Relationship between *Ralstonia Solanacearum* and bioagents recovered from different habitats

Enas A. Hassan[1], Naglaa M. Balabel[2], Abeer E.Ahmed[3], Nerhan A. Eid[3], El shahat M. Ramadan[1]

1 Agric. Microbiol. Dept., Fac. Agric., Ain Shams Univ., Cairo, Egypt
2 Bact. Dis. Res. Dept., Plant Pathol. Res. Instit., Agric. Res. Center, Giza, Egypt
3 Plant Protec., Des. Res. Center, Cairo, Egypt

Corresponding Author : Naglaa M. Balabel, Dr.naglaabalabel@pbrp.gov.eg

Abstract--- Bacterial wilt caused by *Ralstonia solanacearum* phylotype II sequevar I (race 3 biovar 2), is considered one of the most destructive bacterial diseases of potato plants. The aim of this study was to evaluate the potential of some bacterial antagonists on suppressing the causative bacterium. In this regard, 420 isolates of *R.solanacearum* were risolated from different habitats at different Egyptian districts. The variation among these isolates was assessed on the basis of their pathogenic potentialities to tomato seedlings in the greenhouse. The highest potential, as shown by the wilt severity, was obtained by 11 out of 420 isolates. The most aggressiveness isolates were identified by accurate techniques. A collection of 318 isolates of rhizobacteria from rhizosphere of different plants were tested to lay out a biological control protocol against *R.solanacearum* selected strains. *In vitro*, 14 bacterial isolates were more effective in suppressing the pathogen at different levels of relative power of antibiosis. These isolates were categorized into three groups according to their gram-staining reaction, cell morphology and cultural characteristics. Further evaluation under the greenhouse condition has shown that six of the antagonistic isolates were effective in suppressing disease development, as expressed by the Area Under Disease Progress Curve (AUDPC). These isolates were identified as: *Streptomyces toxytricini*, *Stenotrophomonas maltophilia*, *Bacillus pseudomycoides* and *Brevibacillus brevis*.

Keywords: Potato, bacterial wilt , *Ralstonia solanacearum* , biological control, , PCR , PGPR .

1. Introduction

Ralstonia solanacearum is a soil-borne, rod shaped, gram negative, β-proteobacterium that causes bacterial wilt disease to more than 450 plant species including many economically important crops. Due to its wide geographic distribution and unusually broad host range (over 50 plant families) the pathogen is responsible for severe crop losses worldwide (Hayward, 1991 and Saad , 2016).

Although various control measures have been documented, bacterial wilt is still a difficult to control because of wide host range of the pathogen and long survival of the pathogen in soil, especially in deeper layers (Hsu, 1991).Crop rotation with non-host plants, although recommended, is not an efficient method, since *R. solanacerum* has its disseminating and survival phases in the soil and it remains viable for long periods of time. The race and strain diversity of the pathogen has made breeding for resistant cultivars ineffective or with limited value in the control of bacterial wilt (Farag *et al*., 1982; Hanson *et al.*, 1996 and Wang *et al.*, 1998). The use of soil fumigants is environmentally unacceptable, expensive and largely ineffective against the disease (Saddler, 2005). Chemical and soil treatments such as modification of soil pH, heat treatment by solarization, and application of stable bleaching powder, as well as plant resistance inducers as acibenzolar –S-methyl, plant essential oils as thymol, or phosphoric acid have been shown to reduce bacterial populations and disease severity on a small scale (Norman *et al*., 2006 and Abo-Elyousr *et al*., 2012).

Biological control strategies may be either used directly as a practice or after being integrated with other practices for effective disease management at the field level (Myint & Ranamukhaarachchi, 2006). A large numbers of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter* (Ahmed *et al*., 2008), *Klebsiella* (Govindarajan *et al*., 2007), *Serratia* (Gyaneshwar *et al*., 2001) have been reported to enhance plant growth and suppress disease development in various crops.

Several strains of *Pseudomonas fluorescens* have been reported to suppress soil- borne diseases (Weller, 1988). On the other hand, numerous of Actinomycetes and bacteria such as *Stenotrophomonas maltophilia*, *P. glumae*, *Burkholderia cepacia*, *Bacillus* spp., *Erwinia* spp. have been reported to be active control agents against *R. solanacearum* (Messiha *et al*., 2007; Aliye *et al*.,2008 and Xue *et al*., 2009).

The present study aimed to obtain plant growth promoting rhizobacteria (PGPR) from the rhizosphere of

different plants with high biocontrol efficiency against virulent isolates of *R. solanacearum* , which was isolated and identified from different habitats.

2. Material and Methods

2.1. Isolation and growth conditions of *R.solanacearum*:

R. solanacearum isolates in this study were isolated from different habitats, *i.e*. potato tubers, field soil, irrigation water and weeds (*Chenopodium album*, *Portulaca olaraceae*, *Rumex dentatus* and *Solanum nigrum*) obtained from different areas at Al-Gharbia , Al-Behira and Al- Menofia governorates, in Egypt. Isolation was carried out on SMSA medium (Semi Selective Medium of South Africa) according to Elphinstone *et al*. (1996) and incubated at 28±1°C. Typical virulent fluidal, slightly irregular, white colonies with pink centers were selected as *R. solanacearum* (Buddenhagen & Kelman, 1964). The selected isolates were propagated on nutrient agar medium for 48 hours and then were verified serologically using immuno-fluorescent antibody staining (IFAS) (Janse, 1988). Pathogenicity was determined on Super Strain B tomato by stem inoculation .The most virulent pathogenic isolates were selected for further experimentation. The physiological and biochemical tests were made to confirm *R. solanacearum* identity(Hayward *et al.*,1990).Selected isolates were maintained in long-term storage as suspensions in sterile tap water and were revived by plating on tetrazolium chloride (TTC) medium (Kelman, 1954).

2.2. Diagnosis and identification of *R. solanacearum:*

Pathogenicity and identification are the first steps for diagnosis of bacterial brown rot. Accurate identification of *R.solanacearum* race3 biovar2 based on symptomatic or a symptomatic plants along with multiple microbiological and molecular methods were followed.

2.2.a. Pathogenicity test on tomato seedling:

Four weeks old healthy tomato seedlings (*Solanum lycopersicum* cv. Super Strain B) were used as a test plants to determine the virulence of *R. solanacearum* under greenhouse conditions at 20-30°C. Bacterial inoculum was prepared in nutrient broth for 2 days at 28±1°C, centrifuged and suspended in sterile destilled water, and adjusted to be 10^7cfu/ml using spectrophotometer at 620 nm. The seedlings at the three true-leaves stage were inoculated with puncturing the basal part of the stem with the bacterial suspension (Janse, 1988). Five replicates were made for each isolate. Control treatment was prepared by applying few drops of sterile distilled water instead of the bacterium. Disease severity was determined 7 days after inoculation based on the scale of Winstead &Kelman (1952) as follows:
0 = No wilt symptoms; 1 = One or 2 wilted leaves; 2= Three wilted leaves; 3 = All leaves wilted except the tip; 4 = Whole plant wilted and 5= Death (collapse) of whole plant .

Wilt severity was calculated by the following formula:

$$\% \text{ Wilt severity} = \frac{\Sigma (nxv)}{5\,N} X100$$

Where:
n =Number of the inspected samples in each category.
v = Numerical values of each category.
N = Total number of the inspected samples.
5= The highest grade scale.

2.2.b. Identification of *R.solancearum* biovars:

Biovar characterization of the selected pathogenic bacterium (*R. solanacearum*) was done based on the differential ability of pathogen isolates to oxidize sugar and sugar alcohols using standard procedure described by Hayward *et al*. (1990).

2.2.c. Genotypic characterization:

At the sub-species level, identification of *R. solanacearum* isolates can be determined by polymerase chain reaction (PCR) amplification with specific probes and primers. Real-time PCR (Taq-Man) was performed on selected highly pathogenic *R.solanacearum* according to Weller *et al*. (2000) by using the apparatus of Applied Biosystem7500 **.** The reaction mixture consisted of 12.5µl of master mix, 1 µl of primer forward, 1 µl of primer reverse, 1 µl of probe and 7 µl of water and 2.5µl of nucleic acid extract. The sequence of primers and probe used is shown in Table (1), which were provided by OPRON, USA. Positive control of DNA extracted was used and water was used as a negative control.

Table (1): Characteristics of primers and Taq-Man probe used to detect *R. solanacearum* by Real-time PCR.

Primer or probe	Sequence(5'→3')	Length	Dye
RS-I-F	GCA TGC CTT ACA CAT GCA AGTC	22	
RS-II-R	GGC ACG TTC CGA TGT ATT ACT CA	23	

RS-P	AGC TTG CTA CCT GCC GGC GAG TG	23	FAM

2.2.d. **Phylotype analysis of *R. solanacearum* by Multiplex- PCR:**

Representative isolates from observed PCR clusters were further analyzed to assess the phylotype. Analysis of selected isolates was made by using the Opina primers 759/760 as internal markers specific for the *R. solanacearum* strains and a set of four phylotype - specific forward primers with a unique and conserved reverse primer targeted in the 16S-23S Intergentic Spacer region (Opina *et al.*,1997). Table (2) shows the characteristics of the used primers. Suspend one colony of the tested isolates in 100µl of sterile water and heating for 5 min. at 100°C to spin down cell debris. The reaction mixture consisted of 12.5µl of ready master mix, 1 µl from each primer, 3.5µl of water and 2µl of nucleic acid extract. The following cycling program was used in a thermal cycler (Biometra T personal): 96°C for 5 min. and then cycled through 30 cycles of 94°C for 15s, 59°C for 30s and 72°C for 30s, followed by a final extension period of 10 min. at 72°C. A13 µl aliquot of each amplified PCR products was subjected to electrophoresis on 2 % (w/v) agarose gels, stained with ethidium bromide (0.5% µgL-1) and bands were visualized on a UV-transilluminator. This Pmx-PCR amplifies the 280-bp"Universal" *R. solanacearum* specific reference band plus following phylotype-specific PCR products: a 144-bp amplicon from phylotype I strains; a 372-bp amplicon from phylotype II strains; a 91-bp amplicon from phylotype III strains and a 213-bp amplicon from phylotype IV strains (Sagar *et al.*,2014).

Table 2. Characteristics of primers used for Phylotype analysis of *R .solanacearum* by Multiplex-PCR

Primer	Sequence (5`⟶ 3`)	Length
759	GTC GCC GTC AAC TCA CTT TCC	21
760	GTC GCC GTC AGC AAT GCG GAA TCG	24
Nmult:21:1F	CGT TGA TGA GGC GCG CAA TTT	21
Nmult:21:2F	AAG TTA TGG ACG GTG GAA GTC	21
Nmult:23:AF	ATT ACS AGA GCA ATC GAA AGA TT	23
Nmult:22:InF	ATT GCC AAG ACG AGA GAA GTA	21
Nmult:22:RR	TCG CTT GAC CCT ATA ACG AGT A	22

2.3. Isolation of potent antagonistic rhizobacteria:

Potent antagonists were isolated from the rhizosphere of different healthy plants, grown in El-Adlia farm at Sharkia governorate. *Vitis* sp.(grape), *Triticum sp.* (wheat) , *Ocimum basilicum* (basil), *Vicia faba* (faba bean), *Solanum lycopersicum* (tomato), *Alliumo mpeloprasum* (leek), *Zea mays* (corn), *Solanum lacinistum* (solanum), *Ltissima glumerulifora* (solidago), *Cymbogon citrates* (lemon grass), *Salvia officinalis* (salvia), *Calendula officinalis* (pot marigold), *Foeniculum vulgar* (fennel), *Solanum melongena* (eggplant) and *Rosmarinus officinali* (rosemary) . Root samples were vigorously shaken to remove loosely adhering soil (Leben *et al.*, 1968). Five serial 10-fold dilutions were made in sterile phosphate buffer for each sample and spread on glucose nutrient (Dowson,1957), King's B (King *et al.*, 1954) and glycerol nitrate agar (Waksman,1961) media to isolate bacilli and yeasts , fluorescent Pseudomonades and Acyinomycetes, respectively. Plates were incubated at 30 ±1º C for 2-4 days then single colonies were picked up and stored at 4±1º C on the appropriate medium.

2.3.a. Inoculum preparation of the antagonistic rhizobacteria :

Selected bacteria were checked for antagonistic potential, after turbidity standardization. The 48 h. old culture on suitable broth medium was centrifuged at 10.000 g for 10 min. Bacterial pellets were washed twice with sterilized distilled water by centrifugation. The optical density (OD) of the solution was adjusted to 0.45 (620 nm) to obtain $l0^7$cfu /ml (Mortensen, 1999).

2.3.b. Screening of *R. solanacearum* antagonistic rhizobacteria *in vitro:*

Collected isolates of rhizobacteria were tested for their antagonistic potentials against *R. solanacearum* according to the method described by Li *et al*. (2008).Ten ml. of *R. solanacearum* inoculum grown in nutrient broth for 24 h./ 28±1° C and centrifuged were used. The pellets were re-suspended in sterilized saline solution (0.85% NaCl) to a final concentration10^7cfu /ml. then was mixed with 1 litter of lukewarm melted nutrient agar and pouring into sterilized plates. After solidification, each plate was peripherally inoculated with three agar disks (6mm) of antagonist as a standard inoculum and incubated at 28-30°C for72 h. The relative power (RPA) of antibiosis was

used to measure the inhibitory effect of isolates against *R.solanacearum* based on the formulation of inhibition zone around that antagonistic organism .The formula which used to measure this parameter was proposed by Ibrahim *et al.* (1987) as follows:

RPA = Diameter of inhibition zone / Diameter of spotted antagonistic organism.

Bacteria that displayed remarkable inhibition activity were considered antagonistic and selected for further investigations.

2.4. Evaluation of antagonistic potential *in vivo:*

2.4.a. Inoculum preparation of antagonistic rhizobacteria :

The potential of bio-antagonistic rhizobacteria *in vitro* was carried out. The bacteria were inoculated in sterilized suitable broth media individually and incubated at 28-30°C for 2-4 days favorable for each group. After incubation period, the cell density were adjusted to give 10^9cfu/ml .

2.4.b. Greenhouse experiment:

The experiment was carried out under greenhouse conditions for verification bio-control potentials of selected rhizobacteria. Three tomato seedlings (*Solanum lycopersicum* cv. Super Strain B) of 4 weeks old were transplanted in each pot (25 cm. in diameter) filled with sterilized potting mixture (1 soil: 1 peat moss). 50 ml of antagonistic bacterial suspension contained10^9cfu/ ml were add individually into soil for directly transplanting of tomato seedlings. Then, soil-drenching method was applied to infest the plants by bacterial wilt as follows:

The root system of each seedling was wounded with a scalpel and 5 ml. of inoculum (10^8 cfu / ml) per plant were poured on the wounded root system. The planted pots were maintained under greenhouse conditions with 5 replicates for each treatment. The temperature was ranging between 25 to 30°C and the relative humidity between 70 to 90%. Seedlings without pathogen or/and antagonist bacteria were considered as control treatments . The treated plants were examined for disease development over 7 days period after treatment and disease was recorded as disease incidence and bio-control efficiency according to Song *et al.(*2004).

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

Control treatments without either the pathogen or the tested antagonist isolates were considered as negative controls. Complete randomized block design with five replicates for each treatment was followed.

Data were statistically analyzed according to Duncan (1955). LSD test at 5 % level of significance was used for comparison between the means of different treatments.

2.5. Molecular analysis:

The most efficient antagonistic isolates were identified by 16SrRNA sequance. Isolation of cellular DNA was performed as described by Ausubell *et al*. (1987) and amplification of 16S rDNA according to Lane (1991) using the universal 16S primers (F1 5' AGAGTTT(G/C)ATCCTGGCTCAG 3' R1 5' ACGG/C) TACCTTGTTACGACTT 3'). PCR was run on a Gene Amp PCR System 2400 thermal cycler (Perkin Elmer). DNA was amplified over 35 cycles of denaturation for 1 min at 94◦C, annealing at 55°C for 1.5 min and extension at 72°C for 2 min. After the last cycle, DNA was extended at 72◦C for 10 min. Amplification was confirmed by analyzing 5µl of PCR reaction mixture on 1 % agarose gel (Promega). The resulting PCR products sizes were ranged from 1450 to 1500 bp. The PCR-product was purified using QI Aquik PCR Purification Kit (Qia gen). The sequencing was performed in two directions using the previously described primers (Lane, 1991) in GATC Company (Germany). Sequencing data was analyzed by two different computer alignment programs, DNAStar (DNASTAR, Inc., USA) and Sequence Navigator (Perkin, Corp., USA).

2.6. Statistical analysis:

3. Results

3.1. Isolation and characterization of *R. solanacearum* isolates:

Isolation of *R.solanacearum* was made by using SMSA medium, from different habitats collected from Al-gharbia, Al-behira and Al-minufiya governorates. Table (3**)** shows 420 colonies developed typical colonies of *R.solanacearum* on the selective medium (SMSA) from irrigation water, potato tubers, potato stems, soil and weeds. Moreover, IFAS testing was carried out on colonies developed to determine the morphological shape. The cells showed short rod shaped morphology and stained evenly as bright green fluorescent were considered (Fig.1, A and B).The greatest numbers of typical colonies were recognized and selected from potato tubers while, the lowest number was selected from soil samples. On the other hand, approximately similar numbers of typical colonies were selected from potato stems and weeds (Table, 4). The pathogenic potential of the collected isolates (420 isolates) was evaluated for producing wilt to tomato seedlings, 7 days after inoculation under greenhouse conditions presented by disease incidence %**.**

Fig.(2) shows that tuber isolates caused 100% infection to tomato with corresponding wilt severity of 60.0% compared to 13.5% for the potato stem isolates. Soil and irrigation water isolates showed disease

severities of 14.0 and 17.5% , respectively. Moreover, isolates recovered from symptomless weed plants namely *Rumex dentatus*, *Solanum nigrum*, *Chenopodium album* and *Portulaca olaraceae* showed high potentials, being 50.0%, compared to the isolates from potato stems, soil and irrigation water.

3.2. Identification of the causal of bacterial wilt:

The identification of the selected isolates from the different habitats was made based on the morphological properties, biochemical and pathogenicity tests as well as their ability to sugar and sugar alcohol utilization a long with the molecular studies were being considered. All isolates were grown on SMS plates at 72 h. / 28° C and showed the identity to *R.solanacearum*. Observations were made on colony colour, shape, size, surface, margin elevation, opacity consistency, gram reaction and IFAS test.

The results led to the selection of eleven *R. solanacearum* isolates showing discernible disease severity, obtained from potato tubers and weeds .These isolates were identified based on the further investigation.

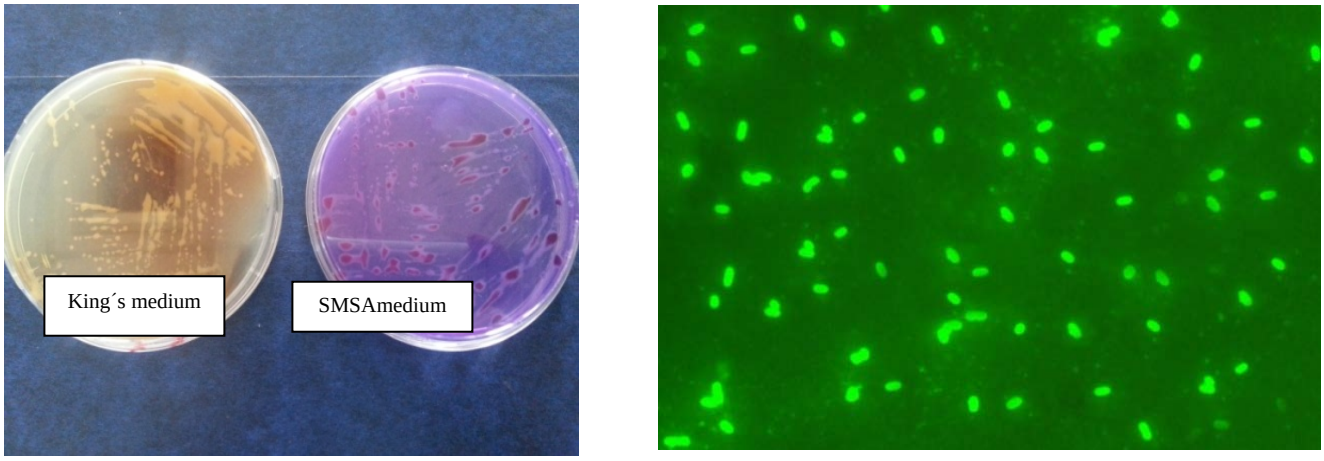
3.2.a. Biovar determination of the pathogen:

The eleven isolates produced acid from maltose, lactose and cellobiose, along with no reaction with sorbitol, mannitol and dulcitol indicating that, the dominant race of *R. solanacearum* in Egypt is race 3, biovar 2**.**

Table 3. Number of *R.solanacearum* isolates on SMSA medium from different habitates:

Governorate	Location	Irrigation Water	Potato tubers	Potato stems	Soil	Weeds*
Al-gharbia	Kafr-yakoub	14	29	12	3	12
	Kafr-hashad	10	47	15	3	14
	Dalgamon	9	17	9	2	16
Al-behira	Kom Hamada	11	16	9	3	11
	El-nigila	13	28	15	2	16
Al-Menufiya	Talia	8	43	20	2	11

*Symptomless *Rumex dentatus*, *Solanum nigrum*, *Chenopodium album*, *Portulaca olaraceae.*



A **B**

Fig. (1 ,A and B): Typical colonies of *R. solanacearum* on King's B and SMSA media (A). Cell morphology of *R. solanacearum*under immunofluorescent (IF) microscope (B).

Table 4. Number of samples, infected samples, selected colonies and number of positive IFAS test recovered from different collected habitats.

Habitat	No. of samples	Infected samples	No. of selected colonies	No. of IF(+)
Potato tubers	60	60	180	180
Potato stems	42	30	80	80
Soil	24	15	15	15
Irrigation water	18	18	65	65
*Weeds	54	35	80	80
Total	198	158	420	420

* Symptomless *Rumex dentatus*, *Solanum nigrum*, *Chenopodium album* and *Portulaca olaraceae*

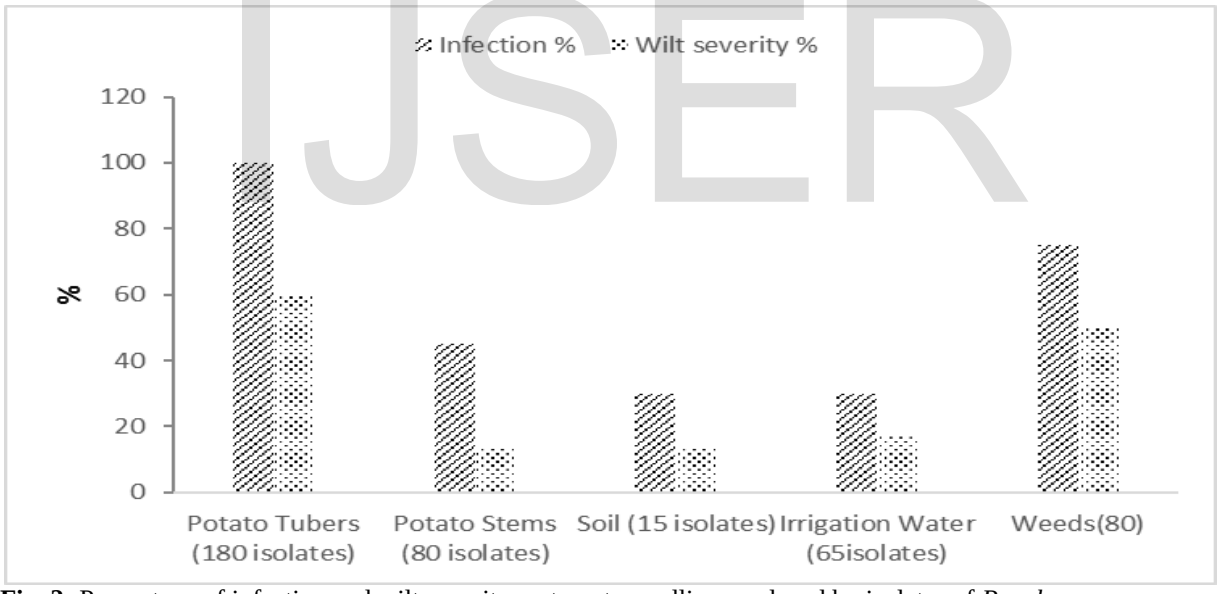


Fig. 2: Percentage of infection and wilt severity on tomato seedling produced by isolates of *R. solanacearum* collected from different sources.

3.3.b. Real-Time PCR assay:

The RS primers and probe were employed to detect all biovars and races of *R. solanacearum*. Positive results were noticed with all tested isolates indicating that, the eleven strains are *R. solanacearum* biovar 2 race 3 (Fig. 3), equivalent to phylotype II ,sequevar I.

3.3.c. Phylotype analysis of *R.solanacearum* by Multiplex- PCR:

Phylotype specific multiplex PCR revealed that all the eleven isolates of *R. solanacearum* belonged to phylotype II as 372- bp amplicon was observed with all tested isolates when Pmx-PCR products of these isolates were subjected to electrophoresis on 2% agarose gel (Fig., 4). These results indicating that, the dominant race of *R. solanacearum* in Egypt is race 3 (phylotype II).

Fig.3: Amplification of DNA extract of eleven selected isolates from different habitats by Taq Man polymerase chain reaction (PCR). Negative and positive controls were included. The curves exceed the threshold considered positive.

Fig. 4: PCR products of 11 isolate of *R. solanacearum* using different primers, 280-bp amplicon from *R.solanacearum* species and 372-bp amplicon from phylotype II strains.

3.4. Screening of antagonistic microorganisms:

A collection of 318 isolates of rhizobacteria from different plants grown at Eladlia farm, Sharkia were *in vitro* screened against the selected eleven isolates of *R.solanacearum* (gave the highest wilt severity). These isolates were evaluated individually against the bacterial wilt pathogen by dual culture inoculation technique. Fourteen isolates showed the highest potential of antagonistic activity against the eleven isolates of *R.*

solanacearum race3 biovae2 as a relative power of antibiosis (RPA) ranging from 5- 9.9 and have a broad spectrum of growth inhibition for all tested pathogenic bacteria (Table. 5). The antagonistic bacteria were categorized into three groups according to their gram staining, cell morphology and culture characteristics .Six antagonistic isolates showed strong antagonism against all tested pathogenic bacteria. Their values of RPA were ranged from 7.9 to 9.9. These isolates showed different morphology including two short rods (M3, M4), two long rods (M5, BG4) and two Actinomycetes (R4, C5).

3.5. Evaluation of biocontrol potential:

Fig. (5) shows disease incidence (%) and biocontrol efficiency (%) of the selected antagonists against the identified *R.solanacearum* race 3 biovar 2 under greenhouse conditions.

The fourteen antagonistic isolates, showing potent *in vitro* inhibition were tested in the greenhouse for their effectiveness in controlling bacterial wilt disease. The fourteen bacterial strains (Fig, 5) were significantly differed in their abilities to suppress bacterial wilt compared to control treatment.

Accordingly, M_3, M_4 (short rod), BG_4, M_5 (long rod) and R_4, C_5 (Actinomycetes) significantly decreased disease incidence from 10 to 40% compared to the control treatment and exhibited the highest percentage of biocontrol efficiency. Isolate M_3, (short rod) was the most effective in reducing the percentage of disease incidence, as it gave a 10% disease incidence with all tested isolates of *R.solanacearum* followed by R_4 and C_5. The corresponding values of biocontrol efficiency was 90%. From the greenhouse experiment, six antagonistic bacterial isolates (M_3, M_4, M_5, BG_4, C_5 and R_4) exhibited high efficient of controlling bacterial wilt and suggested that the existence of these bacterial isolates in the plant rhizosphere might play a good role for the plant to suppress the pathogenic bacterium *R. solanacearum*.

Table 5. Relative power of antibiosis (RPA) of bacterial isolates antagonist against eleven isolates of *R. solanacearum* Race3 Biovar 2 on Kinge´s B agar medium.

Bacterial group	Rhizobacterial isolates	*R.solanacearum* race 3 biovar 2 isolates										
		R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11
Short- rods	B5	8.4 b	7.1 c	8.2 b	7.6 c	8.8 b	7.5 c	8.4 b	8.3 b	8.5 b	8.2 b	5.8 d
	T2	8.5 b	7.2 c	6.8 c	9.5 a	7.8 c	9.1 a	9.1 a	9.1 a	7.5 c	8.1 b	8.7 b
	L6	8.4 b	8.9 a	8.6 a	7.5 c	8.8 b	9.2 a	8.2 b	7.3 c	8.2 b	8.5 b	9.4 a
	S8	8.5 b	9.1a	8.0 b	9.0 a	9.2 a	4.2 d	9.3 a	9.4 a	7.2 c	8.5 b	4.3 e
	M3	8.9 a	9.8 a	9.3 a	9.4 a	9.8 a	9.2 a	9.1 a	9.9 a	8.9 a	8.8 a	8.9 a
	M4	8.9 a	9.7 a	8.9 a	8.8 a	9.7 a	9.3 a	8.9 a	9.7 a	8.7 a	8.9 a	8.9 a
Long- rods	G8	8.3 b	8.5 b	8.2 b	8.8 a	8.5 b	8.3 b	7.8 c	8.5 b	7.8 c	7.8 c	7.8 c
	T6	8.4 b	8.5 b	5.6 c	8.1 b	8.6 b	7.8 c	8.8 a	8.2 b	8.5 b	8.0 b	8.4 b
	T8	8.0c	9.0a	5.3 c	5.0 d	7.7 c	8.3 b	8.2 b	7.8 c	8.0 b	7.2 d	6.4 d
	M5	8.8 a	8.9 a	8.9 a	9.5 a	9.1 a	9.0 a	8.9 a	9.8 a	9.1 e	8.9 a	9.5 a
	BG4	8.9a	9.1 a	8.6 a	9.4a	8.9 b	8.9 a	9.6 a	8.5 b	9.3 a	9.6 a	9.8 a
Actino-mycetes	R4	8.2 b	8.2 b	8.2 b	8.2 b	8.2 b	8.4 b	7.9 c	7.9 c	8.4 b	8.1 b	8.7 b
	C5	9.3 a	9.5 a	9.0 a	9.2 a	9.5 a	9.5 a	9.6 a	8.9 a	8.9 a	9.3 a	9.4 a
	R10	6.2 c	6.1 d	6 c	6.2 d	7.9 c	6 d	5.7 d	6.1 d	6.2 d	6.1 d	6.4 d

Values in the same columns followed by the same letter don´t significantly differ from each other according to Duncan at 5 % level.

3.6. Identification of antagonistic bacteria:

The most potent antagonistic bacteria were identified by amplifying and sequencing the 16Sr DNA techniques by sigma scientific services. The results showed that 16SrDNA of M_3, M_4, M_5 , BG_4, C_5 isolates had 97, 67 , 98,96 and 96% identities with *Stenotrophomonas maltophilia* , *Brevibacillus brevis* , *Bacillus pseudomycoides* , *Streptomyces toxytricini* , respectively. Further studies will be needed to determine the mode of action for these strains with potato plants.

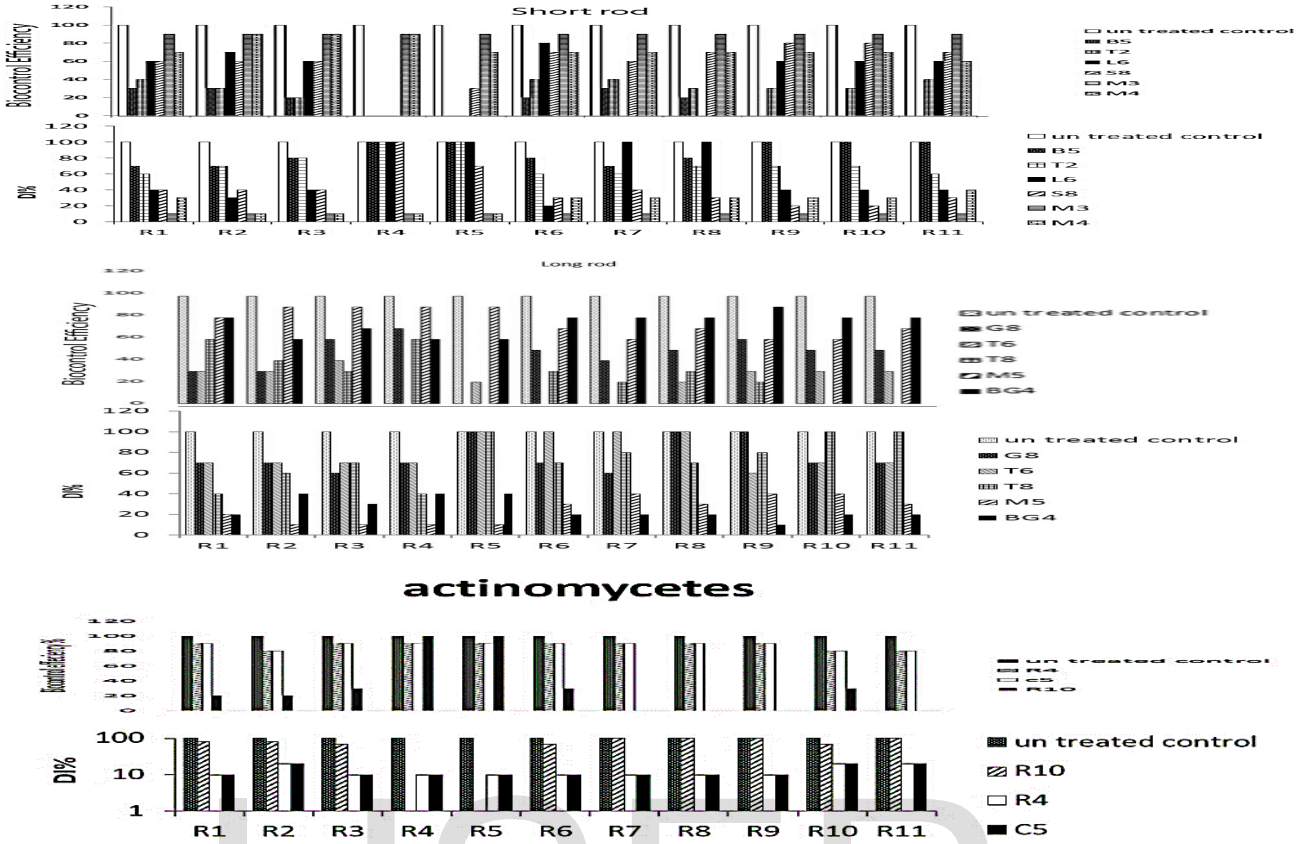


Fig. 5: Biological control of *R.solanacearum* race3 biovar2 by antagonistic bacteria on tomato plant under greenhouse conditions expressed as Disease incidence and biocontrol efficiency of antagonist.

4. Discussion

Potato brown rot, caused by *R. solanacearum* (Yabuuchi *et al.*, 1995), has been reported in Egypt many years ago by Sabet (1961).The disease has created a lot of quarantine problems during the course of exportation of table potatoes to Europe (Farag, 2000).The disease is known to be favored by warm climates; however, serious outbreaks in Europe have been reported by Grouss *et al.*, (1998). Therefore, the origin of the disease in Egypt is thought to be from masked or latent infection(s) introduced with potato seeds imported from Europe. There are a number of "Sheltered sites" where the bacterium might survive such as alternative weed hosts as Solanaceous weeds commonly known as *Solanum dulcamara* (Olsson, 1976a), *S. cinereum* (Graham and Loyd, 1978a) and *S. nigrum* (Hayward, 1975). Certain non Solanaceous weeds, such as *Tropaeolum majus* (Olsson,1976b) and *Portulaca oleracea, Rumex* sp. beside to potato plants and infected plant debris in the deeper soil layers, that mentioned by Graham and Loyd (1978b).

In the present study the isolation of the pathogen was easily made from the most possible sources along with the irrigation water. Limited colony numbers of the pathogen were recovered from the soil compared to other sources that may be attributed to some sort of edaphic factors. The soil type is an important factor affecting survival of *R . solanacearum* (Prior *et al.*, 1993 and Balabel *et al.*, 2005) and different soils may be conducive or suppressive to pathogen survival (French, 1994). Furthermore, Farag *et al.* (1999) reported that, *R. solanacearum* was present in surface (irrigation) water in Egypt. These findings currently show that irrigation water can play an important role in the epidemiology of the brown rot in Egypt.

The pathogenicity of 420 isolates of *R. solanacearum* was evaluated on three-leaved tomato seedlings that are being reported to develop wilt syndrome, 3-7 days after inoculation. In the present study, typical wilting was developed within a week after inoculation depending on the inoculum potential and optimization of condition (Elphinstone *et al.*, 1996). The wilt was reported to fail, however, if night temperature falls below 21ºC (Singh *et al.*, 2014). The disease propensities and wilt severity produced by tuber isolates was greater than those produced by bacteria isolated from potato stems. Moreover, the bacteria isolated from weeds were more virulent than those isolated from water and soil. The greater severity of isolates recovered from potato tubers and weeds were reported earlier by Farag *et al.* (2004) and Balabel *et al.* (2005). Such differences in pathogenic potential may denote the diversity and co extensiveness of strains in nature (Palleroni and Doudoroff, 1971).On the other hand, the co extensiveness of *R. solanacearum* strains in nature and the wide range of hosts either

symptomatic or asymptomatic may be in part attributed to such preferential host effect that generates certain forms with different pathogenic potentials. Such observation may be of value in studying epidemiology of *R. solanacearum* as mentioned before by He *et al.,* (1983), Farag *et al.,* (2004) and Balabel (2006).

The fourteen isolates of the rhizobacteria were evaluated as antagonists against the pathogen that provided an impression on different degrees of antagonism *in vitro* and *in vivo* under greenhouse conditions. Six out of these antagonists gave the highest efficient against pathogenic bacteria that appear in reduced disease incidence (%) and high biocontrol efficiency(%) on tomato plants. These antagonists are affiliated to different species identified as *Streptomyces toxytricini*, *Stenotrophomonas maltophilia*, *Bacillus pseudomycoides* and *Brevibacillus brevis*.

With regard, *Brevibacillus brevis*, is a Gram positive spore-forming bacterium, studies showed that it had significant inhibitory potential on many animal and plant pathogens (Ge *et al*., 2009) and such as *Ralstonia solanacearum* (Che *et al.,* 2012). On the other hand, this bacterium can secrete large amounts of secondary metabolites, as tyrocidine, gramicidin, gratisin (Tamaki *et al.*, 1983), and edeine (Czajgucki *et al.,* 2006). Moreover, this bacterium can secrete non ribosome peptides, which were shown to effectively inhibit the growth of *R.solanacearum* by disturbing cell membrane integrity (Chena *et al.,* 2012). Therefore, *B. brevis* may be considered as a good biocontrol agent against *R. solanacearum.* Previous studies had proved the potential of actinomycetes as effective biocontrol for *Ralstonia solanacearum*. The inhibition of this pathogen may be due to the emission of volatile secondary metabolites secreted by Actinomycetes (Rado *et al.,* 2015). *Stenotrophomonas maltophilia* is a gram - negative bacterium, Initially classified as *Pseudomonas maltophilia*, it was also grouped in the genus *Xanthomonas* before eventually becoming the type species of the genus Stenotrophomonas (Palleroni and Bradbury 1993), Denton and Kerr1998). It is an effective biocontrol agent for the control of some fungal and oomycetous plant diseases (Dal Bello *et al.,* 2002 and Berg *et al.,* 2005). On the other hand, Messiha *et al*., (2007) reported that, this bacterium may be useful for control of brown rot bacterium. *S. maltophilia* produces various antibiotics, for example, maltophilin, a macrocyclic lactam antibiotic, which has antifungal activity, but is inactive against Gram-positive and Gram-negative bacteria (Jakobi *et al.*, 1996).

5. Conclusion:

The present study showed that *R. solanacearum* race3 biovar2 is the dominant pathogenic bacterium under Egyptian conditions causing brown rot .PGPR isolates antagonistic to the causal bacterium could be isolated from local fields and could be used to control the deleterious effect of *R.solanacearum* .Thus, it is hoped that the obtained inoculants antagonists will manage the bacterial wilt disease.

6. References

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