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Biological control of fungal wilt of tomato by plant growth promoting rhizobacteria and *Trichoderma harzianum*

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Abstract

The efficacy of plant growth promoting bacteria i.e. (*Bacillus subtilis, Bacillus polymyxa, Bacillus megaterium, Pseudomonas fluorescence, Azotobacter sp., Azospirillum sp.* and the fungus *Trichoderma harzianum* were studied as bioagents for controlling *Fusarium oxysporum f.sp. lycopersici* and *Verticillium dahliae* the causing wilt disease of tomato. The results of antagonistic activity of the PGP bacteria against Fusarium *oxysporum f.sp. lycopersici* and *Verticillium dahliae in vitro* exhibited higher variation in their abilities to reducing the growth rate of the pathogen and increasing the percentage of pathogen inhibition as compared with the control treatment. Also, the obtained results showed that *Trichoderma harzianum* I, *Pseudomonas fluorescens* and *Bacillus subtilis* gave the best results for controlling *Verticillium dahliae* and *Fusarium oxysporum f.sp. lycopersici.* Under greenhouse conditions, the inoculated tomato seedling with a mixture of (*Azotobacter sp+ Azospirillum sp.+ B. megaterium var. phosphaticum + B. subtilis+ B. polymyxa +Pseudomonas fluorescence+ T. harzianum* I showed the least percentage of infection as compared with the bioagent treatments individually.

Key words: plant growth promoting rizhobacteria, *Azotobacter sp., Azospirillum sp., Trichoderma harzianum, Verticillium dahlia, Fusarium oxysporum f.sp. lycopersici,* fungal wilt disease of tomato.

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Introduction

Tomato (*Lycopersicon esculentum* Mill) is one of the most important vegetable crops in Egypt. The cultivated area of tomato since 1999 to 2013 growing season average reached 498536 acres in old and newly reclaimed lands, which produced about 7964997 million tons average (Source: compiled and calculated by the Ministry of Agriculture and Land Reclamation, Central Administration of Agricultural Economics, Agricultural Economics Bulletin, various issues). Tomato plant are affected by a number of fungal diseases causing substantial losses in yields including root rot vascular wilt and damping–off diseases, which inflict heavy losses in its production. Tomato wilt disease caused by *Fusarium oxysporum* f.sp. *lycopersici* causes reduction in weight of tomato fruits and productivity. It is consider an important pathogen of tomato in Egypt and around the world (Al-Azawi et al., 2012). Plant growth promoting rhizobacteria (PGPR) can produce direct or indirect effects on the host plants , indirect effects are these related to the production of metabolites such as antibiotics, siderophores or cyanogen which increase plant growth by reducing the activity of pathogens. While, the direct effects on plant growth by producing metabolites such as plant growth regulators (PGRs) that directly promote the plant growth or by facilitating nutrient uptake by the plants (Teixeira et al., 2007; Ahmed et al., 2005; Salamone et al., 2001). Currently, There are several PGPR inoculants commercialized those seem to promote plant growth through at least one mechanism, suppression of plant disease (termed Bioprotectants), improved nutrient acquisition (termed Biofertilizers) or phytohormones production (termed Biostimulants) (Tenuta, 2006). The combination with *B. subtilis* and *T. harzianum* showed the highest records of plant growth and macro-nutrients contents in the treatments of tomato inoculation with *Azotobacter chroococcum* (Zaghloul et al., 2007). The objective of this study was to determine the effects of inoculation with rhizobacteria inoculants in controlling tomato wilt diseases caused by *Fusarium oxysporum* and *Verticillium dahlia* and on the growth of tomato plants and yield under greenhouse conditions.

Materials and methods

Isolation of the causal pathogens: Samples of infected tomato seedling and plants showing typical symptoms of fungal wilt diseases were collected during 2013 growing season from different regions of Assiut, Sohag and Luxor Governorates. Target pathogens, *Fusarium oxysporum* f.sp. *lycopersici* and *Verticillium dahlia*, were isolated from diseased plants using tissue transplanting method according to the method of Agrios (1997). Single spore isolation was performed of each isolate to pure cultures on potato dextrose agar (PDA). The isolates were maintained on PDA medium at 4°C. Pathogenicity capability of *F. oxysporum* and *V. dahlia* isolates was carried out. Artificial soil infestation by *F. oxysporum* f.sp. *lycopersici* and *V. dahliae* grown on barley grain medium was carried out as described by Singleton et al. (1992),

Abd-El-Wahab (2004), Bahloul, (2013) and Zaghloul et al. (2015). Disease severity of wilt was recorded after 60 days from sowing date. The arbitrary (0-5) disease index scale as described by Paz-Lago et al. (2000) was adopted.

Isolation of the bioagents: *Trichoderma harzianum* was isolated from the rhizosphere of tomato plants located in Assiut Governorate, Egypt and identified at Mycological Center, Assiut University, Egypt. *Bacillus subtilis, Bacillus polymyxa, Bacillus megaterium* and *Pseudomonas fluorescence* were obtained from Microbial Resources Center, Ain-Shams University, Egypt. *Azotobacter* and *Azospirillum* isolates were isolated from the rhizosphere of tomato plants grown in different locations of Minia, Assiut, Sohag, Luxor Governorates, Egypt. After purification, isolates were tested towards for their efficiency to nitrogen –fixation by growing in modified Ashby′s medium (Abdel-Malek & Ishak, 1968). Then, *Azotobacter* isolates were incubated at 30°C for 2- 5 days, while *Azospirillum* isolates were grown on a semi-solid N-deficient medium (Dobereiner et al., 1976).

Determination of nitrogenase activity of biagents isolates: The nitrogen fixing capability of the isolates was achieved using ambient assay of nitrogenase activity according to Postage (1972). The most efficient nitrogen–fixing bacterial isolates were selected for further studies.

***In vitro* antagonistic effect of certain bioagents against liner growth of the causal pathogens:** Antagonistic properties of plant growth promoting bacteria and *T. harzianum* were tested against *F. oxysporum f. sp. lycopersici and V. dahliae* on Nutrient Agar plates using a dual culture technique. Agar blocks (5mm dia.) containing 5 days old mycelia were placed at the center of Nutrient Agar plates. A loop full culture (24 h old) of bacterial isolates was inoculated linearly at 2 cm juxtaposed to the pathogen of each plate. The fungal pathogens were inoculated centrally on Nutrient Agar plate. Uninoculated plates served as control. All the plates were incubated at 28±1°C for 5 days and colony growth inhibition (%) was calculated according to (Asha et al., 2011) by using the formula:

$$I=100-(100\times(R_2/R_1))$$

Where I is the degree inhibition of vegetative growth of the fungi. R_1 is the radius of the control colony in mm and R_2 is the distance traveled by the pathogenic fungi.

Greenhouse experiments: A pot experiment was carried out under greenhouse condition, Faculty of Agriculture, Al-Azhar University, Assiut, Egypt. The cultivation process was carried out during 2014 growing season using plastic pots of 30-cm in diameter and 35 cm in depth. Seedlings of tomato (cv. Super Jakal) were washed with water and air dried. Suitable number of seedlings was immersed for 20 minutes in the appropriate cell suspension, and then 5% Arabic gum was added to enhance the microbe adhesion to the roots according to the treatment of PGPR. following the protocol described by Rovira (1959), Fages (1990) and Khalifa (2005), Three

replicates were used for each of the following treatments each replicate 3contained plants In this case, using a mixed culture of the PGPR organisms, equal portions of the cells suspensions were thoroughly mixed and similarly used for seedling inoculation. Disease severity on tomato plants was recorded and the results were calculated according to plants was recorded.

Statistical analysis: Data collected were subjected to the statistical analysis according to the standard methods recommended by Gomez and Gomez (1984) using the computer program (Costat)**.** The differences between the mean values of various treatments were compared by Fisher's LSD according to (Gomez & Gomez, 1983).

Results and Discussion

Isolation and identification of causal pathogens: The tomatoes pathogenic fungi causing wilt disease were isolated from various locations in Assiut, Sohag and Luxor Governorates. Thirteen different fungal isolates were obtained which belong to two fungal genera. Fungal isolates were identified as *Fusarium oxysporum f. sp. lycopersici* (8 isolates) and *Verticillium dahlia* (5 isolates) using the morphological features of mycelia and spores as described by Barnet and Hunter (1977), Booth (1977) and Domsch et al. (1980) and confirmed by Mycological Center (AUMC), Assiut University, Assiut, Egypt.

Pathogenicity test: Pathogenicity capability of 8 isolates of ***F. oxysporum*** and 5 isolates of *V.dahlia* was carried out on tomato plants (cultivar Super Jakal) under greenhouse conditions at the farm of Agriculture Faculty, Al-Azhar University (Assiut Branch), Egypt. Data presented in Table (2) shows that *Verticillium dahlia* isolate (No. 1) and *F. oxysporum* isolate (No. 9) were the most virulent among all the tested isolates. In which, they recorded 92 and 91% disease severity, respectively.

Table 1: Pathogenicity tests of 13 fungal isolates on tomato plants cultivar Super Jakal under greenhouse conditions during 2014 growing season.

No. of isolates	Isolates	Disease Severity (%)
1	*Verticillium dahlia*	92.25
2	*Verticillium dahlia*	35.75
3	*Verticillium dahlia*	27.00
4	*Verticillium dahlia*	39.25
5	*Verticillium dahlia*	49.00
6	*Fusarium oxysporum f.sp. lycopersici*	85.00
7	*Fusarium oxysporum f.sp. lycopersici*	48.25
8	*Fusarium oxysporum f.sp. lycopersici*	39.50
9	*Fusarium oxysporum f.sp. lycopersici*	92.00
10	*Fusarium oxysporum f.sp. lycopersici*	43.50
11	*Fusarium oxysporum f.sp. lycopersici*	40.75
12	*Fusarium oxysporum f.sp. lycopersici*	35.75
13	*Fusarium oxysporum f.sp. lycopersici*	34.50
LSD0.05		8.37

Table 2: Nitrogenase activity (nanomole C_2H_4 ml^{-1} hr^{-1}) of either *Azotobacter* or *Azospirillum* isolates.

Azospirillum isolates	Nitrogenase Enzyme activity (n mole C_2H_4/ml/hr)	Azotobacter isolates	Nitrogenase Enzyme activity (n mole C_2H_4/ml/hr)
AZ1	1.04***	A1	112.4
AZ2	0	A2	110.3
AZ3	0.4	A3	112.4
AZ4	0.95	A4	125.7
AZ5	3.11***	A5	112.3
AZ6	.90·	A6	139.5
AZ7	1.14***	A7	182.9
AZ8	0.62	A8	207.3
AZ9	0.52	A9	221.4***
AZ10	0.31	A10	284.7***
AZ11	1.00***	A11	126.50
AZ12	0.62	A12	115.09
AZ13	0.73	A13	453.5***
AZ14	1.04***	A14	325.5***
AZ15	0.62	A15	82.5
AZ16	0.52	A16	41.7
AZ17	0.40	A17	462.5***
AZ18	0	A18	165.2
AZ19	0.73	A19	119.7
AZ20	0.10	A20	200.07

Determination of nitrogenase activity of *Azotobacter* and *Azospirillum* isolates: Twenty isolates of *Azotobacter* and *Azospirillum* were isolated from the rhizosphere and rhizoplane of tomato plants were tested for their efficiency for nitrogen fixation using the ambient assay of Nitrogenase activity and the most efficient five isolates of either *Azotobacter* (AZ17, AZ13, AZ14, AZ10, AZ9) or *Azospirillum* (AS5, AS7, AS14, AS1, AS11), Then these isolates were subjected to further studies as shown in Table (2).

Antagonistic effects of bioagents against target pathogens *in vitro*: The results of the antagonistic activity of the fourteen bacterial isolates as well as of two isolates of *Trichoderma harzianum* - I, *T. harzianum*-II which isolated from tomato plant rhizosphere were tested against *F. oxysporum f.sp. lycopersici* (isolate No.9), and *V.dahliae* (isolate No.1) as shown in Table (3) Data in Table (4). Our results exhibited that the tested bacteria varied in their abilities in reducing radial growth rate of the pathogen and increasing percentage of pathogen inhibition as compared with the control treatment (Table 4).

Evaluation of *Trichoderma harzianum* against target pathogens *in vitro*: The two isolates of *Trichoderma harzianum* - I, *T. harzianum*-II isolated from tomato plant rizosphere were tested against *F. oxysporum* f.sp. *lycopersici*, and *V. dahliae* on PDA medium. Data in Table (3) indicated that all tested isolates of *T. harzianum* I, *T. harzianum* II exhibited different degrees of reaction against *F. oxysporum f. sp. lycopersici* and *V. dahliae*. It was clear from Fig. (1) that the mycelium of *T. harzianum* grew rapidly over the mycelium of the pathogen and prevented its development. *Trichoderma harzianum* isolate No.1 was more effective on the pathogen growth followed by isolate No.2. This is consistent with Zein El-Abdean (2013). There isolates of *T. harzianum*-I was selected for greenhouse studies. Data in Table (4) demonstrated that *B. polymyxa*

was the best isolate since recorded (91.3%) growth reduction of *V. dahliae* followed by *P. fluorescence* (77.6%) growth reduction. The lowest growth reduction of the same pathogens was observed in case of *Azospirillum sp*. (AS11) being 41.6% compared with the control. These results were in agreement with those obtained by Asaka and Shoda (1996) and Sadler (1996).On the other hand, *P. fluorescence* (PF), *Bacillus polymyxa* (BP), *Azotobacter sp* (AZ17) and *Azospirillum sp.* (AS7) were the best isolates and showed the highest antagonistic effect against *F. oxysporum f.sp lycopersici.* These isolates recorded the highest percentages of growth reduction of *F. oxysporum* f.sp. *lycopersici* being (74.6, 66.3, 66, 55.6%) respectively. These results are in harmony with those reported by Martiny and Marme (1995). The variation in the bacterial isolates abilities may be due to the strain type and the type of metabolic materials that are produced by the strains in culture media. In this respect, *Fusarium* and *Verticillium* wilt was suppressed through the activity of PGPR strains. The disease suppressive mechanisms by PGPR include siderophores (mediated competition for iron) (Raaijmakers et al., 1995). Meanwhile, other investigators attributed the disease suppressive mechanisms by PGPR to the competition for nutritional substances or induction of systemic resistance (Van Loon et al., 1998; Fuchs et al., 1997). Nevertheless**,** Albuquerque et al., (2003) reported that the production of HCN by PGPR strains (*Bacillus sp.*) showed antibiosis against soil borne pathogenic fungi. Also, they reported that the PGPR colonize plant organs epiphytically or endophytically and caused enhancing development, protecting the roots from soil borne pathogens and inducing resistance against pathogens.

Table 3: Effect of *T. harzianum* on mycelial growth of the tested pathogens.

Trichoderma isolate No.	Antagonistic ability (scale)*	
	F. oxysporum	*V. dahliae*
I	3	4
II	3	4

*0=negative antagonism, 1=slightly antagonism, 2=moderately antagonism, 3=highly antagonism, 4= over growth.

Effect of seedling treatment of bioagents on incidence of tomato fungal wilt disease under greenhouse conditions: Data in Table (5) showed that the infested soil with either *F. oxysporum f.sp lycopersici* or *V. dahliae* significantly decreased the growth of tomato. Growth characteristics of tomato were significantly increased with the inoculation with PGPR compared to un-inoculated ones. Data in Table (5) showed that the inoculation of tomato with symbiotic N-fixing *Azotobacter sp.* (AZ17) and *Azospirillum sp.* (AS7) exhibited different degrees of reaction against *F. oxysporum f. sp. lycopersici* and *V. dahliae* and significantly decreased the percentage of infected tomato plants compared to the un-inoculated ones. While, the percentage of disease severity significantly decreased with tomato inoculated with symbiotic N-fixing *Azotobacter sp*. and *Azospirillum sp*. These results are in accordance with Hassouna et al., (1998) and Zaghloul et al., (2010). However, lower percentage of disease severity of tomato seedlings were attained in response to treatment with mixture of PGPR (96.84%) against *F. oxysporum f. sp. lycopersici* and (90.26%) against *V.*

dahliae more than the individual one. The beneficial effect of N2-fixers and phosphate dissolving microorganisms on plant growth was also observed by Buchenauer (1998) who concluded that the mechanisms by which PGPR stimulate plant growth via the production of IAA and cytokinins as well as by lowering ethylene level in plants. Also, PGPR induce systemic resistance against root pathogens. This result could be attributed to the synergistic effect in case of dual inoculation. These results are in harmony with those reported by Sanhita et al., (1995), Cal et al., (2004) and Zaghloul et al., (2007).

Table 4: Antagonistic activity of the bacterial isolates against fungal pathogens.

Bacterial isolates	Growth inhibition (%)	
	F. oxysporum f.sp *lycopersici*	*Verticillium dahliae*
Azotobacter sp. AZ_9	27	61.3
Azotobacter sp. AZ_{10}	33	67
Azotobacter sp. AZ_{13}	43.6	70
Azotobacter sp. AZ_{14}	35.6	69
Azotobacter sp. AZ_{17}	66	71
Azospirillum sp. AS_1	49.6	54.6
Azospirillum sp. AS_5	53	53
Azospirillum sp. AS_7	22.3	44
Azospirillum sp. AS_{11}	55.6	41.6
Azospirillum sp. AS_{14}	44	48.3
B. megaterium BM	62.3	62.6
B. subtilis BS	-	71.3
B. polymyxa BP	66.3	91.6
P. fluorescence PF	74.6	77.6
Control	0	0

Table 5: Effect of different rhizobacteria and Trichoderma on incidence of wilt disease of tomato under greenhouse conditions during 2015 growing season.

Treatments	Disease severity (%)	
	F. oxysporum f.sp lycopersici	*Verticillium dahliae*
Azotobacter sp. (AZ17)	51.3	71
Azospirillum sp. (AS7)	64.3	81.6
B. megaterium (BM)	42.6	45
B. subtilis (BS)	64	53.3
B. polymyxa (BP)	30.6	31
P. fluorescence (PF)	24	24.3
T. harzianum-I (T1)	32.6	34
AZ17+AS7+T1	15. 3	29
AZ17+AS7+BM	24.3	36.6
AZ17+AS7+PF	10	15
Mixture	8	8.6
Control	93.3	100
LSD 0.05	58.5	4.41

Our results showed that *B. polymyxa* and *P. fluorescence* were able to reduce disease incidence and severity of *F. oxyproum* and *V. dahliae* in tomatoes by stimulating vegetable growth and root development of the treated plants. From these results it may be concluded that application of PGPB provide a reasonable level of protection against *F.oxysporum* and *V. dahliae* in tomato under greenhouse conditions. Also, the present study provide sufficient evidence to the recommended the use of the mixture of antifungal strains of *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus polymyxa*, *Pseudomonas fluorescence* and *Trichoderma harzianum* in combination with dinitrogen fixers of *Azotobacter sp.* and *Azospirillum sp.* is successful biocontrol agent against soil borne pathogens causing disease of tomato.

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