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STUDIES ON TOLERANCE AND SENSITIVITY OF FUNGAL AND BACTERIAL BIOAGENTS TO THREE PESTICIDES COMMONLY USED IN AGRICULTURE

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ABSTRACT

An investigation was undertaken to appraise the tolerance and sensitivity of bio agents (*Pseudomonas fluorescens*, *Bacillus subtilis*, *Trichoderma viride* and *Trichoderma harzianum*) towards three groups of pesticides using poisoned food technique. Pesticides like Carbenazim, Imidacloprid and Pendimethalin were used at recommended dose and its lower and higher concentration. Tolerance potential of bioagents was assessed under laboratory conditions. Comparatively Imidacloprid found to be more adaptive to all bioagents. However *Trichoderma* spp. was highly sensitive to Carbendazim at all its concentration tested. Among the bacterial bioagents, *P. fluorescens* was found tolerant to Imidacloprid @ 0.02 % was (24.66×10^8 cfu/ml) and Pendimethalin @ 0.3% was (24.33×10^8 cfu/ml) but in Carbendazim at its recommended dose (0.1%), the number of colonies were decreased to 16.00×10^8 cfu/ml. *Bacillus subtilis was* found less sensitive to Carbendazim @ 0.1% (20.00×10^8 cfu/ml) and Imidacloprid @ 0.02% (11.00×10^8 cfu/ml). Mycelial growth of *Trichoderma harzianum* and *Trichoderma viride* were found tolerate to Imidacloprid @ 0.02% *i.e.* 84.66 and 79.66 mm respectively and also tolerate to Pendimethalin @ 0.2% *i.e.* 72.66 mm and 84.66 mm respectively, but both were sensitive to Carbendazim at all concentrations.

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INTRODUCTION

Since the middle of last century, the use of organic synthetic pesticides became a widespread practice, in order to better prevent, control and destroy pests. Despite their usefulness in the increment of food production, the extensive use of pesticides during production, processing, storage, transport or marketing of agricultural commodities can led to environmental contamination and to the presence of residues in food (Massiha *et al*., 2011). Some pesticides are found to be highly persistent in nature thereby causing contamination of soil, ground and surface water. To slow down the deleterious effect of pesticides in ecology of crops, microbes which have efficiency to utilize these pesticides can be explored and utilize in IDM system. So, it is necessary to have strains of biocontrol agent that are compatible and not much sensitive to chemical pesticides and can be successfully incorporated in integrated disease management (IDM), IPM and weed management programme without any reduction in their antagonistic population as well as virulence. Search for potential biocontrol agents for the management of plant diseases has been intensified in recent years to reduce the dependence on ecologically hazardous chemicals (Pandey *et al*., 2006). Thus, *Fluorescent pseudomonas*, group of one of these promising biocontrol agent play an important role in biocontrol of most soil borne plant pathogens. Many of them promote plant growth by suppressing pathogenic microorganisms, synthesizing growth-stimulating plant hormones and promoting increased plant disease resistance (Choudhary *et al*., 2009). The idea of combining biocontrol agents (BCA) with pesticides is for the development or establishment of desired microbes in the rhizosphere (Papavizas and Lewis, 1981). Hence, the present investigation was therefore undertaken to determine the tolerance and sensitivity of bacterial and fungal bioagents to the pesticides and also for determining *in vitro* compatibility of bioagents with the commonly used fungicides, insecticides, herbicides and antibiotics.

MATERIALS AND METHODS

Isolation of bioagents

Soil samples were collected from major kharip crops field in Akola districts of Maharashtra. King's B media (Shweta Sharma *et al*., 2014; Kamei *et al*., 2014; Mina Koche *et al*., 2013) and Nutrient agar media were used for isolation of *P. fluorescens* and *B. subtilis*. One ml of soil suspension from aliquot dilutions 10^5 to 10^8 was aseptically added to sterile Petri plates containing twenty ml of sterile medium and incubated at $28 \pm 2°C$ for 48 h. Colonies of bioagents were identified by biochemical tests. After 48 hrs individual colonies were picked up with sterile loop and transferred to fresh King's B and Nutrient agar slants, the pure cultures so obtained were stored in a refrigerator at 4°C for further use. For isolation of *Trichoderma* soil sample was serially diluted and plated on *Trichoderma* selective media and incubated for 7 to 10 days. Sub culturing with actively growing colony of *Trichoderma* sp. was done on selected and plated on PDA medium (Islam *et al*., 2008). Biochemical characterization of bacterial isolates was done as per Bergey's manual of systemic bacteriology.

Biochemical studies

Biochemical test *viz*., oxidase test, starch hydrolysis, gelatine liquefaction, catalase test and citrate production test were carried out for biochemical conformation of *P. fluorescens* and *B. subtilis* (Gade *et al*., 2014; Aneja, 2003).

Oxidase test

An inoculating loop was taken. A well isolated colony was spreaded on an oxidase disk. The reaction was observed within two minutes at 20-30^{0}C. Deep purple blue indicates positive reaction (Tariq *et al*., 2016).

Starch hydrolysis

Starch is a complex carbohydrate of the polysaccharide type hydrolysed by bacterium. The positive test indicates the presence of amylase enzyme utilized for the hydrolysis of starch. Inoculate the bacteria on the starch agar plates and incubated for two days. After incubation flooded the plates with Lugol's iodine solution. Presence of starch hydrolysis was indicated by the appreance of clear reddish zone *i.*e. the starch was partially hydrolysed to dextrin (Poonam Kumari and Veena Khanna, 2016).

Gelatin liquefaction

Filter paper discs were dipped in a day old culture suspension and were placed on Petri dishes containing gelatin nutrient agar medium. The Petri dishes were incubated at 30^{0}C for two days and then flooded with 12.5 per cent $HgCl_2$ solution. The development of yellow halo around the growth indicates utilization of gelatine (Rashi Jaisingh *et al*., 2016).

Citrate utilization test

This test was performed by inoculating the microorganism into an organic synthetic medium, Simmon's citrate agar, where sodium citrate is the only source of carbon and energy. Bromothymol blue was used as an indicator. When citrate acid was metabolized, the CO_2 generated and combined with sodium and water to form sodium carbonate, which changed the colour of indicator from green to blue and this constitutes a positive test (Venkateswar Reddy *et al*., 2016).

Catalase test

This test was used to indicate the presence of catalase enzyme. Inoculate the petriplate with the bacteria and incubate it for three days. A bit of growth was removed from the plate and placed on a slide, to which 3% H_2O_2 was added. Appearance of bubbles showed the positive test for catalase (Tariq *et al*., 2016).

Growth promoting activity

Siderophore production

Evaluation of isolates with universal Chromeazurol assay (CAS) helps in detection the siderophore by *Fluorescens pseudomonas*. This assay mainly depends on the colour zone i.e. orange zone against dark blue background, a positive indication for the presence of siderophore. All the isolates were screened by CAS method (Schwyn and Neilands, 1987) for their ability to produce siderophore.

IAA production

The IAA test is performed by inoculating a bacterium into tryptone broth, the indole produced during the reaction is detected by adding Kovac's reagent which produces a cherry red reagent layer (Sharma *et al*., 2014).

Tolerance and Sensitivity test for isolated bioagents

Three pesticides *viz*., Imidacloprid, Carbendazim and Pendimethlin were tested against isolated biocontrol agents using poisoned food technique. Bacterial isolates were tested *in vitro* to check their compatibility to pesticides at its three concentrations. The bacterial suspensions, at the concentration of 10^8 cfu/ml, were pipette out in Petri dishes containing King's B agar and Nutrient agar amended with the Carbendazim @ 0.05%, 0.1% and 0.2%, Imidacloprid @ 0.01%, 0.02% and 0.03% and Pendimethalin @ 0.2%, 0.3% and 0.4%. The suspensions were dispersed over the medium and the Petri plates were then incubated at 25^{0}C for 48 hr. After 48 hrs number of colonies counted and compared with control. While for the fungal bioagents, pesticides amended potato dextrose media were poured on petriplate and allowed them to solidify. Thereafter, the plates were seeded centrally with a 5 mm disc of 4 days old culture of *Trichoderma* species. Plate with fungi alone served as a control. The radial growth of mycelium in each treatment was measured and compared with the control.

RESULTS AND DISCUSSION

The experiment was carried out to study the tolerance/ sensitivity of *P. fluorescens, B. subtilis*, *T. viride* and *T. harzianum* to different pesticides. The step wise regression analysis was done at different a concentration of pesticides in

Table 1: Morphological and biochemical reactions of selected Isolates

Sr. No.	Characters	Reactions of isolates					
		Pseudomonas fluorescens			*Bacillus subtilis*		
		Pf 1	Pf 2	Pf 3	B1	B2	B3
Morphological properties of bacterial isolates							
1	Shape	Rod	Rod	Rod	Rod	Rod	Rod
2	Fluorescence	+ve	+ve	+ve	-ve	-ve	-ve
3	Gram reaction	-ve	-ve	-ve	+ve	+ve	+ve
Biochemical properties of bacterial isolates							
4	Gelatine liquefaction	+ve	+ve	+ve	+ve	+ve	+ve
5	Oxidase test	+ve	+ve	+ve	-ve	-ve	-ve
6	Starch hydrolysis	+ve	+ve	+ve	+ve	+ve	+ve
7	Citrate utilization	+ve	+ve	+ve	-ve	-ve	-ve
8	Catalase test	+ve	+ve	+ve	+ve	+ve	+ve

+ ve - Positive, - ve – Negative

Table 2: Growth promoting character of bacterial isolates

Sr. No.	Bacterial isolates		Siderophore production	IAA production
1	*Pseudomonas fluorescens*	Pf1	+ ve	+ ve
		Pf2	+ ve	+ ve
		Pf3	+ ve	+ ve
2	*Bacillus subtilis*	B1	-ve	+ ve
		B2	-ve	+ ve
		B3	-ve	+ ve

+ ve - Positive, - ve – Negative

Tables 3: Tolerance and sensitivity of *Psudomonas fluorescens* to different pesticides

Sr. n.	Treatments	C Concentration (%)	Number of colonies (cfu/ml 108)
1)	T1(Imidacloprid)	0.01	33.66
	T2(Imidacloprid)	0.02	24.66
	T3(Imidacloprid)	0.03	15.66
2)	T4(Carbendazim)	0.05	21.33
	T5 (Carbendazim)	0.1	16.00
	T6 (Carbendazim)	0.2	9.00
3)	T7(Pendimethalin)	0.2	32.00
	T8(Pendimethalin)	0.3	24.33
	T9 (Pendimethalin)	0.4	14.66
4)	T10(Control)		40.33
	SE(m) ±		1.10
	CD (P = 0.01)		4.80

Average of three replications

Table 4: Tolerance and sensitivity of *Bacillus subtilis* to different pesticides

Sr.No.	Treatments	Concentration(%)	Number of colonies(cfu/ml 10^8)
1)	T_1 (Imidacloprid)	0.01	17.33
	T_2 (Imidacloprid)	0.02	11.00
	T_3 (Imidacloprid)	0.03	5.66
2)	T_4 (Carbendazim)	0.05	24.30
	T_5 (Carbendazim)	0.1	20.00
	T_6 (Carbendazim)	0.2	7.66
3)	T_7 (Pendimethalin)	0.2	3.33
	T_8 (Pendimethalin)	0.3	0.00
	T_9 (Pendimethalin)	0.4	0.00
4)	T_{10} Control	-	35.00
	SE(m) ±		1.00
	CD (P = 0.01)		4.40

Average of three replications

which dependent variance in case of bacteria was number of colonies and in case of fungi was growth colonies in mm and independent variance was pesticides concentrations. This analysis was used to obtain an optimum concentration of pesticides and maximum count of bacteria.

The results of biochemical tests revealed that all isolates of *P. fluorescens* were rod shaped and gram negative and produced yellow colonies on king' B medium, showed a positive reaction for gelatine liquefaction, starch hydrolysis, oxidase test, citrate utilization test and catalase test (Table 1). The biochemical tests *i.e.* gelatin liquefaction, H_2S production, starch hydrolysis, casein hydrolysis and chrome-azurol assay for siderophore production further confirmed to be *P. fluorescens* (Asha *et al*., 2011). All thirty isolates were positive to catalase test and nineteen isolates were positive for gelatin liquefaction and six were able to hydrolysis starch (Koche *et al*., 2011; Armarkar, 2011). These results in present findings collaborates with the results of Dibua *et al*. (2014) also reported that *Pseudomonas* sp. was gram negative and motile rod shaped bacteria whereas *Bacillus subtilis* were gram positive, motile and spore forming rod. The results are also corroborates with the finding made by Gade *et al*., 2008.

Isolates of *Bacillus subtilis* were gram positive, rod shaped, white colour flat colony with serrated margin and showed positive response for gelatin liquefaction, starch hydrolysis and catalase test and showed negative response for citrate utilization test (Table 1) (Jayasri *et al*., 2015). Similar type of work was also carried out by Ratna kumari *et al*. (2012) who reported that the *B. subtilis* isolates were gram positive, rod shaped and showed a positive reaction for catalase test, starch hydrolysis, casein hydrolysis and gelatin liquefaction. Similarly Khan *et al*. (2011) also given the same result that *B. subtilis* were gram positive, rod shaped showed positive reaction for starch hydrolysis

Table 5: Tolerance of *Trichoderma viride* to different

Sr. No.	Treatments	Concentration(%)	Mycelium growth (mm) (DAI) 3rd	5th	7th
1)	T_1(Imidacloprid)	0.01	44.66	62.66	86.33
	T_2 (Imidacloprid)	0.02	33.00	56.00	79.66
	T_3 (Imidacloprid)	0.03	28.66	51.66	78.00
2)	T_4 (Carbendazim)	0.05	0.00	0.00	0.00
	T_5 (Carbendazim)	0.1	0.00	0.00	0.00
	T_6 (Carbendazim)	0.2	0.00	0.00	0.00
3)	T_7(Pendimethalin)	0.2	35.00	51.00	80.00
	T_8(Pendimethalin)	0.3	24.33	40.33	72.66
	T_9(Pendimethalin)	0.4	28.66	29.00	54.00
4)	T_{10} Control	-	47.33	73.66	89.33
	SE(m) ±		0.90	0.70	0.60
	CD (P = 0.01)		3.70	3.20	2.70

Average of three replications

Table 6: Tolerance of *Trichoderma harzianum* to different pesticides

Sr. No.	Treatments	Concentration (%)	Mycelium growth (mm)(DAI) 3rd	5th	7th
1)	T_1(Imidacloprid)	0.01	45.33	64.33	86.66
	T_2(Imidacloprid)	0.02	35.33	61.00	84.66
	T_3(Imidacloprid)	0.03	30.00	55.33	81.00
2)	T_4 (Carbendazim)	0.05	0.00	0.00	0.00
	T_5 (Carbendazim)	0.1	0.00	0.00	0.00
	T_6 (Carbendazim)	0.2	0.00	0.00	0.00
3)	T_7 (Pendimethalin)	0.2	21.33	52.66	81.33
	T_8 (Pendimethalin)	0.3	13.00	44.00	77.00
	T_9 (Pendimethalin)	0.4	10.00	26.00	41.33
4)	Control	-	48.66	71.00	89.66
	SE(m) ±		0.70	0.60	0.70
	CD (P = 0.01)		3.00	2.50	2.80

Average of three replications

Siderophore production and IAA production

Appearance of zone around the bacterial colony after 48 hrs of incubation indicated the strains of *P. fluorescens* have the ability to chelate Fe^{3+} from chromoazurol S agar medium. All collected isolates of *Pseudomonas fluorescence* showed positive reaction for siderophore production test. Formation of a red color layer over the tryptone broth which inoculated with bacterial inoculum confirmed the positive reaction for IAA production test. All the isolates of *P. fluorescens* showed a positive reaction for IAA production test (Table 2). These results in present findings collaborates with the results of Gate (2009) who assayed ten isolates of *P. fluorescens* and found that all isolates were positive for siderophore production. Twenty isolates were able to produce siderophore and seventeen isolates showed positive test for IAA production (Koche *et al.*, 2011). Similarly Armarkar *et al.* (2011) also reported that eight isolates were able to produce siderophore and thirteen isolates showed positive test for IAA production. Isolates of *Bacillus subtilis* showed negative reaction to siderophore and positive reaction to IAA production (Table 2). Production of indole substance or well known as Auxin synthesis is one of the important factor in plant metabolism Raupach and Kloepper (1998). Agarwal and Shruti Agarwal (2013) were also reported that all the isolates showed positive character in IAA production.

Tolerance and sensitivity test for isolated bioagents

Maximum tolerance concentration of *P. fluorescens* was found to be at lower concentrations of pesticides *i.e.* Imidacloprid @ 0.01% (33.66×10^8 cu/ml), Pendimethalin @ 0.2% (32.00×10^8 cu/ml) and Carbendazim @ 0.05% (21.33×10^8 cu/ml), these seem to be the safe tolerance limit to *P. fluorescens* (Table 3).

The colonies in the pesticide amended medium gradually started to decrease when the concentration of pesticides increased. The growth of bioagents were good even at its recommended dose *i.e.* T_2 (24.66×10^8cfu/ml), T_5 (16.00×10^8cfu/ml), T_8 (24.33×10^8cfu/ml) (Table 3). These results in present findings collaborates with the results of Report from Mathew (2003) indicated that *Pseudomonas fluorescens* was very well compatible to Imidacloprid. The observation of Laha and Venkataraman (2001) also reported that Carbendazim was very much compatible with *P. fluorescens*. The similar observation in result had been reported by Sing and Dube (2010) that *Psudomonas fluorescens* was compatible with all fungicides (Metalaxyl, Ridomil MZ, Captan, Difolatan, Thiram and Bavistin) at both 0.3 and 0.6% concentrations.

The maximum tolerance showed by *B. subtilis* were at the lower concentration of pesticides *i.e.* Imidacloprid @ 0.01% (17.33×10^8cfu/ml), Carbendazim @ 0.05% (24.30×10^8cfu/ml) and was moderately sensitive to Pendimethalin at its lower concentration *i.e.* @ 0.2% (3.33×10^8cfu/ml). *B. subtilis*

showed a moderate growth in recommended doses of pesticides *i*.e. Imidacloprid @ 0.02% (11.00 × 10^8cfu/ml), Carbendazim @ 0.1% (20.00 × 10^8cfu/ml). *Bacillus subtilis* whereas at certain extent sensitive to Imidacloprid @ 0.03% (5.66×10^8cfu/ml) (Table 4). Similarly Nasrin Sabourmo haddam *et al*. (2014) also studied the ability of the bacterial isolates *Bacillus* sp. to tolerate imidacloprid. Similar result had given by Shetti *et al*. (2014) that *Bacillus weihenstephanensis* was compatible to Imidacloprid. Geeta *et al*. (2014) also reported the similar findings that the nine bacterial isolate to tolerate pesticide like Endosulfan, Imidacloprid and Carbendazim.

T. viride showed maximum radial growth of mycelium in lower dose of Imidacloprid @ 0.01% (86.33 mm) and Pendimethalin @ 0.2% (80.00 mm) after one week of incubation, but showed a sensitive nature to Carbendazim fungicide in its all doses. *T. viride* showed good mycelial growth even for recommended dose of pesticides *i*.e. Imidacloprid @ 0.02% (79.66 mm) and Pendimethalin @ 0.3% (72.66 mm) (Table 5). Maximum tolerance concentration of *T. harzianum* was at the lower concentration of pesticides *viz*., Imidacloprid @ 0.01% (86.66 mm) and Pendimethalin @ 0.2% (81.33 mm) after one week of incubation, but *Trichoderma harzianum* was highly sensitive to Carbendazim *i*.e. there was no growth of mycelium (Swati Pandey *et al*., 2012). *T. harzianum* showed good mycelial growth even for recommended dose of pesticides *i*.e. Imidacloprid @ 0.02% (84.66 mm) and Pendimethalin @ 0.3% (77.00 mm) (Table 5). The results are in conformity with Islam *et al*. (2011) that the growth of *Trichoderma was* inhibited by Carbendazim. Similar observation had been reported by Deepika Saxena *et al*. (2014) who reported that *T. harzianum*, showed good radial growth of mycelium on Pendimethalin up to 250μl a.i./ml. The report of Desai and Kulkarni (2004) were also similar that *T. harzianum* showed inhibitory growth on Carbendazim. Khalko *et al*. (2005) also reported that the fungicide Bavistin and Dithane M-45 completely inhibited the growth of *T. harzianum* and *T. viride*.

The result of present study suggest that the bioagents which were isolated were able to grow in presence of added pesticides (Carbendazim, Imidacloprid and Pendimethalin) in the growth media and may thus be used for Bioremediation of pesticicide contaminated soil study resulted that *P. fluorescens* was able to tolerate Pendimethalin. Whereas, *B. subtilis* showed compability with Carbendazim.

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