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Deciphering multifarious properties of Pseudomonads suppressing tomato (*Solanum lycopersicum*) wilt

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ABSTRACT

Wilt is the most common disease encountered in tomato crop. To devise an eco-friendly management module against *F. oxysporum* f. spp. *solani*, the causal organism, native isolates of *Pseudomonas* spp. were isolated from agricultural fields. The present experiment was conducted at SKUAST-Jammu between 2014-15. Biochemical screening of isolates revealed that antimicrobial compounds, viz. 2,4-DAPG and siderophore production were maximum in isolate PS 1 (217. 70 µg/ml and 32. 25 µg/ml) and HCN production was highest in isolate PS 6 (0. 11 µg/ml). Isolates PS 1 to PS 20 produced ferrate hydroxamate type siderophores whereas PS 21 to PS 30 produced catecholate type siderophores. Growth promoting substances, viz. IAA, GA and phosphorus solubilization were maximum in PS 7 (583. 63 µg/ml), PS 15 (652. 07 µg/ml) and PS 30 (358. 25 µg/ml) respectively. TLC of 2,4-DAPG extracted from various isolates showed R*f* values in the range of 0. 7 to 0. 89, close to the relative mobilty (R*f*) of phloroglucinol (0. 89). PS 1 reported highest seed germination (75%) with vigour index of 1275. Pot experiment revealed that isolate PS 4 increased plant height, plant growth parameters and further reduced wilt incidence (34. 25%) as compared to the seedling treated with *Fusarium oxysporum* (60%).

Key words: 2,4-DAPG, GA, HCN, IAA, Pseudomonads, Tomato Wilt

Increasing global population has created threat to food security and to cope with requirement of sufficient food for all, strategic management of diseases in crops is vital. Tomato is an important vegetable crop and is infected by several soil borne fungal pathogens causing serious diseases such as wilts (Abdel-Monaim 2010). Biocontrol of plant pathogens by antagonistic microbes is a potential non-chemical, cheap, eco-friendly and effective method for the management of plant diseases (Gupta *et al.* 2016a, Gupta *et al.* 2016b, Sharma *et al.* 2018). Over the past years, studies have demonstrated that several metabolites produced by antagonistic bacteria playa key role in the control of various plant pathogens (Paulsen 2005). Members of the genus *Pseudomonas* have been known for their potential role in control of various fungal diseases (Sharma *et al.* 2016; Angayarkanni *et al.* 2005). The present experiment was conducted to devise an eco-friendly management module against *F. oxysporum* f. spp. *solani* that causes tomato wilt.

MATERIALS AND METHODS

The present experiment was conducted at SKUAST-J and the isolates were collected from the research fields of the University. Isolation of *Pseudomonas* spp**.** was done following Sharma *et al.* (2018).

Biochemical and morphological characterization: The isolates were subjected to various biochemical tests and examined for their colony morphology, pigmentation, epifluorescence, cell shape and gram reaction based on Hort *et al.* (1994).

Physiological characterization: Twenty four hours old culture of the test organisms grown in NB (Nutrient Broth) were spotted on the Trypticase Soy Agar (TSA) plates and incubated for 24 to 48 h at 4°C and 41°C and the observations on growth were recorded at the end of incubation period.

Production of antimicrobial compounds

Siderophore production assay: Qualitative analysis of siderophore production was performed as per the methodology of Bano and Musarrat (2003). Quantification of siderophore production was done using a modified CAS indicator solution prepared according to Schwyn and Neilands (1987).

Hydrogen cyanide (HCN) production: The isolates of *Pseudomonads* were tested for hydrogen cyanide (HCN) production by picric acid method given by Wei *et al.* (1991). HCN was quantified as per the modified method of Alonso-Amelot *et al.* (2000).

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Production of plant growth promoting compounds

Indole acetic acid (IAA): Quantitative estimation of crude Indole Acetic Acid was made following the method of given by Gordon and Paleg (1957).

Gibberellic Acid (GA): Quantitative estimation of crude Gibberellic acid (GA) was estimated as per Paleg (1965).

Inorganic phosphorus (P_i) solubilization: Estimation of inorganic phosphorus (P_i) released from TCP (Tri Calcium Phosphate) was done following phosphomolybdic blue colour method given by Jackson (1973).

Production of 2,4-DAPG and its conformation through TLC

2, 4-DAPG was extracted from *Pseudomonas* spp. by following the methodology of Rosales *et al.* (1995). Samples (20 µl) were loaded on thin layer chromatography plates coated with a 250 µm layer of silica gel and developed in acetonitrile/methanol/water (1:1:1). Spots were visualized by spraying with diazotized sulphanilic acid. *Rf* values of the spots were compared with Phloroglucinol (50 mg/ml methanol).

In vitro evaluation of antagonistic activity of Pseudomonas isolates against Fusarium oxysporum

Promising isolates of *Pseudomonas* spp. were tested against *Fusarium oxysporum* using dual culture technique as described by Huang and Hoes (1976) and expressed as inhibition percentage using the formula given by Vincent (1947).

Preparation of the antagonist cell suspension

King's B broth culture of *Pseudomonas* spp. isolates was taken and harvested in 10 ml sterile distilled water. The cell suspensions of antagonists were serially diluted from 10^{-1} to 10^{-5}. The cell concentration in the final dilution was adjusted to 10^8 cfu/ml in SHIMAZDU UV Vis Spectrophotometer (OD 0.5 at 425 nm).

Seed germination test by paper towel method

Seeds of tomato (var. lehar) were surface sterilized with sodium hypochlorite for 1 min and washed several times with tap water to remove traces of NaOCl. The dried seeds were soaked in prepared cell suspension for 2 h. These seeds were then placed equidistantly on towel paper @ 20 seeds/petri plate. The towel papers were made sufficiently wet and incubated in the BOD at 26 ± 2°C for 6-7 days. Vigour index was calculated by using formula proposed by Woodstock (1969).

Evaluation of plant growth promotion through pot experiments

Preparation of fungal and bacterial inoculants: For fungal inoculants, 500 ml glass bottles were filled (2/3) with sorghum seed and 150 ml distilled water. The sorghum bottles were sterilized through autoclaving for 1 h at 121°C. Each bottle was inoculated with two to three discs (4 mm diameter) of the test pathogenic fungi and incubated at 25 ± 2°C for 3 weeks. After incubation, the bottles were emptied and the growth paste was slowly dried under room temperature to allow it for maturation (Al-Juboory and Juber 2013). For *Pseudomonas* isolates, 48 h old broth culture kept at 28 ± 2°C on rotary shaker (150 rpm) was taken.

Pot experiment: These were carried out using tomato seedlings (var. lehar) under greenhouse conditions. Potting mixture consisting of 3 parts soil and 1 part FYM was autoclaved for 1 h at 121°C. Sterilized potting mixture was transferred to plastic pots (30 cm diameter), each containing 8 kg potting mixture. Inoculation of soil was carried out with addition of 20 g pathogen inoculum per pot. Similarly, broth bacterial culture with a cfu of 10^8 was used for inoculating *Pseudomonas* isolates to the soil. Thereafter, tomato seedlings were transplanted in these pots. Observations on wilt incidence of the plants in pots were recorded and after three months of transplanting, growth parameters, viz. shoot and root dry weight were measured to observe the antagonistic and growth promotion properties of *Pseudomonas* isolates.

RESULTS AND DISCUSSION

Isolation and morpho-physiological characterization of isolates: Thirty isolates which showed fluorescence under UV light were selected for further studies. The isolates formed irregular and non-spreading type of colonies with slimish cream colour and yellow-green diffusible pigment of variable intensities on *Pseudomonas* specific media i.e. King's B media. Microscopic observations revealed them to be rod shaped and the isolates fluoresced under UV light. On TSA, the isolates showed positive growth at 41°C and no growth at 4°C. These characters reveal the identity of the isolates as *Pseudomonas* spp.

Biochemical characterization: All the selected thirty isolates were positive for catalase production, phosphorus solubilization, arginine hydrolysis, indole production and gelatin liquefaction but negative for starch hydrolysis and urease activity with varying intensities. These biochemical tests confirmed the isolates to be *Pseudomonas* spp. as reported by earlier workers (Meera and Balabaskar 2012).

Antimicrobial traits and plant growth promoting substances

Siderophores Production Assay: Involvement of more than one mechanism of bio-control like ability has been reported in fluorescent *Pseudomonads* (Tripathi and Johri 2002). Siderophore production in our isolates ranged from 21.97 to 32.25 µg/ml with a mean production of 26.02 µg/ml (Table 1). Maximum siderophore production was observed in isolate PS 10 (32.25 µg/ml) followed by PS 18 (31. 53 µg/ml) and PS 19 (30.97 µg/ml). The isolates from PS 1 to PS 20 produced ferrate hydroxamate type of siderophores as they showed peak between 420-450 nm whereas the isolates from PS 21 to PS 30 may be catecholate type of siderophores as they showed peak at 495 nm. Our results are in corroboration with the results obtained by Kumar *et al.* (2002)

HCN production assay: Hydrogen cyanide (HCN)

Table 1 Production of antimicrobial and plant growth promoting substances *Pseudomonas fluorescens* isolates

Isolate	Siderophore (µg/ml)	Hydrogen cyanide (µg/ml)	IAA (µg/ml)	GA (µg/ml)	Phosphorus (µg/ml)
PS1	32.25 ± 1.39	0.06 ± 0.01	47.40 ± 1.95	207.28 ± 1.70	245.72 ± 2.34
PS2	24.71 ± 1.27	0.10 ± 0.02	29.12 ± 1.90	150.88 ± 2.80	136.86 ± 2.63
PS3	26.20 ± 1.48	0.04 ± 0.01	33.28 ± 0.95	112.49 ± 1.18	190.42 ± 1.74
PS4	25.81 ± 0.43	0.04 ± 0.01	41.22 ± 1.05	104.15 ± 1.81	188.24 ± 0.57
PS5	24.45 ± 1.89	0.03 ± 0.01	33.32 ± 1.9	111.89 ± 1.35	209.56 ± 2.18
PS6	25.76 ± 1.22	0.11 ± 0.01	31.99 ± 1.46	130.34 ± 1.78	156.13 ± 1.07
PS7	25.03 ± 1.40	0.01 ± 0.00	583.63 ± 3.26	531.94 ± 1.48	150.33 ± 1.21
PS8	24.63 ± 1.73	0.03 ± 0.01	385.77 ± 1.22	516.60 ± 1.28	128.20 ± 1.32
PS9	28.44 ± 1.23	0.01 ± 0.00	109.56 ± 1.67	321.22 ± 1.26	175.64 ± 1.52
PS10	24.18 ± 1.55	0.04 ± 0.01	537.28 ± 1.40	574.77 ± 1.66	153.26 ± 2.08
PS11	23.84 ± 1.32	0.05 ± 0.01	471.32 ± 1.70	454.70 ± 1.28	153.82 ± 1.88
PS12	25.46 ± 1.32	0.04 ± 0.01	33.48 ± 1.51	71.56 ± 1.53	207.69 ± 1.85
PS13	23.64 ± 1.62	0.03 ± 0.01	94.17 ± 2.66	480.14 ± 2.80	118.53 ± 1.67
PS14	23.56 ± 1.62	0.03 ± 0.01	42.58 ± 1.38	83.98 ± 0.66	135.12 ± 1.64
PS15	23.82 ± 2.14	0.04 ± 0.02	309.65 ± 2.78	652.07 ± 1.36	138.05 ± 1.49
PS16	24.42 ± 1.62	0.04 ± 0.01	195.56 ± 2.50	216.88 ± 1.36	89.79 ± 2.36
PS17	28.06 ± 1.31	0.03 ± 0.01	431.63 ± 3.25	427.17 ± 2.22	48.77 ± 1.83
PS18	31.53 ± 1.65	0.04 ± 0.01	214.38 ± 1.12	550.10 ± 2.04	100.70 ± 2.07
PS19	30.97 ± 1.47	0.03 ± 0.01	60.16 ± 2.04	223.26 ± 1.20	148.71 ± 2.35
PS20	26.29 ± 1.55	0.04 ± 0.01	112.16 ± 2.82	397.85 ± 0.61	133.19 ± 1.71
PS21	28.46 ± 1.94	0.03 ± 0.01	83.75 ± 2.21	400.54 ± 1.10	117.79 ± 2.55
PS22	24.65 ± 2.11	0.03 ± 0.01	376.38 ± 2.55	574.78 ± 0.66	111.93 ± 2.29
PS23	26.03 ± 1.33	0.03 ± 0.01	35.62 ± 1.22	131.40 ±1.28	178.51 ± 2.03
PS24	25.84 ± 2.32	0.03 ± 0.01	223.73 ± 1.67	211.78 ± 0.68	138.05 ± 1.62
PS25	27.40 ± 1.44	0.01 ± 0.00	89.14 ± 2.42	519.80 ± 1.44	187.36 ± 1.72
PS26	23.36 ± 1.62	0.03 ± 0.01	174.90 ± 0.92	425.10 ± 1.64	112.05 ± 1.61
PS27	24.75 ± 1.49	0.03 ± 0.02	215.90 ± 0.53	417.13 ± 1.50	149.89 ± 2.75
PS28	21.97 ± 2.19	0.04 ± 0.02	174.24 ± 2.62	281.95 ±1.73	126.64 ± 2.29
PS29	26.01 ± 1.75	0.04 ± 0.01	90.46 ± 1.96	543.69 ±1.55	89.42 ± 2.06
PS30	28.96 ± 1.58	0.03 ± 0.01	129.94 ± 2.51	569.39 ± 1.87	358.25 ± 1.79

effectively blocks the cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms (Hassanein *et al.* 2009). The production of HCN by certain fluorescent *Pseudomonas* is believed to be involved in the suppression of root pathogens (Ramette *et al.* 2003). All the selected isolates of *Pseudomonas* spp. produced HCN. HCN production ranged from 0.01 to 0.11 µg/ml. Isolate PS 6 exhibited maximum HCN production i.e. 0.11 µg/ml whereas isolates PS 9 and PS 25 produced minimum HCN production, i.e. 0.01 µg/ml (Table 1). Similar results were obtained by Voisard *et al.* (1989).

Indole acetic acid production assay: Fluorescent *Pseudomonad* offers an interesting biological system with their ability to promote plant growth through production of plant hormones and through control of plant pathogens and deleterious organisms or both (Vanpeer and Schippers 1989). The isolates of fluorescent *Pseudomonad* selected for the present study were able to produce IAA ranging from 29.12 µg/ml to 583.63 µg/ml with a mean production of 179.72 µg/ml (Table 1). Out of the thirty isolates, highest IAA production (crude) was recorded in isolate PS 7 (583.63 µg/ml) and the minimum was recorded in isolate PS 2 which exhibited 29.12 µg/ml. Our results are in agreement with Megha (2006) who reported that the amount of IAA produced

ranged from 80-760 µg/l of broth.

Gibberellic acid production assay: Selected isolates of fluorescent *Pseudomonad* of the present study were able to produce GA. Isolate PS 15 showed highest GA production, i.e. 652.07 µg/ml and minimum was recorded in isolate PS 12, i.e. 71.56 µg/ml (Table 1). The results of the present studies are in agreement with Jagdish (2006) who reported a strain *Pseudomonas* spp. B-25 that produced copious amounts (290 g/l) of plant growth promoting substances.

Phosphorus solubilization assay: It has been reported that higher concentrations of phosphate-solubilizing bacteria are commonly found in the rhizosphere soil as compared to non-rhizospheric soil (Reyes and Valduz, 2006). Out of the selected thirty isolates, PS 30 showed maximum phosphorus production of 358.25 µg/ml (Table 1). Similar observations have been made with fluorescent *Pseudomonads* and other organisms by various workers (Das *et al.* 2003, Suneesh 2004).

Table 2 R_f values of 2,4-DAPG extracted from *Pseudomonas fluorescens* isolates

Isolate	R_f value	2,4-DAPG (µg/ml)	Inhibition (%) of mycelial growth of *F. oxysporum*
PS1	0.75	2157.70	65.45
PS2	0.70	303.00	55.56
PS3	0.74	56.00	52.45
PS4	0.74	1184.00	60.25
PS5	0.76	68.00	51.46
PS6	0.73	571.00	56.75
PS7	0.85	859.00	59.64
PS8	0.76	457.00	55.98
PS9	0.84	676.00	57.85
PS10	0.86	752.00	57.45
PS11	0.84	529.00	56.65
PS12	0.85	129.00	52.47
PS13	0.83	251.00	54.63
PS14	0.82	186.00	53.46
PS15	0.84	300.00	56.54
PS16	0.83	171.00	53.42
PS17	0.80	574.00	57.65
PS18	0.76	313.00	56.78
PS19	0.78	342.00	57.85
PS20	0.80	373.00	57.95
PS21	0.84	603.00	58.65
PS22	0.86	862.00	59.68
PS23	0.85	469.00	57.65
PS24	0.81	725.00	58.84
PS25	0.88	575.00	58.65
PS26	0.89	564.00	57.64
PS27	0.80	949.00	59.45
PS28	0.88	529.00	57.34
PS29	0.89	857.00	58.65
PS30	0.82	966.00	59.35

Comparison of Rf values of 2,4-DAPG extracted from different isolates of Pseudomonas spp. : Antibiotic-producing PGPR have been studied intensively and special attention has been given to 2,4-DAPG-producing *Pseudomonas* spp. because of their ability to control a wide variety of soil-borne plant pathogens (Tehrani *et al.* 1998). In our studies distinct spots of 2,4-DAPG having R*f* values in the range of 0.70 - 0.89 which was close to the R*f* value of synthetic phloroglucinol (0.89) were obtained (Table 2). Similar results were obtained by Rosales *et al.* (1995) who reported distinct spots at R*f* of 0.88. On the basis of the performance of isolates of *Pseudomonas* spp. in the various biochemical tests and antimicrobial (2,4-DAPG) and growth promoting substances production assays, two isolates, viz. PS 1 and PS 4 were selected for further studies so as to evaluate their efficacy against *Fusarium oxysporum*.

Dual culture test for in vitro assay: Among the isolates, both PS 1 and PS 4 *Pseudomonas* isolates were effective with an inhibition of over 60% against *Fusarium oxysporum*. However, maximum inhibition (65.45%) was noticed in isolate PS 1 followed by isolate PS 4 that recorded 60.25% growth inhibition of fungal mycelia (Table 2). Thus, from dual culture assay, it was clear that the *Pseudomonas* spp. has the ability to inhibit the growth of *F. oxysporum.*

Seed germination test using paper towel technique: Seeds of tomato (var. lehar) soaked in the cell suspension of 48 hr. old culture of *Pseudomonas* isolates showed increased germination rate as compared to control. The data presented in Table 3 revealed that in case of PS 1 treated seeds, 75% germination and vigour index of 1275 was recorded whereas PS 4 treated seeds exhibited 71% germination and a vigour index of 1118.25.

Evaluation of bioagent through pot experiment: Results presented in Table 4 showed that the growth parameters viz. plant height, number of branches and dry weight of tomato plants were highly affected by the applications of different bio-treatments. Maximum shoot dry weight (9.34 g) and root dry weight (1.43 g) were found in tomato seedlings treated with isolate PS 4 in soil infected with *F. oxysporum* whereas isolate PS 1 treated seedling reported shoot dry weight (7.10 g) and root dry weight (0.68 g). Thus, it is concluded from the study that the application of *Pseudomonas* spp. isolates result in the effective control

Table 3 Effect of *Pseudomonas* spp. culture filtrate on the germination rate and vigour index of tomato seeds

Treatment	Germination (%)	Shoot length (cm)	Root length (cm)	Vigour Index (%)
P.fluorescens (PS 1)	75.00	9.00	8.00	1275.00
P.fluorescens (PS 4)	71.00	8.25	7.5	1118.25
Control (D.W)	45.00	4.20	7.00	504.00
Control (KBB)	35.00	8.90	7.70	581.00

Table 4 Effect of *Pseudomonas* isolates on growth paramaters and disease expression of tomato plants infected with *Fusarium oxysporum*

Treatment		Growth parameter				Wilt incidence (%)
Fungal treatment	Bacterial inoculant	Height (cm)	No. of branches	Dry weight (g/plant)		
				Shoot	Root	
Untreated	Un-inoculated	50.2^{ab}	10.8^{c}	8.07^{cd}	1.01^{cd}	0
	PS 1	47.3^{ab}	16.3^{de}	4.36^{bc}	0.45^{ab}	0
	PS 4	44.3^{b}	15.1^{d}	3.34^{ab}	0.24^{a}	0
Treated	Un-inoculated	22.4^{b}	5.8^{b}	3.54^{ab}	0.65^{bc}	60
	PS 1	52.6^{ab}	18.65^{de}	7.10^{bcd}	0.68^{bc}	27.65
	PS 4	52.4^{ab}	23.6^{ef}	9.34^{cd}	1.43^{cde}	34.25

Values in the same column sharing the same letter don't differ significantly, according Tukey's test at 5% level.

of wilt incidence as well as plant growth promotion in the seedlings infected with *F. oxysporum.* Moreover, there was an increase in germination per cent and seedling vigour of tomato when treated with *Pseudomonas* spp. Results of this study also are in line with Saber *et al.* (2015) who reported that soil infested with *F. oxysporum* gave 100% and 60% post damping off and wilt incidence.

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