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EVALUATION OF SOME PLANT GROWTH-PROMOTING YEASTS AND RHIZOBACTERIA FOR PROTECTING TOMATO PLANT AGAINST FUSARIUM WILT

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Abstracts: Three plant growth-promoting yeasts (PGPY) and two rhizobacteria (PGPR) were tested for controlling tomato wilt under greenhouse and field conditions. Before transplanting, seedlings were treated with suspension of the tested antagonistic local isolate of PGRY or PGPR. Under greenhouse and field conditions, all treatments significantly reduced disease severity of tomato wilt relative to the infected control. The highest disease reductions in pots (75.0, 67.4 %) and field (52.5, 42.4%) were achieved by *Azospirillum brasilense* and *Bacillus subtilis* in comparison with seedlings inoculated with the pathogen alone. Also, these two treatments produced the highest tomato yield compared to the control plants inoculated with the pathogen under field condition. The lowest disease reductions were caused by *Candida sake* and *Pichia membranifaciens* (40.9 and 41.0%, respectively). All inoculation treatments with tested strains scored significant increases in shoot fresh and dry weight of tomato plants compared to the infected control, and the most promotive were those inoculated with *A. brasilense* followed by *B. subtilis*.

Key wards: Biocontrol, *A. brasilense*, *B. subtilis*, *C. sake*, *P. membranifaciens*, Tomato wilt.

Introduction

Fusarium oxysporum f. sp. *lycopersici* (Sacc.) Snyder & H.N. Hansen, is economically an important wilting pathogen of tomato in Egypt (Eraky-Amal *et al.*, 2007). Management of this pathogen is difficult due to their endophytic growth and persistence in soil (Alström, 2001). Several disease management strategies are available e.g. resistant cultivars, biological control, crop rotation and chemical fungicides. Furthermore, new races of pathogen that overcome plant resistance have continued to appear (Rodríguez-Molina *et al.*, 2003). A promising strategy for replacement of chemicals has been the

implementation of biocontrol technology, either used individually or as an Integrated Pest Management (IPM) component (Mao *et al.*, 1998).

Different strains of rhizosphere bacteria, called plant growth promoting rhizobacteria (PGPR), stimulate plant growth mainly by directly affecting plant metabolism and/or the availability of nutrients (Bashan and Levanony, 1990). Other PGPR strains promote plant growth indirectly by suppressing soil-borne pathogens, or by stimulating plant natural defences, by a mechanism called induced systemic resistance (ISR) (Kloepper *et al.*, 1993). The following mechanisms of PGPR to protect plant against pathogens are: (1) to promote the production of extracellular growth-promoting chemical substances (Horemans *et al.*, 1986), ironchelating siderophores (Schippers *et al.*, 1987), antibiotics (Weller, 1988) and HCN (Voisard *et al.*, 1989) that enhance plant growth or inhibit soilborne plant pathogen, (2) induce plant resistance and mineralize soil nutrients (Okon and Kapulnik, 1986), and (3) reduce the population of major root pathogens by competing for energy-yielding nutrients (Elad and Chet, 1987).

Isolates of *Azospirillum* species are possibly the most studied PGPR bacteria. Although they are not known to induce systemic resistance (ISR) in plants, there are some reports of their biocontrol activity (Bashan and De-Bashan, 2002). *Azospirillum* can reduce the incidence and severity of damping off caused by *Rhizoctonia solani* Kohn, possibly by bacterial colonization of the sclerotia (Gupta *et al.*, 1995). Romero *et al.* (2003) reported that canker severity was not affected by *Azospirillum* seed treatments. However, leaf-and plant-death were delayed on Azospirillum-treated plants compared with nontreated controls. Also, certain strains of *Bacillus subtilis* proved to be very active biocontroller, Dass and Teyegaga (1996) found that an isolate of *Bacillus subtilis* inhabited the growth of 5 wood decay fungi. Also, *Bacillus subtilis* was used to biocontrol the causal agent of leaf blight of *Wigna aconifolia* (Majumdar *et al.*, 1996).

According to authors' information, no data are available concerning the use of yeasts and *Azospirillum brasilense* against fusarium wilt of tomato. Therefore, the aim of the present work is to investigate the influence of plant growth promoting rhizobacteria (*Azospirillum brasilense* Tarrand, krieg and Döbereiner and *Bacillus subtilis* Cohn) as well as plant growth promoting yeasts (*Saccharomyces cerevisiae*, *Candida sake* (Saito et Ota) van Uden & Buckley Nov. Comb and *Pichia membranifaciens* Wicherham) as biocontrol agents against Fusarium wilt of tomato disease.

Material and Methods

Seeds and seedlings growth:

Tomato seeds (*Lycopersicon esculentum* Mill) cv. Prichard (highly susceptible to Fusarium wilt) were obtained from the Ministry of Agriculture, Egypt and used in this study. Seeds were planted in pots 30 cm diameter and placed on a bench in a conditioned greenhouse at 30±5°C with 68-80% RH and watered as required. Superphosphate was added at rate of 50 kg P_2O_5/feddan plus supplementary N-fertilizer, in urea form, at a rate of 90 kg N/feddan.

Preparation of fungal pathogen:

Pathogenic isolate of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) was isolated from naturally infected roots of naturally diseased tomato plants showing wilt symptoms grown in Assiut Governorate, Egypt. The obtained fungal isolates were grown on PDA slants and kept at 4°C until used. Inocula of the pathogen was prepared by inoculation sterilized milk bottles 0.5 L. containing Barley medium (75g Barley + 25g pure sand + 2g sucrose + 0.1g yeast extract + 100ml water) with the tested fungi and incubated at 28 °C for two weeks.

Preparation of PGPY and PGPR inocula:

Three yeast strains (*Saccharomyces cerevisiae*, *Pichia membranifaciens* and *Candida sake*), were used in this investigation to test their efficacy to control tomato wilt under greenhouse and field conditions. These strains were previously isolated from composite sample of the clay soils of Assiut Experimental Farm planted with grape plants (Mohamed, 2006). They were identified based on their morphological and physiological characteristics including their ability to utilize all carbon and nitrogen sources as well as fermentation of carbon sources according to Barnett *et al.* (2000). Cultures of the bacterial species, *Azospirillum brasilense* and *Bacillus subtilis* were obtained from Soils and Water Department, Faculty of Agriculture, Assiut University, Egypt.

Separate cultures of the yeast strains, *Azospirillum brasilense* and *Bacillus subtilis* were respectively grown on 100 ml aliquots of malt-yeast-glucose-peptone (YM) medium, nitrogen-free NFb semisolid medium (Döbereiner *et al.*, 1995) and nutrient broth medium, respectively in 250 ml Erlenmeyer flasks. The flasks were incubated at 25, 37 and 28 C for 5 days for yeast strains, *A. brasilense* and *B. subtilis*, respectively. The counted numbers of viable cells in cultures at the time of use for inoculation were $2.1x10^7$, $1.1x10^9$ and $7x10^8$ CFU/ml for yeast strains, *A. brasilense* and *B. subtilis* respectively.

Greenhouse Experiments:

The trials were carried out in the Greenhouse of Plant Pathology Dept., Faculty of Agriculture Assiut Univ. Two pot experiments were conducted in 2007 and 2008 seasons to investigate the influence of seedling inoculation with each of the previous strains as a biocontrol agent against Fusarium tomato wilt disease. Tomato seeds, cv. Prichard were sown in trays (30 x 50 cm, 10 cm deep) containing sieved clay soil mixed with 3 % peat moss, and watered twice a week. After 45 days, similar healthy seedlings (15 cm in length) were uprooted, inoculated or un-inoculated with separate culture of the tested strains before transplanting in black pots, 30 cm in diameter containing 5 kg sieved clay soil collected from Assiut Experimental Farm.

Infestation of soil in pots with the pathogenic fungus was done by applying the prepared inoculum, as described before, to pots at rate of 3 % (w/w), mixed thoroughly with the soil, then watered and left for one week to insure establishment and distribution of the inoculum in soil. Pots containing non infested soil were used as control treatment.

Tomato seedlings (45 days old) were dug off seedling trays and the root thoroughly washed by running water to remove any adherent particles, then treated by dipping the root in broth culture of one of the tested PGPY or PGPR strains for one hour. The treated, tomato seedlings were then transferred to the pathogen infested pots. Two seedlings were transplanted in each pot and 5 replicates were planted for each particular treatment. Also, untreated seedlings were transplanted in pots containing infested soil (infected control). Plants were irrigated when needed and fertilized as usual.

After 8 weeks from transplanting, plants of five replicates from each treatment were uprooted, washed thoroughly with running water, blotted with tissue paper, weighed to determine fresh weights, and then oven dried at 70°C for 72 h for dry weights. The nitrogen content of dried shoots was determined by semi-microkjeldahl technique (Bremner and Mulvaney, 1982).

Disease severity assessments:

The plants in pots (10 plants/treatment) were examined for determination disease severity % (DS%) after 8 weeks from transplanting, as a wilting percent using the rating scale in which infected plants were classified according to a numerical grades ranging from 0 to 4 as follows:

0 = healthy, 1 = > 25 of plant leaflets are yellow and of vascular root bundles are dark brown, 2 = < 26 – 50 of plant leaflets are yellow and of vascular root bundles are dark brown, 3 = < 51 – 75 of plant leaflets are yellow and of vascular root bundles are dark brown, 4 = <76–100 of plant leaflets are

yellow and of vascular root bundles are dark brown.

Disease severity percentage (DS%) was calculated according to the following formula:

DS% = $\sum$(1A +2B + 3C + 4D)/ 4T X 100 where, A, B, C and D are the number of plants corresponding to the numerical grade, 1, 2,3 and 4 respectively and 4T is the total number of plants (T) multiplied by the maximum discoloration grade 4, where T= A+B+C+D.

Field Experiment:

Two field experiments were conducted at the Experimental Farm of Faculty of Agriculture, Assiut University, Assiut, Egypt in 2007 and 2008 growing seasons. Prichard cultivar and *Fusarium oxysporum* f. sp. *lycopersici* (FOL) were used in this study during the winter growing seasons. Before the transplanting, roots of transplants were dipped into broth culture of each bioagents and then transplanted to field soil artificially infested with the pathogen. Soil was artificially infested with pathogen fungi grown on Barley medium at rate 100 gm /m^2 soil. The experimental field plot area was 3 x 3.5 meter (1/400 feddan) containing 4 ridges each 2.5 meter long. Six seedlings were transplanted in each ridge at 25 cm in between distance; the approximate plant populations were 9600 plant/feddan. The experimental design was a complete randomized block design with 3 replicates for each treatment. Disease was recorded after 8 weeks from planting as the total percentage of plants showing any wilt symptoms due to the pathogen. From each treatment 10 plants were randomly selected and used as replicates. Weight of ripened collected fruits was recorded until the end of the season after four months from transplanting for yield determination.

Statistical analysis:

All experiments were performed twice. Analyses of variance were carried out using MSTATC computer programme. Analysis showed no significant difference between the two test seasons for any of the treatments. So, results for duplicate tests were combined for final analysis. The wilt percentage was first transformed angularly and then analysed by a single factor ANOVA. Least significant difference (LSD) was calculated at $P \leq 0.05$ according to Gomez and Gomez (1984).

Results and discussion

Effect of inoculation with biocontrol agents on growth of tomato plant, in pots.

The obtained results in Table (1) show that the phytopathogen caused significant reductions in plant height, shoot and root fresh and dry weight of tomato plants

compared with the healthy control and results in non-significant reductions in number of branches/plant and total nitrogen uptake. The inhibitory effect of the phytopathogen might be attributed to the phytotoxic effect of pathogen toxins (Orolaza *et al.*, 1992). Compared with the infected control, all inoculation treatments with tested strains scored significant increases in shoot fresh and dry weight of tomato plants. The most promising treatment was that inoculated with *A. brasilense* followed by *B. subtilis*. Inoculation with either of these two strains scored significant or highly significant increases in all measured plant growth parameters and total shoot nitrogen.

Table(1): Effect of inoculation with PGPY and PGRR on growth of Tomato plants and total nitrogen in shoot of plants, in pots.

Treatments	Plant height (cm)	Shoot weight (g/plant)		Root weight (g/plant)		Number of branches/ plant	Total N in shoot (mg/plant)
		Fresh	Dry	Fresh	dry		
Healthy control	45.55	40.15	14.01	4.63	0.83	6.5	462.2
Infected control	41.12	36.03	12.61	3.64	0.64	5.0	404.6
A. brasilense	45.25	45.08	15.86	5.71	1.02	8.0	572.1
B. subtilis	45.47	44.47	15.25	4.67	0.83	7.8	541.1
S. cerevisiae	40.45	41.31	14.55	3.79	0.67	7.3	454.2
C. sake	40.95	43.27	15.00	4.19	0.74	6.2	492.9
P. membranifaciens	42.17	42.18	14.81	3.74	0.68	5.8	478.8
L.S.D. 0.05	2.18	3.58	1.50	0.80	0.13	2.00	87.6

The enhancement of growth parameters imposed by *A. brasilense* inoculation may due to N_2-fixation (Shabaev *et al.*, 1991), or/and to direct hormonal effects on root mass. The reports of other investigators on *Azospirillum* had attributed the improvements in growth and yield of agronomically important crops to the production of growth promoting substances such as IAA, gibberellins, and possibly cytokinins, thus improving uptake of water and nutrients by inoculated plants (Bashan *et al.*, 1990; Dobbelaere *et al.*, 1999). Similar responses were reported by Romero *et al* (2003); they found that tomato plants bacterized with *A. brasilense* Sp7 and *Azospirillum* sp. BNM-65

were taller, had more leaves and had higher shoot and root dry weight, than non bacterized plants.

Also, *B. subtilis* had increased fresh and dry weight of tomato shoots and roots than infected control, such results may be due to increase of plant hormones. Arkhipova *et al.*, (2005) observed a general increase in the hormonal content of lettuce roots inoculated with a cytokinin-producing *B. subtilis* strain. They suggested that the change in root hormone content was a result of the indirect effect of the inoculated bacterium on the ability of plants themselves to produce cytokinins and other phytohormones, such as IAA and ABA.

Effect of different biocontrol agents on developments of Fusarium wilt of tomato plants under greenhouse conditions

Results presented in Table 2 show that seedling treatment with any of the tested bioagents significantly reduced wilt percent in tomato plants.

Table(2): Effect of inoculation with PGPY and PGRR on developments of Fusarium wilt in tomato plants cultivar cv Prichard under greenhouse conditions.

Treatments	Disease severity (%)	Wilt reduction (%) relative to infected control
Healthy control	0	0
Infected control	68	0
A. brasilense	17	75
B. subtilis	22.2	67.4
S. cerevisiae	31.2	54.1
C. sake	40.2	40.9
P. membranifaciens	40.1	41
L.S.D. at 0.05	4.5	-

The highest disease severity reduction was observed with *A. brasilense* and then *B. subtilis*, 75, 67.4 %, respectively, and the lowest reductions were caused by

C. sake and *P. membranifaciens* (40.9 and 41.0%, respectively).

The observed reduction in the disease severity and increased vegetative growth of tomato plant by *Bacillus subtilis* and *A. brasilense* compared to infected control may be due to stimulative for motion and length of root hairs, and thus the root surface area as reported by Dobbelaere *et al.* (2001) in treatment with *A. brasilense* also, in agreement with our results. Ibrahim (1990) reported that the antagonistic effect of *Bacillus subtilis* is due to production of extracellular antifungal agents that inhibited the growth of *Cephalosporium maydis*, the causal pathogen of late wilt disease of maize.

All three tested yeast species significantly reduced wilt disease under glasshouse conditions. These results are agree with those reported by El-Tarabily (2004) who mentioned that isolates of *Candida valida*, *Rhodotorula glutinis* and *Trichosporon asahii*were capable of colonizing sugar beet roots, promoting growth of sugar beet and protecting the seedlings and mature plants from *R. solani* diseases. The mechanisms by which the yeasts involved in controlling plant diseases and their role in the biocontrol activity included; competition for space and nutrients (Filonow, 1998), production of antifungal diffusible metabolites (Masih *et al.*, 2001), volatile compounds (Payne *et al.*, 2000), production of cell-wall degrading enzymes such as b-1,3-glucanase (Masih and Paul, 2002) and mycoparasitism (Wisniewski *et al.*, 1991).

Effect of different biocontrol agents on development of Fusarium wilt of tomato plants under field conditions

The results presented in Table 3 indicate that all inoculation treatments significantly reduced the disease severity and increased tomato yield relative to control infected with *Fusarium oxysporum* f.sp. *lycopersici*. The treatments inoculated with *A. brasilense* or *B. subtilis* resulted in highest reductions in disease severity as well as produced the highest tomato yield compared to the control plants inoculated with the pathogen; the reductions in disease incidence were 52.5 and 42.4%, respectively. The lowest disease reductions were attained when seedlings were treated with *C. sake* and *P. membranifaciens* recording 12.5 and 11.7% respectively (Table 3), but these two treatments resulted in 100 and 110 % increases in fruts yield.

Table(3): Effect of inoculation with PGPY and PGRR on development of Fusarium wilt disease and fruit yield of tomato plants under field conditions.

Treatments	Wilt (%)	Reduction (%) relative to infected control	Yield of ripen fruits /Feddan Ton/faddan	% increase in yield relative to infected control
Healthy control	0.0	0.0	19	0
Infected control	75.00	0.0	10	0
A. brasilense	35.6	52.5	26	160
B. subtilis	43.2	42.4	22	120
S. cerevisiae	53.00	29.3	22	120
C. sake	65.6	12.5	20	100
P. membranifaciens	66.2	11.7	21	110
L.S.D. at 0.05	0.5	-	1.1	-

Our study showed that tomato plants treated with PGPR and PGPY caused high reductions in disease severity and produced higher fruit yield compared to the untreated control plants. *Bacillus* isolates have been reported to promote the growth of a wide range of plants (Kokalis- Burelle *et al.*, 2002). Treatment with *Bacillus pumilus*, was reported to induce a rapid lignification in cucumber plants in response to ingress of *Colletotrichum orbiculare*, and total peroxidase and superoxide dismutase activities were increased more than those in the buffer control (Jetiyanon *et al.*, 1997). These responses may be due to the production of siderophores, antibiotics, wall appositions and defense enzymes, which adversely affect the pathogens. Also, our results agree with those reported by Hassan and Abd El-Rehim (2002), who reported that dipping onion seedlings before transplanting and foliar spray with *Saccahromyces cerviciea*, after 45 days from transplanting significantly influenced the exportable, total and culls onion bulb yield as well as reduced incidence of neck rot disease.

It may be concluded from the results of the present

investigation that application of PGPY and PGPR provide a reasonable level of protection against *Fusarium oxysporum* f.sp. *lycopersici*, especially in organic farming system, where plant nutrition and disease control are the main limiting factors.

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تقييم بعض سلالات من الخمائر والبكتريا المشجعة للنمو على حمايه نباتات الطماطم ضد الذبول الفيوزاريومى

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[1] قسم أمراض النبات- كلية الزراعة- جامعة أسيوط- أسيوط- مصر

[2] قسم الاراضى والمياه- كلية الزراعة- جامعة أسيوط- أسيوط- مصر

تحت ظروف الصوبة والحقل تم دراسة استخدام ثلاث انواع من الخمائر المشجعة للنمو وكذلك اثنين من البكتريا المشجعة للنمو على مقاومة مرض الذبول الفيوزاريومى فى الطماطم . تم معاملة الشتلات بالغمر فى معلق كل من الخمائر والبكتريا المعزولة محليا. وتحت ظروف الصوبة ادت جميع المعاملات الى خفض شدة المرض بالمقارنة بالنباتات المعدية والغير معاملة. وكانت اعلى نسبة للحد من المرض المعاملة بعزلات *Azospirillum brasilense* ثم *Bacillus Subtilis* حيث كان مقدار الخفض فى المرض فى تجارب الاصص 75% ، 67.4 ٪ و تحت ظروف الحقل 52.5% و 42.4 % على التوالي فضلا عن انتاج اعلى محصول من الطماطم تحت ظروف الحقل مقارنة بالنباتات الغير معاملة والمعدية بالمسبب المرضى. وكانت اقل المعاملات فى الحد من شدة المرض هى المعاملة بـ *Candida sake* , *Pichia membranifaciens* حيث خفضت شدة المرض بمقدار 41.0 % و 40.9 ٪ على التوالي فى تجارب الاصص.

ادت جميع المعاملات الى زيادة فى الوزن الاخضر و الجاف لنباتات الطماطم بالمقارنة بالنباتات المصابة. وكانت اكثر العزلات فى زيادة الوزن الرطب والجاف العزلات *A. brasilense* تليها *B. Subtilis*. وايضا ادت هذه العزلات الى زيادة ملحوظة فى المحتوى الكلى للنتيروجين فى النباتات.