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POTENTIAL OF SOME RHIZOSPHERIC BACTERIAL STRAINS TO MANAGE BACTERIAL WILT OF TOMATO

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ABSTRACT

Bacterial wilt induced by '*Ralastonia solanacerum'* is a major constraint in vegetable production worldwide. In this study, potential of some rhizospheric bacterial strains to manage bacterial wilt disease under greenhouse was evaluated. The strains were applied by soil drench method. Strains PM12 and PM29 provided maximum controls and reduced disease index up to 70% on tomato plants, compared with the pathogen control. These two strains also induced tomato plants for higher inducible production of defense related biochemicals like total phenolics, peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) when were used to inoculate the root. Furthermore these strains significantly promoted growth of tomato under greenhouse conditions. Taken all together, the present study concludes that these two strains have great potential to manage bacterial wilt of tomato and can be successfully used in our conventional agriculture system.

Keywords; Tomato, Bacterial wilt, Rhizospheric, Induced systemic resistance

INTRODUCTION

Broad research on use of microbes in conventional agriculture for control of plant diseases is in progress. Researchers are in an effort to improve plant vigor and growth by using plant growth promoting rhizobacteria (PGPR), which is thoughtas an alternativeapproach to chemical pesticides and fertilizers (Hanif *et al.,* 2014). Performance of PGPR under greenhouse conditions has been very effective but their potential under field conditions is still questionable. This can be attributed to improper selection, characterization and varied environmental conditions in which they are supposed to perform (Raaijmakers *et al.,* 1995; Silva *et al.,* 2006; Radjacommare *et al.,* 2010). Hence in current investigation, we envisioned on characterization of rhizospheric *Pseudomonas* strains having antimicrobial activity for pathogen along with plant growth promoting and disease suppressing traits.

Rhizosphere-inhabiting *Pseudomonas* play important role in the biological control of soilborne plant pathogens (Jankiewicz and Koltonowicz 2012). These agents face pathogens by multiple mechanisms. These include production of antibiotics, siderophore, and inducing systemic resistance (ISR). PGPR can confer plants to increased defensive capacity against a wide array of pathogens which is known as ISR (van Loon *et al*., 2007; Ongena and Jacques 2008). ISR protects plants by activation of inducible defense mechanisms in a very similar way as against pathogenic microbes. Some mechanisms responsible for ISR include massive biochemical changes inside plant body, reinforcements of physical defense barriers like cell walls, production of biochemicals toxic to pathogens like phytoalexins and over production of pathogenesis-related (PR) proteins (Pieterse and van Loon 1999; van Loon and Pieterse 2006; Kavino 2007; Kavino 2008). PGPR promote plant growth by producing phytohormoneas, phosphate solubilization, siderophore and indirectly by competing with the phytopathogens for space and nutrition at rhizosphere (Gupta *et al.,* 2000).

Tomato (*Lycopersicon esculentum* Miller) is an important vegetables crop of Pakistan. This is the second major vegetable produced in Pakistan. Bacterial wilt disease can completely destroy the crop or cause significant yield losses (Halila and Strange, 1996).. Chemical control of wilt has not been effective because pathogen is both soil and seed borne. In order to achieve good production of tomato, it is important to select biocontrol agents having multiple traits through which they can suppress the diseases and promote plant growth. In this regard, in current investi-gation, we report the screening and characteri- zation of some rhizospheric strains which with ability of these strains capable to induce systemic resistance against bacterial wilt of tomato along with growth promotion of tomato plants.

MATERIALS AND METHODS

Management of Bacterial wilt of tomato under greenhouse conditions:

Preparation of bacterial inoculum: Rhizospheric non-pathogenic bacterial strains were procured from bacterial conservatory of Institute of Agricultural Sciences, University of the Punjab, Lahore Pakistan. These bacterial strains (Table-1) were grown on KB broth medium with constant shaking at 100 *g* for 48 h at room temperature (28±2°C). Bacterial cells were harvested by centrifugation at 12,000 *g* for 15 min and bacterial cells were resuspended in PB (0.01 M, pH 7.0). The concentration was adjusted to approximately 10^8cfu/mL^{-1} (OD595=0.3) with a spectrophotometer and used as bacterial inoculum (Thompson, 1996).

Table-1: Details of bacterial strains used in this study.

Bacterial Species	Strains	Rhizosphere sources
Bacillus megaterium	OSR3	Maize
B. megaterium	MCR7	Maize
B. megaterium	MCR8	Maize
B. megaterium	ZMR4	Maize
B. megaterium	ZmR6	Maize
Pseudomonas putida	FBL12	Sorghum
P. flourscene	FBL02	Tomato
B. fortis	FBL01	Wheat
B. subtilis	FBL10	Wheat
B. subtilis	FBL11	Wheat

Preparation of pathogen inoculum: Virulent strain of *Ralastonia solanacerum f*.sp. *lycopersici* was also obtained from bacterial conservatory of Plant Biotechnology Lab, Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan. The bacterial inoculum was prepared by adopting the same methodology of Thompson (1996).

Growth of tomato plants: Tomato seeds of bacterial wilt susceptible variety (Fine Star) were obtained from Federal Seed Certification Authority, Lahore. Seeds were surface-sterilized with 2% sodium hypochlorite for 2min, washed thoroughly with sterilized water, and planted into pots of sterilized soil. After 4 weeks, seedlings were trans-planted into pots containing sterilized sandy loamy soil.

Evaluation of PGPR strains in control of bacterial wilt under greenhouse conditions: Selected bacterial strains were evaluated under greenhouse conditions for control of tomato bacterial wilt. Bacterial suspensions were watered into the soil around the tomato roots using 50 mL /plant at the time of transplanting into soil containing. After one week of bacterial application, pots were provided with 50 mL of pathogen inoculum prepared previously. After 30 days of incubation under green house, data were taken.

Disease severity was recorded on a 0–4 visual scale of the shoots and root according to Rothrock (1987), which 0 = rhizome and root with no symptom, 1 = 25% damage 2 = 25–50% damage, 3 = 50–75% damage, 4 = 75–100 damage %. Disease index and biocontrol effect were calculated according to the method of Li *et al*., (2008).

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

$$\text{Biocontrol effect (\%)} = \frac{\text{(Disease index of pathogen control- diseased index of bacterial control)}}{\text{Disease index of pathogen control}} \text{X100}$$

Potential of bacterial strains to induce systemic resistance in tomato plants: Another independent greenhouse experiment was performed to assess the potential of bacterial strains to induce systemic resistance in tomato plants. For that purpose, tomato seedlings were again raised as previously described. These were transferred in plastic pots containing sterilized sandy loamy soil. These were provided with 50 mL of bacterial cell suspension prepared as described in above section. After bacterial inoculation, defense related biochemicals like total phenolics, peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase(PAL) were quantified in tomato plants at different intervals viz 0, 2, 4, 8 days post inoculation (DPI).

Total phenolics content was assayed by the method of Zieslin and Ben-Zaken (1993). One gram shoot sample was extracted with 10 mL of 80% methanol at 70°C for 15 minutes. Reaction mixture consisted of 1 mL methanolic extracts, five mL of distilled sterilized water and 250 µl of

1 N Folin Ciocalteau reagent. The absorbance of the developed blue color was measured using a spectrophotometer at 725 nm. The amount of phenolics was expressed as milligram gallic acid/ gram plant material.

For estimation of defense related enzymes, leaf samples were taken from the plants at regular intervals for enzymes assays. One gram of leaf sample was homogenized with 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) in ice bath for enzyme assays. The homogenates were then centrifuged at 10,000 g for 10 min. Supernatants were used to analyze the PO, PPO and PAL activities.

PPO activity was determined according to the procedure described by Mayer *et al*., (1965). The reaction mixture comprised of 200 µl enzym*e* extract and 1.5 mL of 0.01 M catechol. Activity was expressed as changes in absorbance at 495 nm $min^{-1}mg^{-1}$ protein. PAL activity was determined by the method of Burrell and Rees (1974). The reaction mixture containing 1 mL of 0.015 M L-phenylalanine, 1.9 mL of 0.1 M Tris–HCl buffer (pH 8.5), and 0.1 mL enzyme extract was incubated at 30°C for 15 min. The reaction was terminated by the addition of 200 µl of 6 M HCl, and absorbance was measured at 290 nm.

Peroxidase activity was assayed by the method of Fu and Huang (2001). Reaction mixture contained 50 µl of enzyme, 2.85 ml of 0.1 M phosphate buffer (pH 7.0) and mixed with 50 µl of 20 mMguaiacol reagent and 20 µl of 40 mM hydrogen peroxide. Rate of increase in absorbance was measured at 470 nm. All activities were expressed as change in absorbance for 1 g fresh weight per minute.

Effect of bacterial strains on growth of tomato plants: An independent pot experiment was conducted to assess beneficial effects of our bacterial strains on plant growth and development. Plastic pots of 10 inch diameter were filled with sterilized sandy loamy soil as growth media. Ten seeds of a tomato were sown in each pot. Bacterial inocula were prepared by growing them in LB broth media overnight. Bacterial cells were collected by centrifugation and re-suspended in distilled sterilized water at concentration of $1x10^8$cfu/ml by taking OD at 600 nm. One hundred ml of bacterial inoculum was provided in each pot. Control treatment was provided with 100 mL of distilled sterilized water. All the treatments were arranged in complete randomized block design with five replications and performed twice. After forty days of incubation under greenhouse environment, harvesting of plants was done and different growth parameters were recorded like shoot length, root length, total fresh and dry biomass.

Data analysis: The obtained experimental data was statistically analyzed by performing One-way ANOVA and subsequently DNMRT test (Steel and Torrie 1980) at $P = 0.05$ and with the help of computer aided software "DSASTAT".

RESULTS

Evaluation of PGPR strains to manage bacterial wilt under greenhouse conditions: Selected pseudomonas strains applied as a soil drench, significantly reduced disease index of Bacterial wilt disease on tomato plants, compared with the pathogen control. The changes of Disease indexes and Biocontrol effect of applied strains in the greenhouse trial are shown in Table 2. Here strains FBL11 and MCR8 strongly reduced disease index and provided maximum significant biocontrol efficacies of 81.97 and 77.89%, respectively. These both strains reduced disease index up to 76.31 and 68.23% as compared to pathogen control.

Table -2: Potential of bacterial strains to manage bacterial wilt of tomato.

Bacterial strains treatments	**Disease Index (%)**	**Control Effect (%)**
FBL01	77.67±05.69ab	10.39±00.91f
FBL02	71.33±08.67b	13.98±00.73d
FBL10	44.67±03.81d	51.39±03.92b
FBL11	14.83±00.93gh	81.97±05.83a
FBL12	64.31±07.03c	23.56±02.54d
OSR3	79.52±05.18ab	08.64±06.07fg
MCR7	33.55±02.15ef	47.81±02.82bc
MCR8	21.06±03.77g	77.89±05.61a
ZMR4	72.00±04.73b	09.15±01.40fg
ZMR6	36.05±04.51e	54.87±02.31b
Pathogen control	83.02±07.34a	-
Untreated control	-	-

Mean ± standard deviation. Values with same letter differ non-significantly (P>0.05) as governed by ANOVA and DNMRT.

Potential of bacterial strains to induce systemic resistance in tomato plants: With the aim of investigating biochemical markers normally associated with induced systemic resistance defense response, the accumulation of total phenolics, PO, PPO and PAL was studied in tomato plants under influence of selected *Pseudomonas* strains in time course manners. Figure presents results showing change in quantities of these defense related biochemicals at specific time intervals.

Like previous experiment, here also strains performed best. Results showed that plants provided with FBl11 and MCR8 produced a robust and transitory accumulation of Total phenolics, PO, PPO and PAL within the third(1.0 dpi) and forth (2.0 dpi) time intervals exhibiting a maximum production of all these defense related biochemicals (Fig. 1). Here total phenolics accumulated rapidly and was easily detectable at 2 dpi interval. It maintained increasing trend up to 2 dpi and values reached at peak levels at 4 dpi interval. A decline was seen at later intervals in case of both bacterial strains.

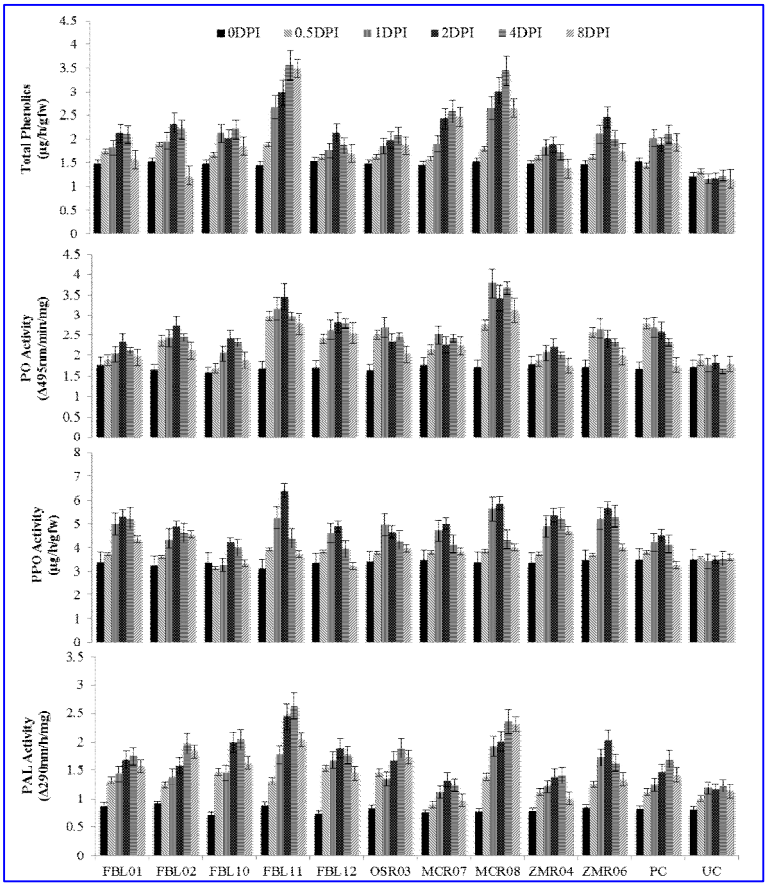

Figure-1: Change in defense related biochemicals of tomato under influence of bacterial strains.

Similar kinetics were observed in case of PO, PPO and PAL in tomato plants receiving FBL11 and MCR8. These also started increasing at initial time intervals then either maintained induced quantities or declined at later intervals for both strains.Some inducible reflects in quantities of all defense related biochemicals were also observed in case of pathogen treated plants. Untreated control plants maintained constant lower levels of all these defense related biochemicals.

Effect of bacterial strain on growth of tomato plants under greenhouse: Bacterization of tomato plants showed statistically higher values in all variables measured in this study as compared to control plants (Table 3). However, the magnitude of growth promotion varied among the both strains as was observed in seedling assay. Similarly to denote changes in growth parameters, average values were used obtained from data of all three tomato varieties. Symbiotically grown plants with FBL11and

MCR8 conferred 28.1 and 37.3% more shoot length respectively as compared to control (Table 2). In the same direction, root length showed prominent increases for FBL11 (68.5%) and MCR8 (51.8%) as compared to control (Table 2). Bacterial treated plants exhibited dense root network upon uprooting as compared to control (Table 2). Analysis of % age increase in total biomass showed increase of 67.9, 38.2% and 48.1 and 29.4% for fresh and dry biomass when plants were provided with FBL11 and MCR8 respectively (Table 2).

Table-3: Effect of different rhizospheric bacterial strains on growth of tomato plants.

Bacterial strains treatments	Shoot length (cm)	Root length (cm)	Wet weight (g)	Dry weight (g)
FBL01	14±1.0cd	5±0.3d	0.212±0.05cd	0.101±0.003d-f
FBL02	21±1.7a	12±1.0a	0.244±0.03b	0.111±0.007bc
FBL10	23±1.1a	12±1.3a	0.312±0.02a	0.117±0.005ab
FBL11	22±1.9a	11±0.7ab	0.305±0.01a	0.121±0.008a
FBL12	12±0.6de	3±0.1ef	0.123±0.04f	0.106±0.008d
OSR3	13±0.9c-e	3±0.2ef	0.225±0.08bc	0.113±0.010b
MCR7	20±0.7ab	4±0.6de	0.215±0.02b-d	0.011±0.001g
MCR8	19±1.4b	4±0.9de	0.206±0.06de	0.119±0.009ab
ZMR4	15±1.4cd	5±0.7d	0.211±0.05de	0.105±0.002d
ZMR6	17±1.8bc	7±0.5c	0.231±0.02bc	0.106±0.004d
Control	16±1.2bc	10±0.8b	0.241±0.01b	0.103±0.005de

Mean ± standard deviation. Values with same letter differ non-significantly (P>0.05) as governed by ANOVA and DNMRT.

DISCUSSION

In current investigation, the biocontrol potential of some rhizospheric bacterial strains was assessed using some bacteriological methods. In first phase of study, greenhouse experiments were performed to check disease management ability of selected bacterial strains along with induction of systemic resistance in tomato plants. Here two strains viz: FBL10 and MCR8 provided maximum suppressions against bacterial wilt disease. These two strains showed remarkable changes in defense related biochemical of tomato.

Diseases are an important reason for losses in agricultural crop commodities. It is estimated that world faces nearly 13% losses in agriculture produce because of plant diseases caused by number of pathogens (Thakore 2006). Fungal diseases cause a severe reduction in production and subsequently lower economic return to grower (Strange and Scott 2005).The inherent plant resistance is one of the efficient means to control disease. It is observed that with the passage of time, many fungal pathogens have developed acquired resistance against these chemicals (Raupach and Kloepper 2000; Strange and Scott 2005). Moreover these pesticides have harmful effects on human being and environment (Ruy 2003; Domico 2006). Keeping in view the detrimental effects of these fungicides, it becomes necessary to find out some safe strategy to manage diseases. This innate resistance may be improved by the inoculation of some biological inducers such as non-pathogenic rhizospheric bacterial strains (Hallman et al., 1997; Gray and Smith 2005). The present studies are advancement in the similar way. As it involves enhancement of innate resistance factors of tomato plants by treating with rhizospheric bacterial strains.

Resistance against pathogens and variation in defense related biochemicals in plants treated with rhizospheric bacteria have been found in many vegetable crops (Hammerschmidt *et al.*, 1982; Wei *et al.*, 1996; Hofte *et al.*, 1997; Zehnder *et al.*, 2001). According to (Ahn *et al.*, 2002), the application of bacterial bio-control agents to induce systemic resistance in crops is the most significant method of disease management. This technology has shown its significance in many crops (Nurnberger *et al.*, 2004; Nicaise and Zipfel 2009). Plants have innate defense system comprising of phenolic compounds which remain quiescent and are activated on getting signals generated by biotic or abiotic elicitors. Different enzymes like PO, PPO and PAL has a potential role in the biosynthesis of phenolics and phytoalexins (Daayf *et al.*, 1997). These phenolic compounds and rest of the phytoalexins restrict growth and establishment of pathogen inside host plants (Burrell 1974; Berg 2009; Akram and Anjum; 2011). Pieteres *et al.*, (2000) found increased activity of total phenolics

and defense related enzymes viz: PO, PPO and PAL in *Arabidopsis* on treating with rhizospheric *Pseudomonas* strains. Increased activity of PAL and PO in cucumber plants by application of *Psudomonas* strains against *Pythium aphanidermatum* have also been reported by Chen *et al.,* (2000). Enhanced PAL activity and salicylic acid has also been revealed in beans treated with *P. aeruginosa* TNSK2 (De Meyer and Hofte 1997). Ramamoorthy *et al.,* (2002) also noted increased activity of PPO, PAL and PO in tomato cultivars treated with *P. fluorescens* isolates Pf1 and enzymatic activity arrived at peak on 3rd day post inoculation. Zdor and Anderson (1992) also found increased enzymatic activity in beans inoculated with *P. fluorescens*. PPO is involved in oxidation of phenolic compounds and therefore decreases disease intensity (Tortel-Aziz *et al.,* 2008).

In our current study another independent greenhouse experiment was performed in which tomato plants were co-cultivated with these bacterial microbes. Since these strains were previously approved for ISR properties, we anticipated that these strains can also promote growth of tomato. To prove this hypothesis, different growth parameters of tomato plants were observed. In this greenhouse experiment root inoculation of our bacterial strains significantly promoted growth attributes of tomato plants of all three tomato varieties. These strains provided significant increases in all growth parameters of tomato plants under observations. Here also strain viz: FBL10 and MCR8 got superior in these traits.

Some bacterial strains have been previously reported to promote growth of different plants like apricot, sweet cherry and apple along with protection against different diseases by inducing systemic resistance (Esitken *et al.,* 2002, 2003, 2006; Pirlak *et al.,* 2007; Aslantas *et al.,* 2007). Same types of results were found when different crops like barley, wheat and corns were exposed to PGPB. Berg (2009) reported that root inoculations of sugarcanes with PGP bacterial strain promoted yield, cane length and number of clusters per plant. In the same direction two bacterial strains OSU142 and M3 promoted growth and fruit quality of sugar beet (Cakmakci *et al*., 2001) and tomatoes (Turan *et al.,* 2004).

CONCLUSIONS

Use of bio-control agents for induction of systemic resistance and consequentially disease management in plants is an excellent substitute for synthetic chemicals. Biological control efficacy of FBL10 and MCR8 was significant, so that these strains can be considered for registration as a new pesticide. But, the application methods of these bacterial microbes should be studied in detail before this process. Additional information is also required to understand the complex process of biological control of bacterial wilt.

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