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PSEUDOMONAS AS A PROSPECTIVE CANDIDATE FOR MINIMIZATION IN USE OF CHEMICAL PESTICIDES OR THEIR GRADUAL REPLACEMENT WITH BIOCONTROL AGENTS ON AGRICULTURAL FIELDS

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Isolates of *Pseudomonas* species obtained from rhizosphere, rhizoplane and endophytic habitats of various plants were tested for their antagonistic activity against plant pathogenic *Fusarium oxysporum* isolated from the roots of a diseased pea plant. *Pseudomonas* isolate P5 showed best activity against *F. oxysporum* followed by P4, P22 and P17. Some isolates of *Pseudomonas*, P24, P25 and P26, grew unrestrictedly and completely covered the *Fusarium* colony. The antagonistic effect of *Pseudomonas* on *F. oxysporum* observed in this research can be attributed to the ability of antibiotic synthesis by *Pseudomonas* and also the ability to counter the toxic metabolites of *Fusarium oxysporum*.

Key words: plant disease, *Fusarium oxysporum*, biological control, *Pseudomonas*

Human has developed an agricultural ecosystem which is an artificial ecosystem for the cultivation of plants and animals appropriate for food. This artificial ecosystem has disturbed a steady balance between the host plant and pathogen allowing development of organisms pathogenic to the host crop plant in excess; therefore, the cultivated plants are more vulnerable to disease (Oku 1994). To overcome this problem, new varieties of crops are developed. But, after a period of time these new varieties also become susceptible to the pathogen, as new races of the pathogen develop with time (Oku 1994; Trigiano *et al*. 2004).

Fusarium wilts and root rot diseases have been a problem throughout the world for a long period of time. Padwick (1938), Buxton and Storey (1954), Haware (1971), Clarkson (1978), Manzies and Koch (1990), Keinath (1994) and many other researchers have discussed *Fusarium* wilt as a major problem in several crops. The problem still exists in the present times. Walsh *et al.* (2010), Polizzi *et al.* (2011) and several researchers have reported the spread of *Fusarium* wilt diseases to new host varieties of crops and in new regions of the world. A number of *Fusarium* species have been implicated in this problem, majorly *F. oxysporum*. The control of this organism has been an important confront as the pathogenic forms of *Fusarium oxysporum* show great genetic variability (Leslie & Summerell 2006).

There is an increasing consideration in using physical methods and cultural practices in disease management as alternatives to pesticides for the control of soil-borne pathogens (Katan 2000). But, the physical methods being unfeasible for the general masses of farmers and unsuitable for large areas may not be acceptable in the long run. Crop rotation is a cultural method of disease control which may be effective in some cases.

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Chemical treatment has been the most effective and popular method of disease control till date. Fungicides such as methyl bromide, captan and carbendazim have been used effectively against *Fusarium oxysporum* (Melero-Vara *et al*. 2005; Mukhtar 2007). But with time, the uncontrolled use of chemicals has posed many environmental problems and risks to the health of humans and other organisms. Therefore, there is a need to minimize the use of hazardous chemicals and gradually increase the use of natural and safe methods of controlling pathogen.

Widespread investigation on the biological interactions that occur in the rhizosphere of plants has revealed a number of agents which are able to control plant diseases. The mechanisms involved by these biocontrol agents have been classified as antibiosis, competition, parasitism and induced resistance to plant diseases. The concept of rhizosphere competence of these agents is a major consideration. These micro-organisms called biological control agents are also implicated in plant growth promotion (Whipps 2001). A number of bacteria and fungi have been studied which are known to control pathogenic micro-organisms in the rhizosphere. Among fungi, *Trichoderma* has been considered as a promising biocontrol agent (Harman *et al*. 2004; Zeilinger & Omann 2007; Sharma 2011). Bacteria such as *Bacillus subtilis, B. cepacia Serratia plymuthica*, *Pseudomonas putida* and *P. fluorescens, P. aureofaciens* (Duijff *et al*. 1999; Whipps 2001) have also been evaluated with promising results.

These bacterial agents especially *Pseudomonas* spp. are known to show strong antibiosis to pathogens. They produce antibiotics such as butyrolactones, HCN, kanosamine, viscosinamide and zwittermycin A and many other antibiotics some of which are yet to be characterized (Kim *et al*. 1999; Whipps 2001). Another mechanism of biological control is the competition for nutrients. In case of *Pseudomonas,* it is recognized that bacterial iron chelators can impound iron when it is present in limited quantity and make it unavailable to pathogenic fungi (Loper & Henkels 1999). The pathogenic forms of *Fusarium oxysporum* require available Fe for the germination and penetration of root tips of susceptible host. Both, fluorescent *Pseudomonas* and *F. oxysporum* produce specific Fe chelating compounds (siderophores). But, siderophores of *F. oxysporum* have less affinity for Fe than the siderophores of *Pseudomonas*. Thus, *Pseudomonas* can easily deprive *F. oxysporum* of Fe under conditions of iron scarcity and result in decreased likelihood of root infection (Baker 1986). Parasitism is yet another mechanism involved in biocontrol. Certain bacteria such as *Arthrobacter* are known to parasitize the fungus *Pythium debaryanum* and cause complete lysis of fungal host (Mitchell & Hurwitz 1965). Rhizosphere bacteria, specifically, *Bacillus* and *Pseudomonas* are believed to induce systemic resistance in plants by enhanced expression of stress related genes. Some of these bacteria are also able to colonize root tissue internally (van Loon 1998; Whipps 2001). The role of rhizospheric bacteria in plant health promotion may be indirect by suppressing pathogens in the rhizosphere of plants or by the supporting growth of mycorrhizal fungi. Plant growth enhancement may also be achieved directly by associative nitrogen fixation and solubilization of nutrients by these bacteria. Thereafter, these nutrients are made available to plants (Whipps 2001). With such an intensive investigation on the attributes of bacterial agents and their promising prospective in agriculture attempts to popularize biological methods of pest control is necessary. This research aims at reinforcing faith in biocontrol agents and a gradual reduction in use of synthetic chemicals in agriculture.

MATERIAL AND METHODS

The pathogen taken under study was *Fusarium oxysporum* (isolate FP-02/G) isolated from the diseased pea plant root. The pathogen has been deposited at ARI, Pune (India) under accession no. NFCCI-2195.

Isolation and screening of microbes to obtain potential antagonists of the pathogen

Fungi and bacteria both were screened to isolate antagonists of the pathogen *F. oxysporum*. A similar line of investigation was followed for both. Amongst fungi, several strains of *Trichoderma* showed considerable activity against *F. oxysporum*. Activity of *Trichoderma* has been discussed in another paper (Sharma 2011). The present paper focuses on bacterial isolates showing biocontrol potential.

The screening for bacterial agents was performed in two phases. In the first as well as the second phase screening, healthy pea plants and other plants from var-

ious regions of Jabalpur district (India) were arbitrarily selected. The roots of these plants were employed for isolation of microbial agents from rhizosphere, rhizoplane and endophytic habitats with expected potential to control the growth of pathogen under study. All the isolated microbes were screened for their antagonism on the pathogen. Dual culture technique was followed for the first and second phase screening. All isolations in the first phase were done on nutrient agar medium (3.0 g Beef Extract, 5.0 g Peptone, 2.0 g Sodium chloride, 15.0 g Agar) and the inoculated medium plates were incubated at 30°C for 24 hours. Modified methods were adopted for isolation from rhizosphere and rhizoplane (Pandey & Palni 1998; Hasan 2002).

a) Isolation of microbes from rhizosphere: Roots of freshly obtained plants were taken and the soil clumps adhered to roots were shed off. The roots were washed in a minimum amount of sterilized distilled water for 10 to 15 seconds. This washing was taken as sample suspension containing rhizosphere microbes; 0.1 ml of suspension was spread on the medium.

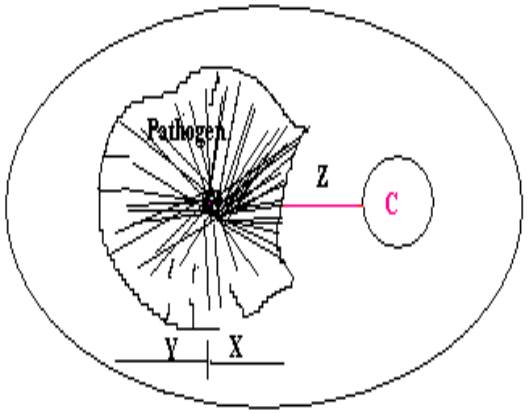
b) Isolation of microbes from rhizoplane: Roots were washed under tap water for 2–3 minutes and cut into segments of 3–4 centimetre. The segments were washed in sterilized distilled water several times and left in sterilized distilled water for 5 minutes. Thereafter, the segments were placed on the medium.

c) Isolation of endophytes: Modification of method by Paul *et al*. (2007) was adopted. Root segments were washed as mentioned in b) and then treated with 0.05% of $HgCl_2$ for 30 seconds to one minute depending on the kind of root. The segments were rinsed several times in sterilized distilled water. About a half centimetre portion was cut off from either side of each root segment and then the segment was placed on the medium.

Test of antagonism by dual culture technique on 90 mm petri-plate (modified methods adopted from Dhingra and Sinclair 1995; Nair and Anith 2009)

The isolates obtained by the above method were subjected to dual culture technique to test their activity against the pathogen. Isolates showing zone of inhibition against pathogen were tentatively identified to be *Pseudomonas* when subjected to the gelatine liquefaction test. Also, they showed growth below 10°C (King *et al*. 1954; Blazevic *et al*. 1973).

Based on the results obtained in the first screening phase, second screening phase was planned. In the second screening phase, *Pseudomonas* spp. were specifically isolated on *Pseudomonas* selective medium (for fluorescein) [HiMedium, M120, Lot-24035] and subjected to the dual culture technique. The test was performed on potato dextrose agar medium (Central Drug House (P) Ltd., JO 0013, Batch 51037). The fungal pathogen was point inoculated and 200 µL broth culture of the expected control agent was aliquoted in a well (7 mm diameter) at a distance of 12 mm opposite to the pathogen (Figure 1).



C – control agent, Z – zone of inhibition
X – growth towards control agent
Y – growth in the opposite direction
Z is directly proportional to difference (Dfp) between X and Y
% Inhibition = (Dfp / Y) × 100

Figure 1. Diagrammatic representation of biocontrol activity

Analysis of observations recorded in Pseudomonas-Fusarium dual culture

Observations were taken as the pathogen approached the well. The difference (Dfp) in growth of the pathogen towards the well (control agent) from the point of inoculation and in the opposite direction from point of inoculation was associated with the zone of inhibition (Z) formed by the control agent against pathogen. This relation was analysed statistically by MS Excel Data Analysis ToolPak application for Correlation.

Identification of Pseudomonas

Pseudomonas was cultured in *Pseudomonas* selective (for fluorescein) broth medium for 48 hours till visibly sufficient density was obtained; thereafter, glycerol (20% v/v) was added to the culture and was stored at about 10°C. *Pseudomonas* was

tentatively identified on the basis of its growth on *Pseudomonas* selective (for fluorescein) agar medium, gram staining, and gelatine liquefaction test (King *et al.*1954; Blazevic *et al.* 1973).

RESULTS AND DISCUSSION

Forty five isolates of *Pseudomonas* were tested in both the phases of screening. Twelve isolates obtained from rhizoplane and eleven isolates obtained from rhizosphere of plants showed considerable results. In this research, endophytic isolates did not show comparably good results. Table 1 shows the potential of these twenty-three isolates. Unidentified antibiotics released from *Pseudomonas* culture aliquot in the well, diffused into the surrounding agar medium and inhibited the growth of *F. oxysporum*. This was evident by measuring the growth of *F. oxysporum* towards and away from *Pseudomonas* culture aliquot and relating the growth

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Analysis of antagonistic activity of *Pseudomonas* spp. on *Fusarium oxysporum*

Source plant / habitat rhizoplane (rp) rhizosphere (rs)		Isolate no.	Growth difference Y – X = Dfp [mm]	Zone of inhibition, Z [mm]	% inhibition of growth posed by *Pseudomonas* sp. on *F. oxysporum* (Dfp / Y) ×100
Abelmoschus esculentus	rp	P5	4.50	4.38	44
Datura spp.	rp	P4	3.50	3.50	36
Annona squamosa	rp	P22	2.38	3.38	24
Brassica oleracea	rp	P17	2.63	3.00	24
Solanum molongera	rp	P19	2.13	2.88	19
Solanum lycopersicum	rs	P21	2.25	2.63	24
Carica papaya	rs	P23	1.88	2.63	18
Pisum sativum	rp	P18	1.63	2.38	16
Musa paradisiaca	rp	P6	1.63	2.25	15
Bougainvillae “Barbara K”	rp	P1	1.25	2.13	13
Bergera Koenigii	rs	P7	1.50	2.00	15
Mangifera indica	rs	P16	1.13	2.00	10
Psidium guajava	rs	P9	1.00	1.75	9
Vinca rosea	rp	P11	1.25	1.75	13
Glycine max	rs	P10	0.79	1.63	8
Azadirachta indica	rp	P13	0.88	1.63	9
Cicer arietinum	rp	P20	0.91	1.63	9
Bougainvillae “Buttiana”	rs	P12	0.63	1.25	7
Ocimum sanctum	rs	P15	0.75	1.25	7
Brassica juncea	rs	P2	0.75	1.13	8
Tagetes patula	rp	P3	0.76	1.13	7
Aloe vera	rs	P8	0.31	1.00	3
Tagetes erecta	rs	P14	0.25	0.55	3

Isolates of *Pseudomonas* showing visibly considerable inhibition to *Fusarium* were selected and recoded
X = Growth of *Fusarium* towards *Pseudomonas*
Y = Growth of *Fusarium* away from *Pseudomonas*
Data given is an average of quadruplicates
Arranged in the order of zone of inhibition measurements
Readings were taken after about 55 hours of beginning the experiment when the zone of inhibition was visible distinctly
Positive correlation (0.96568) between Dfp and Z obtained through MS Excel Data Analysis ToolPak application

difference to the zone of inhibition. Several isolates showed clearly distinct zone of inhibition.

Zone of inhibition in relation to the growth of Fusarium oxysporum

The difference in the growth of *F. oxysporum* towards and away from *Pseudomonas* (Dfp) quite well corresponded to the zone of inhibition (Z). An overall directly proportional relationship between Dfp and Z was observed with a correlation value of 0.96568. This confirmed the inhibitory effect of *Pseudomonas* isolates on *F. oxysporum*. *Pseudomonas* isolate P5 showed best activity against *F. oxysporum* followed by P4, P22 and P17 as evident (Table 1). The effect of *Pseudomonas* on *F. oxysporum* can be visualized in Figure 2 (a & b).

Most of the *Pseudomonas* isolates were not able to grow on PDA medium used for dual culture, but some isolates (P24, P25 and P26) grew unrestrictedly and completely covered the *Fusarium* colony. Although, such a swathe of *Pseudomonas* interfered with the growth of *F. oxysporum* but it was not confirmed if it completely restricted the growth of *F. oxysporum* beneath the swathe.

The antagonistic effect of *Pseudomonas* on *F. oxysporum* observed in this research may be attributed to the ability of antibiotic synthesis by *Pseudomonas*. *Pseudomonas* species are known to produce a number of antibiotics such as Amphisin, Oomycin A, Tensin, Tropolone and cyclic lipopeptides (Compant *et al*. 2005). Pyoluteorin, Pyrrolnitrin, hydrogen cyanide, phenazine-1-carboxylate are also produced by *Pseudomonas*. Lactone, 2,3-de-epoxy-2,3-didehydra-rhizoxin is also in the list (Shalini & Srivastava 2008).

Zone of inhibition in relation to growth of Pseudomonas

The effect of *F. oxysporum* on *Pseudomonas* has not been evaluated in this work. The interaction is assumed to show unidirectional antibiosis. Since *Pseudomonas* was cultured separately and then inoculated in the wells of the dual culture plate after about 48 hours, *Pseudomonas* is assumed to synthesize enough antibiotics to inhibit *F. oxysporum* by this time period. Therefore, evaluating the effect of *F. oxysporum* on *Pseudomonas* in this experiment might be futile. The broth culture of *Pseudomonas* aliquot in wells may still produce antibiotics in the initial stages of inoculation until the broth soaks and diffuses around the well with time and *F. oxysporum* begins to release toxins to interfere with antibiotic production by *Pseudomonas*. Although, in cases where *Pseudomonas* isolates were overwhelming the *Fusarium* colony, it is interpreted that *F. oxysporum* was unable to restrict the growth of *Pseudomonas*.

Other researchers such as Duffy and Defago (1997) found in their experiment that biosynthesis of impor-

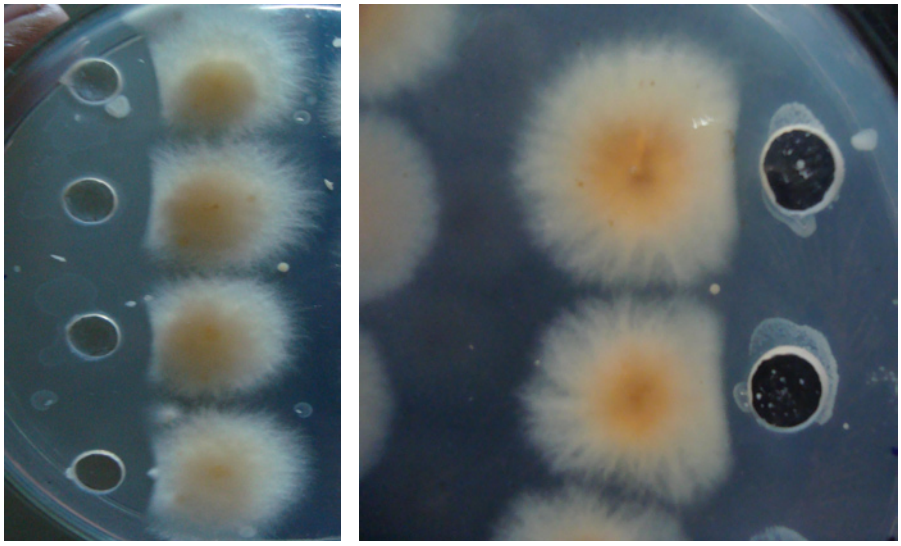

Figure 2 (a & b). Zone of inhibition created by bioagent *Pseudomonas* sp. Isolate no.P5 and P22 against pathogen *Fusarium oxysporum* after 48 hours of experiment

tant antibiotics, 2,4-diacetylphloroglucinol and pyoluteorin, by *Pseudomonas* is retarded even at low concentrations of fusaric acid. They stated that toxin fusaric acid produced by *Fusarium* spp. affected the biosynthesis of antibiotics but not preformed antibiotics. Also, bacterial growth was not affected even at high concentrations of fusaric acid.

Tentative identification of bacterial biocontrol agent

The Gram negative rod-shaped bacterial isolates showed fluorescence on *Pseudomonas* selective agar medium (Figure 3) and also, yellow green pigmentation was noticed in *Pseudomonas* selective broth medium. The isolates grew profusely at about 30°C.

On the basis of literature available, *Pseudomonas aeruginosa* build colonies surrounded by a yellow to greenish-yellow zone due to fluorescein production which fluoresces under UV light. If pyocyanin is also synthesized, a bright green colour is produced. Most pyocyanin-producing *Pseudomonas* strain synthesize fluorescein also and others produce just one of the pigments. Temperature can be a determining factor as most fluorescent strains will not grow at 35°C and higher. Rather, they grow between 25°C and 35°C (King *et al*. 1954; Blazevic *et al*. 1973).

The findings in this research reconfirm the biocontrol potential of *Pseudomonas*. Research by several workers over a period of time has elicited interest and faith in the prospects of bacterial agents like *Pseudomonas* in plant disease control. Production of antibiotics, inhibition of several pathogens such as *Fusarium oxysporum,* solubilization of inorganic phosphate and plant growth promotion are the attributes investigated upon by several researchers (Landa *et al*. 2002; Sarathchandra *et al.* 1993; Compant *et al*. 2005; Hallman *et al*. 1997; van Loon *et al*.1998; Negi *et al*. 2005). Production of siderophores and competition for nutrients is another mechanism of pathogen control (Buysens *et al*. 1996).

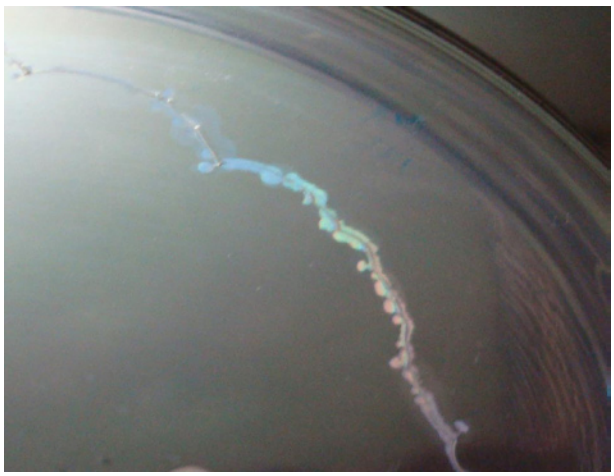

Figure 3. Glimpse of fluorescence noticed in *Pseudomonas* sp. Isolate no. P5

Though *Pseudomonas* and other biocontrol agents have been studied in detail over the years, often the results obtained in laboratory experiments may not be reproducible on agricultural lands due to various reasons or for the fact that the effect of biocontrol agent may be seen after repeated application of the agent on field over a period of time. Under such circumstances where using biocontrol agent alone may not be very effective, Integrated Pest Management (IPM) can be adopted.

Integrated Pest Management is a strategy that focuses on long-term solution of the pests through a combination of techniques such as biological control (*Pseudomonas* and *Trichoderma*), habitat manipulation, modification of agronomic practices, and use of resistant varieties (Birthal & Sharma 2004). Preferably, pesticides should be used as a last remedy in IPM programmes because of their potential negative effect on the environment. Botanical pesticides can be used as raw crushed plant leaves, extracts of plant parts or chemicals purified from the plants (Birthal & Sharma 2004; Ehler 2006).

CONCLUSION

In the present research, several isolates of *Pseudomonas* showed inhibitory effect on the growth of pathogenic *Fusarium oxysporum*. *Pseudomonas* Isolates P5, P4, P22 and P17 gave best results followed by other listed isolates.

In the present time, natural antagonists of agricultural pests are an important alternative to synthetic pesticides. Bacterial and fungal agents have been studied as biocontrol agents with promising results. Proper strategy of implementation and commercialization of these agents is necessary for their efficient outcome in agricultural fields.

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