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Biological Control of Bacterial Wilt of Tomato by Plant Growth Promoting Rhizobacteria

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Abstract: Seven isolates of *Ralstonia solanacearum* were isolated from naturally wilted roots of tomato plants grown in Assiut governorate. All isolates were pathogenic to tomato plants and produced typical symptoms of wilt. Isolate No. 5 exhibited the highest virulence followed by isolate No. 4. *Pseudomonas fluorescens*, *P. putida*, *Bacillus subtilis* and *Enterobacter aerogenes* were isolated from tomato rhizosphere and tested against *R. solanacearum* *in vitro* and *in vivo*. Results showed that all PGPR strains except *E. aerogenes*, increased seed germination up to 15% over untreated control. Under greenhouse conditions, *P. fluorescens* exhibited the highest disease reduction of tomato bacterial wilt disease followed by *P. putida* and then *B. subtilis* while *E. aerogenes* showed the lowest disease reduction percentage. Also tomato plants treated with all PGPR isolates significantly stimulated plant growth promotion under greenhouse conditions. In field trails, addition of *P. fluorescens*, *P. putida* and *B. subtilis* at concentration of 10^8 cfu mL⁻¹ showed that *Pseudomonas fluorescens* caused the highest disease reduction percentage of tomato bacterial wilt disease while *P. putida* exhibited the lowest disease reduction percentage.

Key words: Bacterial wilt, tomato, biological control, *Pseudomonas fluorescens*, *P. putida*, *Bacillus subtilis*, *Enterobacter aerogenes*

INTRODUCTION

In Egypt, tomato (*Lycopersicon esculentum* Mill.) is considered one of the most important vegetable crops. The cultivated area is 250,000 ha producing 10,000,000 tones with a productivity of 400,000 kg ha⁻¹ (FAO, 2009). Bacterial wilt of tomato caused by *Ralstonia solanacearum* (Yabuuchi *et al.*, 1995), causes a considerable amount of damage to tomatoes and many other crops in tropical, subtropical and warm temperate regions of the world (Ji *et al.*, 2005), that limits production of diverse crops such as potato, tomato, eggplant, pepper, banana and peanut (Williamson *et al.*, 2002). The pathogen is a widespread and economically important bacterial plant pathogen (Horita and Tsuchiya, 2001).

Various strategies have been developed to control bacterial wilt include the use of host-plant resistance and cropping systems (Dalal *et al.*, 1999), transgenic resistant plant (Jia *et al.*, 1999) and biological control includes Vesicular-arbuscular Mycorrhizae (VAM) (Halos and Zorilla, 1979), a virulent mutants of *R. solanacearum* (Dong *et al.*, 1999), genetically engineered antagonistic bacteria (Kang *et al.*, 1995), some naturally occurring antagonistic rhizobacteria such as *Bacillus* spp. (Da Silveira *et al.*, 1995) or integration of these strategies (Anith *et al.*, 2004).

Applying chemical pesticides is generally considered as the most effective and fastest strategy for plant disease management, however, no effective chemical product is available for *Ralstonia* wilt. In recent years, Plant Growth Promoting Rhizobacteria (PGPR) has been suggested as a potentially attractive alternative disease management approach since PGPR are known for growth promotion and disease reduction in crops (Jetiyanon and Kloepper, 2002). PGPR is a mixture of beneficial microorganisms which can increase the crop yield, plant growth and also protect against plant pathogens (Ramanathan *et al.*, 2002; Ramamoorthy *et al.*, 2002; Nandakumar *et al.*, 2001; Higa, 1999). Among PGPR, fluorescent pseudomonads have been reported to be effective against a broad spectrum of plant pathogens, including fungi, bacteria and viruses in many plant species, e.g., bean, carnation, cucumber, radish, tobacco and tomato (Van Loon *et al.*, 1998). Similarly, the sporulating Gram-positive bacteria like *Bacillus* spp. have also been used successfully for plant disease control (Kloepper *et al.*, 2004).

The objectives of this study were to evaluate the efficacy of PGPR strains against *Ralstonia solanacearum* *in vitro* and determine the efficacy of PGPR strains to reduce tomato bacterial wilt caused by *R. solanacearum* under greenhouse and field conditions.

MATERIALS AND METHODS

Isolation and identification of the causal pathogen:

Ralstonia solanacearum were isolated from naturally diseased tomato plants showing wilt symptoms, collected from different localities of Assiut governorate. Sections of discoloured tissue from the vascular strands in stems of tomato were removed, suspend in a small volume of 50 mM phosphate buffer and were leaved for 5 to 10 min. A series of decimal dilutions of the suspension 50-100 μ L was transferred to a Kelman's tetrazolium medium, streak with an appropriate dilution plating technique was used. The plates were incubated for two to six days at 28°C. Pure cultures of presumptive isolates were identified using morphological and biochemical tests according to the methods of Lelliott and Stead (1987), Klement *et al.* (1990) and Schaad (2001).

Pathogenicity tests: Pathogenicity of bacterial isolates were carried out by inoculating the susceptible tomato cultivar GS by each isolates. Bacterial isolates were grown on NA agar medium for two days at 30°C, suspended in sterile distilled water and an optical density of 0.1 at 600 nm wavelength (using spectrophotometer model 6405UV/VIS), approximately 10^8 cfu mL⁻¹ was adjusted. Inoculation was made at the three to four true leaf stages by puncturing the stem at the axils of the third fully expanded leaves from the apex with a needle dipped in inoculum (Winstead and Kleman, 1952). Eight tomato plants were used for each isolates. Plants inoculated with sterile water served as negative control. Inoculated plants were kept in a climate chamber with 30/27°C day/night temperature and 85% relative humidity. Plants were watered well, with avoided wetting the foliage for 24 h (Williamson *et al.*, 2002). The experiment was undertaken with completely randomized design and repeated twice.

Wilt intensity has been calculated after inoculation by 21 days according to Winstead and Kelman (1952), using the following formula:

$$I\% = \left[\frac{\sum (n_i \times v_i)}{V \times N} \right] \times 100$$

where, I = wilt intensity (%); n_i = number of plants with respective disease rating; v_i = disease rating (following scale: 1 = no symptoms; 2 = one leaf wilted; 3 = two to three leaves wilted; 4 = four or more leaves wilted; 5 = whole plant wilted); V = the highest disease rating; and N = the number of plants observed.

Isolation and identification of the PGPR strains: For isolation of PGPR strains from potato and tomato

rhizosphere in Assiut governorate, plant roots were gently washed twice in sterile water to remove adhering soil and then root sections of approximately 1 g were added to 200 mL sterile water in flasks and shaken on rotary shaker at 150 rpm for 30 min. Then serial dilutions of the root suspension were plated on Nutrient Agar (NA) (King *et al.*, 1954) and incubated at 28°C for 48 h. When the bacterial colony appeared on the medium, representative isolates were picked for this study.

Pure cultures of PGPR strains were identified using the morphological and physiological characteristics according to the methods of Lelliott and Stead (1987), Klement *et al.* (1990) and Schaad (2001).

Preparation of PGPR strains inoculums: Two days grown cultures of the selected strains of PGPR were harvested from agar plates by flooding with sterile deionized water. Concentration of the inoculum was determined spectrophotometrically at 600 nm, Four concentrations were used 10^8 , 10^7 , 10^6 and 10^5 cfu mL⁻¹ for each isolate.

Evaluation of PGPR strains for their antagonistic effect

in vitro: *In vitro*, antagonistic effects of plant growth-promoting rhizobacteria (*Pseudomonas fluorescens*, *P. putida*, *Bacillus subtilis* and *Enterobacter aerogenes*) against *R. solanacearum* were carried out by using the filter paper disk method. NA medium was used in order to favor the growth of *R. solanacearum* and the potential growth-promoting rhizobacteria. The experimental designs were design in Complete Randomized Design (CRD) with four replications.

Effects of PGPR strains on seed germination in vitro:

To ensure that the bioagents have not any harmful effect on seeds and seedlings, the following experiment was carried out, tomato seeds were immersed in the bioagents suspensions separately which were containing 10^8 cell mL⁻¹ of different bioagents. Sterile Distilled Water (DW) was used as control. Seeds were gently shaken for 2 h then, blot dried, plated on wet blotters and the germination was tested by filter paper method. The treated seeds were incubated at 28°C for one week. Periodically, the water contents were readjusted and samples were taken for assaying germination (Abdul-Baki and Anderson, 1973).

Greenhouse experiments

Growth of plants: Seeds of tomato cultivar GS were obtained from the Ministry of Agriculture, Egypt and used in this study. Tomato seeds were surface-sterilized with 2% sodium hypochlorite for 2 min (Guo *et al.*, 2004),

washed thoroughly with sterilized water and planted into pots of sterilized soil. After 4 weeks, seedlings were transplanted into pots containing experimental soil amendments and grown in the greenhouse at 25-35°C.

Colonization capacity of PGPR strains: Colonization capacity of PGPR strains were tested in separate experiment. Ten days after transplanting, three rhizosphere samples from each treatment were carefully collected from the experimental pots. Each sample consisted of the whole root system with tightly adhering soil of three individual plants. To harvest bacterial cells from the rhizosphere soil and the rhizoplane, one gram of the root samples was soaked in 9 mL of 50 mM phosphate buffer with shaking at 150 rpm for 30 min. To determine the CFU counts of the PGPR strains, serial dilutions of the cell suspension were made and plated on NA medium. Three plates were used for each dilution. The plates were incubated at 28°C for 2 days before the number of colonies was counted. The level of root colonizing bacteria was calculated as colony forming units/g root (cfu/g root).

Evaluation of PGPR strains in control of bacterial wilt: Four PGPR strains were evaluated under greenhouse conditions for control of tomato bacterial wilt. For each strain, four concentrations were tested (10^8 , 10^7 , 10^6 and 10^5 cfu mL⁻¹). Tomato seedlings roots were treated separately with each concentration for 30 min at the time of transplanting. Pathogen inoculation was carried out as described before onto each seedling. All treated and inoculated seedlings were maintained as previously mentioned and the disease index was recorded 3 weeks after inoculation. Four replicates were used, each replicate consisted of two seedlings and the experiment was repeated twice.

Biological control efficacy was calculated using the following formula:

$$\text{Biocontrol efficacy} = \frac{(\text{Disease incidence of control} - \text{Disease incidence of antagonist-treated group})}{\text{Disease incidence of control}} \times 100\%$$

Plant growth promotion assessment: The strains of PGPR were tested for their ability to promote plant growth in separate experiment. At the end of the experiment (2 months after transplanting), plants including the roots were harvested from the pots and fresh weight of stems and roots recorded, was measured to determine the effects of bacterial treatments on plant growth. Healthy plants were counted and uprooted separately and their weights recorded to measure growth promotion, compared with the untreated control (Lim and Kim, 1997):

$$\text{Biomass increase} = \frac{(\text{Average fresh weights of plants treated with antagonist} - \text{Average fresh weights of control plants})}{\text{Average fresh weights of control plants}} \times 100\%$$

Field experiments: Based on greenhouse screening, the most promising four concentrations treatments were selected to follow-up their effects against the pathogen under field conditions. The field trials were conducted at the Experimental Farm of Faculty of Agriculture, Assiut University, Assiut, Egypt in 2011 growing season. Field plots (3-3 m) comprised five rows and six holes/row arranged in a completely randomized block design. Three plots were used as replicates for each treatment as well as for the untreated control treatment. Application of PGPR strains was carried out as in greenhouse experiments and Percentage of disease reduction was evaluated. From each treatment, 10 plants were used for evaluating tomato plants yield.

Statistical analysis: Data were subjected to statistical analysis using analysis of variance and means were compared using the LSD (P 70:05) test according to Gomez and Gomez (1984).

RESULTS

Isolation, identification and pathogenicity test: Seven isolates of *R. solanacearum* were tested with tomato plants under greenhouse conditions. All isolates were pathogenic on tomato plants and produced typical symptoms of wilt. Isolate No. 5 exhibited the highest disease incidence (96% wilting) followed by isolate No. 4 which achieved (80% wilting). Isolate No. 1 caused the lowest percentage (40% wilting) followed by isolate No. 2 after four weeks from inoculation (Fig. 1). According to the above results isolate No. 5 was used in the following experiments.

Seven pure cultures of pathogen isolates have typical cultural characteristics and were proving to be pathogenic and causing wilt symptoms to tomato plants were identified as *Ralstonia solanacearum* according to their morphological and physiological characteristics as reported by Hayward (1964) and Krieg and Holt (1984).

Identification of the PGPR strains: Pure cultures of non-pathogenic bacteria isolated from tomato rhizosphere (*Pseudomonas fluorescens*, *P. putida*, *Bacillus subtilis* and *Enterobacter aerogenes*) were identified according to their morphological, cultural and physiological characteristic as stated in Bergey's Manual of Systematic Bacteriology (Schaad, 1980; Krieg and Holt, 1984; Klement *et al.*, 1990).

Evaluation of PGPR strains for antagonism *in vitro*: *Pseudomonas fluorescens*, *P. putida* and *Bacillus subtilis* had broader inhibition zone against the pathogenic isolates of *R. solanacearum* Isolate No. 5 showed less

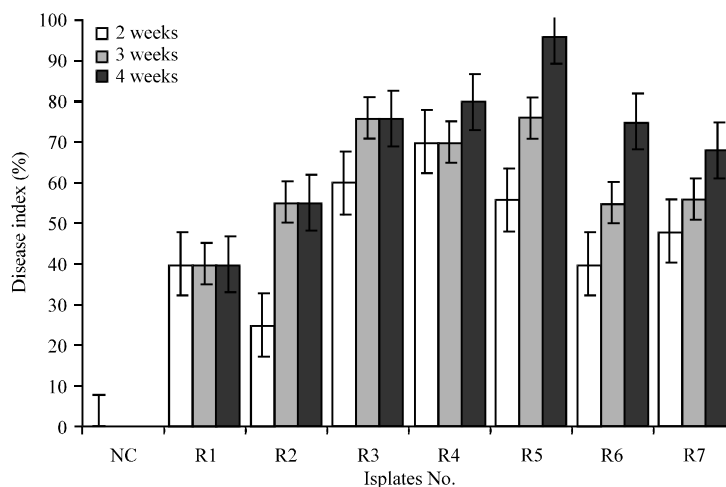


Fig. 1: Pathogenicity tests of seven isolates of *Ralstonia solanacearum* on GS tomato plants cultivars. Bars indicate the standard error

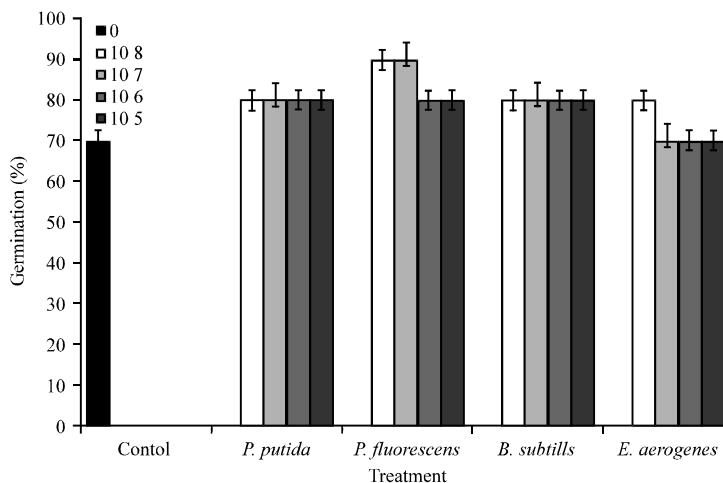


Fig. 2: Effects of different concentration of PGPR strains (*Pseudomonas fluorescens*, *P. putida*, *Bacillus subtilis* and *Enterobacter aerogenes*) on seed germination of GS tomato plants cultivars after 7 days, Bars indicate the standard error

than 0.3 cm of inhibition zone width, in other hand, *Enterobacter aerogenes* had much stronger inhibition to *R. solanacearum* strains with more than 0.3 cm of inhibition zone width.

Effects of PGPR strains on seed germination in vitro:

Results illustrated in Fig. 2 show that, seed inoculation with different bioagents significantly enhanced seed germination. However, the rate of enhancement varied with PGPR strains. All PGPR strains except *Enterobacter aerogenes*, increased seed germination up to 15% over nontreated control.

Colonization capacity of PGPR strains under greenhouse conditions: Colonization of the rhizosphere is required for

a PGPR strain to consistently influence plant growth and health (Chin-A-Woeng *et al.*, 2000). In order to support these biocontrol data, the evolution of PGPR strains populations was evaluated on roots of tomato plants grown under greenhouse conditions. For the four different inocula tested, the population level was assessed by agar plate count 30 days after inoculation. Populations observed for the different PGPR strains were found to progressively converge and decrease, stabilizing around 2.5×10^5 . *Pseudomonas fluorescens* show the highest root-colonization ability of tomato plants with all tested concentrations followed by *Pseudomonas putida*, while *Enterobacter aerogenes* gave the lowest ability of root-colonization (Table 1).

Plant growth promotion assessment: Results of this experiment show that bacterial isolates have been stimulated plant growth promotion under greenhouse conditions and indicated that tomato plants treated with PGPR strains significantly grew better than control. Biomass increase of tomato plants treated with PGPR strains are shown in Table 2. *P. fluorescens* exhibited the highest biomass increase of treated tomato plants (166.7%) followed by *P. putida* (157.8%). On the other hand, mixture of different PGPR strains recorded the lowest biomass increase (18.6%) compare with control followed by *Enterobacter*

aerogenes (37.7%), respectively. Concentration (10^8 cfu mL⁻¹) of all PGPR strains exhibited highest biomass increase of treated tomato plant, while concentration (10^5 cfu mL⁻¹) of all PGPR strains exhibited lowest biomass increase.

Table 1: Comparison of the root-colonization abilities of PGPR strains after inoculation under greenhouse conditions

Treatments	Conc. of treatment	Rhizosphere sample (cfu g ⁻¹)
<i>Pseudomonas putida</i>	10 ⁸	4.8×10 ⁷ B
	10 ⁷	3.2×10 ⁷ E
	10 ⁶	2.89×10 ⁷ H
	10 ⁵	2.3×10 ⁴ N
<i>Pseudomonas fluorescens</i>	10 ⁸	5.3×10 ⁷ A
	10 ⁷	3.9×10 ⁷ C
	10 ⁶	6.54×10 ⁷ G
	10 ⁵	5.33×10 ⁴ M
<i>Bacillus subtilis</i>	10 ⁸	3.8×10 ⁷ D
	10 ⁷	7.8×10 ⁶ F
	10 ⁶	2.6×10 ⁷ I
	10 ⁵	8.42×10 ⁴ L
<i>Enterobacter aerogenes</i>	10 ⁸	2.23×10 ⁷ J
	10 ⁷	1.9×10 ⁵ K
	10 ⁶	1.32×10 ⁴ O
	10 ⁵	9.17×10 ³ P
Control		2.00×10 ² Q

Values in the same column followed by a similar letter are not significantly different as determined by the LSD test (p = 0.05)

Evaluation of PGPR strains in control of bacterial wilt under greenhouse conditions:

Results of this experiment (Table 3) show that *Pseudomonas fluorescens* exhibited the highest disease reduction percentage of tomato bacterial wilt disease (55.3%) followed by *Pseudomonas putida* (46.1%) and then *Bacillus subtilis* (38.2%) while *Enterobacter aerogenes* showed the lowest disease reduction percentage (25%). A concentration of (10^8 cfu mL⁻¹) of all PGPR strains exhibited highest disease reduction percentages of tomato bacterial wilt disease, while concentration (10^5 cfu mL⁻¹) of all PGPR strains exhibited the lowest disease reduction percentages.

Field experiments:

Results in Fig. 3 indicated that *Pseudomonas fluorescens* exhibited the highest disease reduction percentage of tomato bacterial wilt disease (80%) followed by *Pseudomonas putida* and then *Bacillus subtilis*. To further evaluate the growth stimulation *Pseudomonas fluorescens* recorded the highest increase percentage of yield per plant (348%) followed by *Pseudomonas putida* while *Bacillus subtilis* achieved the lowest increase percentage (91%) (Fig. 4).

Table 2: Biomass increase of tomato as influenced by inoculation with plant growth promoting rhizobacterial strains

Treatment	Conc.	Treated with pathogen		Untreated	
		Fresh weight (g)	Biomass increase (%)	Fresh weight (g)	Biomass increase (%)
<i>Pseudomonas putida</i>	10 ⁸	76.57A	188.07	81.06B	204.97
	10 ⁷	66.13BCD	148.80	71.76CD	169.98
	10 ⁶	66.00BCD	148.31	69.90DE	162.98
	10 ⁵	65.37CD	145.94	67.31EF	153.24
<i>Pseudomonas fluorescens</i>	10 ⁸	79.71A	199.89	91.03A	242.48
	10 ⁷	70.12B	163.81	79.23B	198.08
	10 ⁶	68.13BC	156.32	74.80C	181.42
	10 ⁵	65.62BCD	146.88	69.28DE	160.65
<i>Bacillus subtilis</i>	10 ⁸	67.19BC	152.78	70.10DE	163.73
	10 ⁷	62.05DE	133.45	68.89DE	159.18
	10 ⁶	58.96E	121.82	63.38F	138.45
	10 ⁵	50.57F	90.26	58.91G	121.63
<i>Enterobacter aerogenes</i>	10 ⁸	44.21G	66.33	42.88H	61.32
	10 ⁷	38.79H	45.94	39.94HIJ	50.26
	10 ⁶	35.73HI	34.42	39.56HIJ	48.83
	10 ⁵	27.69JK	4.18	38.55IJ	45.03
Mixture of PGPR strains	10 ⁸	36.73H	38.19	42.46HI	59.74
	10 ⁷	31.65IJ	19.07	38.60IJ	45.22
	10 ⁶	30.10JK	13.24	37.68J	41.76
	10 ⁵	27.57JK	3.72	36.09J	35.78
Healthy control		26.58K		26.58K	
Infected control		20.07L	-24.50	20.07L	-24.50

Values in the same column followed by a similar letter are not significantly different as determined by the LSD test (p = 0.05)

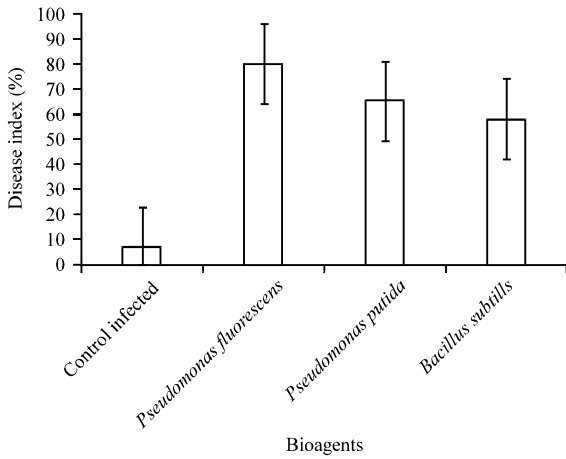


Fig. 3: Disease reduction percentage of treated tomato plants with PGPR strains under greenhouse conditions

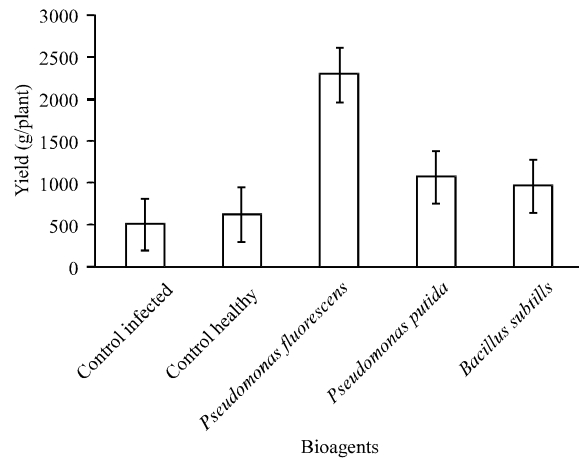


Fig. 4: Effect of PGPR strains on yield per plant (g) at the end of experiment under field conditions

Table 3: The average disease incidence and disease reduction percentage of treated tomato plant with PGPR strains

Treatment		Disease incidence (%)	Disease reduction (%)
PGPR	Conc.		
<i>Pseudomonas putida</i>	10 ⁸	45FG	52.63
	10 ⁷	45FG	52.63
	10 ⁶	50EFG	47.37
<i>Pseudomonas fluorescens</i>	10 ⁵	65BCDE	31.58
	10 ⁸	40G	57.90
	10 ⁷	40G	57.90
<i>Bacillus subtilis</i>	10 ⁶	45FG	52.63
	10 ⁵	45FG	52.63
	10 ⁸	45FG	52.63
<i>Eutrobacter aerogenes</i>	10 ⁷	60CDEF	36.84
	10 ⁶	60CDEF	36.84
	10 ⁵	70BCD	26.32
Mixture of PGPR strains	10 ⁸	80AB	52.63
	10 ⁷	55DEFG	42.11
	10 ⁶	50EFG	47.37
Infected control	10 ⁵	65BCDE	31.58
		95A	

Values in the column followed by a similar letter are not significantly different as determined by the LSD test (p = 0.05)

DISCUSSION

Results reported herein indicate that the seven bacterial isolates obtained from naturally diseased tomato plants collected from different localities of Assiut governorate proved to be pathogenic and able to infect tomato plants causing wilt symptoms and varied in their pathogenicity. They were identified as *Ralstonia solanacearum*. Present results agreed with those reported by El-Ariqi *et al.* (2005) and Abo-Elyousr and Asran (2009).

The effect of different bioagents treatments on tomato seed germination showed that all treatments increased percentage germination; the highest level of germination was achieved when tomato seeds were subjected to *Pseudomonas fluorescens*. It has been reported that fluorescent pseudomonads also play a role in growth promotion by production of plant hormones and other growth promoting substances such as auxins (Loper and Schroth, 1986), gibberellins (Ramamoorthy *et al.*, 2002) and 1-amino-cyclopropane-1-carboxylate deaminase (Jacobson *et al.*, 1994).

Under greenhouse conditions, results clearly confirm that plants treated with PGPR isolates significantly reduced disease compared to infected control, as well as caused greater amounts of biomass (fresh and dry weight) compared to the control. Disease reduction by PGPR in colonization of plant roots may occur directly, through competition for space, nutrients and ecological niches or production of antimicrobial substances and indirectly, through Induction of Systemic Resistance (ISR) (Kloepper and Beachamp, 1992; Liu *et al.*, 1995). PGPR may induce plant growth promotion by direct or indirect modes of action (Kloepper *et al.*, 1988; Beachamp, 1993; Lazarovits and Nowak, 1997). The mechanism involved in PGPR-mediated plant growth promotion is directly by production of plant growth regulators (auxins, cytokinins, gibberellins) and facilitation of the uptake of nutrients (nitrogen fixation, solubilization of phosphorus). The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of plant pathogens on plants by production of inhibitory substances (antibiotics, antifungal metabolites, iron-chelating siderophores, cell wall-degrading enzymes and competition for sites on roots) or by increasing the natural resistance of the host (induced systemic resistance).

Field results clearly confirm that application of PGPR as potential bioagents in controlling tomato bacterial wilt under field condition. Present results were agree with those reported by Guo *et al.* (2004), who reported that *R. solanacearum* wilt disease reduction and yield increase of tomato plants after treatment by *Bacillus* spp. and fluorescent pseudomonads. Also Priou *et al.* (2005), recorded 80% reduction of the tomato bacterial wilt disease under greenhouse conditions using *Pseudomonas putida*.

In the present study, PGPR strains have highly root-colonizing capacity achieved high record of reduced disease severity, biomass and yield increased under greenhouse and field conditions. One of the most important problems in biocontrol using microbial products is the storage time of living microbes. A short shelf life can be a serious obstacle in the development of biocontrol products with living microbes.

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