Suppression of Bacterial Wilt Disease of Tomato Plants Using Some Bacterial Strains

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Abstract: Six isolates of *Ralstoniasolanacearum* were isolated from naturally wilted roots of tomato plants grown in Minufiya governorate. All isolates were pathogenic to tomato plants and produced typical symptoms of wilt. Isolate No. 6 exhibited the highest virulence followed by isolates No. 4. Characterization of strains of *Ralstoniasolanacearum*, were performed based on pathogenicity, Biochemical and physiological tests. *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Bacillus cereus*, *Pseudomonas syrinagae*, *Pseudomonas stutzeri*, and *Bacillus thuringiensis* were isolated from tomato rhizosphere as biocontrol agents and tested against *Ralstonia solanacearum (R. solanacearum) in vitro*and *in vivo*. All the bio-control agents tested reduced the bacterial wilt disease to various degrees. the physiological and biological characters of six isolates revealed similar characters. Under greenhouse conditions, *Pseudomonas aeruginosa (P. aeruginosa*) and *Bacillus thuringiensis*exhibited the highest disease reduction of tomato bacterial wilt disease (89%, 86% respectively) followed by *P. putida, Bacillus cereus,* and *P. stutzeri* (70%, 68% and 65%) while *P. syrinagae* showed the lowest disease reduction (61%). Two bacterial strains which showed highly antagonistic activity towards tomato bacterial wilt were identified using specific polymerase chain reaction (PCR) of 16S rDNA gene. The 16SrDNA sequence analysis showed that the 1st strain belongs to the genus *Pseudomonas*, with closest similarity to *Pseudomonasaeruginosa* (100% similarity). The 2nd strain identified as *Bacillus*, with closest similarity to *Bacillus thuringiensis* (99%).
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Key words: Bacterial wilt disease, *Ralstoniasolanacearum*, Tomato, Biological control, *Pseudomonasaeruginosa*, *Bacillus thuringiensis*

Introduction

Bacterial wilt caused by *Ralstoniasolanacearum* **(Yabuuchi *et al*., (1995)** is primarily a soil borne disease of wide distribution in the tropics, subtropics and warm temperate regions of the world **(Buddenhagen *et al.,* 1962).** *R. solanacearum*is a rod shaped, gram negative, β proteo-bacterium that causes bacterial wilt in more than 200 plant species including many economically important crops**.** In Egypt, tomato plants (*Lycopersiconesculentum* Mill.) is considered one of the most important vegetable crops **(FAO, 2009).** Bacterial wilt of tomato caused by *Ralstoniasolanacearum* 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). It is difficult to control bacterial wilt disease due to high variability of the pathogen, limited possibility for chemical control, high capacity of the pathogen to survive in diverse environments and its extremely wide host range (Anonymous, 2004). The use of resistant varieties has been used to reduce disease (Dalal *et al*., 1999). However, crop resistance is often overcome by the genetic diversity of the pathogen as well as genotype x environment interactions (Wang *et al.,* 1998). Disease control is being attempted with crop rotation, intercropping, organic manuring and use of resistant cultivars. However, crop rotation-based control of bacterial wilt is often hampered by the pathogen's wide host range. Applying chemical pesticides is generally considered as the most effective and fastest strategy for plant disease management (Kloepper*et al*., 2004), however, no effective chemical product is available for *Ralstonia* wilt.

Biological control is still in its research phase (van Overbeek*et al*., 2002), with few studies reported for bacterial wilt (Shekhawat *et al.,* 1993; Lwin and Ranamukhaarachchi, 2006; Messiha *et al.,* 2007). Biological control not only increases crop yield and suppresses disease but also avoids environmental pollution. It is important to develop methods for evaluating antagonistic microorganisms and incorporating them into successful disease management. Research on microbial antagonists, has shown promise for bacterial wilt control (Lwin and Rnamukhaarachchi, 2006). Toyota and Kimura (1996) reported the suppressive effect of some antagonistic bacteria on *R. solanacearum.* Furthermore, Ciampi-Panno*et al.* (1989) showed that antagonistic pathogens were effective in suppressing *R. solanacearum*under field conditions. Several

antagonists have been evaluated with variable success (Shekhawat *et al.,* 1993). Lwin and Ranamukhaarachchi (2006) reported a satisfactory suppression of the bacterial wilt pathogen by the application of a commercially available mixture of effective microorganisms (EM). Further studies have identified many microorganisms with the potential of suppressing bacterial wilt, although they have not yet been evaluated for effectiveness (Hoang *et al.*, 2004).

Biological control agents are able to limit the growth and the activity of bacterial phytopathogens in two main ways, production of anti-microbial substances and competition for space and nutrients at specific sites on the plant surface (site of competition). Anti-microbial compounds are of three main types; antibiotics, bacteriocins and siderophores. These are distinguished in terms of their chemical nature, anti-microbial activity and means of detection during *in vitro* culture (Strauch *et al.*,2001).

The objective of this research was to isolate and evaluate potential soil-borne antagonists for their ability to suppress the growth of *R. solanacearum in vitro* and *in vivo* conditions.

2.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 Minufiya governorate. Infected tomato stems were cut into small pieces and placed in test tubes containing 5 ml of sterile distilled water for standard isolation (Hildebrand *et al.,* 1988). Bacteria were allowed to flow from the vascular bundles for 5 to 10 minutes. One loopful of the bacterial suspension was streaked onto Kelman'stetrazolium medium (Kelman, 1954) and incubated at 28^{o}C for 48 h.

Physiological and biochemical testof the causal pathogen:

Six bacterial isolates of tomato were characterized by using the following tests: oxidation/fermentation, starch hydrolysis, indole production and nitrate (NO_3) reduction (Hayward, 1964; Lelliott and Stead, 1987; Hildebrand *et al.,* 1988). Additionally, the tests such as oxygen relation, levan production, urease test, gelatin liquefaction, tween 80 hydrolysis, catalase production, sodium chloride (5 and 7%) tolerance, oxidase test and growth on potato slice were also performed according to Lelliott and Stead (1987), Hildebrand *et al.*, (1988). Furthermore, some tests were made on arginine, dihydrolase, motility, citrate utilization and ammonia production following the method of Hildebrand *et al.,* (1988).

Hypersensitive reaction of the causal pathogen:

All ten bacterial wilt isolates of tomato tested for hypersensitive reaction (HR) on tobacco leaf. The bacterial suspension was prepared and adjusted to 0.2 OD (optical density) at 600 nm by Spectonic 20 (Bausch and Lomb, Co. Ltd.), which was about 108 colony forming unit (cfu) per ml. One side of completely expanded tobacco leaves was infiltrated with 1.0 ml of bacterial suspension and the opposite sides with water as a control. The HR was observed daily for 5 days after infiltration of bacterial suspension (He *et al.,* 1983).

Pathogenicity tests:

Pathogenicity of bacterial isolates were carried out by inoculating the susceptible tomato cultivar GS by each isolates. Bacterial isolates were grown on nutrient 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. Clay loam soil was autoclaved for 3 hours pots of 30 cm in diameter sterilized by soaking in 5% phenol. Healthy seedling tomato (*Lycopersiconesculentum* Mill. Cv Marmand), were planted and placed in greenhouse. All test plants were allowed to grow for 6-8 weeks or until they were 15-20 cm high for each isolate (Five plants of each plot, and four replicates). Inoculation was made at the three to four true leaf stages by puncturing the stem at the axis of the third fully expanded leaves from the apex with a needle dipped in inoculum (Winstead and Kleman, 1952). Plants inoculated with sterile water served as negative control. Inoculated plants were kept in a climate chamber with 27/30°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:

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

where, I = wilt intensity (%); ni = 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 = plant dead); V = the highest disease rating; and N = the number of plants observed.

Isolation and identification of the Biological strains:

For isolation of biological strains from tomato rhizosphere soil samples of tomato were

collected from various areas in Minufiya governorate. subsample of 10 g was taken from the soil samples from each site, placed in 250 ml Erlenmeyer flasks with 100 ml sterilized distilled water (DW) and mixed for 10 min with a magnetic shaker, From this suspension, a dilution series up to 6-10 were prepared (James *et al.,* 1990). When the bacterial colony appeared on the medium, representative isolates were picked for this study. Pure cultures of biocontrol agent 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).

In-vitro evaluation of potential antagonists:

Six antagonists namely *Pseudomonas aeruginosa, P. putida, P. syrinagae, P. stutzeri, Bacillus cereus and B. thuringiensis* were evaluated against the bacterial wilt pathogen *in vitro*. The experimental designs were complete randomized design (CRD) with four replications. Cross culture method and filter paper disk method were used in first experiment and second experiment, respectively. PDA medium was used in both experiments in order to favor the growth of *R. solanacearum*and the potential antagonists. Of these antagonists, two most effective antagonists were selected based on the degree of inhibition of pathogen and growth rate of antagonist for *in-vitro* evaluation studies.

***In-vivo* evaluation of potential bio-control agents**:

The Six selected potential antagonists ***(****P. aeruginosa, P. putida, P. syrinagae, P. stutzeri,B. cereus and B. thuringiensis)* were evaluated *in vivo* against *R. solanacearum*in the greenhouse using susceptible tomato variety marmand**,** The experimental designs were complete randomized design (CRD) with five Replications (3seedlind/plot). The temperature and relative humidity of the greenhouse were set at 30ºC and 80% respectively in order to favour the disease development. In pot experiment, the antagonists were introduced one week before the pathogen inoculation. Six selected antagonists were applied to 21-days-old tomato seedlings growing in separate pots filled with sterilized soils. Antagonists were applied regularly up to 6 times at one-week interval. To apply antagonists, 15 mL of suspension at a concentration of 109 cfu/mL of each of the six selected antagonists were used. After 60 days of planting percentage of disease reduction was evaluated from each treatment, fresh and dry weight of shoot, fresh and dry weight of root, 10 plants were used for evaluating tomato plants yield.

Statistical analysis:

The obtained data were statistically analysed according to the method of Gomez and Gomez (1984).

Bacterial Identification Using 16s rRNA Gene Amplification:

For 16S rDNA sequencing, DNA templates were prepared for PCR amplification as described by Marmur (1961). DNA coding for 16S rRNA regions was amplified by PCR with *Taq*Polymerase as described by Kawasaki *et al.* (1993), Yamada *et al.* (2000) and Katsura *et al.* (2001). PCR product for sequencing 16S rDNA regions was amplified using two primers DNA 20F 5'-GAGTTT GAT CCT GGC TCA G-3', position 9-27 on 16S rDNA by the *E. coli* numbering system (Brosius *et al*., 1981), and 1500R 5'-GTT ACC TTG TTA CGA CTT-3', position 1509-1492 on 16S rDNA by the *E. coli* numbering systems (Brosius *et al*., 1981). PCR amplification was conducted with DNA Engine Dyad Thermal Cycler (Bio-Rad Laboratories, USA). The PCR product was analyzed by 0.8% (w/v) agarose gel electrophoresis and purified with a QIAquick PCR purification kit (QIAGEN GmbH, Germany). Then the direct sequencing of 16S rDNA of the single-banded and purified PCR products [*ca.* 1500 bases, on 16S rDNA by the *E. coli* numbering system (Brosius *et al*., 1981)] was conducted. Sequencing of the purified PCR products were carried out with an ABI PRISM Big DyeTM Terminator Ready Reaction Cycle Sequencing Kit (version 3.0, Applied Biosystems, USA). The primers of 20F, 520R (5'-GTA TTA CCG CGG CTG CTG-3', positions 519-536) were used for partial sequencing of 16SrDNA, and additional 1500R, 520F (5'-CAG CAG CCG CGG TAA TAC-3', positions 926-907) for full length sequencing. DNA sequencing was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). In the sequence analysis, the nucleotide sequences obtained from all primers were assembled using Cap contig assembly program, as accessory application in Bio Edit Program (Anonymous, 2007). Homology search was performed by using the standard nucleotide BLAST (BLASTn) from the NCBI web server (Anonymous, 2009) against previously reported sequences as the Gen Bank/EMBL/DDBJ database for determination of the nearest sequences.

The method D1/D2 domain of 26S ribosomal RNA sequence was carried out for yeast coded LR10. The isolation of DNA for PCR was carried out by boiling cells with lysis buffer according to Maniatis *et al.* (1982). The divergent D1/D2 domain of 26S rDNA was amplified with primers NL-1 (5'-GCA TAT CAA TAAGCG GAG GAA AAG-3') and NL4 (5'-GGT CCGTGT TTC AAG ACG G-3') (Kurtzman and Robnett, 1998). The nucleotide sequences of D1/D2 domain of 26S rDNA were directly determined using PCR products according to Kurtzman and Robnett (1998). Cycle

sequencing of the D1/D2 domain was used with forward primer NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3'), and reverse primer NL4 (5'-GGT CCGTGT TTC AAG ACG G-3'), by ABI Prism TMBigDyeTM Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems, USA). The sequences of D1/D2 domain of 26S rDNA were compared by BLASTn Homology Search (Anonymous, 2009).

3. Results:

Isolation, identification, Physiological, biochemical test of the causal pathogen:

Six strains of *R. solanacearum* isolated from naturally diseased tomato plants were characterized using Physiological and biochemical tests**.** All six strains were arginine dihydrolase negative and oxidase, catalase and urease positive. All of them oxidized citrate within 4-5 days of inoculation by changing blue media into green. On the other hand, none of the strains neither hydrolyzed starch or produced indole and liquefied gelatin. Strains were highly sensitive to NaCl at 5% but not at 7%. All the strains produced nitrate and ammonia after 2-3 days of inoculation and they showed positive reactions in levan production, motility. Biochemical test of all 6 bacterial wilt strains oxidized disaccharides, maltose, lactose and cellobiose by changing color of the medium from green to yellow. On the other hand, the strains failed to oxidize hexose sugar alcohols, mannitol, sorbitol and dulcitol, even after 28 days of inoculation (Table 1) according to Hayward (1964) and Krieg and Holt (1984).

Table 1. Characterization of *Ralstoniasolanacearum*strains isolated from bacterial wilt infected tomato plants inMinufiya governorate

	Characteristic Result					
	Strain1	**Strain2**	**Strain3**	**Strain4**	**Strain5**	**Strain6**
Maltose	+	+	+	+	+	+
Lactose	+	+	+	+	+	+
Cellobios	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-

Biochemical/Physiological test

Starch hydrolysis	-	-	-	-	-	-
Indole production	-	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-
Nitrate production	+	+	+	+	+	+
Levan production	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Uerase	+	+	+	+	+	+
Oxidative	+	+	+	+	+	+
Fermentative	-	-	-	-	-	-
Arginine dihydro	-	-	-	-	-	-
Growt	+	+	+	+	+	+
Motility	+	+	+	+	+	+
Ammonia production	+	+	+	+	+	+
Citrate utilization	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Salt tolerance at 5%	+	+	+	+	+	+
Salt tolerance at 7%	-	-	-	-	-	+

+ Positive reaction or growth; - Negative reaction or no growth

Hypersensitive reaction of the causal pathogen:

Virulence test of pathogenic strains was detected in greenhouse. Results indicated that all six strains of*R.solanacearum,* were virulent (pathogenic) and gave yellowish discoloration (necrosis) was observed (positive reaction) when Tobaco leaves

were infiltreated with *R.solanacearum* suspension at infilterated area 5 days after inoculation.

Pathogenicity tests:

Six 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. 6 exhibited the highest disease incidence (98.3% wilting) followed by isolate No. 4 which achieved (96.5% wilting). Isolate No. 5 caused the lowest percentage (45.5% wilting) followed by isolate No. 3,2,1 after five weeks from inoculation (Fig. 1).

Isolation and identification of the Biological strains:

Pure cultures of biocontrol agent isolated from tomato rhizosphere (*Pseudomonas aeruginosa, P. putida, P. syrinagae, P. stutzeri, Bacillus cereus and Bacillus thuringiensis*) were identified according to their morphological, cultural and physiological characteristic as stated in Bergey's Manual of Systematic Bacteriology (Table 2). According to the above results isolate No. 6 was used in the following experiments.

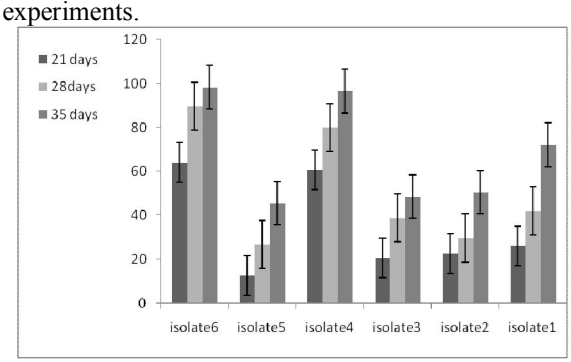


Fig (1): Pathogenicity tests of six isolates of *Ralstoniasolanacearum* on marmand tomato plants cultivar. Bars indicate the standard error.

Table 2.Characterization and identification of the six bacterial strains.

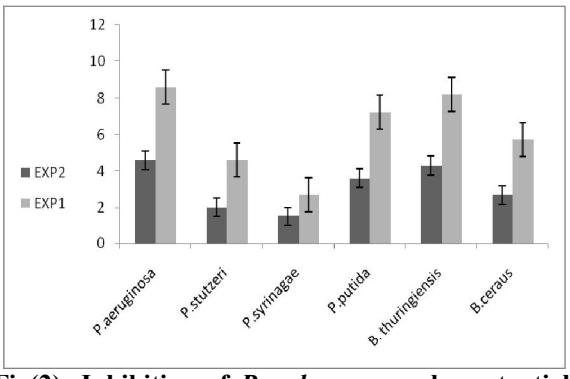
Characteristic Result	Strain1	Strain2	Strain3	Strain4	Strain5	Strain6
Shape of cells	Rod	Rod	Rod	Rod	Rod	Rod
Size	Short	Short	Short	Short	Long	Long
Gram'sstaining	-	-	-	-	+	+
Sporulation	-	-	-	-	+	+
Motility	+	+	+	+	+	+
Pigmentation	+	+	+	+	-	-
Utilization of sugar:						
Mannitol	-	-	-	-	A	-
Fructose	A	A	A	A	A	A
Sucrose	A	A	A	A	A	A
Arabinose	-	-	-	-	-	-
Glucose	A	A	-	A	A	A
Galactose	A	A	A	A	-	A
Lactose	-	A	A	A	-	-
Maltose	A	A	-	A	A	A
Dextrose	A	-	A	-	A	A
Glycerol	A	A	A	A	-	A
Menthol	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	+	+
Gelatin liquefication	-	-	-	-	+	+
Indole formation	-	-	-	-	-	-

+: Positive reaction or growth -: Negative reaction or no growt A: Acid

***In-vitro* evaluation of potential antagonists:**

All biocontrol agent were screened against the plant pathogen *R. solanacearum* antagonism activity towards it. All biocontrolagent showed its ability to inhibit the *R. solanacearum*with different distances of inhibition zone. *Pseudomonas aeruginosa* and *Bacillus thuringiensis* were able to significantly reduction of the growth of pathogen and by far superior to others showed more inhibition zone (8.6,8.2mm) respectively in Cross culture method and

(4.6,4.3 mm respectively in filter paper disk method), while *P. stutzeri* and *P. syrinagae* showed the lowest inhibition zone of pathogen(4.6, 2.7 mm) in Cross culture method and (2.0,1.5 mm) in filter paper disk method as shown in Fig.2.



Fig(2): Inhibition of *R. solanacearum*by potential antagonists in *in-vitro* studies. Bars indicate the standard error.
EXP1: Cross streak method
EXP2: Filter paper disc method

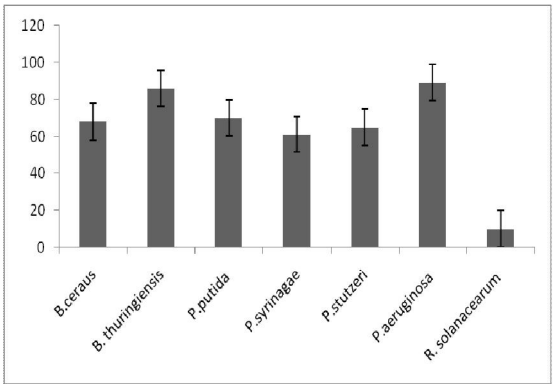


Fig 3: Disease reduction percentage of treated tomato plants with potential antagonists under greenhouse conditions. Bars indicate the standard error.

***In-vivo* evaluation of potential bio-control agents**

The results presented indicate that all biocontrol agent had the ability to reduce the growth of *R. solanacearum*. The most effective reduction of disease symptoms were obtained by using *Pseudomonas aeruginosa* and *Bacillus thuringiensis*. Fig.3 showed the reduction of disease symptoms by using these two organisms. The effects on fresh and dry weight of shoot and on fresh and dry weight of root after control the disease by *P. aeruginosa* and *B. thuringiensis* were shown in (Fig. 4,5) and (Fig 6,7) respectively.

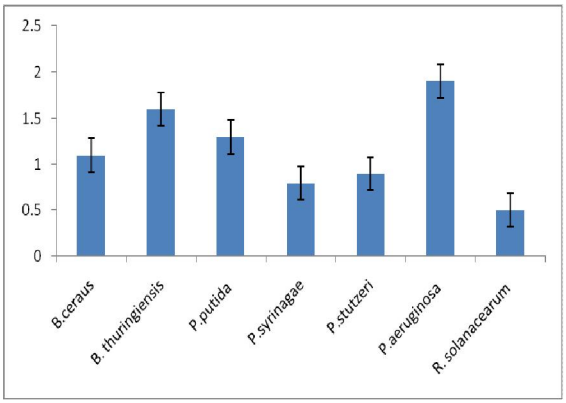


Fig. 4: Effect of potential antagonists on dry weight of root tomato plants. Bars indicate the standard error.

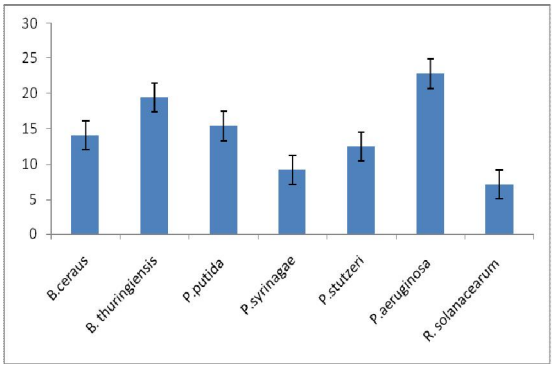


Fig. 5: Effect of potential antagonists on fresh weight of root tomato plants Bars indicate the standard error.

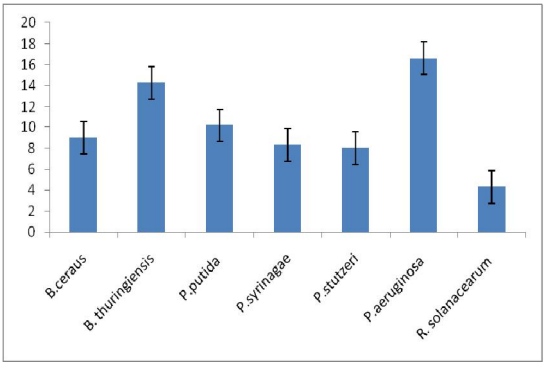


Fig 6: Effect of potential antagonists on dry weight of shoot tomato plants Bars indicate the standard error.

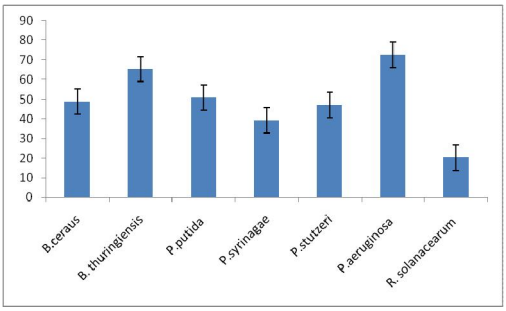


Fig 7: Effect of potential antagonists on fresh weight of root tomato plants. Bars indicate the standard error.

Bacterial Identification Using 16s rRNA Gene Amplification:

Using partial 16S rDNA sequencing analysis showed that the 1st strain belongs to the genus *Pseudomonas*, with closest similarity to *Pseudomonas aeruginosa* 100% similarity (Fig. 9) while the 2nd strain belongs to the genus *Bacillus*, with closest similarity to *Bacillus thuringiensis*99% similarity (Fig. 10).

AGAACCTTGGGGTGATGAAGGTCCTAGGGTTGTAAAGCTCTTTCATCCGTGAAATATATGATGGTAGG
CGAATGAAGAATCCCCGGCTAACTCCAGTGTTTAGCAGCCGCGCCTAATATGAAGGGGGCTAGCGTGA
TGTGVCAAATTTACTGGGCTAAAGCGCACGCTAGGCGGATCGATCAGTCAGGGGTGAAATCCCAGGG
CTCAACCCTGGAACTGCCTTTGATACTGTCGATCTGGAGTATGGAAGAGGTGAGTGGAATTCCGAGTG
TAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGTCCATTACTGA
CGCATGAGGTGCGAAAGCGTGGGGGAGCAAACAGGATGGAGATACCCTGGTAGTCCGGACGCCGTAA
ACGATGAATGTTAGCCCTCGGGCATTATACTGTTCGGTGGCGCAACTAAGGCATTAAACATTCCGCCT
GGGGGAGTACGGACGCAAGATTAAAAACTCAAAGGAATTGCAAGGGGCCCCGCAGAAAGCGGTGGA
GCATGTGGTTTAATTCGAAAGCAACGAGAACAATCTTACCAGGCCTTGCATGCCCGGCTAACCTGCCG
AGATGCAGGGGGTCCCTTCGGGGACCGGGACACAGGTGCTGCATGGCTGTCTTCAGCTCGTGTACGAG
ATATTGGGTTAAGTCCCGCAACGAGCGCGACCCTCGCCCTTATTTGCCAGCATTTAGTTGGGCACTCTA
CC

Fig. 8: Sequence of the 16S rRNA gene of isolate Ab-453 (*Pseudomonas aeruginosa*)

GGCGGATGTTAAGTTGTTTCTTTGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTGAACCTACCT
ATGAAGACTGGAATAACTTCGGGAAACCGGAGCTAATGCCGGATAACATTTTGGGAACCGCATGGTTC
TAAAGTAAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCAGTATTAGCTTTATTGGTAAGTCC
GACGGCTTTTTTAAGGCAAACTATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGAACTGAG
AGACGGTCCAGACTCCTACGGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGG
AAGTAACGCCGCGTGAGTGATGAAGGTTTTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACAAAAT
GTGTAAGTAACTGTGCACATTTTGACGGTACCCAATCAGAAAGCCAGGGGCTAACTACTGGCCAGCAG
CCGCGGTAATACGTAGGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTT
CTTAAGTCTCGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGAAACTGGGGAAACTTGAGTA
CAGAATGATTGA

Fig 9. Sequence of the 16S rRNA gene of isolate Ab-1 (*Bacillus thuringiensis)*

4. Discussion

In this study, results indicate that the six bacterial isolates obtained from naturally diseased tomato plants collected from different localities of Minufiya governorate proved to be pathogenic and able to infect tomato plants causing wilt symptoms and varied in their pathogenicity. They were identified as *Ralstoniasolanacearum*. Present results agreed with those reported by El-Ariqi*et al*., (2005) and Seleim*et al.,*(2011). They said that *Ralstoniasolanacearum* produced fluidal and irregular colonies with pink or light red at centers at 30oC after 48 h of incubation.

All bio-control agents tested showed their ability to reduce the severity of bacterial wilt disease and increased percentage of germination. The highest level of germination was achieved when tomato seeds were subjected to *Pseudomonas aeruginosa* and *Bacillus thuringiensis*. Biological control by using antagonistic fluorescent *Pseudomonas* strains against soil-borne tomato diseases has been reported (Vogt and Buchenauer 1997; Anith*et al.,* 2004). *Pseudomonads* spp. are metabolically very active and have a high growth and aggreesively colonize root systems (Burr *et al.,* 1978). Some of these specifically belonging to *P.aeruginosa*caused substantial increase in plant growth and yield. They would fall under the category of plant growth-promoting bacteria (PGPB). *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).On the other hand *Bacillus*spp.specifically those belonging to the*Bacillus thuringiensis*play a rolein biological control of bacterial wilt of tomato due to its rapid growth in broth culture, high thermal tolerance, and ready formation to resistance spores(Broadbent *et al.,*1971).In vitro conditions, results clearly confirm that plants treated with *P. aeruginosa* and *B. thuringiensis* significantly reduced disease compared to infected control. Disease reduction by *P. aeruginosa* and *B. thuringiensis* 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 Beauchamp, 1992; Liu *et al.,* 1995). *P. aeruginosa* and *B. thuringiensis* may induce plant growth promotion by direct or indirect modes of action (Kloepper*et al.,*1998; Beauchamp, 1993; Lazarovits and Nowak, 1997). Directly by production of plant growth regulators (auxins, cytokinins, gibberellins) and facilitation of the uptake of nutrients (nitrogen fixation, solubilization of phosphorus). The indirect by *P. aeruginosa* and *B. thuringiensis* 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).

In vivo results clearly confirm that application of *P. aeruginosa* and *B. thuringiensis* as potential bioagents in controlling tomato bacterial wilt under greenhouse 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 *Pseudomonas* spp. Also (Seleim*et al.,* 2011) recorded 96% reduction of the tomato bacterial wilt disease under greenhouse conditions using *Pseudomonas* spp.

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