J Agric. Res., 2017, Vol. 55(3): 525-536
www.jar.com.pk
Agriculture Department,
Government of Punjab

PLANT PATHOLOGY

POTENTIAL OF RHIZOSPHERIC *PSEUDOMONAS* STRAINS TO MANAGE FUSARIUM WILT OF TOMATO

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Article received on:
25/02/2016
Accepted for publication:
01/06/2016

ABSTRACT

Fusarium wilt induced by *Fusarium oxysporum* f. sp. *lycopersici* (FOL) has hampered tomato production worldwide. The present research work was conducted at the Institute of Agricultural Sciences, University of Punjab, Qauid-e-Azam Campus, Lahore, Pakistan during the year 2013-14 to search for a biological control strategy to combat this disease. For this purpose indigenous non-pathogenic rhizospheric isolates of *Pseudomonas* spp. were collected to test their potential to induce resistance in tomato against Fusarium wilt. On preliminary grounds 31 isolates of *Pseudomonas* spp. were tested for *in vitro* antibiosis. Eight isolates that efficiently inhibited the growth of *F. oxysporum* were selected for further experimentation. Greenhouse study revealed that two bacterial isolates i.e. PM12 and PM29 significantly reduced disease index ≥ 60% in two Fusarium wilt susceptible tomato varieties viz: Nagina and Rio- Grande. In addition these isolates resulted in higher accumulation of defense related enzymes and phenolic compounds in tomato plants. During the two growing seasons of 2013 and 2014, sugarcane press-mud based formulations were developed for field evaluations. Combined application of PM12 and PM29 gave the lowest disease index (18.17%) as compared to the pathogen control. Identification based on amplification of ITS region revealed PM12 and PM29 as *P. aeruginosa* and *P. putida.* Results clearly highlights the significance of *Pseudomonas* strains in suppression of *F. oxysporum* and promoting plant growth thus indicating their possible use for the management of Fusarium wilt in tomato cultivation.

KEYWORDS: ***Solanum lycopersicum;*** **tomato;** ***Pseudomonas;*** **induced resistance; Fusarium wilt; phenolics; antibiosis; Pakistan.**

INTRODUCTION

Fusarium wilt of tomato (*Solanum lycopersicum* L.) caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder & Hans, is one of the devastating diseases responsible for economic losses in tomato production (2). In Pakistan tomato yield is less than that of other developed countries (17). The annual production of tomatoes in Pakistan was estimated at 530,000 tons, covering an area of about 52,300 hectares (37). *Fusarium oxysporum* is soil borne fungus causing vascular wilt. Management strategies rely on the use of chemical fumigants and resistant varieties. However excessive use of chemicals not only pollutes the environment but it is also detrimental to human health (5). In the same way using resistant cultivars is not an effective strategy because there are chances of mutation in *Fusarium* spp. (18). Recently, due to increased awareness about the hazards of pesticides, research efforts are being confined on searching strategies that are ecofriendly and economical (1, 2, 3, 4). Biological control using microorganisms or their metabolites serves as an alternative to chemicals for management of plant diseases (42). Among the biocontrol agents most studies are conducted on bacteria belonging to the genera *Bacillus, Streptomyces, Pseudomonas, Burkholderia*, and *Agrobacterium* (27).

Plant growth promoting bacteria (PGPR) belonging to genus *Pseudomonas* helps in protecting the plants from different foliar and soil-borne pathogens (33). These not only antagonize the pathogens but also induce systemic resistance in plants (44). Induced systemic resistance is governed by strengthening of cell walls, triggering of pathogenesis related proteins and phenolic compounds in plants (10). PGPR strains like *Pseudomonas* have been found to be involved in plant growth promotion (9, 20, 43). Now PGPR

formulations are commercially available such as Victus and Conquer formulated with Pseudomonas fluorescens strains, for potato, apple, strawberry and tomato crops (7, 11).

The purpose of this study was to assess the potential of indigenous non-pathogenic *Pseudomonas* strains in suppression of Fusarium wilt in tomato and induction of defense enzymes and compounds.

MATERIALS AND METHODS

This study was conducted at the Institute of Agricultural Sciences, University of Punjab, Quaid-e-Azam Campus, Lahore, Pakistan during the year 2013 and 2014.

Isolation of Pseudomonas strains: *Pseudomonas* strains were isolated from rhizosphere of healthy looking tomato plants growing by dilution plate technique using *Pseudomonas* specific media (*Pseudomonas* Isolation Agar). After incubated at 35ºC for 48 hours, single bacterial colonies well separating from the other ones were restreaked on a new LB (Luria Bertani) agar plate with an inoculating loop.

Antagonistic bioassay: Bacterial isolates were tested for their ability to inhibit *F. oxysporum* on agar plates. Dual culture plate method was used to determine the antifungal activity of bacterial strains (14). A mycelial plug of actively growing FOL (*Fusarium oxysporum* f. sp. *lycopersici*) was placed into the center of potato dextrose agar medium and bacterial strain was streaked 2 cm away on either side of mycelial plug. Plates were then incubated at 28°C for about seven days. Percent inhibition of growth was recorded by measuring radial growth of fungal mycelium.

Bacterial and fungal inoculum: Bacterial strains that inhibited fungal growth upto 75 percent in antagonistic bioassay were selected for greenhouse experiment. Bacteria were cultured on King's B media with continuous shaking at 100 rpm for 48 hours at room temperature (28±2°C). Bacterial cells were harvested by centrifugation at 12000 rpm for 15 minutes and pellets obtained were re-suspended in Phospahte Buffer (0.01 M, pH 7.0). The concentration was adjusted to approximately 10^8 cfu mL^{-1} (OD595=0.3) with a spectrophotometer and used it as bacterial inoculum (47).

A virulent strain of FOL was used. One week old culture plates of FOL on Malt Extract Agar (MEA) medium were used for preparing micro conidial suspension. Concentration was adjusted to 1000 conidia mL^{-1} with haemocytometer.

Plant cultivation: Seeds of two Fusarium wilt susceptible tomato varieties (Rio Grande and Nagina) were surface-sterilized with 2% $NaOCl_2$ solution for two minutes followed by thorough washings with sterilized distilled water and planted in pots containing sterilized sandy loam soil. After four weeks, seedlings were transplanted to sterilized plastic pots (6 inches diameter) containing sterilized sandy loamy soil and then biocontrol potential of selected *Pseudomonas* strains was checked under greenhouse conditions.

Biocontrol of tomatoes wilt in greenhouse: Bacterial strains selected after the antagonistic bioassay were evaluated under greenhouse conditions to control fusarium wilt disease in tomatoes. Bacterial suspensions were provided as soil drench method in pots @ 50 mL/plant. Pathogen inoculum (50mL) was supplied to the pots after one week of bacterial application. Data were recorded after 30 days of incubation under greenhouse conditions.

Disease severity was recorded on a 0-4 visual scale of the shoots and root according to Rothrock (8). Disease index and biocontrol effect were calculated according to the method of Li *et al.* (26) as given below:-

$$\text{Disease index (\%)} = \sum \frac{\text{(Grade of disease severity + diseased plants of this grade)}}{\text{Total plants assessed} \times \text{Highest Grade of disease severity}} \times 100$$

$$\text{Biocontrol effect (\%)} = \frac{\text{(Disease index of pathogen control – disease index of bacterial control)}}{\text{Disease index of pathogen control}} \times 100$$

Induction of systemic resistance by potential Pseudomonas strains: A second greenhouse study was performed to check the efficacy of selected *Pseudomonas* strains for inducing systemic resistance in tomato plants. Selected bacterial strains were used against a single susceptible variety. Time course study was performed to assess biochemical basis of resistance through quantification of total phenolics and enzymes involved in 'phenylpropanoid' pathway viz: phenylalanine ammonia-lyase (PAL), poly phenoloxidase (PPO) and peroxidase (PO). Assays were performed by taking root samples which were excised into small pieces. Samples were washed to remove any soil particles and dried between two layers of blotting paper to remove tap water. The roots were ground with liquid nitrogen in pestle and mortar and then further processed for the analysis of phenolic compounds and defence related enzymes.

Total phenolic content was assayed by the method of Zieslin and Ben-Zaken, (50) with some modifications. One gram root sample was mixed with 80 percent methanol solution and agitated at 70°C. After 15 minutes of agitation 1mL of this extract was incubated with 5mL of distilled water and 250 µL of 50% Folin Ciocaltueau reagent (1N) in dark for ½ hour at 25°C. After incubation, absorbance was measured at 725 nm using spectrophotometer. Catechol was used as a standard. Quantification was done by comparing with standard curve as 'µg catechol/mg protein.

For estimation of defense related enzymes, 1 g of washed root sample was ground in pre-chilled pastel and mortar containing five mL of ice cold 100 mM phosphate buffer (pH 7.0). After crushing, whole material was transferred into a falcon tube and centrifuged at 10000 rpm for 15 minutes at 4°C. Supernatant obtained after centrifugation was used for the quantification of enzymes.

PO activity was determined by the method of Fu and Huang (19) with some modifications. Reaction mixture comprised 50 µL of supernatant, 0.1M phosphate buffer (pH 7.0), 20 mM guaiacol reagent and 40 mM hydrogen peroxide. Changes in absorbance were noted spectrophotometrically at 470 nm. PPO activity was analyzed by using method of Mayer *et al.* (29) with some modifications. For this purpose supernatant was mixed with 0.01M catechol and left it for one hour. After incubation absorbance was recorded at 495 nm.

PAL activity was measured according to the method of Burrell and Rees (29) with some modifications. Reaction mixture consisted of 0.2 mL of supernatant, 0.03M L- Phenyl alanine and sodium borate buffer (pH 8.8) which was kept at 37°C for one hour. After incubation 1M trichloro acetic acid was added to the reaction mixture and quantity of trans- cinnamic acid formed was measured at 290 nm.

Development of bioformulation: Two most efficient bacterial strains selected from greenhouse study were evaluated in this experiment. The ability of selected bacteria to suppress fusarium wilt was studied in the field. For this purpose formulations of bacteria were prepared with sugarcane pressmud. Randomized split plot design was adopted for field trials and each treatment was replicated thrice. Main plots were subdivided into 3×4 m^2 subplots.

Two strains viz: *P. aeruginosa* and *P. putida* were grown for 24 hours at 35°C in LB broth media. Bacterial cultures were centrifuged at 4000 rpm for 15 minutes, the resulting pellet was washed two times with 1 mL of 0.01 M phosphate buffer saline, pH 7.0. Pellet was dissolved in sterile distilled water and concentration was adjusted to 10^4cfu/ml by taking OD of 0.1 at 600nm. Three treatments were made viz. T_1 (*P. aeruginosa*), T_2 (*P. putida*) and T_3 (*P. aeruginosa* + *P. putida*). Hundred ml of these formulations were mixed with 100g of sterilized sugarcane pressmud carrier.

Biocontrol of wilt in field grown tomatoes: Field trials were conducted at the experimental station of Institute of Agricultural Sciences, University of Punjab, Lahore, during February – June 2013 and 2014. Seedlings of tomato variety "Rio- Grande" were raised in sterilized media in pots. Fungal inoculum was developed on sorghum grains and applied @ 100g/ plot and left for one week to make the field infected. After one week of pathogen establishment seedlings were transferred to the field and sugarcane pressmud based bio-formulation was applied @ 100g/ plot. Data of disease index and biocontrol effect were recorded after two months of seedling transplantation. Tomato fruits were harvested three times and biomass (fresh & dry) was calculated at final harvest.

Molecular typing: The 16S rRNA gene was amplified from two *Pseudomonas* strains (PM12 and PM9), exhibited higher suppression efficacy using bacterial universal oligonucleotide primers as described by Tambong *et al.* (46). This primer pair amplifies a fragment within the highly variable regions of the 16S rRNA. Sequencing was done and submitted to NCBI. The 16S rRNA gene sequences were compared with those in GenBank using the BLAST programme and the strains showing maximum similarity were aligned using the CLUSTAL W program

Data analysis: All the data were statistically analyzed performing one-way ANOVA followed by DNMRT test (45) at $P = 0.05$ and with the help of computer aided software "DSSTAT". All the experiments were performed in a randomized complete block design and each treatment contained three replicates.

RESULTS AND DISCUSSION

Antagonistic bioassay

Among 31 isolates tested for antagonistic activity in lab conditions eight strains performed efficiently in reducing mycelial growth of fungus. Three strains i.e. PM4, PM 12 and PM17 showed 84.02, 82.77 and 81.85 percent inhibition (Table 1). Five strains viz. PM6, PM25, PM28, PM29 and PM31 showed more than 75 percent biocontrol effect. In this research bacterial isolates were collected from the roots of healthy tomato plants because it has been studied earlier (48) that potential of rhizospheric strains to combat the disease is more if collected from the target crop. The possible reason of rhizobacterial isolation from the same crop on which pathogen attacks may involve the host plant specificity by some of the naturally occurring antagonists already present in the soil. Findings of this study showed that about 25 percent (8 out of 31) isolates showed antagonistic activity against FOL *in vitro* conditions which are in agreement with the findings of Eleftherio *et al.* (16) and Zheng *et al.* (49).

Biocontrol of tomatoes wilt in greenhouse

Selected *Pseudomonas* strains provided significant control of fusarium wilt disease on tomato. Disease index and biocontrol effect observed under the influence of these strains is described in Table 2. Here strains PM12 and PM29 strongly reduced disease index and provided maximum significant biocontrol efficacies in both susceptible varieties i.e. Nagina and Rio-Grande. The results revealed that PM12 provided maximum control of fusarium wilt in Nagina (67.12%) and Rio-Grande (73.04%) (Table 2) followed by PM29 (60.41 and 64.24%) and reduced disease index to 14.78 and 22.43 percent, respectively. PM6 showed 59.54 and 63.06 percent control on Fusarium wilt. These results agree to the findings of Kumar *et al.* (24), who found that *P. fluorescens* isolates efficiently reduced early blight disease on tomato to 18-42 percent. Similarly *P. fluorescens* strain-B5 reduced Verticillium wilt of cotton to 60 percent (28).

Table 1. Effect of selected Pseudomonas strains on mycelial growth of Fusarium oxysporum *in vitro* conditions

Pseudomonas strains	Inhibition percentage	*Pseudomonas* strains	Inhibition percentage
PM1	52.13±05.14^{F}	PM17	81.85±07.32^{A}
PM2	21.64±02.03^{MN}	PM18	08.16±00.78^{O-Q}
PM3	42.37±03.03^{F-I}	PM19	65.91±04.32^{CD}
PM4	84.02±06.18^{A}	PM20	48.51±03.88^{FG}
PM5	12.42±01.06^{N-P}	PM21	32.44±02.97^{I-K}
PM6	77.37±07.21^{AB}	PM22	21.18±01.84^{MN}
PM7	35.61±02.77^{IJ}	PM23	61.36±05.18^{C-E}
PM8	08.23±00.74^{Q}	PM24	31.05±02.77^{I-L}
PM9	15.19±02.11^{NO}	PM25	78.10±06.51^{AB}
PM10	09.05±01.44^{O-Q}	PM26	68.81±05.61^{B-D}
PM11	45.82±04.13^{F-H}	PM27	22.43±01.71^{MN}
PM12	82.77±07.45^{A}	PM28	77.28±06.31^{AB}
PM13	61.55±07.51^{C-E}	PM29	71.60±05.07^{BC}
PM14	36.72±03.20^{IJ}	PM30	14.42±01.28^{NO}
PM15	24.40±02.12^{M}	PM31	74.51±06.43^{BC}
PM16	12.67±00.95^{N-P}		

Dual culture plate assay was performed under laboratory conditions to check the antagonistic activity of selected *Pseudomonas* strains against *Fusarium oxysporum* f. sp. *lycopersici*. After seven days radial growth of fungal mycelium was measured and percentage inhibition in growth was estimated. Values with same letters differ non-significantly (P>0.05) as governed by ANOVA and DNMRT. Values with ± signs represent standard error between different replicates of the same treatment. Whereas PM stands for *Pseudomonas* and numbers denote different strains of *Pseudomonas.*

Table 2. Effect of selected *Pseudomonas* strains to suppress fusarium wilt disease on two tomato varieties under greenhouse conditions

	Disease index and bio-control effect			
Treatments	**Nagina disease index (%)**	**Bio control effect (%)**	**Rio-Grande disease index (%)**	**Bio control effect (%)**
PM12	10.85f(±00.75)	67.12a(±06.04)	14.91g(±01.19)	73.04a(±05.11)
PM29	14.78e(±01.33)	60.41b(±05.44)	22.43f(±01.74)	64.24b(±04.47)
PM6	14.98e(±00.74)	59.54b(±02.16)	23.14f(±00.92)	63.06b(±05.67)
PM25	29.04cd(±02.32)	43.34cd(±03.01)	34.91d(±02.77)	45.41d(±03.62)
PM4	30.84cd(±02.46)	46.12c(±03.15)	35.45d(±02.13)	43.22d(±02.16)
PM17	30.87cd(±02.37)	45.12c(±02.17)	44.12c(±03.52)	36.55f(±03.28)
PM28	40.26b(±02.14)	37.01ef(±01.83)	52.12b(±04.61)	50.71c(±04.01)
PM31	34.87c(±03.07)	41.28ce(±03.78)	31.95de(±02.18)	40.02de(±02.06)
PC	88.76a(±06.97)	-	84.06a(±05.87)	-
UC	-	-	-	-

Tomato plants were co-cultivated with selected *Pseudomonas* strains and disease index was recorded after 30 days of pathogen challenge. Data shown represent average values from three independent experiments. Values with same letters differ non-significantly (P≥0.05) as governed by ANOVA and DNMRT. Values with ± signs represent standard error between different replicates of the same treatment. Whereas PM stands for *Pseudomonas* and numbers denote different strains of *Pseudomonas*, PC = Pathogen control, UC = Untreated control

Induction of systemic resistance by potential *Pseudomonas* strains

Changes in quantities of defense related biochemicals were studied at specific time intervals. Plants treated with bacterial strains PM12 and PM29 produced a robust and transitory accumulation of phenolics in initial intervals and reaching at peak level after four days of post inoculation and then started declining (Fig. 1). Strains PM12 and PM29 showed 2.6 and 2.5 folds increase at four days of post inoculation (dpi) as compared to 0 dpi (Fig. 1).

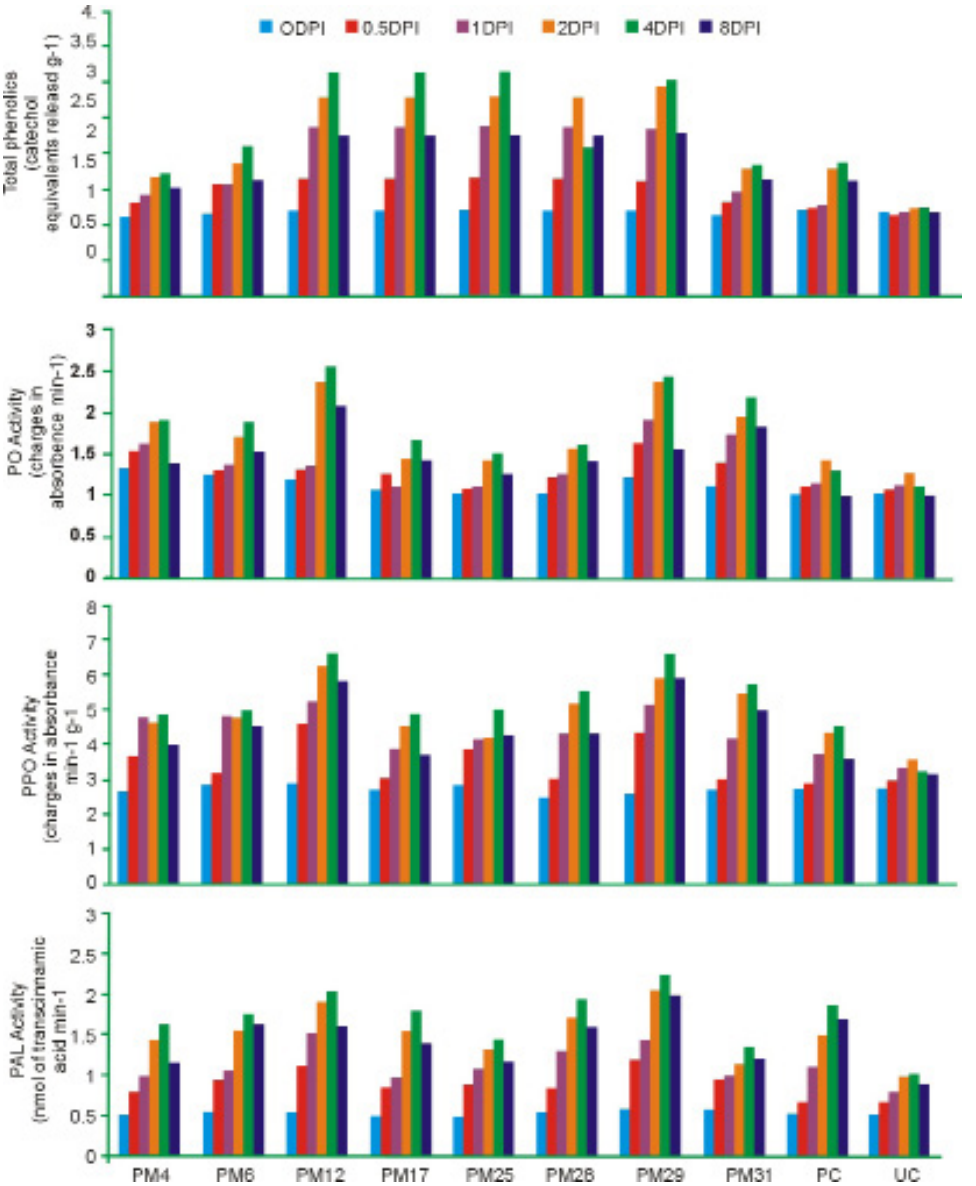

Fig. 1. Development of plant defense related substances in greenhouse tomatoes exposed to various *Pseudomonas* strains. Tomato plants were co-inoculated with bacterial strains following pathogen challenge as described in experimental procedures. Activities of phenyl ammonia lyase, polyphenoloxidase, peroxidase and phenolic compounds were analyzed from the leaf tissues in a time course manner at intervals of 0, 0.5,1, 2, 4 and 8 days of post inoculation (dpi) after pathogen challenge. Data represent average values from three independent experiments, with error bars depicting standard error.

In the same way time course study revealed that tomato plants challenged with bacterial strains PM12 and PM29 exhibited an increasing trend of PO, PPO and PAL activity that showed maximum activity at 4 dpi and then started to reduce at 8 dpi. In case of PO activity PM12 and PM29 showed 2.16 and 2.02 folds upregulation of phenolics at 4 dpi as compared to 0 dpi. Plants treated with PM12 and PM29 showed maximum activity of PPO at 4 dpi and found to be 2.28 and 2.58 folds more as compared to 0 dpi. Similarly highest activity of 3.9 and 3.8 folds was noted for PM12 and PM29 at 4 dpi as compared to 0 dpi. Pathogen treated plants also showed some inducible changes in defence related enzymes activity. Un-treated control plants maintained constant low levels of defence biochemical at all time intervals. Rhizobacteria helps in defending plants from pathogen attack

by stimulating defence mechanisms of the plants through accumulation of PR proteins, developing structural barriers and inducing phenolic compounds synthesis (6, 10, 31). In the present investigation tomato plants treated with bacterial strains PM12 and PM29 showed significantly high levels of phenolic compounds as compared to untreated control. Similar findings have also been reported by Seleim *et al.* (41) who treated tomato plants with *P. fluorescens* and *P. putida* and observed induction of defense related proteins, viz. PO, PAL and PPO against *Ralstonia solanacearum*. Similarly in another study (32) three isolates of *P. fluorescens* were found to induce systemic resistance against bacterial wilt of tomato through increased activity of PO, PPO, PAL, β-1, 3-glucanase and phenolics.

Enzymes like PO, PPO and PAL that are involved in phenylpropenoid pathway also induce resistance in plants (21, 35). These enzymes are helpful in degrading pectolytic enzymes released by the pathogens and also induce the plants for production of quinones and phytoalexins (22, 25). In this study increased levels of these enzymes were recorded when the tomato plants were treated with bacterial strains. Similarly in another study (3) high levels of these enzymes in tomato plants were documented under the influence of *Bacillus* strains.

Biocontrol of wilt in field grown tomatoes

The treatment where mixture of strains (*P. aeruginosa* + *P. putida*) was used provided maximum control of fusarium wilt. Biocontrol potential of this treatment was 65.24 and 74.85 percent in field trials conducted during 2013 and 2014 (Table 3). These bacterial strains not only managed the disease but also enhanced plant growth and yield. Significant increase ($P > 0.05$) in plant height and biomass was recorded when plants were treated with bacterial inducers in comparison to untreated control. Plants treated with combination of strains showed average increase in plant height to 48.16 percent over untreated control during both season field trials (Table 4). In the same way significant average increase of 51.58 percent in number of fruits was seen in plants co-inoculated with both bacterial strains as compared to untreated control during the years 2013 and 2014, respectively (Table 4).

An average increase of fresh and dry biomass to 49.04 and 43.07 percent was recorded under the influence of combined bacterial strains. Similarly consortium application of strains provided significant increase in average yield of 49.76 percent during two years.

Table 3. Effect of sugarcane press mud based bio-formulation of selected *Pseudomonas* strains on suppression of fusarium wilt disease on tomato variety "Rio-Grande" under field conditions during the years 2013 & 2014

	2013		2014	
Treatments	**Disease index (%)**	**Suppression efficacy (%)**	**Disease index (%)**	**Suppression efficacy (%)**
PC	62.31a (±8.12)	-	71.47a (±9.71)	-
UC	-	-	-	-
T_1	21.54c (±3.05)	58.62b (±6.24)	27.72c (±3.77)	63.47b (±7.41)
T_2	28.74b (±3.11)	54.03bc (±7.22)	34.22b (±4.52)	47.02c (±5.45)
T_3	18.76c (±2.14)	65.24a (±8.76)	17.58d (±2.54)	74.85a (±9.44)

Disease parameters were taken after one month of application of bio-formulation in the field. Data shown represent average values from three independent experiments. Values with same letters differ non-significantly ($P>0.05$) as governed by ANOVA and DNMRT. Values with ± signs represent standard error between different replicates of the same treatment. Where PC = Pathogen control, UC = Untreated control, T1= Plants treated with *P. aeruginosa*, T2 = Plants treated with *P. putida*, T3 = Plants treated with *P. aeruginosa* + *P. Putida*.

Table 4. Effect of sugarcane press mud based bio-formulation on growth and yield of tomato variety "Rio-Grande" under field conditions during two subsequent years.

Treatment	2013				
	Plant height (cm)	Total biomass (g)		Number of fruits	Yield (Kg)
		Fresh	Dry		
PC	27.16cd (±02.14)	92.04f (±11.54)	13.68e (±01.22)	14.05d (±01.08)	2.12d (±0.18)
UC	31.85c (±03.01)	119.90e (±09.87)	16.36d (±01.32)	16.23c (±02.27)	3.44c (±0.26)
T_1	43.45b (±3.88)	157.02b (±14.37)	20.46b (±01.93)	21.08ab (±02.07)	4.58a (±0.35)
T_2	42.14b (±3.65)	146.42c-d (±11.38)	19.33bc (±02.11)	19.42b (±02.01)	4.07b (±0.41)
T_3	46.53a (±4.11)	175.11a(±13.61)	23.19a (±02.34)	24.24a (±02.11)	5.12a (±0.47)
		2014			
PC	25.47cd (±02.42)	87.94e (±11.09)	11.64cd (±01.64)	14.54d (±01.32)	2.53d (±0.19)
UC	29.85c (±02.66)	102.40d (±10.13)	13.52c (±01.71)	15.78c (±02.16)	4.46c (±0.33)
T_1	42.11a (±03.09)	138.62b (±10.21)	17.94ab (±02.13)	21.52ab (±01.71)	6.02ab (±0.53)
T_2	40.64ab (±05.19)	127.25bc (+12.17)	16.45b (±01.65)	19.01b (±02.37)	5.43b (±0.41)
T_3	44.84a (±02.74)	155.67a (±13.24)	19.52a (±02.53)	24.27a (±03.62)	6.72a(±0.55)

Growth parameters were taken after one month of application of bio-formulation in the field whereas yield was recorded after three months. Data shown represent average values from three independent experiments. Capital letters represent levels of significance (P>0.05) as governed by ANOVA and DNMRT. Values with ± signs represent standard error between different replicates of the same treatment. Whereas PC = Pathogen control, UC = Untreated control, T1= Plants treated with *P. aeruginosa*, T2 = Plants treated with *P. putida*, T3 = Plants treated with *P. aeruginosa* + *P. Putida*.

Field experiment showed that combination of bacterial strains (PM12 + PM29) provided maximum disease suppression efficacy as compared to other treatments. Synergistic effect of inoculating two *Pseudomonas* strains may lead to higher accumulation of defence related biochemicals and metabolites than individual application of these strains. Similar results have been documented by Saravanakumar *et al*. (44). According to their findings combination of *Pseudomonas* strains i.e. Pf1, TDK1 and PY15 was found effective in reducing sheath rot disease in rice plants caused by *Sarocladium oryzae*.

Fluorescent pseudomonad species such as *Pseudomonas fluorescens* (33), *Pseudomonas putida* (13), *Pseudomonas chlororaphis* (12) and *Pseudomonas aeruginosa* (4) have been used to manage pathogens as well as to promote growth and yield in many crop plants. *Pseudomonas fluorescens* has been shown to increase seed germination, root and shoot length and seedling vigour in several instances (15, 23, 36). In a study corn plants inoculated with a strain of *Pseudomonas putida* was found to increase biomass of corn plants in addition to having antagonistic activity against *Fusarium* (30). All these quoted findings agree to the present results because parameters like plant height and biomass were significantly increased under the influence of bacterial strains in field experiments.

Molecular typing

The most active strains (PM12 and PM29) were identified by targeting ITS regions, amplified regions subjected to sequencing. These strains were identified as *P. aeruginosa* (PM12) and *P. putida* (PM29) and were submitted to NCBI under the accession numbers KT966743 and KT966744. Dendrogram showed the similarity of these strains with already submitted strains in the gene bank using Clustal W (Fig. 2A & B).

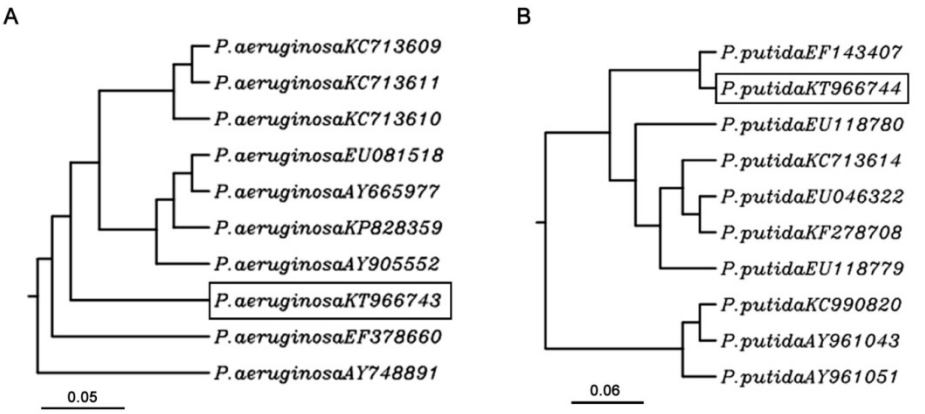

Fig. 2. Rooted neighbor-joining tree based on partial 16s rDNA gene sequences. The scale bar indicates nucleotide substitutions per nucleotide position. The accession numbers of selected bacterial strains were shown in boxes. A= *Pseudomonas aeruginosa* PM12, B= *P. putida* PM29.

CONCLUSION

The study concludes that *Pseudomonas* strains can be used to replace the chemicals keeping in view the potential of these strains for induction of systemic resistance and plant growth promotion under both greenhouse and field conditions. So there is dire need to produce these strains on commercial scale to manage Fusarium wilt in tomato that will be beneficial both for the farmers and the environment.

REFERENCES

1. Agbenin, N.O., A.M. Emechebe and P.S. Marley. 2004. Evaluation of Neem seed powder for Fusarium wilt and Meloidogyne control on tomato. Arch. Phytopthol. Plant. Protect. 37(4):319-326.
2. Agrios, G. N. 2005. Plant Pathology. 5th Ed. Burlington, MA. Elsevier Academic Press.
3. Akram, W., T. Anjum, B. Ali and A. Ahmad. 2013. Screening of native bacillus strains to induce systemic resistance in tomato plants against fusarium wilt in split root system and its field applications. Int. J. Agric. Biol. 15(6):1289-1294.
4. Anjaiah, V., P. Cornelis. and N. Koedam. 2003. Effect of genotype and root colonization in biological control of Fusarium wilts in pigeonpea and chickpea by *Pseudomonas aeruginosa* PNA1. Can. J. Microbiol. 49(2):85–91.
5. Baysal, O., M. Caliskan and O. Yesilova. 2009. An inhibitory effect of a new *Bacillus subtilis* strain (EU07) against *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Physiol. Mol. Pl. Pathol. 73 (1-3):25-32.
6. Benhamou, N., R. Belanger and T. Paulitz. 1996. Ultrastructural and cytochemical aspects of the interaction between *Pseudomonas fluorescens* and Ri T-DNA transformed pea roots: host response to colonization by *Pythium ultimum Trow*. Planta. 199(1):105-117.
7. Bhattacharyya, P.N. and D.K. Jha. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World, J. Microbiol. Biotechnol. 28(4):1327-1350.
8. Burrell, M.M and T. A. Rees. 1974. Metabolism of phenylalanine and tyrosine in rice leaves infected by *Pyricularia oryzae*. Physiol. Pl. Pathol. 4(4):497-508.
9. Carlier, E., M. Rovera, A. Rossi Jaume and S.B. Rosas. 2008. Improvement of growth, under field conditions, of wheat inoculated with *Pseudomonas chlororaphis* subsp. *aurantiaca* SR1. World, J. Microbiol. Biotechnol. 24(11):2653-2658.
10. Chen, C., R. Belanger, R. N. Benhamou and T. C. Paulitz. 2000. Defence enzymes induced in cucumber roots by treatment with

plant growth promoting rhizobacteria (PGPR) and *Pythium aphanidermatum*. Physiol. Mol. Pl. Pathol. 56(1):13-23.
11. Chet, I and L. Chernin. 2002. Biocontrol, microbial agents in soil. *In:* Bitton G (ed.) Encyclopedia of environmental microbiology. Willey, New York, USA. p. 450-465.
12. Chin-A-Woeng, T.F.C., G.V. Bloemberg, A.J. Vander Bij, K.M.G.M. Vander Drift, J. Chripsema, B. Kroon, R.J. Scheffer and C. Keel. 1998. Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis lycopersici*. Mol. Pl. Microbe. Interact. 11(11):1069–1077.
13. De Freitas, J.R and J.J. Germida. 1991. *Pseudomonas cepacia* and *Pseudomonas putida* as winter wheat inoculants for biocontrol of Rhizoctonia solani. Can. J. Microbiol. 37(10):780-784.
14. Dennis, C and J. Webster. 1971. Antagonism properties of species groups of *Trichoderma*, III. Hyphal interaction. Trans. Br. Mycol. Soc. 57(3):363-369.
15. Egamberdieva, D. 2008. Plant growth promoting properties of Rhizobacteria isolated from wheat and pea grown in loomy sand soil. Turk. J. Biol. 32(1):9-15.
16. Eleftherios, C.T., D.I. Tsitsigiannis and S.E. Tjamos. 2004. Selection and screening of endorhizosphere bacteria from solarized soils as biocontrol agents against *Verticillium dahlia* of solanaceous hosts. Eur. J. Pl. Pathol. 110(1):35-44.
17. Farooq, A.M., B. Tabassum, I.A. Nasir and T. Husnain. 2010. Androgenesis induction, callogenesis, regeneration and cytogenetic studies of tomato haploid. J. Agric. Res. 48(4):457-470.
18. Fravel, D., C. Olivain and C. Alabouvette. 2003. *Fusarium oxysporum* and its biocontrol. New. Phtytol. 157(3):493-502.
19. Fu, J.H.B. 2001. Involvement of antioxidants and lipid peroxidation in the 284 adaptation of two cool-season grasses to localized drought stress. Environ. Exp. Bot. 45(2):105-114.
20. Haas, D and G. Défago. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat. Rev. Microbiol. 3(4):307-319
21. Jourdan, E., G. Henry, F. Duby, J. Dommes, J.P. Barthelemy, P. Thonart and M. Ongena. 2009. Insights into the defense-related events occurring in plant cells following perception of surfactin-type lipopeptide from *Bacillus subtilis*. Mol. Plant-Microbe. Interact. 22(4):456-468.
22. Kavino, M., S. Harish, N. Kumar, D. Saravanakumar and R. Samiyappan. 2008. Induction of systemic resistance in banana (*Musa* spp.) against banana bunchy top virus (BBTV) by combining chitin with root-colonizing *Pseudomonas fluorescens* strain CHA0. Eur. J. Pl. Pathol. 120(4):353–362.
23. Khalid, A., M. Arshad and Z.A. Kahir. 2004. Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. Appl. Soil. Ecol. 96(3):473-480.
24. Kumar, P., N. Kaushal and R. C. Dubey. 2015. Isolation and identification of plant growth promoting rhizobacteria (*Pseudomonas* spp.) and their effect on growth promotion of *Lycopersicon esculentum* L. Academia Arena. 7(5):44-51.
25. Li, L and J.C. Stiffens 2002. Over expression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. Planta. 215(2):239-247.
26. Li, W., J.C. Hu and S. J. Wang. 2008. Growth-promotion and biocontrol of cucumber fusarium wilt by marine *Bacillus subtilis* 3512A. J. Shenyang Agric. Univ. 39(2):182-185.
27. Lucy, M. E., Reed and B. R. Glick. 2004. Applications of free living plant growth-promoting rhizobacteria. Rev. Ant. Van Lee. 86 (1): 1–25.
28. Mansoori, M., A. Heydari, N. Hassanzadeh, S. Rezaee and L. Naraghi. 2013. Evaluation of *Pseudomonas* and *Bacillus* bacterial antagonists for biological control of cotton Verticillium wilt disease. J. Pl. Prot. Res. 53 (2):154-157.
29. Mayer, A.M., E. Harel and R.B. Shaul. 1966. Assay of catechol oxidase a critical comparison of methods. Phytochem.

5(4):783-789.
30. Mehnaz, S and G. Lazarovits. 2006. Inoculation effects of *Pseudomonas putida, Gluconacetobacter azotocaptans*, and *Azospirillum lipoferum* on corn plant growth under greenhouse conditions. Microb. Ecol. 51(3):326-335.
31. Mpiga, P., R. R. Belanger, T.C. Paulitz and N. Benhamou. 1997. Increased resistance to *Fusarium oxysporum* f.sp. *radicis-lycopersici* in tomato plants treated with the endophytic bacterium *Pseudomonas fluorescens* strain 63-28. Physiol. Mol. Plant. Pathol. 50(5):301-320.
32. Murthy, K., N. Uzma, F. Chitrashree and C. Srinivas. 2014. Induction of Systemic Resistance in tomato against *Ralstonia solanacearum* by *Pseudomonas fluorescens*. Amer. J. Pl. Sci. 5(12):1799-1811.
33. Ownley, B. H., B.K. Duffy and D.M. Weller. 2003. Identification and manipulation of soil properties to improve the biological control performance of phenazine-producing *Pseudomonas fluorescens*. Appl. Environ. Microbiol. 69 (6):3333–3343.
34. Paul, P.K. and P.D. Sharma. 2002. *Azadirachta indica* leaf extract induces resistance in barley against leaf stripe disease. Physiol. Mol. Pl. Pathol. 61(1):3-13.
35. Radjacommare, R., S. Venkatesan and L. Samiyappan. 2010. Biological control of phytopathogenic fungi of vanilla through lytic action of *Trichoderma* species and *Pseudomonas fluorescens*. Arch. Phytopathol. Pl. Prot. 43 (1): 1–17.
36. Ramamoorthy, V., R. Viswanathan, T. Raghuchander, V. Prakasam and R. Samiyappan. 2001. Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pest and diseases. Crop. Prot. 20 (1): 1-11.
37. Ramzan, A., T.N. Khan, N.N. Nawab, A. Hina, T. Noor and G. Jillani. 2014. Estimation of genetic components in F1 hybrids and their parents in determinate tomato (*Solanum lycopersicum* L.). J. Agric. Res. 52(1): 65-75.
38. Rothrock, C.S. 1987. Take-all of wheat as affected by tillage and wheat-soybean double cropping. Soil. Biol. Biochem. 19 (3): 307–311.
39. Sakthivel, N and S.S. Gnanamanickam. 1987. Evaluation of *Pseudomonas fluorescens* for suppression of sheath rot disease and for the enhancement of grain yields in rice (*Oryza sativa* L.). Appl. Environ. Microbiol. 53(9):2056-2059.
40. Saravanakumar, D., N. Lavanya, K. Muthumeena, T. Raguchander and R. Samiyappan. 2009. Fluorescent pseudomonad mixtures mediate disease resistance in rice plants against sheath rot (*Sarocladium oryzae*) disease. Biocontrol. 54(2):273-286.
41. Seleim, M. A., K. A. Abo-Elyousr, A. A. A. Mohamed and H. A. Al-Marzoky. 2014. Peroxidase and polyphenoloxidase activities as biochemical markers for biocontrol efficacy in the control of tomato bacterial wilt. J Plant Physiol Pathol 2(1):1-4.
42. Sindhu, S.S., Y.S. Rakshiya and G. Sahu. 2009. Rhizosphere bacteria and their role in biological control of plant diseases. Pest Techol. 3:10-21.
43. Srinivasan, K., G. Gilardi, A. Garibaldi and M.L. Gullino. 2009. Bacterial antagonists from used rockwool soilless substrates suppress Fusarium wilt of tomato. J. Pl. Pathol. 91(1):147-154.
44. Srivastava, R., A. Khalid, U.S. Singh and A.K. Sharma. 2010. Evaluation of Arbuscular mycorrhizal, fluorescent Pseudomonas and *Trichoderma harzianum* formulation against *Fusarium oxysporum* f. sp. lycopersici for the management of tomato wilt. Biol. Control. 53(1):24-31.
45. Steel, R.G.D and J.H. Torrie. 1980. Principles and Procedures of Statistics. A Biometrical Approach. 2nd Ed., McGraw Hill Inter, Book Co, Tokyo, Japan.
46. Tambong, J. T., K.N. Mwange, M. Bergeron, T. Ding, F. Mandy, L. M. Reid and X. Zhu. 2008. Rapid detection and identification of the bacterium *Pantoea stewartii* in maize by TaqMan real-time PCR assay targeting the cpsD gene. J. Appl. Microbiol. 104(5):1525–1537.

47. Thompson, D.C. 1996. Evaluation of bacterial antagonist for reduction of summer patch symptoms in Kentucky blue grass. Pl. Dis. 80(8):856-862.
48. Weller, D.M. 1988. Biological control of soilborn plant pathogens in the rhizosphere with bacteria. Ann. Rev. Phytopathol. 26: 379-407.
49. Zheng, Y., Q.Y. Xue, L.L. Xu, Q. Xu, S. Lu, C. Gu and J. H. Guo. 2011. A screening strategy of fungal biocontrol agents towards Verticillium wilt of cotton. Biol. Control. 56(3):209- 216.
50. Zieslin, N and R. Ben-Zaken. 1993. Peroxidase activity and presence of phenolic substances in peduncles of rose flower. Pl. Physiol. Biochem. 31(3):333- 339.

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