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Efficacy of native fluorescent pseudomonads in management of bacterial wilt of tomato under the mid hill conditions of Meghalaya, India

Janshame Tariang and Dipali Majumder

Abstract
Bacterial wilt caused by *Ralstonia solanacearum* is of common occurrence in commercially grown solanaceous vegetables in acidic soils. The extent of damage ranges from 60-80% depending on the crop variety and growing season in North East India. The wide host range, diverse race and biovar of *R. solnacearum*, the management of bacterial wilt through resistant cultivar is not so encouraging. In order to develop efficient bio-control management strategy of *R. solanacearum*, two biocontrol agents *viz. Pseudomonas fluorescens* (USR 9.2) and *P. putida* (PC) with ability to produce antibiotic, metabolite and native to the soils of Meghalaya, India were used in this study. *In-vivo* evaluation on tomato plant at Nongpoh and Umiam, Meghalaya, India against *R. solanacearum* was carried out following different methods of application of the bio-agent *i.e*. individual application as seed treatment or root dip treatment or soil application and in combination as seed treatment + root dip treatment and as seed treatment + root dip treatment + soil application. Both pseudomonad fluorescents were found to have suppressive effect against *R. solanacearum* isolates under *in-vivo* in the mid hills condition of Meghalaya. The study revealed that soil treatment for individual application was most effective, whereas seed treatment + root dip treatment + soil application was found most effective for application methods in combination. However, the effect of the bio-agent on the yield was found maximum when applied in combination *i.e.* seed treatment + root dip treatment and seed treatment + root dip + soil application rather than single application alone.

Keywords: Meghalaya, pseudomonad fluorescent, bacterial wilt, bio-agent, tomato

1. Introduction
Bacterial wilt disease caused by *Ralstonia solanacearum* (Smith) (Yabuuchi *et al*., 1995) [56] is one of the major production constraints in many crop plants of the tropical, sub-tropical and temperate regions worldwide. Economic loss due to this disease has been observed in Meghalaya as well. The pathogen is known for broad host range belonging to solanaceae, musaceae, zingiberaceae family including ornamental crops. The disease is favoured by high temperature (severe at 24-35 °C) and seldom found in temperate climates where the mean temperature for any winter month falls below 10 °C. High soil moisture and periods of wet weather or rainy seasons are associated with high disease severity and survival of the pathogen. Soil moisture is also one of the major factors affecting reproduction (Nesmith and Jenkins, 1985) [39]. The species of *R. solanacearum* is subdivided into five biovars based on the utilization of the disaccharides cellobiose, lactose, maltose and oxidation of the hexose alcohols dulcitol, mannitol and sorbitol (Hayward, 1964) [22] and into five races, based on host range. In India, bacterial wilt is one of the important production constraints and is of common occurrence in Kerala, Karnataka, Himachal Pradesh, Sikkim, West Bengal and North Eastern states including Assam, Meghalaya and Tripura. The disease out-break is very severe during peak monsoon accompanied with warm and humid weather causing total crop loss. The disease was first reported from West Bengal (Das and Chattopdhay, 1955) [11] and caused up to 90% yield loss in solanaceous vegetables. In Meghalaya also the disease limits the vegetable production up to 60-80% and causes great economic loss depending on the variety and the growing season. The management of bacterial wilt by cultural method was found not much effective as the disease occurred even in first planting in barren land (Kelman, 1953) [26]. The use of resistant varieties has not yet become a feasible method due to the location-specific nature of the resistance to bacterial wilt, high variability of the pathogen and wide host range. Biological control can be an alternative as it is less destructive to the ecosystem than the

chemical pesticides and maintains the sustainable management practices (Cook and Baker, 1983; Barea and Jeffries, 1995) [10, 6]. *Pseudomonas fluorescens* is known as an effective biocontrol agent against soil borne pathogen in both green house and field condition (Anuratha and Gnanamanikam, 1990) [4] due to their production of antibiotics (Gutterson *et al*., 1988) [19], phenazine-1-carboxylic acid (PCA) metabolite (Tuner and Messenger, 1986) [52], siderophores (Kloepper and Schroth, 1981) [30], indole acetic acid (IAA), hydrogen cyanide (Defago *et al*., 1990) [14], enzymes like proteases, glucanases (Jensen *et al*., 1980; Hamamoto *et al*., 1994) [24, 21], ability to compete for space and nutrients in the soil (Elad *et al*., 1987; Haas and De'fago, 2005) [16, 20] and compounds(VOC) (Raza *et al*., 2016) [45]. Plant growth promoting rhizobacteria (PGPR) isolated from rhizosphere of different crops were found to be effective when they are inoculated with *R. solanacearum* (Amara *et al*., 1996; Maji and Chakrabartty, 2014) [2, 34]. Antagonist *P. fluorescens* was reported to have suppressive effects on *R. solanacearum* (Mulya *et al*., 1996; Messiha *et al*., 2007; Vanitha *et al*., 2009; Upreti and Thomas, 2015) [38, 36, 54, 53]. Considering the above facts, the study was taken up to evaluate the efficacy of the potential antagonist and growth promoting action of native antibiotic and metabolite producing strains of *Pseudomonas fluorescens* and *P. putida* against *R. solanacearum* under field condition of Meghalaya, India.

2. Materials and Methods

2.1 Collection and isolation of bacterial wilt pathogen from solanaceous vegetables

Wilted tomato, potato, capsicum and brinjal were collected from Ri-Bhoi District of Meghalaya, a state in the North Eastern part of India located between 90°55'15 to 91°16' latitude and 25°40' to 25°21' longitude with an average elevation of 485 metres. Wilted plant samples were collected during the month of April-May as during this month the disease is at its peak due to favourable temperature and moisture. Ooze test was done against each sample prior to isolation. The associated pathogen was isolated from infected plant parts using Pseudomonas solanacearum (PS) medium (Himedia) + 1% Triphenyltetrazolium chloride (TZC) solution (Kelman, 1954) [27].

2.2 Pathogenecity test

Pathogenicity test was done against each isolates by root dip method (Winstead and Kelman, 1952; Prior *et al*., 1990) [55, 41]. The 48 h old pure culture of each isolates were used to prepare bacterial suspension of 10^8cfu/ml. Healthy tomato (Rocky), capsicum (California wonder), chilli (Pikadon), brinjal (Pusa purple long) seedling were uprooted, washed in water, root tip were clipped (so as to make injury) and immersed in the respective bacterial suspension for 15-20 min and then planted in 20 cm diameter earthen/plastic pots (one plant per pot) filled with sterilized soil. Pathogenicity test for the isolates collected from wilted potato plants were carried out in susceptible tomato cultivar "Rocky". Plants inoculated with sterile water served as control. The inoculated plants were examined for wilt symptom development.

2.3 Characterization of bacterial wilt pathogen

Morphological and biochemical characterization of bacterial wilt isolates were carried out. A 48 h old colony of each isolate was picked up (loopful), mixed with 10 ml of sterile water in a culture tube and vortex. Serial dilutions were made upto 10^{-7}cfu/ml and from the final dilution, 0.1 ml was pipette out in Petri plates containing PS medium. The plates were incubated at 28±2 °C for 48 h and the colony characters of each isolate was recorded. Biochemical characterization of the isolates were carried out for the identification and confirmation of *R. solanacearum* by referring the guidelines described by Stanley *et al*. (2005) [50] and Chaudhry and Rashid (2011) [9]. The test conducted are outlined as follows:

2.3.1 Gram staining

Gram staining was carried out for all the isolates to differentiate the bacteria as Gram positive or Gram negative by using the gram staining kit (K00-1Kt, Himedia). A sterile slide was taken on which, a loopful of 48 h old bacterial isolates were smeared, air dried and heat fixed with the help of a very low flame (spirit lamp). The smear was flooded with crystal violet for one min followed by washing in a gentle stream of water. Gram's iodine solution was then flooded for another one min which was again followed by washing and then decolorized with 95 percent ethyl alcohol. The smear was again washed with water and counter stained with safranin for 30 secs. Finally the smear was washed again with water, dried and observed microscopically at 10X, 40X and 100X (using oil) for Gram positive or negative reactions and the shape of the cells was recorded.

2.3.2 KOH solubility test (3%)

KOH solubility test was done for all the isolates for further confirmation of Gram reaction of the tested bacteria. Few drops of KOH (3% solution) were dropped on the clean glass slide. A loopful of fresh colony was picked up with the help of inoculating loop and mixed with the solution and observed for formation of slime threads (Suslow *et al*., 1982) [51].

2.3.3 Kovacs oxidase test

The test was carried out by using oxidase disc (DD018, Himedia), where 24 h old bacterial culture was picked up with the help of a tooth pick and rubbed on the oxidase disc. A change in colour of the disc was observed and recorded.

2.3.4 Catalase test

A loopful of the test bacterial culture (18-24 h) and a drop 3% hydrogen peroxide (H_2O_2) were mixed on a clean glass slide. Observation was made for the production of gas bubbles with naked eye and under a compound microscope of 40X (Schaad, 1980) [48].

2.3.5 Arginine dehydrolase

Arginine dehydrolase agar medium was prepared and five ml was dispensed into culture tubes and autoclaved. The tubes were stab inoculated with 24 h grown culture and incubated at 30±1 °C for 4 days. Observation of the change in colour of the medium was recorded.

2.3.6 Levan production

Freshly streaked 24 h old single colony was selected and then inoculated on Levan agar medium and incubated for four days at 30±1 °C. Observations were made for production of cleared whitish swelling on top of the bacterial growth.

2.3.7 Starch hydrolysis

The test was done to detect the ability of the isolates to degrade starch by the production of amylase enzyme. The fresh test cultures were picked up and streaked on the starch agar plates and incubated at 30±1 °C for 48 h. After

incubation, the plates were flooded with iodine solution and observation was recorded.

2.3.8 Denitrification

Culture tubes containing inverted Durham's tube were filled up with Nitrate broth. Care was taken so that there was no air bubble inside the Durham's tube. Broth were inoculated with a loopful of overnight grown culture of the test bacterium and incubated at 28±2 °C. After one week of incubation, the inverted Durham's tubes were observed for accumulation of gas.

2.3.9 Production of fluorescent pigment

King's Medium B base (King *et al*., 1954) [28] plates were streaked with the test bacterium and incubated for 48 h at 30±1ºC. After incubation these plates were observed for the production of fluorescence with the help of a UV transilluminator.

2.4 Molecular identification of the isolates

Molecular identification of purified isolates was carried out by using *R. solanacearum* specific primer 759/760 (F 5'-GTCGCCGTCAACTCACTTTCC-3', R 5' GTCGCCGTCA GCAATGCGGAATCG-3') (Opina *et al*., 1997; Romano *et al*., 2012) [40, 46]. A loopful of each of the fresh cultures (48 h) grown on PS agar medium were mixed with 20 µl of nuclease free water in a 0.5 ml micro tubes, vortexed and incubated at 95 °C for 6 minutes. PCR was performed using a Gradient Master Cycler 5331 (Eppendorf Make, Germany). The reaction mixture (total volume 25 µl) consisted of 10X PCR buffer (1 µl), 25 mM $MgCl_2$ (1.4 µl), 10 mM dNTPs (0.2 µl), 1 U Taq DNA (0.05 µl), nuclease free water (Himedia, India), 0.16 µl of each forward and reverse primer and 2 µl of bacterial suspension mixed. The Master Cycler was programmed using the following protocol: denaturation temperature was set at 94 °C for 3 min, annealing temperature at 53 °C for 1 min and extension at 72 °C for 1 min 30 sec., followed by 30 cycles of 94 °C for 15 sec., 60 °C for 15 sec., 72 °C for 15 sec. and final extension of 72 °C for 5 min (Opina *et al*., 1997) [40]. PCR products were electrophoresed onto agarose gels 0.8% in 1X TBE buffer at 90 V for 45 min using a mini gel electrophoresis assembly (HU10, Sci-plas, Hongkong) and visualized with UV light using gel documentation system (Bio-Rad, CA, USA) after gel red (Gel-Red Fluorochrom stain, Biotium, USA) staining.

2.5 Fluorescent pseudomonads strains

Two native fluorescent pseudomonads strains USR 9.2 (*P. fluorescens*) and PC (*P. putida*) which have been screened for anti-biotic and metabolite production (Devi, 2012) [15] were obtained from Microbial Culture Laboratory, College of Post-Graduate Studies, Umiam, Meghalaya, India. These two native strains were evaluated for their efficacy under *in-vivo* conditions against *R. solanacearum* causing bacterial wilt in tomato.

2.5.1 Preparation of antagonistic bacterial inoculum

USR 9.2 and PC were grown on King's medium B agar medium and were harvested in sterile water by centrifugation at 12,000 rpm for 10 min. The pellet was resuspended in distilled water and bacterial concentration of 1×10^8 cfu/ml was prepared by adjusting with distilled water to A_{610} nm (OD=0.45) using UV visible spectrophotometer (Mortensen, 1999; Vanitha *et al*., 2009) [37, 54].

2.6. *In-vivo* evaluation for efficacy of native pseudomonads fluorescent by different method of application of the bio-agent against *R. solanacearum* and on the yield

2.6.1 Field evaluation

The experiment was conducted at the farmer's field, Nongpoh and at the experimental field of College of Post Graduate Studies (CPGS), Umiam, Ri-Bhoi District, Meghalaya, India. The representative isolate of *R. solanacearum* which was found comparatively most virulent based on the pathogenecity test *i.e.* "BRT 1"was tested against *P. fluorescens* (USR 9.2) and *P. putida* (PC). Ramdomized block design (RBD) was adopted for field experiment. Six treatments used in this experiment were seed treatment (T_1), root dip treatment (T_2), soil application (T_3), seed treatment + root dip (T_4), seed treatment + root dip + soil application (T_5) and control (T_6). Three replications were taken for each treatment. Bacterial wilt susceptible tomato variety (Rocky) was used. The tomato seeds were treated with USR 9.2 and PC suspension (10^8cfu/ml) before sowing for seed treatment application and at the time of transplanting for other methods of application of the bio-agent. After 20 days of transplanting the tomato plants were inoculated with *R. solanacearum* "BRT 1" suspension (10^8cfu/ml) for the soil drenching treatment. Daily observation was done for occurrence of bacterial wilt incidence. For evaluation of efficacy of the bio-agent on the yield, plant height and number of branches per plant were taken at 60 days after transplanting. Number of fruits per plant and total fruit weight per plant were also recorded.

$$\% \text{ of Bacterial wilt incidence} = \frac{\text{Number of wilted plants} \times 100}{\text{Total number of plants receiving that treatment}}$$

2.6.2 Preparation of bacterial pathogen inoculums

Inoculum of *R. solanacearum* was grown on PS agar medium for 48 h at 28±2 °C. The bacterial cells were harvested with sterile distilled water by centrifugation at 12,000 rpm for 10 min. The pellet was resuspended in distilled water and bacterial concentration of 1×10^8 cfu/ml was prepared by adjusting with distilled water to A_{610} nm (OD=0.45) using UV visible spectrophotometer (Mortensen, 1999; Vanitha *et al*., 2009) [37, 54].

2.6.3 Experimental design and data analysis

Data analysis for field evaluation of *P. fluorescens* and *P. putida* against bacterial wilt were statistical measured by using the data of the percentage of affected plants and transformed to arcsine square root equivalents prior to a one-way analysis of variance (ANOVA). Means were separated using Duncan's multiple range test (DMRT; P = 0.05) using SPSS software. The significant difference, if any, among the treatment means were compared using critical difference (CD) at *P*=0.05.

3. Results

3.1 Collection and isolation of bacterial wilt pathogen

Out of the twenty five samples collected fifteen samples (7 of tomato, 1 of potato, 6 of capsicum and 1 of brinjal) showed positive response to ooze test and were further isolated on TZC medium. Fluidal light pink to dark pink centred colonies (BRT 1, MT 1, MT 3, MT 6, MT 7, MT 8, MT 10, UPR 1, CC 1, CC 2, UC 1, MC 1, MC 2, MC 3 and CB) were picked and purified on PS medium(Table 1).

Table 1: Pathogenicity of bacterial wilt pathogen

Isolates	Inoculated host plant	No. of days required to show wilt symptom after inoculation of bacterial wilt pathogen [α]
BRT 1	Tomato	10±1.4
MT 1	Tomato	12±1.4
MT 3	Tomato	13±0.7
MT 6	Tomato	15±1.0
MT 7	Tomato	14±0.7
MT 8	Tomato	11±1.0
MT 10	Tomato	13±0.7
UPR 1	Tomato	16±0.7
CC 1	Capsicum	13±0.7
CC 2	Capsicum	12±1.4
UC 1	Capsicum	14±1.0
CB	Brinjal	15±0.7

[α] Values are the mean (±SE) of five replicates

3.2 Pathogenecity test

All the 15 isolates (BRT 1, MT 1, MT 3, MT 6, MT 7, MT 8, MT 10, UPR 1, CC 1, CC 2, UC 1, MC 1, MC 2, MC 3 and CB) showed positive response to pathogenecity test. BRT 1 was found most virulent isolate based on the number of days taken for wilt symptom to develop (Table 3). Hence, BRT 1 was further used as a representative isolate for field experiment under the investigation.

3.3 Characterization of bacterial wilt pathogen

The morphological characters of the isolates were studied in terms of the colour of the colony on PS medium, intensity of colour of pigment on TZC, shape of bacterium, colony elevation and colony consistency. All the 15 isolates were observed rod shaped, creamish white in colour on PS medium. Most of the isolates produced dark pink pigment (11), others produced light pink (4) colour pigment on TZC medium. The colonies were all fluidal and raised (Table 1).

Biochemical characterisation for all the 15 isolates was carried out to confirm their identity as *R. solanacearum.* Nine biochemical properties of the isolates for *R. solanacearum* was studied including Gram staining, KOH solubility test (3%), Kovac's oxidase test, catalase test, arginine dehydrolase test, levan production, starch hydrolysis, denitrification test and production of fluorescent pigment. All the 15 isolates resulted negative to Gram staining, arginine dihydrolysis, levan production, starch hydrolysis, fluorescent production test and positive to KOH 3% test, Kovac's oxidase test, catalase test and denitrification test (Table 2).

Table 2: Morphological characters of bacterial wilt pathogen

Isolates	Morphological characters [α]				
	Cell shape	Colony colour	Pigment colour	Consistency	Elevation
BRT 1	R	CW	DP	F	Rs
MT 1	R	CW	DP	F	Rs
MT 3	R	CW	LP	F	Rs
MT 6	R	CW	DP	F	Rs
MT 7	R	CW	DP	F	Rs
MT 8	R	CW	DP	F	Rs
MT 10	R	CW	DP	F	Rs
UPR 1	R	CW	DP	F	Rs
CC 1	R	CW	LP	F	Rs
CC 2	R	CW	DP	F	Rs
UC 1	R	CW	DP	F	Rs
MC 1	R	CW	DP	F	Rs
MC 2	R	CW	LP	F	Rs
MC 3	R	CW	LP	F	Rs
CB	R	CW	DP	F	Rs

[α] R = Rod, CW = creamish white, DP = dark pink, LP = light pink, F = fluidal, Rs = raised

Table 3: Biochemical characterization of bacterial wilt pathogen

Isolates	Biochemical test [α]								
	Gram Staining	KOH 3%	Kovac's oxidase test	Catalase test	Arginine dihydrolysis	Levan production	Starch hydrolysis	Denitrification	Fluorescent pigment production
BRT 1	-	+	+	+	-	-	-	+	-
MT 1	-	+	+	+	-	-	-	+	-
MT 3	-	+	+	+	-	-	-	+	-
MT 6	-	+	+	+	-	-	-	+	-
MT 7	-	+	+	+	-	-	-	+	-
MT 8	-	+	+	+	-	-	-	+	-
MT 10	-	+	+	+	-	-	-	+	-
UPR 1	-	+	+	+	-	-	-	+	-
CC 1	-	+	+	+	-	-	-	+	-
CC 2	-	+	+	+	-	-	-	+	-

UC 1	-	+	+	+	-	-	-	+	-
MC 1	-	+	+	+	-	-	-	+	-
MC 2	-	+	+	+	-	-	-	+	-
MC 3	-	+	+	+	-	-	-	+	-
CB	-	+	+	+	-	-	-	+	-

α " + " = positive; "–" = negative

3.4 Molecular identification of the isolates

Molecular detection by using *R. solanacearum* species specific primer 759/760 revealed that out of 15 isolates only 6 isolates (MT 3, MT 10, BRT 1, CB, CC1 and UC1) were confirmed as *R. solanacearum* with an amplicon size of 280bp (Fig.1).

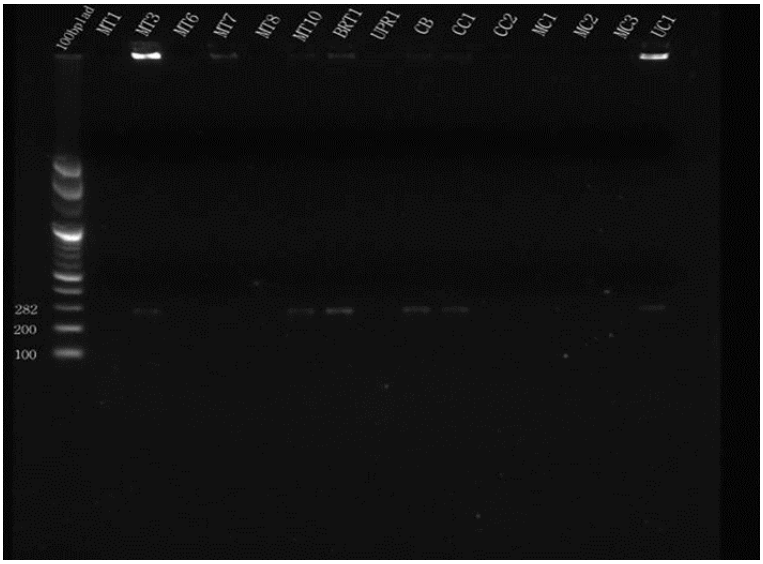

Fig 1: Six isolates: Lane 3(MT3), Lane 7(MT!0), Lane 8(BRT1), Lane 10(CB), Lane 11(CC1), Lane 16(UC1) amplified at 282 bp size and were confirmed to be *R. solanacearum*

3.5 Field evaluation of *P. fluorescens* (USR 9.2 and *P. putida* (PC) under different methods of application

3.5.1 Effect of *P. fluorescens* (USR 9.2) on bacterial wilt of tomato

USR 9.2 was found very effective when applied as soil application and in combination of application methods as seed treatment + root dip treatment + soil application with no record of bacterial wilt incidence. The control plots were recorded with the highest bacterial incidence upto 14.58%. Application method of the bio-agent as seed treatment recorded bacterial wilt incidence of 8.33%. Root dip treatment and seed treatment + root dip treatment both recorded bacterial wilt incidence of 4.17% (Table 4).

Table 4: Comparative evaluation of application methods of biocontrol agents {*P. Fluorescens* (USR 9.2) and *P. putida* (PC)} on bacterial wilt incidence (%) induced by *R. solanacearum* in tomato

Bioagent application methods	Per cent incidence of bacterial wilt[α]	
	USR 9.2 treated	PC treated
Seed treatment	8.33% 3.19±0.21[b]	12.50% 3.61±0.37[b]
Root dip treatment	4.17% 2.32±0.66[ab]	6.25% 3.19±0.21[b]
Soil application	0.00% 1.00±0.12[a]	0.00% 1.00±0.17[a]
Seed + root dip treatment	4.17% 2.32±0.66[ab]	0.00% 0.60±0.31[a]
Seed + root dip + soil application	0.00% 1.00±0.06[a]	0.00% 1.00±0.09[a]
Control	14.58% 3.73±0.13[b]	20.83% 3.11±0.26[b]

aValues are mean±SEM, values followed by different letters indicated statistically significant difference at ><0.05 as determined by one-way ANOVA.
Values in bold-face fonts indicate raw data on bacterial wilt incidence.
Values in normal-face fonts indicate bacterial wilt incidence derived from raw data using (logx+1) transformation.

3.5.2 Effect of *P. putida* (PC) on bacterial wilt of tomato

PC was found equally effective when applied as soil application, seed treatment + root dip and as seed treatment + root dip treatment + soil application with no record of bacterial wilt incidence. Application method of the bio-agent as seed treatment and root dip treatment recorded bacterial wilt incidence of 12.50 and 6.25% but they were found to be at par with each other. However, the control plots recorded maximum bacterial wilt incidence of 20.83% (Table 4).

3.5.3 Effect of *P. fluorescens* (USR 9.2) and *P. putida* (PC) on yield of the tomato crop at farmer's field, Nongpoh, Meghalaya

At farmer's field in Nongpoh, the effect of USR 9.2 on the fruit weight per plant was recorded maximum (1.8 kg/plant) when applied in combination as seed treatment + root dip treatment. Methods of application such as seed treatment, root dip treatment and soil application recorded a lower fruit weight of 1.5, 1.6 and 1.7 kg/plant respectively which were observed to be at par with each other (Table 5). Similarly, the

effect of PC on the fruit weight per plant was recorded maximum when the bio-agent was applied in combination method as seed treatment + root dip + soil application and as seed treatment + root dip treatment with 1.8 and 1.9 kg/plant respectively. A comparatively lower yield was recorded in the other methods of application as well as in the control plot (Table 5).

Table 5: Comparative evaluation of application methods of biocontrol agents (*P. Fluorescens* (USR 9.2) and *P. putida* (PC)) on yield of the tomato crop at farmer's field, Nongpoh, Meghalaya

Bioagent application methods	Concentration (cfu/ ml)	Plant height (cm)		No. of branches		No. of fruits per plant		Weight of fruits per plant (kg)	
		USR 9.2	PC	USR 9.2	PC	USR 9.2	PC	USR 9.2	PC α
Seed treatment	10^8	59.0^{ab}	56.8^{a}	4.8^{a}	4.5^{a}	39.5^{b}	26.5^{a}	1.5^{ab}	1.7^{ab}
Root dip treatment	10^8	59.0^{ab}	57.8^{ab}	4.7^{a}	4.6^{a}	41.4^{b}	24.9^{a}	1.6^{ab}	1.6^{ab}
Soil application	10^8	59.7^{ab}	55.6^{a}	4.8^{a}	4.4^{a}	41.3^{b}	21.3^{a}	1.7^{ab}	1.6^{ab}
Seed + root dip treatment	10^8	57.0^{a}	57.8^{ab}	5.2^{a}	4.5^{a}	42.9^{b}	30.3^{a}	1.8^{b}	1.8^{b}
Seed + root dip + soil application	10^8	56.9^{a}	59.0^{ab}	4.9^{a}	4.6^{a}	43.1^{b}	28.0^{a}	1.7^{ab}	1.9^{b}
Control	10^8	61.6^{b}	61.8^{b}	4.5^{a}	4.4^{a}	28.5^{a}	24.5^{a}	1.4^{a}	1.4^{a}

αValues are mean±SEM, values followed by different letters indicated statistically significant difference at $P<0.05$ as determined by one-way ANOVA. Values in column having same alphabetical letters do not differ significantly at ($P = 0.05$)

3.5.4 Effect of *P. fluorescens* (USR 9.2) and *P. putida* (PC) on yield of the tomato crop at CPGS Experimental field, Umiam, Meghalaya

At CPGS Experimental field, Umiam the effect of USR 9.2 on the fruit weight per plant recorded maximum when the bio-agent was applied in combination methods as seed treatment + root dip + soil application and as seed treatment + root dip treatment with 1.7 and 1.6 kg/plant respectively but were statistically found to be at par with each other. Application method of the bio-agent as seed treatment, root dip treatment and soil application were observed with a lower fruit weight per plant *i.e.* 1.3 kg/plant for each method of application (Table 6). Similarly, the effect of PC on the fruit weight per plant was recorded maximum when the bio-agent was applied in combination as seed treatment + root dip + soil application and as seed treatment + root dip treatment with 1.6 kg/plant in both the cases. Single method of application of the bio-agent as root dip treatment recorded a fruit yield of 1.4 kg/plant. Seed treated, soil application and the control plots were found to give minimum fruit weight/plant with 1.3 kg/plant in all cases (Table 6).

Table 6: Comparative evaluation of application methods of biocontrol agents (*P. fluorescens* (USR 9.2) and *P. putida* (PC)) on yield of tomato crop at CPGS Experimental field, Umiam, Meghalaya

Bioagent application methods	Concentration (cfu/ ml)	Plant height (cm)		No. of branches		No. of fruits per plant		Weight of fruits per plant (kg)	
		USR 9.2	PC	USR 9.2	PC	USR 9.2	PC	USR 9.2	PC α
Seed treatment	10^8	53.5^{a}	51.8^{a}	4.5^{ab}	3.9^{a}	22.8^{ab}	24.5^{a}	1.3^{a}	1.3^{a}
Root dip treatment	10^8	54.0^{a}	48.0^{a}	4.2^{a}	4.0^{a}	23.0^{ab}	26.4^{a}	1.3^{a}	1.4^{a}
Soil application	10^8	56.1^{a}	54.5^{a}	4.8^{b}	4.6^{a}	25.5^{b}	28.8^{a}	1.3^{a}	1.3^{a}
Seed + root dip treatment	10^8	55.1^{a}	54.6^{a}	4.1^{a}	4.0a	24.5^{ab}	28.6^{a}	1.6^{b}	1.6^{b}
Seed + root dip + soil application	10^8	57.9^{a}	56.7^{a}	4.4^{ab}	4.4a	27.0^{b}	29.0^{a}	1.7^{b}	1.6^{b}
Control	10^8	58.6^{a}	58.3^{a}	4.1^{a}	4.1^{a}	19.4^{a}	25.0^{a}	1.1^{a}	1.3^{a}

αValues are mean±SEM, values followed by different letters indicated statistically significant difference at $P<0.05$ as determined by one-way ANOVA. Values in column having same alphabetical letters do not differ significantly at ($P = 0.05$)

4. Discussion

The suppressive effect of USR 9.2 a strain, belongs to *P. fluorescens* biovar V isolated from rice rhizosphere against *R. solanacearum* may be correlated with the fact that it has the ability to produce antibiotic 2, 4-diacetylphloroglucinol (DAPG) and HCN, whereas PC which belong to *P. putida* isolated from pea rhizosphere might be due to the fact that it could produce antimicrobial metabolites *i.e*. HCN, antibiotic DAPG and pyrrolnitrin (prn) as studied by Devi (2012) [15]. In addition to the production of HCN, DAPG and prn, rhizospheric bacteria *P. fluorescens* and *P. putida* are well known for the production of metabolite such as PCA (Tuner and Messenger, 1986) [52], siderophores (Kloepper *et al*., 1981) [30], enzymes like proteases, glucanases (Jensen *et al*., 1980; Hamamoto *et al*., 1994) [24, 21] which might have contributed to the suppressive effect on *R. solanacearum*. Kipgen *et al*. (2012) [29] also reported successful *in-vitro* inhibition of *R. solanacearum* by native fluorescent pseudomonads isolates of Meghalaya, which were found positive for the presence of bio-synthetic gene that encoded for production of the antibiotic prn. Similarly, Savithiry and Gnanamanickam (1987) [47], Anuratha and Gnanamanickam (1990)[4], Das and Bora (2000) [12], studied the zone of inhibition on KMB medium and observed copious affectivity of *P. fluorescens* in suppressing *R. solanacearum* under *in-vitro* plates.

Strains USR 9.2 and PC that showed antagonism against *R. solanacearum* under field condition and was found to have a high potential in the management of bacterial wilt in Meghalaya. The application method of the bio-agent as soil treatment and in combination of application methods as seed treatment + root dip treatment + soil application were found most effective with no record of bacterial wilt incidence. The findings of the present investigation can be supported with the findings of Chakravarty and Kalita (2011, 2012) [7, 8] who found that application method of *P. fluorescens* formulation as seed + root + soil method could reduce the bacterial wilt of brinjal in field experiment up to 83.33%. Combined application of *P. putida* and Actigard (acibenzolar-S-methyl) was also observed to reduce bacterial wilt incidence to a great extent (Anith *et al*., 2004) [3]. Mulya *et al*. (1996)[38] reported that *P. fluorescens* strain PfG32 isolated from rhizosphere of

onion actively suppressed the occurrence of bacterial wilt disease in tomato in vermiculite amended natural soil and produced antibiotic substance and siderophores.

Although the combination of seed treatment + root dip treatment + soil application showed best performance in management of bacterial wilt under field condition in tomato, but except soil application in both the cases *i.e*. USR 9. 2 and PC other application methods such as seed treatment and root dip treatment alone did not show much effect. This might be attributed to the fact that the pathogen is well known as soil dweller (Agrios, 1997) [1], hence application of the bio-agent in the soil was most effective. Jinnah *et al*. (2002) [25] reported that the management of bacterial wilt of tomato in the field by soil drenching with *P. fluorescens* reduced the disease incidence greatly. The report of Prior and Fegan (2005) [42] on reduction of bacterial wilt up to 80% in tomato by using *P. putida* also supported the findings of the present investigation. Bakker *et al*. (2007) [5] reported that the mode of action of fluorescent pseudomonads for suppression of diseases included siderophore-mediated competition for iron, antibiosis, production of lytic enzymes and induced systemic resistance (ISR). Fluorescent pseudomonads were reported to produce metabolite salicyclic acid which was suggested to trigger induced resistance (Leeman *et al*., 1996; De Meyer and Hofte, 1997; Maurhofer *et al*., 1998) [32, 13, 35]. These characters might also be the reason for suppression of bacterial wilt disease in addition to the production of the antibiotic and antimicrobial metabolites by the two fluorescent pseudomonad strains. Many other experiments were also carried out in field conditions on the evaluation of the efficacy of *P. fluorescens* to control bacterial wilt and were reported for successful management of bacterial wilt disease in tomato (Messiha *et al*., 2007; Kuarabachew *et al*., 2007; Vanitha *et al*., 2009) [36, 31, 54].

P. fluorescens (USR 9.2) and *P. putida* (PC) were found to have a great effect on yield of the tomato plant when the bio-agent was applied in combination *i.e.* seed treatment + root dip treatment and seed treatment + root dip treatment + soil application. Combination of methods used for application of USR 9.2 did not show much effect on the plant height and number of branches but was found to have significant effect on parameters such as number of fruits per plant and the fruit weight per plant. The effect of application of PC on the growth parameters *viz.*, height of the plant, number of branches and number of fruits were found not effective but had great effect on the fruit weight/yield of the tomato plant. This might be due to ability for phosphorous solubilization, IAA, auxins, gibberellins production by the two fluorescent pseudomonad strains. The results of the present investigation on the yield agreed with those reports made by Loper and Schroth (1986) [33] that fluorescent pseudomonads enhance growth promotion in plant by production of plant hormones such as auxins, gibberellins (Ramamoorthy *et al*, 2002) [43] and 1-amino-cytlopropane-1-carboxylate deaminase (Jacobson *et al*., 1994) [23]. It has also been reported that pseudomonads have a significant impact on plant growth by providing the plant with compounds that is synthesized by the bacterium or facilitating the uptake of nutrients from the environment (Glick, 1995; Glick *et al*.¸1999) [17, 18]. Pseudomonads strains was also found to increase the yield of brinjal upto 75% (Ramesh *et al*., 2009) [44]. The increase in the fruit weight can be correlated with the findings of Seleim *et al*. (2011) [49] where they found that there was increased in the biomass of the tomato fruit when treated with strains of fluorescent pseudomonads.

5. Conclusion

Bacterial wilt caused by *R. solanacearum* is a major disease in commercially grown solanaceous vegetables in Meghalaya. Biovar 3 and 5 of R. solanacearum are most prevalent in the state. Two native strains *P. fluorescens* (USR 9.2) and *P. putida* (PC) were found to have high potential in management of bacterial wilt disease in Meghalaya and for promoting yield. Application of the bio-agent in combination of application methods i.e. seed treatment + root dip treatment + soil application might be the most effective application method for managing bacterial wilt and in promoting the yield. These two fluorescent pseudomonad strains could serve as potential bio-agent against bacterial wilt of solanaceous vegetables in Meghalaya which needs further field trials under different agro-ecosystems of the state.

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