoderma* load was about $1×10^7$ cfu/g of formulation. The agent was stored below 20°C. The formulation was found suitable as even after six months

the viable load did not fall below $1×10^5$ cfu/g.

Islam et al. (2007), used organic carrier material for colonization and multiplication of *Trichoderma* strains consisting of cow dung, poultry refuse, sand and maize meal. They obtained high density of the agent after 15 days and mentioned that storage temperature and light radiations influenced viability. Low temperature (4°C) was more suitable than room temperature and blue colour polythene bags were more efficient in maintaining viability than transparent bags or dark coloured bags as reflection of blue light induced conidiation (Horwitz et al., 1984; Berrocal-Toto et al., 1999; Islam et al., 2007). Ranasingh et al. (2006) used cow dung slurry and farmyard manure and found the preparation efficient for growth of *Trichoderma*.

Pseudomonas was stored in *Pseudomonas* selective broth medium supplemented with excess of glycerol (20% v/v glycerol) and stored at about 10°C.

Manikandana et al. (2010), tested trehalose, polyvinylpyrrolidone and glycerol for liquid formulation of *Pseudomonas fluorescens*. They found that glycerol supported the organism better that the other two agents. It was found suitable for seed treatment in controlling *Fusarium* wilt disease of tomato. The researchers also found that there was increase in tomato fruit yield.

Preliminary test of compatible combination of biocontrol agents against *Fusarium* on pea seeds

Before testing the selected biocontrol agents in greenhouse pot trials, a preliminary *in vitro* test was performed. The effect of interaction among the pathogen; *F. oxysporum*, and the biocontrol agents; *Trichoderma* and *Pseudomonas* in combination was evaluated on the seeds of pea. The application of biocontrol agents was found to be effective in controlling *Fusarium* infection of seeds. 100% germination and about 90% control of disease was obtained.

The results obtained in this experiment could not be very well reproduced in greenhouse pot trial. Various factors responsible for this are discussed in the study.

The enzymes and antibiotics produced by *Trichoderma* species and other biocontrol agents are strongly influenced by the substrate on which the agent is grown and conditions in the laboratory may occur seldom in nature or may not occur at all (Howell, 2003). Field trials are necessary to evaluate biocontrol agents and thereafter repeated application of the agent on the selected field may be required to obtain desired results

Greenhouse pot trial and garden trial to evaluate efficiency of biocontrol agents

Results obtained in the eleven sets of treatments were analysed. All the treatments were compared to Treatment set 7 in terms of disease control and

Treatment set 11 in terms of growth enhancement. Growth parameters (seed germination, fresh weight, dry weight and length of shoot and root) were evaluated in unwilted plants. Set 7 showed very clear symptoms of disease severity when compared to Set 11. In Set 7, every parameter studied reflected effect of pathogenic *F. oxysporum*. All the other treatments, in the experiment, used to control the pathogen showed significant effect but the magnitude of significance was not consistent in all the studied parameters of each treatment. The results did not fit in the frame of expectations completely.

The biocontrol agents, singly and in combination (Treatment set 1, 2 and 3) showed significant difference in terms of controlling disease symptoms when compared to Set 7 indicating partial control on *Fusarium* infection as per observation data (Table 2A, Figures 1 and 2). Set 1 (only *Trichoderma*) showed 62.6% disease control, Set 2 (only *Pseudomonas*) showed 36.1% control and Set 3 (*Trichoderma* + *Pseudomonas*) showed 50.6% control. Compared to all these sets the best disease control was observed in Set 4 (carbendazim) with 83.1% result. When evaluating biocontrol agents as growth promoters (Set 5 and 6) expected results were not obtained on comparing with Treatment set 11. But Set 3 showed significant increase in fresh weight and length of root. On an average, the biocontrol agents did not show considerable enhancement in growth of plants (Table 2A).

The significant decrease in disease symptoms obtained in Sets 1,2 and 3 may be attributed to various reasons like lowering of effective pathogen density due to presence of other microbes (the applied biocontrol agent) in the rhizosphere and rhizoplane of seedling; on account of a competitive edge. The other reasons may be direct antagonism shown by the biocontrol agents through production of inhibitory metabolites by both the control agents (*Trichoderma* and *Pseudomonas*). *Trichoderma* may also have shown parasitism under suitable conditions.

Garden trial of *Trichoderma* on pea

In greenhouse pot trials, use of autoclaved soil and measured densities of pathogen as well as control agents limits the arena of interactions and may not give a fair prediction of the performance of biocontrol agent on agricultural fields where the conditions and interactions may be several folds complex.

To study growth promoting property of *Trichoderma* and *Pseudomonas*, a garden trial was performed in February 2010. Observations were taken prematurely due to unfavourable climatic conditions. Significant results were obtained for root weight enhancement but not for shoot weight and length of shoot and root (Table 2B). The results obtained were poor in terms of growth enhancement of pea plants. This may be attributed to one or more factors mentioned subsequently.

Environmental factors influencing biotic interactions under *in vitro* and *in vivo* conditions vary greatly. These differences may in turn show variations in the results obtained under *in vitro* and *in vivo* conditions (Kucuk and Kivanc, 2003). In agricultural soil, density of pathogen, antagonist (biocontrol agent), occurrence of other soil microflora (qualitative and quantitative), soil composition, texture, temperature, pH, moisture content and other biotic and abiotic factors show a complex interaction with one another as well as with the seed or plant present amidst all these interacting factors. Therefore, there is a possibility of differences in results obtained in laboratory experiments, in greenhouse pots containing autoclaved soil and in agricultural fields of various regions. Hence, keeping in mind these factors repeated application of biocontrol agents and other soil ammendments over a period of time may be important to obtain desired results on field.

Inam-ul-Haq et al. (2009), obtained favourable results in their experimental field after four months of continuous application of *Trichoderma harzianum* to the field. These workers stated that the time of application was important according to climatic conditions of the area. As suggested by the authors, in areas where field remains fallow during summer season and there is intermitted rain, *Trichoderma* spp. can be applied to control *F. oxysporum* but this application is also dependent on the texture of soil as sandy clay loam soil gave better results than clay loam soil. Also, Spadaro and Gullino (2005), interpreted in their experiment that temperature, soil moisture and soil type affected efficacy of a biocontrol agent. In general, *Trichoderma* spp. are favoured by acidic soil conditions (Chet and Baker, 1981; Papavizas, 1985). pH values higher than 6.5, 6.0 and 5.2 are needed for maximal linear growth, conidiophore formation and conidium germination, respectively (Sivan et al., 1984; Geypens and Feys, 1976). Nutritional conditions in soil may also influence production of lytic enzymes by *Trichoderma* and play a role in pathogen suppression or enhancement (Kucuk and Kivanc, 2008; Nemeskeri and Gyori, 2005). Higher amounts of soluble K, P, Mg and total C and N in soil increase conduciveness to *Fusarium* infections of pea (Oyarzun et al., 1998). Sivan and Chet (1986) studied long term effect of *T. harzianum* on *Fusarium* wilt of cotton using successive plantings. The antagonist persisted in soil throughout three consecutive plantings, reducing *Fusarium* wilt incidence in each growth cycle. This implicates that immediate control of pathogen is not obtained as the biocontrol agent requires to establish itself in soil and gradually controls the pathogen over a period of time. Also, specific density of the agent should be maintained in soil for effective biocontrol. Increased concentration of *Trichoderma* in soil results in decreased rhizosphere colonization of *F.oxysporum* suggesting a competitive mechanism of biocontrol. Biocontrol agent may colonize root tip region and decrease probability of infection by *Fusarium* and

Table 2A. Effect of various treatments on growth of pea plants in greenhouse pot trial.

Parameter		Seed germ	Fresh wt (g)		Dry wt (g)		Length (cm)		Wilt (%)	Disease control (%)
		Out of 25 sown	Shoot	Root	Shoot	Root	Shoot	Root		
Treatment set no.	Treatments									
1	F+T	22.33^A	0.773^A	0.127^{AL}	0.060^A	0.027^{AL}	11.67^A	8.07^A	31	62.65
2	F+P	21.33^A	0.803^A	0.127^{AL}	0.063^A	0.043^A	10.70^A	8.63^A	53	36.14
3	F+T+P	22.00^A	0.757^A	0.237^{A,BL}	0.057^{AL}	0.048^A	10.23^A	13.90^{A,B}	41	50.60
4	F+CARB	23.67^A	0.800^A	0.217^{A,BL}	0.062^A	0.043^A	11.20^A	12.50^A	14	83.10
5	T	24.00	0.717	0.147	0.050	0.047	11.02	11.93	9.7	-
6	P	24.67	0.663	0.130	0.047	0.035	9.93	8.97	5.4	-
7*	F	16.00	0.500	0.030	0.025	0.007	6.73	2.07	83.3	0
8	T MED	24.33	0.787	0.140	0.062	0.037	11.63	9.80	5.5	-
9	P MED	22.33	0.677	0.103	0.050	0.032	9.67	6.60	6.0	-
10	F MED	23.67	0.813	0.107	0.062	0.038	10.60	7.67	1.4	-
11*	No treatment	24.33	0.823	0.137	0.060	0.047	11.77	10.53	4.1	-
P value		0.00147	0.00029	0.00013	0.018264	0.00713	0.000376	6.95E-11		
Turkey's HSD		5.82	0.201	0.109	0.034	0.033	3.05	2.86		
LSD		3.37	0.117	0.063	0.019	0.019	1.77	1.66		
α = 0.05 (Level of significance)										

*All treatments were compared to Set 7 and 11. A = Significant in terms of disease control; when compared to Set 7 as per HSD and LSD. B = Significant in terms of plant growth to Set 11 as per HSD and LSD. AL / BL = Significant only as per LSD. Set 8 to 10 did not show significant positive difference in readings; when compared to Set 11.

other pathogens that penetrate root tip (Sivan and Chet, 1989). The exudates from roots of plant species and their varieties influence biocontrol activity of *Trichoderma* (Badri et al., 2007). Root colonization by *Trichoderma* also enhances root growth and development, crop productivity, resistance to abiotic stresses and uptake of nutrients (Harman et al., 2004). Altomare et al. (1999), and also Sivan and Harman (1991) reported solubilization of phosphates and micronutrients by *Trichoderma harzianum* and also mentioned that *Trichoderma* is capable of colonizing and growing along the whole root system. And, can store solubilized phosphate gradually providing it to the plant throughout the life of the plant.

Pseudomonas have also been evaluated as promising biocontrol agents. According to van Loon et al. (1998), Lemanceau et al. (1993) and Duijff et al. (1993), *Pseudomonas putida* WCS358 was found to exhibit biocontrol activity on *F. oxysporum* on account of its iron chelating ability by secreting a pyoverdin type siderophore named pseudobactin 358. Similarly, *P. fluorescens* WCS417 was shown to be twice as effective against *F. oxysporum* as the previous strain. Maurhofer et al. (1995)

reported that *P. fluorescens* CHAO is a PGPR with effective mechanisms of disease suppression; behaves as an endophyte and produces several toxic metabolites against pathogen. Research has shown compatibility of *Trichoderma* strains with bacterial antagonists. Wahid (2006) found that using biocontrol agents in combination gave better results than using them singly. *Trichoderma viride* and *T. harzianum* were proved to be compatible with *P. fluorescens* and suppressed seedling disease of chilli and tomato tomato significantly when they were applied together (Rini and Sulochana, 2007; Chaube and Sharma, 2002). But,

Figure 1. Treatment 5, *Trichoderma* (left) and Treatment 7, *Fusarium* (right); wilting was observed in Treatment 7 but yellowing was not prominent.

Figure 2. Treatment 5, *Trichoderma* (left) and Treatment 3, *Fusarium* + *Trichoderma* + *Pseudomonas* (right).

Table 2B. Evaluation of effect of *Trichoderma* on plant growth in a garden trial.

Treatment	Fresh weight (g)		Dry weight (g)		Length (cm)	
	Shoot	Root	Shoot	Root	Shoot	Root
Trichoderma	0.959	0.2955[B]	0.18	0.065	13.35	11.12
No treatment	1	0.195	0.0835	0.0485	13.04	10.64
P value	0.565	0.04	0.271	0.038	0.683	0.603
Turkey's HSD	0.147	0.096	0.181	0.016	1.57	1.91
LSD	0.147	0.096	0.181	0.015	1.57	1.91
α = 0.05						

B = Significant in terms of plant growth promotion as per HSD and LSD.

in the present research the combination of selected strains of *Trichoderma* and *Pseudomonas* did not show better result than their separate applications. This suggests that the best compatible match needs to be selected before the agents are used in combination.

On using *Trichoderma* in combination with chemical fungicides, it is necessary to test the compatibility of the fungicide with *Trichoderma*. In this research it was found that carbendazim inhibited *Trichoderma* and *Fusarium* alike. Other workers have also found similar results where the category and dosage of chemical pesticide was important. Wang et al. (2005) found that fludioxonil inhibited *Fusarium* strongly but showed little effect on *Trichoderma*. Singh and Singh (2007) evaluated five pesticides (carbendazim, captan, vitavax, monocrotophos and thiram) against seven biocontrol agents, namely, *T. viride-1, T. viride-2, T. harzianum-1, T. harzianum-2, T. harzianum-3, Glioclodium virens* and T-35 (*T. harzianum*) and found that all the pesticides inhibited growth of all these biocontrol agents to varying extends.

The success of biocontrol agents is greatly influenced by the competence of pathogen to establish itself in soil and the opportunity to infect the host plant. Kraft and Wilkins (1989) studied severity of *Fusarium* disease in terms of soil compaction. The author stated that soil compaction restricts root growth and branching which limits nutrient availability and also allows pathogens to germinate under the influence of root exudates. This increases the chances of root tip infection as the roots take longer time to grow away from pathogen. Other than soil compaction, deficient or excess soil moisture and high temperature increase severity of *Fusarium* root rot. Under stress conditions *Fusarium* infection may progress faster and show symptoms early. This suggests, proper tillage and unrestricted growth of roots is an important factor in reducing *Fusarium* wilt disease.

Therefore, an integrated pest management (IPM) schedule by a balanced application of biological, chemical and physical methods is necessary. In each of the IPM schedules followed year after year, the proportion of chemical pesticides should be reduced gradually.

Effect of pesticide carbendazim on microbial flora of soil

In the greenhouse pot trial, the best disease control activity was shown by carbendazim. Although the chemical is very effective in controlling the target organism; the pathogen but it also hinders growth of other micro organisms present in the agricultural ecosystem. Tables 3A and 3B show the effect of this pesticide on the pathogen, the potential biocontrol agent and other microbes as well.

Carbendazim (methyl benzimidazol-2-yl carbamate) is a systemic fungicide. It acts by interfering with microtubule formation during mitosis in fungal cells. Carbendazim has been categorized as a mutagen (Food and Protection Act, 1985, Part III, Evaluation on carbendazim, Pesticides Safety Directorate, Kings Pool.).

In this research, Tables 3A and 3B clearly indicate that chemical pesticide carbendazim is mainly non selective in nature. The response shown by microbes at varying concentrations of carbendazim is discussed further. In control set (0.0 mg/ml carbendazim), normal growth of *Fusarium* and *Trichoderma* (Figure 3) as well as of other microbes was noted. In treatment set; 0.05 mg/ml carbendazim, growth of *Fusarium* and *Trichoderma* both was very effectively restricted for the first 48 h, but thereafter, within one week both *Fusarium* and *Trichoderma* showed very difficult recovery from pesticide inhibition (Figure 4). The growth of *Fusarium* was about 1 mm in radius. The mycelia did not spread out in the medium, instead the organism clumped itself on the point of inoculation. It seemed as if it was difficult for *Fusarium* to derive nutrition from the medium yet it was stacking on its own mycelia clump. After about one month there was slight increase in the radius of growth (1.5 mm). This indicated that the organism strived to overcome the effect of pesticide although there was no spread of mycelia.

Under such stress conditions there are chances of mutations in fungi as pesticides are known to induce

Table 3A. Effect of carbendazim on plant pathogenic *Fusarium oxysporum* and potential biocontrol agent *Trichoderma harzianum*.

Organism / Pesticide (mg/ml)		*Fusarium*	*Trichoderma*
			Radius
0.0	48 h	8.5 mm	20 mm
	1 week	25 mm	Exceeds 45 mm, plate overwhelmed
	1 month	30 mm	Rising on walls of plate with heavy sporulation
0.05	48 h	No growth even after heavy inoculation	No growth even after heavy inoculation 1 mm;
	1 week	Probability of very feeble growth, hardly differentiated by naked eyes	Within one week white mycelial growth seen in the form of compact clump; Sporulation and maturation of spores in one week
	1 month	1.5 mm	No further visible development
0.25	48 h	No growth	No growth
	1 week	1 mm	Within one week white mycelial growth in the form of clump; Sporulation and maturation of spores in one week
	1 month	2 mm	No further visible growth
1.25	48 h	No growth	No growth
	1 week	No growth	No growth
	1 month	No growth	No growth

mutations in biological systems (Deising et al., 2008). At 0.05 mg/ml carbendazim, there was inhibition of growth but the fungi was not killed, indicating its potential to overcome the effect of pesticide. Also it had potential to become resistant to this pesticide in course of time. Similarly, *Trichoderma* strived to survive and, like *Fusarium*, managed to show feeble growth in the form of clump restricted to its point of inoculation.

Within the limit of 1 mm radius of that clump it showed sporulation and maturation of spores and avoided spread of mycelia. This organism also shows potential of mutation under stress conditions (Serfling et al., 2007; Deising et al., 2008). Such possibility occurs when concentrations of pesticides are fungi-static and not fungi-cidal. D'Mello et al. (2000), in a similar experiment, studied graded concentrations of carbendazim on mycelia growth and toxin production of *Fusarium* and found that the effect was dose related. This phenomenon was also observed by Aggarwal et al. (2005) in their experiment on fungicides.

Further in this research, at 0.25 mg/ml carbendazim, *Fusarium* showed about 2 mm radius of growth which is slightly more as compared to 1.5 mm at 0.05 mg/ml concentration. Although, the reason for this seems to be inexplicable in the present research, induced variations in physiology by random mutations and environmental conditions could be responsible. In case of *Trichoderma*, the response at 0.25 mg/ml carbendazim was similar to that at 0.05 mg/ml.

At 1.25 mg/ml, carbendazim proved to be effectively fungicidal for both the fungi even after one month no signs of growth were observed. The fungal inoculums

from these plates showed no growth when streaked on to pesticide free medium.

Pathogenic and non-pathogenic, beneficial and non beneficial fungi as well as bacteria, all were, more or less, affected by chemical pesticide carbendazim. The effect of carbendazim on *Rhizobium* was also evaluated. It was found that at 1.25 mg/ml there was noticeable decrease in number of colonies although at lower concentrations (0.05 and 0.25 mg/ml) pesticide did not pose much deleterious effect. Therefore, as interpreted from Table 3B, it is clear that *Rhizobium* is not free from the effect of pesticide carbendazim. Walley et al. (2006) in their research found that chemical pesticides affect nitrogen fixation and nodulation by *Rhizobium* in pulse crops. Similarly, Ebbels (1967) also found that pesticides show deleterious effect on nodulation in *Pisum sativum L.* and may also be lethal to *Rhizobium*.

In the present research, on studying the effect of pesticide on general microbial flora of soil yeasts were found to be the most resistant organisms to pesticide carbendazim, although, they were affected at higher concentration (1.25 mg/ml). With increase in concentration of pesticide there was marked decrease in number of microbial colonies in general, as shown by Table 3B. At 1.25 mg/ml, moulds were the most affected followed by actinomycetes. Yeasts and bacteria were comparatively less affected. But, at this concentration, a predominant colony of *Fusarium* was observed (Figure 5). This is a very strong indication that strains of *Fusarium* (and probably other fungi) exist in nature which are resistant to carbendazim and can proliferate even at high concentrations of this pesticide. The excessive use

Table 3B. Effect of carbendazim on general microbial flora of soil.

| Pesticide (mg/ml) \ Organism	*Rhizobium* (On NA medium)	Soil microflora (On NA medium)	Soil microflora (On PDA medium)
	(Number of colonies observed after 48 h)		
0.0	Confluent growth, overlapping colonies, slimy, water droplet appearance, transparent to translucent colonies.	Confluent growth, mixed culture of bacteria (rods, cocci, Gram positive and Gram negative).	Countless yeasts and actinomycetes, some bacterial colonies as well. Moulds grew later- *Mucor* and *Aspergillus* dominated, other unidentified moulds also appeared throughout the plate.
0.05	Confluent growth, visibly more distinct and less overlapping colonies yet abundant to count, colony size- 1-1.5 mm in diameter.	ca. 200 large colonies of yeast, 2 mm diameter *ca,* 350 small colonies of yeasts and bacteria.	ca. 60 yeasts and 15 actinomycetes, *ca,* 250 very minute colonies of bacteria and yeasts, one prominent colony of *Fusarium,*; 3 cm diameter.
0.25	Abundant colonies visibly more distinct and less overlapping, translucent, slimy, water droplet like colonies, 1-1.5 mm diameter.	90 large colonies of yeast, 2 mm diameter, ca, 150 small colonies of yeasts and bacteria.	45 yeasts, 2 colonies of *Mucor*, 1 colony of *Aspergillus,* 3 actinomycetes on an average yeasts, moulds and actinomycetes appeared but in lower number.
1.25	Abundant colonies, non-overlapping, comparative decrease in density, marked decrease in size of colonies, pinhead sized colonies, less than 1 mm diameter, transparent to translucent , minute water droplet-like colonies.	110 colonies of yeasts, 2-3 mm in diameter and a few bacteria after 1 week *Mucor* was observed.	6 colonies of yeasts, 3-4 mm in diameter One colony of *Fusarium*, 1.5 cm diameter.

ca. = circa
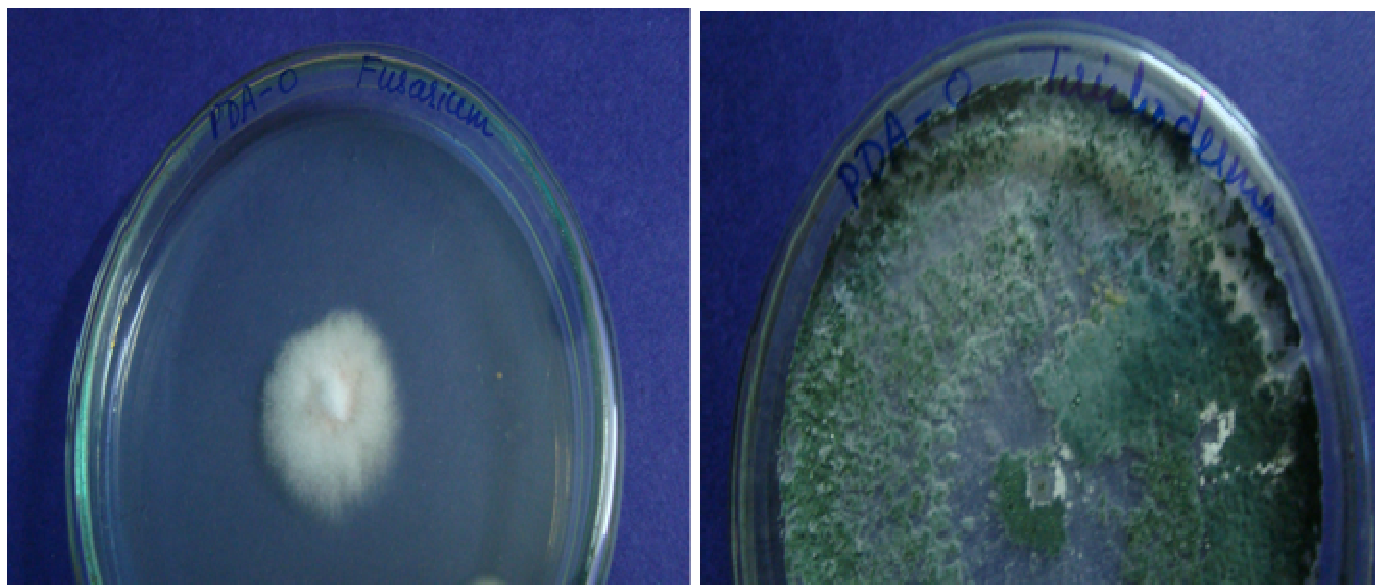
Figure 3. Unrestricted growth of *Fusarium* (left) and *Trichoderma* (right) on PDA medium without carbendazim.

Figure 4. Very feeble growth of *Fusarium* (left) and *Trichoderma* (right) on PDA medium incorporated with   0.05 mg/ml of carbendazim.
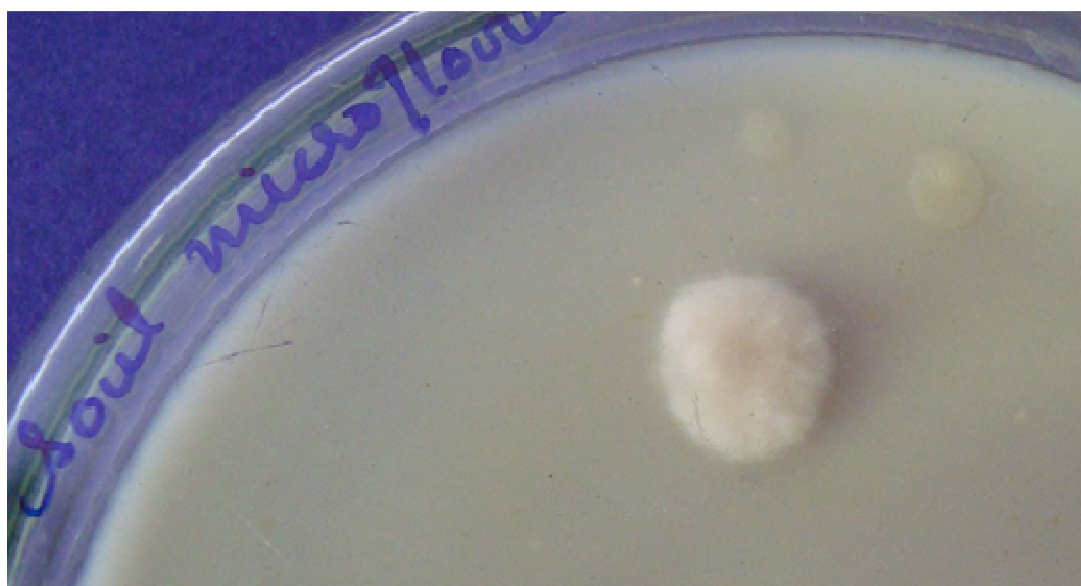

Figure 5. A resistant strain of *Fusarium* species growing on PDA medium incorporated with 1.25 mg/ml of Carbendazim. Excessively high concentration of carbendazim gives milky appearence to the medium.

of pesticides can encourage high densities of such resistant fungi resulting in occurrence of new strains of pathogens (Karaca and Kahveci, 2010). This phenomenon may become a bigger threat to agriculture. Chen and Zhou (2009) stated that benzimidazole fungicides particularly, carbendazim have been consistently used for over a period of thirty years or more in the Asian subcontinent. The effectiveness of such popular fungicides is being threatened by the emergence of resistant pathogen population in the field.

Deising et al. (2008), discussed occurrence of fungicide resistance under two categories- qualitative and quantitative and stated that qualitative resistance was due to mutations in genes encoding fungicide targets where as quantitative resistance was due to sub lethal fungicide stress. In the category of quantitative resistance, several different mechanisms may be involved such as keeping the intracellular concentration of fungicide low by synthesis of efflux transporters that can secret fungicide to the extra cellular space. Other mechanisms involve modifications of plasma membrane causing reduced fungicide permeability or synthesis of enzymes that degrade fungicide molecules (Deising et al., 2008). Environmental imbalances and irremediable damage to living beings is also a major concern.

Material safety data sheet for Bavistin (carbendazim) issued by CRPCARE (2007) categorizes this product under hazardous substance and states that the product may cause heritable genetic damage, may impair fertility and cause harm to unborn child. Studies on animals

indicate that prolonged exposure to carbendazim can cause damage to liver, kidney and thymus.

Therefore, the most popular chemical pesticides in the present times could, possibly, become a major threat to human health and environment in future. Under such circumstances, biological control of plant pathogens can be an option to look up to.

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