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Biochemical Characterization of Native *Pseudomonas fluorescens* Strains followed by their Compatibility with Agrochemicals and Phyto-extracts under i*n vitro*

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ABSTRACT

Pseudomonas fluorescens (Pf), a Plant Growth Promoting Rhizobacteria (PGPR) has a wide scope for commercialization as it can be used in the production of biofertilizers. A total of fifty four Pf strains were characterized for HCN, ammonia, IAA, siderophore production. All Pf strains showed positive reaction to ammonia production, except Pf 8 strain. A total of thirty two strains showed IAA production. HCN and siderophore production results were encouraging for selective strains only. The percentage PGP characteristics were found to be 98.1%, 59.3%, 27% and 7.4% of ammonia, IAA, HCN and siderophore, respectively. As a step forward, the compatibility response of all P. fluorescens strains with commercially available agrochemicals viz., fungicides, herbicides and insecticides were screened under in vitro. All Pf strains were compatible with fungicides, except with copper oxychloride and mancozeb. Among the tested herbicides, compatibility was found with atrazine and imazethapyr. Our attempts for compatibility studies against plant extracts were affirmative. Thus, the aim to screen-out commercial agrochemical and phytoextract tolerant strains for integrated disease management was achieved.

Key words: *Pf strains, incompatibility, integrated disease management.*

INTRODUCTION

Agriculture is increasingly dependent on the use of chemical fertilizers, growth regulators and pesticides to realize potential of the yield of the crop. This dependence is associated with problems like environmental pollution, health hazards, interruption of natural ecological nutrient cycling and destruction of biological communities. Therefore, the immediate demand is to attain crop improvement and disease management in shorter intervals of time with favorable inputs.

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This can be achieved with the use of microbes replacing or reducing chemical pesticides.

Plant growth promoting rhizobacteria (PGPR) can actively colonize plant roots and increase plant growth and yield thereby act as a potential tool to provide sustainable benefits to agriculture[30]. *P.fluorescens* (*Pf*) strains are PGPR that promote plant growth with their ability to produce phytohormones against phytopathogenic microorganisms through the production of siderophores[9,24]. *Pf* strains act as efficient biocontrol agents[21] in view of their capacity to produce antibiotics, enzymes and fungicidal compounds[1]. *Pf* strains could affect seed germination and seedling growth of various agronomically important crops[3,26]. Biological control by *Pf* strains is important in integrated disease management (IDM), and it is gaining impetus and encompasses all the available control methods. *Pf* strains can be applied as seed treatment / soil drench application for biological control against soil borne pathogens.

Among the available IDM practices: cultural, biological, host plant resistance and combined use of chemical is a common phenomenon. However, combined use of chemical and bioagent, has become a necessary component in the IDM[14,8,10]. Using *Pf* strains to replace many chemical pesticides are gaining importance now. Similarly, medicinal plant extracts are identified to be non-mutagenic and less toxic on plant tissues and are the source of natural pesticides that make excellent leads for pesticide development[17,19]. Many workers have used crude extracts of various plants against several plant pathogens[29]. Several higher plants and their constituents have been successfully used in plant disease control[28]. The active principles from plants were proved effective against several plant pathogenic fungi *in vitro*[27]. Further, it has been identified that not much work has been carried out on the compatibility testing of *P. fluorescens* strains with commonly used synthetic agrochemicals and plant extracts, therefore it is a vital need.

MATERIAL AND METHODS

Isolation of *P. fluorescens* strains

Soil samples from the rhizosphere of different crops were collected in the year 2015 from different districts of Telangana region of Andhra Pradesh, India. From 10 g of soil, dilutions of the suspension were made with 100 ml of distilled water and around 0.1 mL was spread on Kings B media (Himedia) selective for *P.fluorescens* and incubated at 28ºC until the bacterial colonies were formed. After incubating for 24 h, the plates were detected under U.V. transilluminator by observing fluorescence effect in Kings B medium and picked fluorescens colonies for purification. Chromosomal DNA from the cultures was extracted to confirm strains as fluorescent *Pseudomonas*, 16S-23S rRNA intervening sequence-specific primers used for RAPD analysis[12].

Characterization of Pf strains

Fifty four *Pf* strains were screened for their plant growth promotion activity by assaying the following attributes: HCN, ammonia, IAA, siderophore production. The ability to produce HCN was assessed according to Bakker and Schipper, 1987 and ammonia production test by Egamberdiyeva[9]. IAA production test was done according to Brick *et al*., 991. Siderophore production was tested following the CAS plate assay[23].

Compatibility of agrochemicals and plant extracts

Solutions of different fungicides (carbendizam, copper oxychloride, hisulphur, hexaconazole, mancozeb, propioconazole and tricyclazole) and herbicides (atrazine, glyphosate, imazethapyr, paraquat dichloride and pendimethalin) at 1 - 4 % w/v concentrations were prepared. The insecticides imidacloprid (0.6, 0.7, 0.8 and 0.9% w/v) and thiomethoxam (0.1, 0.2, 0.3 and 0.4% w/v) were prepared by dissolving them in sterile distilled water. Dried and coarsely powdered leaves of *Physalis peruviana* (1.3 kg), *Abutilon indicum* (600 g), *Evolvulus alsinoides* (2.3 kg) and *Carthamus tinctorius* petals (750 g) were extracted separately using methanol in a Soxhlet extractor for 48 h. The methanolic

extract obtained was concentrated under reduced pressure and the yield was calculated to be 27 % w/w (*P. peruviana*), 19.0 % w/w (*C. tinctorius*), 10.8 % w/w (*A. indicum*), and 11.5 % w/w (*E. alsinoides*). The solvent free dry residues of different plant extracts were tested at 1000, 3000, 5000 and 8000 ppm. Different concentrations were prepared by dissolving the extract in sterile distilled water mixed with methanol (9.5:0.5). The final solutions were carefully made free from methanol by evaporation under reduced pressure.

Pf strains were screened for their resistance towards different agrochemicals and plant extracts through disc-diffusion method. Filter paper discs (5 mm diameter) were made by adding four different concentrations of prepared stock solutions to evaluate the concentrations equivalent to, above and below the recommended dose of test agrochemicals and plant extracts. A 100 µL of individual bacterial culture (10^7 cells/mL harvested at early logarithmic growth) was spread on KB agar plates with sterile glass spreader aseptically. These discs were placed at equidistance on the agar surface in duplicate after drying. Plate with filter paper disc dipped in sterile distilled before it was used as control. The plates were incubated for 2-3 days at 28 ± 2º C and the diameter of the inhibition zones were measured in mm.

RESULTS AND DISCUSSION

A total of eighty three bacteria were isolated from the rhizosphere soils of different crops. Among them, fifty four strains were identified as *P. fluorescens* and twenty nine as non-*P. fluorescens* bacteria using a U.V. transilluminator, by observing fluorescence in Kings B medium and as well as with specific ITS1F and ITS2R primers. Under HCN production test, only fifteen *Pf* strains (Table 1) showed positive with the formation of dark brown color, the rest of the strains retained yellow color on Whatman No.1 filter paper, inferring the absence. In case of ammonia production, all strains were observed positive except strain *Pf* 8 which was isolated from rice crop. While thirty two strains showed IAA production by developing pink color upon addition of ortho-phosphoric acid to their culture supernatant, only four strains were found positive for siderophore production, which was identified with the formation of orange colored zone on CAS blue agar plates.

The effect of hazardous agrochemicals such as fungicides, herbicides, insecticides and plant extracts on *Pf* strains had also been investigated. The paper disc method helped in quick screening of *Pf* strains for sensitivity to widely used commercial agrochemicals and plant extracts. All the tested *Pf* strains were compatible with carbendizam, hisulphur, hexaconazole, propiconazole and tricyclazole. Fungicides like mancozeb and copper oxychloride were bactericidal at 1, 2, 3 and 4 % concentrations for few strains. Mancozeb showed incompatible reaction with fourteen *Pf* strains and the average zone of inhibition was recorded to be 1-5 mm diameter at 3 and 4 % concentration. Only four *Pf* strains were incompatible with copper oxychloride out of which two strains, *Pf* 38 (3 mm) and *Pf* 43 (7 mm) were susceptible even at minimum concentration (1%).

Although all the tested fifty four *Pf* strains were resistant to atrazine and imazethapyr, susceptibility reaction was observed with rest of the herbicides like paraquat dichloride, pendimethalin and glyphosate. While it was only four strains that were inhibited with pendimethalin and two strains with glyphosate, a total of twenty *Pf* strains were inhibited by paraquat dichloride. An average zone of inhibition of around 1-13 mm diameter was recorded with paraquat dichloride, pendimethalin and glyphosate (Table 2). Insecticides, thiomethaxone and imidacloprid, generally used for seed treatment purpose, showed resistance reaction with all *Pf* strains tested at different concentrations (0.2 – 1.0%) with no zone of inhibition around filter paper disc impregnated with these insecticides. The extract of *P. peruviana* (MEPP), *C.*

tinctorius (MECT), *A. indicum* (MEAI) and *E. alsinoides* (MEEA) at different concentrations (1000, 3000, 5000 and 8000 ppm) had no inhibitory effect on any of the tested *Pf* strains with remarkable growth around filter paper disc impregnated with plant extracts.

Pseudomonas fluorescens are efficiently used to control plant pathogens in different crops. In IDM, compatibility of biocontrol agents with plant extracts and agrochemical sprays is important for them to be effective. The effectiveness of such agents mainly depends on sufficient density and appropriate distribution of agent population of host plant, which may be compromised by agrochemicals. It is a general belief that fungi or bacteria used as biocontrol or plant growth promoting agents should not be mixed together with any chemical pesticides and farmers are advised in the same way. Such a recommendation will limit the use of these agents in crops like ornamental plants where chemical pesticides are frequently applied as combined spray. Hence, studying the compatibility of isolated *Pf* strains with commonly used agrochemicals was felt necessary and reports regarding the compatibility were available[25].

Microbial production of HCN is an important antifungal trait to control root infecting fungi[20]. With this, the HCN producing *Pf* strains were ascertained to possess the capability of producing antifungal substances. Production of ammonia was exhibited by all the isolates except *Pf* 8. IAA may function as important signal molecule in the regulation of plant development. Out of fifty four strains, thirty two were positive for IAA production. The majority of fluorescent Pseudomonads are siderophore producers. Siderophores efficiently deplete iron from the environment, making it less available to certain competing microorganisms including plant pathogens[11]. Among the tested strains, *Pf* 21, *Pf* 23, *Pf* 34 and *Pf* 36 were identified to produce siderophores. The percentage PGP characteristics were found to be 98.1% of ammonia, 59.3% of IAA, 27% of HCN and 7.4% of siderophore. Eight strains were identified to exhibit more than three PGP traits, which could emerge as potential candidate for the development of bioinoculants for crop plants.

Compatibility of agrochemicals and plant extracts had minimal effect on growth of *Pf* strains. Deleterious effect was found to some extent with herbicides followed by fungicides while no inhibition was observed with insecticides and plant extracts that allows them to use in combination with *Pf* strains for seed treatment. Among the tested fungicides, mancozeb and copper oxychloride demonstrated incompatibility with some *Pf* strains. While atrazine and imazethapyr showed resistivity, the other commercially used herbicides like paraquat, pendimethaline and glyphosate proved susceptibility with some of the tested strains. Lack of knowledge of the compatibility of antagonist microorganisms with agrochemicals of natural origin like plant extracts may contribute to the failure of biocontrol to perform as expected. Leaf extracts of *Physalis peruviana, Abutilon indicum* and *Evolvulus alsinoides* are reported to possess antifungal activities[7,3]. Safflower petals, richest source of gamma linolenic acid were reported as good antifungal agent[18]. Sajeli Begum[22] reported that leaf extracts and steroidal saponin of *Cestrum diurnum* L. inhibited the growth of plant pathogenic fungi. All the four plant extracts were found to be compatible with *Pf* strains. Based on the observed results in consideration to the above discussions, it has been identified that most of the *Pf* strains are showing resistance and can multiply around disc saturated with varying concentrations of agrochemicals and plant extracts. Combined applications of biocontrol agents with small quantities of agrochemicals and plant extracts may reduce the biomass of antagonists and also the relative costs of formulation.

Table 1: Biochemical characterization of *Pseudomonas fluorescens* strains isolated from different crops cultivated in Telangana State, India

				Biochemical characterization			
Strain	Crop	Location	District	[a]	[b]	c	d
Pf 1	Rice	Tudkurthy (Nagarkarnool)	Mahabubnagar	-	+	+	-
Pf 2	Rice	Tudkurthy (Nagarkarnool)	-do-	-	+	+	-
Pf 3	Castor	Palem (Bijnepally)	-do-	-	+	+	-
Pf 4	Cotton	Palem (Bijnepally)	-do-	-	+	+	-
Pf 5	Chilli	Manganoor (Bijnepally)	-do-	-	+	+	-
Pf 6	Groundnut	Elikacherla (Thimmajipet)	-do-	-	+	+	-
Pf 7	Rice	Lattupally (Bijnepally)	-do-	-	+	+	-
Pf 8	Rice	Lattupally (Bijnepally)	-do-	-	-	+	-
Pf 9	Groundnut	Lattupally (Bijnepally)	-do-	-	+	+	-
Pf 10	Redgram	Vasanthapuram (Bijnepally)	-do-	-	+	+	-
Pf 11	Castor	Lingasanipally (Bijnepally)	-do-	-	+	+	-
Pf 12	Rice	Kottalgadda (Bijnepally)	-do-	-	+	+	-
Pf 13	Sunflower	Lingasanipally (Bijnepally)	-do-	+	+	-	-
Pf 14	Rice	Kottalgadda (Bijnepally)	-do-	+	+	+	-
Pf 15	Maize	Lingasanipally (Bijnepally)	-do-	+	+	+	-
Pf 16	Rice	Thimmajipet (Thimmajipet)	-do-	+	+	+	-
Pf 17	Groundnut	Vattem (Bijnepally)	-do-	-	+	-	-
Pf 18	Cotton	Edirepally (Thimmajipet)	-do-	-	+	-	-
Pf 19	Rice	Kodkurthy (Bijnepally)	-do-	-	+	+	-
Pf 20	Rice	Vattem (Bijnepally)	-do-	-	+	+	-
Pf 21	Rice	Edirepally (Thimmajipet)	-do-	-	+	-	+
Pf 22	Castor	Nagarkurnool	-do-	-	+	+	-
Pf 23	Groundnut	Vattem (Bijnepally)	-do-	+	+	-	+
Pf 24	Redgram	Palem (Bijnepally)	-do-	-	+	+	-
Pf 25	Chilli	Nadigadda	-do-	+	+	+	-
Pf 26	Maize	Thadoor (Thadoor)	-do-	-	+	+	-
Pf 27	Chilli	Karvanga (Telkapally)	-do-	+	+	-	-
Pf 28	Sunflower	Yadireddipally (Thadoor)	-do-	-	+	-	-
Pf 29	Sunflower	Tallapally (Telkapally)	-do-	+	+	-	-
Pf 30	Chilli	Tallapally (Telkapally)	-do-	+	+	-	-
Pf 31	Cotton	Indrakol (Nagarkarnool)	-do-	+	+	-	-
Pf 32	Groundnut	Chennur (Gopalpet)	-do-	-	+	-	-
Pf 33	Groundnut	Nellikonduru	-do-	-	+	+	-
Pf 34	Groundnut	Sudhakal	-do-	+	+	-	+
Pf 35	Groundnut	Gundoor	-do-	+	+	+	-
Pf 36	Groundnut	Thadoor (Thadoor)	-do-	+	+	-	+
Pf 37	Chickpea	Manganoor (Bijnepally)	-do-	-	+	+	-
Pf 38	Sorghum	Kondapur (Narayanked	Medak	-	+	+	-
Pf 39	Rice	Peddapendyala (Dharmasagar)	Warangal	-	+	+	-
Pf 41	Maize	Peddapendyala (Dharmasagar)	Warangal	-	+	+	-
Pf 42	Sugarcane	Kondapur (Narayanked)	Medak	-	+	-	-
Pf 43	Sunflower	Kondapur (Narayanked)	Medak	-	+	-	-
Pf 44	Rice	Wanaparthy (Wanaparthy)	Mahabubnagar	-	+	+	-
Pf 45	Chickpea	Kondapur (Narayanked)	Medak	-	+	+	-
Pf 46	Maize	Chinnakarupumala (Peddakothapally	Mahabubnagar	-	+	+	-
Pf 47	Chilli	Peddapendyala (Dharmasagar)	Warangal	-	+	-	-
Pf 48	Rice	Chinnakarupumala (Peddakotapally)	Mahabubnagar	-	+	-	-
Pf 49	Sorghum	Atchampet (Achampet)	-do-	-	+	-	-
Pf 50	Sorghum	Palem (Bijnepally)	-do-	-	+	-	-
Pf 51	Parthenium	Nagarkurnool (Nagarkurnool)	-do-	-	+	+	-
Pf 52	Parthenium	Palem (Bijnepally)	-do-	+	+	-	-
Pf 53	Rice	Vanasthalipuram (Hyderabad)	Hyderabad	-	+	-	-
Pf 54	Rice	Lingotam (Achampet)	Mahabubnagar	+	+	-	-

a = HCN, b = Ammonia, c = IAA, d = siderophore

-do- = same as above, + = Positive, - = Negative

Table 2: Sensitivity of *Pseudomonas fluorescens* strains to different agrochemicals and plant extracts

Agrochemical	No. of strains Tested	No. of strains showing resistance concentration (%)			
Fungicides		**1**	**2**	**3**	**4**
Carbendizam	54	+	+	+	+
Copperoxychloride	50	3	4	4	4
Hisulphur	54	+	+	+	+
Hexconazole	54	+	+	+	+
Mancozeb	40	8	13	14	14
Propioconazole	54	+	+	+	+
Tricyclazole	54	+	+	+	+
Herbicides		**1**	**2**	**3**	**4**
Atrazine	54	+	+	+	+
Glyphosate	52	1	1	1	2
Imazethapyr	54	+	+	+	+
Paraquat dichloride	34	19	19	20	20
Pendimethaline	50	4	4	4	4
Insecticides		**0.4**	**0.6**	**0.8**	**1**
Imidachlopid	54	+	+	+	+
Thiomethoxam	54	+	+	+	+
Plant extracts		**1000 ppm**	**3000 ppm**	**5000 ppm**	**8000 ppm**
MEPP	54	+	+	+	+
MECT	54	+	+	+	+
MEAI	54	+	+	+	+
MEEA	54	+	+	+	+

+ - 100 % resistance; MEPP – Methanolic extract of *P. peruviana*; MECT – Methanolic extract of *C. tinctorius* ; MEAI – Methanolic extract of *A. indicum*; MEEA – Methanolic extract of *E. alsinoides*.

CONCLUSION

Our studies revealed that under *in vitro* conditions, *P. fluorescens* strains tested in the present study were compatible with most of the commercially used agrochemicals and none of the plant extract used could inhibit the same. Hence, our aim to screen-out agrochemical and phytoextract tolerant strains for integrated disease management was achieved.

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