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Effect of pesticide amended soil on population of *Pseudomonas fluorescens* under green house conditions

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Abstract
Pseudomonas fluorescens is an important beneficial microorganism that enhances growth of a crop plant and is used as a bioagent. It is ecofriendly and inhabitant of soil, which play an important role in integrated disease management. Isolates of *P. fluorescens* were selected on the basis of morphological and biochemical tests. All isolates were Gram negative and rod shaped. They were positive for arginine dihydrolysis, catalase and H_2S gas production. Amongst them, four isolates showed their ability to hydrolyze starch**,** two isolates showed citrate utilization, 13 isolates showed KOH test and 12 isoates showed urease test whereas, four isolates showed oxidase test. Among fifteen isolates, Pf_2 gave maximum growth inhibition of *Fusarium oxysporum* f. sp. *ciceri* (60.36%) and also of *Rhizoctonia bataticola* (60.50%). Enumeration of *P. fluorescens* population was high in the soil where *P. fluorescens* was added @ 15g/kg in pesticides amended soil. Whereas, *P. fluorescens* population was increased up to 60 days after incubation and decline at 90 days. All pesticides under test were compatible with *P. fluorescens*.

Keywords: *Pseudomonas fluorescens*, pesticides, fungicides, insecticides, herbicides, antibiotics

Introduction
Modern agriculture is highly dependent on chemicals for plant disease, pest and weed management. Residues of these pesticides remain in soil which cause environmental and soil pollution. 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) [22]. Thus, fluorescent pseudomonas, group of one of Thee 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) [6]. 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) [23]. Further, the antagonism of BCA was also influenced by the addition of fungicides (Kay and Stewart, 1994; Naar and Kecskes, 1999) [14, 20]. Since pesticides may have deleterious effects on antagonists, an understanding of their effect on antagonists, would provide information on the selection of selective pesticides and pesticides resistant antagonists for compatibility studies. Hence, the present study was therefore undertaken to determine *in vitro* compatibility of P. fluorescens with the commonly used fungicides, insecticides, herbicides and antibiotics.

Materials and Methods
Morphological Studies
The confirmation of the *P. fluorescens* isolates was made by streaking pure culture of isolates on King's B medium, separately. The individual colonies were examined for shape, size, pigmentation, and structure of colonies. The Gram reactions of the selected isolates were seen according to Buchanon and Gibbeson (1974) [5].

Physiological and biochemical properties
Physiological and biochemical properties of isolates were studied as per methods described in the Practical Bacteriology (Deshpande and Papdiwal, 1979) [7].

Oxidase test
A well isolated colony was touched and spread on an oxidase disk (Disk contains N, N-dimethyl-p-phenylenediamine oxalate and α-naphthpol). The reaction was observed within two minutes at 25-30ºC. Deep purple blue indicated positive reaction.

Arginine test
For Arginine test decarboxylase test media was prepared according to the Fay and Berry (1972) [8]. Purple colour indicated positive reaction and yellow colour or no colour change indicated negative reaction.

Starch hydrolysis
Starch is a complex carbohydrate of the polysaccharide type of hydrolyzed by bacterium. The positive test indicates the presence of amylase enzyme utilized for hydrolysis of starch. Inoculation of the bacteria on the starch agar plates was done and incubated for 2 days. After incubation the plates were flooded with Lugol's iodine solution. Presence of starch hydrolysis was indicated by the appearance of clear reddish zone indicated that starch was partially hydrolyzed to dextrin.

Citrate Utilization test
The citrate test is performed by inoculating the microorganisms 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 the citrate acid was metabolized, the CO_2 generated and combined with sodium and water to form sodium carbonate an alkaline product, which changed the colour of the indicator from green to blue and this constitutes a positive test.

Urease test
Urease test was performed by growing the test organisms on urea broth or agar medium containing the pH indicator phenol red (pH 6.8). During incubation microorganisms possessing urease, produced ammonia that raise the pH of the medium/ broth. As the pH becomes higher, the phenol red changes from a yellow colour (pH 6.8) to a red or deep pink (cerise) colour. Failure of the development of a deep pink colour due to no ammonia production is evidence of a lack of urease production by the microorganism.

Catalase Test
This test was used for indicating presence of catalase enzyme. Inoculated the nutrient agar tubes with bacteria and incubated for three days. A bit of growth was removed from the slants and placed on a slide, to which 3% H_2O_2 was added. Appearance of bubbles showed positive test for catalase.

Dual culture test
Antagonistic activity of *P. fluorescens* was examined against fungal plant pathogens i.e. *Fusarium oxysporum* f. sp. *ciceri* and *Rhizoctonia bataticola* by dual culture test and inhibition zone was observed after 7 days of incubation at 30^0C.

Enumeration of *Pseudomonas fluorescens* population (cfu/g soil) from pesticide amended soil
Carrier based culture of bioagent was prepared by using eight days old broth cultures in 1:2 proportion of culture and sterile talcum powder. Plastic pots having capacity of 1 kg soil were disinfected with sodium hypochloride. Sterilized soils were inoculated with carried based *P. fluorescens*. Pesticides *viz*. fungicides, insecticides, herbicides and antibiotics were added individually at single concentration in the soil. Carrier based culture of *P. fluorescens* was added @ 10 g/kg and 15g/kg in respective soil. Isolation of *P. fluorescens* was done by serial dilution method on King's B medium (King *et al*., 1954) [15]. Enumeration of population count of *P. fluorescens* was done by observing the bacterial colonies under *in vitro.*

Results and discussion
Morphology and staining reaction of fluorescent Pseudomonas
Morphological study was undertaken to identify the bioagent as *P. fluorescens*. Bacterial cells of all fifteen isolates were rod shaped, occurring singly or in pairs and Gram negative. The colonies were irregular and yellow green on King's B medium (King *et al*. 1954) [15] (Table 1).

Physiological and Biochemical characters
Physiological and biochemical properties of 15 isolates with respect of oxidase test, arginine dihydrolysis, starch hydrolysis, citrate utilization, urease, H_2S production, catalase and KOH were studied (Table 1).
All isolates were positive for arginine dihydrolysis, catalase and H_2S gas production. Out of 15 isolates, only four isolates showed their ability to hydrolyze starch**,** which was evident from the zones formed. Only two isolates showed positive reaction against citrate utilization. 13 isolates showed positive reaction against KOH test and 12 isoates showed positive reaction against urease test. Whereas, four isolates showed positive reaction for oxidase test (Table 1).
All fifteen isolates of Fluorescent Pseudomonas were, rod, gram negative which produced round to irregular colonies with yellowish, dull yellowish and greenish yellowish, water soluble pigment production (Gate, 2009) [9]. These isolates were found positive for oxidase, catalase, urease and only three isolates were able to hydrolyse starch (Tiwari and Thrimurthy, 2007; Siddiqui and Shakeel, 2009) [27, 25]. 15 isolates were found capable of H_2S production (Mahesh, 2007 and Gate, 2009) [18, 9] and two were positive to citrate utilization (Shivani Bhatia *et al*. 2005; Gate, 2009; Nisharani Urkade, 2010; Belkar and Gade, 2012) [24, 9, 21, 3]. All isolates were positive for arginine Dihydrolysis activity (Ipper *et al*. 2005; Yeole and Dube, 2000) [12]. The results were also matching with the characteristics published in Bergey's Manual of Determinative Bacteriology, 7th edition (Breed *et al*. 1957) [4].

Effect of *Pseudomonas fluorescens* on growth of *Fusarium oxysporum* f.sp. *Ciceri* and *Rhizoctonia bataticola*
Observations on average colony diameter and percent growth inhibition were recorded. All isolates under test were efficient to check the mycelial growth of *Fusarium oxysporum* f. sp. *ciceri* and *R. bataticola* (Table 2). Data presented in table 2 indicate that Pf_2 was found effective to arrest the growth of *Fusarium oxysporum* f. sp. *Ciceri* (60.36%) whereas isolate Pf9 gave least percent growth inhibition (42.47%) with maximum mycelium growth (51.77mm). In case of *R. bataticola*, Pf2 showed maximum growth inhibition (60.50%) followed by Pf4 (43.70%) and Pf5 (43.70%) whereas, least growth inhibition was found by isolate Pf1 (32.46%) with maximum mycelia growth (60.78mm). Present findings were

also in line with Vidyasekaran and Muthamilan (1995) [28] who reported that the strain *P. fluorescens* Pf1, Pf27, Pf12 and Pf21 were inhibitory *in vitro* to *Fusarium oxysporum* f. sp. *ciceri* with inhibition zone of 41, 41, 35 and 14 mm, respectively. In dual bottom plate's assay, Fluorescent Pseudomonads (FPs) isolates *viz.*, AMET1039, AMET1041, AMET1042, AMET1055 and AMET1064 exhibited maximum mycelial growth inhibition of *Rhizoctonia solani* MML4001 due to the production of HCN (Jayaprakashvel *et al.*, 2010) [13].

Enumeration of *Pseudomonas fluorescens* population at different intervals from pesticides amended soil (10^8cfu/g soil)

The experiment was conducted to study the enumeration of population of *P. fluorescens* from pesticides amended soil at different intervals *i.e.*30, 60, and 90 DAI with application of *P. fluorescens* 10 g/kg soil and 15 g/kg soil.

Initially, the population of *P. fluorescens* was found o decrease at 30th day of observation whereas, it was found to increase at 60th day when used as 10 g/kg soil and 15 g/kg soil. Among all pesticides, Metalaxyl was better utilized by the *P. fluorescens* which resulted to get maximum count 17.97×10^8 cfu/g soil followed by COC (27.33×10^8 cfu/g soil) and benomyl (15.27×10^8 cfu/g soil) at different levels of application of fungicides amended soil with *P. fluorescens* applied @ 10 g/kg soil and 15 g/kg soil, respectively (Table 3). This is in accordance with the results of Guang *et al.* (1999) [10] who reported that application of carbendazim in soil enhanced the population of *P. fluorescens* while Mathew (2003) [19] reported that *P. fluorescens* was highly compatible with carbendazim and Mancozeb. Suslow and Schroth (1981) [26] reported that seed treatment with fungicides used for general disease control had no effect on *P. fluorescens* survival and benomyl, Thiram, carboxin, oxycrboxin and dizoben did not affect the growth of *P. fluorescens in vivo.* The results are in agreement with these findings.

In second set of experiment, the tolerance level of *P. fluorescens* was tested against insecticides where, maximum count was recorded in quinolphos (12.38×10^8 cfu/g soil) followed by spinosad (8.4×10^8 cfu/g soil) and in imidacloprid (15.10×10^8 cfu/g soil) followed by emamectin benzoate (14.45×10^8 cfu/g soil) after 60 days interval of incubation from insecticides amended soil where *P. fluorescens* was incorporated in soil @ 10 g/kg and 15 g/kg soil, respectively (Table 4). According to, Ahemad and Khan (2011) [1] *Pseudomonas putida* PS9 grew with the varying concentration of insecticides and showed varying degree of tolerance levels against the tested insecticides might be due to utilization of these insecticides as the only energy source.

Data presented in table 5 indicate that all herbicides *viz.*, imazethapyr, 2, 4-D and pendimethalin were compatible with *P. fluorescens.* Amongst them, maximum count was recorded in 2,4-D *i.e.* 15.10×10^8 cfu/g soil and 15.94×10^8 cfu/g soil under herbicides amended soil where *P. fluorescens* was incorporated @ 10 g/kg and 15 g/kg soil, respectively. Kumar *et al*., (1996) [16] stated that microorganisms can grow at higher concentration of herbicides. Beethi and Rajendra (2008) [2] reported that 2,4-D was compatible with *P. fluorescens.* Hung *et al.*, (2009) [11] reported that *P. fluorescens* strain IM-4 was capable of degrading imazethapyr as this strain could utilize Imazethpyr as the sole carbon and energy source.

Incorporation of antibiotics in soil showed maximum population of *P. fluorescens* was at 60 DAI. Maximum count was recorded in streptomycin sulphate (4.43×10^8 cfu/g soil) and in combination with tetracycline + streptomycin sulphate (8.40×10^8 cfu/g soil) @ 10 g/kg soil and 15 g/kg soil *P. fluorescens* in antibiotic amended soil (Table 6). This result also in line with Lindaw *et al.*, (1996) [17] who reported that *P. fluorescens* strain A506 appears to be completely compatible with subsequent application of streptomycin sulphate. The aim of the experiment was fulfilled by detecting tolerance level of *Pseudomonas fluorescens* against pesticides which are being commonly used in plant protection system where these microbes can be better utilize to support plant and soil health.

Table 1: Morphological and biochemical properties of selected *Pseudomonas fluorescens* isolates

Sr. No.	Characters	Pf_1	Pf_2	Pf_3	Pf_4	Pf_5	Pf_6	Pf_7	Pf_8	Pf_9	Pf_{10}	Pf_{11}	Pf_{12}	Pf_{13}	Pf_{14}	Pf_{15}
Morphological properties																
1	Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
2	Pigmentation	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
3	Gram reaction	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Physiological and Biochemical properties																
4	Starch hydrolysis	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
5	Citrate utilization	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
6	Catalase activity	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
7	H_2S production	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
8	Urease test	+ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
9	KOH test	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
10	Arginine dihydrolysis	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
11	Oxidase test	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Table 2: Efficacy *of Pseudomonas fluorescens* isolates against *Fusarium oxysporum* f.sp. *Ciceri* and *Rhizoctonia bataticola*

Isolates	Mycelium growth(mm) (7 DAI)		Growth inhibition % (7 DAI)	
	FOC	*R. bataticola*	FOC	*R. bataticola*
Pf_1	46.00	60.78	48.88	32.46
Pf_2	35.67	35.55	60.36	60.5
Pf_3	46.89	52.00	47.9	42.22
Pf_4	40.67	50.67	54.81	43.7
Pf_5	45.00	50.67	50.00	43.7
Pf_6	51.0	51.33	43.33	42.96
Pf_7	41.00	90.00	53.71	32.46

Pf_8	46.11	Sig	48.76	60.5
Pf_9	51.77	0.7	42.47	42.22
Control	90.00	3.09		
SE(m)±	0.4	35.55		
CD (P=0.01)	1.57	52.00		

Table 3: Enumeration of *Pseudomonas fluorescens* (10g/kg and 15g/kg soil) population at different intervals from fungicides amended soil. (10^8cfu/g soil)

Tr. No	Treatment	Concentration	30 DAI		60 DAI		90 DAI	
			10g/kg	15g/kg	10g/kg	15g/kg	10g/kg	15g/kg
T_1	Metalaxyl	0.1%	2.33	3.03	17.97	15.27	3.47	4.00
T_2	Mancozeb	0.2%	0	2.73	1.4	2.13	2.17	1.87
T_3	Benomyl	0.1%	2.4	2.53	4.87	15.27	2.67	4.07
T_4	COC	0.2%	1.47	27.33	2.47	13.13	2.73	3.83
T_5	Carbendazim	0.1%	1.43	2.47	11.0	3.93	3.93	4.17
T_6	Thiram	0.2%	1.47	2.43	4.5	6.87	2.37	3.93
T_7	Control		23.67	32.00	37.07	37.50	9.83	11.83
S.E.(m)±			0.06	0.07	0.04	0.11	0.10	0.09
C.D. (p=0.01)			0.27	0.28	0.18	0.48	0.44	0.38

Table 4: Enumeration of *Pseudomonas fluorescens* (10g/kg and 15g/kg soil) population at different intervals from insecticides amended soil. (10^8cfu/g soil*)*

Tr. No	Treatment	Concentration	30 DAI		60 DAI		90 DAI	
			10g/kg	15g/kg	10g/kg	15g/kg	10g/kg	15g/kg
T_1	Quinolphos	0.1%	3.13	4.70	12.38	11.88	4.85	3.18
T_2	Imidacloprid	0.01%	1.8	4.70	8.4	15.10	5.3	2.80
T_3	Spinosad	0.02%	1.2	1.95	8.4	3.58	4.23	3.25
T_4	Emamectin Benzoate	0.03%	4.8	9.68	5.8	14.45	5.23	2.40
T_5	Control		24.63	30.75	37.25	35.98	9.9	11.50
S.E.(m)±			0.11	0.12	0.11	0.42	0.11	0.09
C.D. (p=0.01)			0.49	0.49	0.48	1.76	0.46	0.39

Table 5: Enumeration of *Pseudomonas fluorescens* (10g/kg and 15g/kg soil) population at different intervals from herbicides amended soil. (10^8Cfu/g soil)

Tr. No	Treatment	concentration	30 DAI		60 DAI		90 DAI	
			10g/kg	15g/kg	10g/kg	15g/kg	10g/kg	15g/kg
T_1	Imazethapyr	0.1%	2.06	3.26	10.9	13.70	4.00	3.02
T_2	2,4-D	0.2%	15.10	14.30	13.40	15.94	3.82	3.24
T_3	Pendimethalin	0.3%	1.94	3.48	3.92	15.12	4.9	2.48
T_4	Control		24.10	31.36	36.80	37.55	9.94	10.18
S.E.(m)±			0.06	0.11	0.44	0.11	0.1	0.11
C.D. (p=0.01)			0.25	0.45	1.82	0.48	0.42	0.49

Table 6: Enumeration of *Pseudomonas fluorescens* (10g/kg and 15g/kg soil) population at different intervals from antibiotics amended soil. (10^8cfu/g soil)

Tr. No	Treatment	concentration	30 DAI		60 DAI		90 DAI	
			10g/kg	15g/kg	10g/kg	15g/kg	10g/kg	15g/kg
T_1	Tetracycline + streptomycin sulphate	0.01%	2.10	4.03	2.39	8.40	3.20	3.31
T_2	Streptomycin sulphate	0.01%	2.17	4.86	4.43	2.44	4.27	4.31
T_3	Control		23.37	31.07	36.86	37.07	9.77	10.79
S.E.(m)±			0.11	0.06	0.11	0.12	0.045	0.11
C.D. (p=0.01)			0.48	0.25	0.44	049	0.18	0.48

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