Plant Pathol. J. 25(2) : 199-204 (2009)

The Plant Pathology Journal

Note

Biological Control of Fusarium Wilt in Tomato by Plant Growth-Promoting Yeasts and Rhizobacteria

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(Received on September 11, 2008; Accepted on May 15, 2009)

Three plant growth-promoting yeasts and two rhizobacteria were tested for controlling tomato wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* under greenhouse and field conditions. Under greenhouse and field conditions, all treatments were 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* compared to infected control. Under field condition all treatments produced the highest tomato yield compared to the control plants inoculated with the pathogen.

Keywards : *Azospirillum brasilense*, *Bacillus subtilis*, biocontrol, *Candida sake*, *Pichia membranifaciens*, tomato wilt

Fusarium oxysporum f. sp. *lycopersici* (Sacc.) Snyder & H.N. Hansen is economically 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, used individually or as an integrated pest management (IPM) component (Lemessa and Zeller, 2007).

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 defenses, by a mechanism called induced systemic resistance (ISR) (Siddiqui, 2006). The mechanisms of PGPR to protect plant against pathogens are promote plant growth or inhibit soilborne plant pathogens include the production of extracellular growth-promoting chemical substances (Horemans et al., 1986), iron chelating siderophores (Szczech and Shoda, 2006), antibiotics (Weller, 1988) and HCN (Schmidt et al., 2004), induce plant resistance and mineralize soil nutrients (Okon and Kapulnik, 1986) and reduce the population of major root pathogens, compete for energy- yielding nutrients (Elad and Chet, 1987).

Azospirillum sp. is possibly the most studied PGPR bacteria. They are not known as ISR activating bacteria (Bashan and De-Bashan, 2003), although there are some reports of their biocontrol activity. *Azospirillum* can reduce the incidence and severity of damping-off caused by *Rhizoctonia solani* Köhn, possibly by bacterial colonization of the sclerotia (Gupta et al., 1995). Also, certain strains of *Bacillus subtilis* proved to be the most active biocontroller, Omar et al. (2006) found that an isolate of *B. subtilis* inhabited the growth of *Fusarium oxysporum* f.sp. *radicis-lycopersici*, the causal organism of fusarium crown and root rot of tomato. In addition, *B. subtilis* was used to biocontrol the causal agent of tomato damping-off caused by *R. solani* (Szczech and Shoda, 2006).

There is no information concern 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 (*A. brasilense* Tarrand, krieg and Döbereiner and *B. subtilis* Cöhn) 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.

Seeds and seedlings growth. Tomato seeds (*Lycopersicm esculentum* Mill) cultivar 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 121 kg P_2O_5/

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hectar plus supplementary N-fertilizer, in urea form, at a rate of 215 kg N/hectar.

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 Potato dextrose agar (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 (75 g Barley, 25 g pure sand, 2 g sucrose, 0.1 g yeast extract per 1000 ml water) with the tested fungi and incubated at 28°C for two weeks.

Inoculum of PGPY and PGPR preparation. Three yeast strains (*Saccharomyces cerevisiae*, *Pichia membranifaciens* and *Candida sake*) were used to test their efficacy to control tomato wilt under greenhouse and field conditions. They were isolated from composite sample of the clay soils of Assiut Experimental from planted with grape plants. 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). *Azospirillum brasilense* and *Bacillus subtilis* were obtained from Soils and Water Department, Faculty of Agriculture, Assiut University, Egypt.

Yeast strains, *A. brasilense* and *B. subtilis* were separately 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°C, 28°C, and 37°C for 5 days for yeast strains, *A. brasilense* and *B. subtilis*, respectively. The counted numbers of viable cells of cultures at the time of use for inoculation were 2.1×10^7, 1.1×10^9 and 7×10^8 colony forming units/ml for yeast strains, *A. brasilense* and *B. subtilis*, respectively.

Greenhouse Experiments. The trials were carried out in the Greenhouse of Plant Pathology Department, Faculty of Agriculture Assiut University. A pot experiments were conducted in 2007 and 2008 seasons to investigate the influence of seedling inoculation with each of the previously strains as a biocontrol agent against Fusarium tomato wilt disease. Tomato seeds cv. Prichard were sown in trays (30×50 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 cultures 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 seeds were sown in trays (30×50 cm, 10 cm deep) containing sieved clay soil mixed with 5% peat moss, and watered twice a week, After 45 days old, similar healthy seedlings (15 cm in length) 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 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. In addition, untreated seedlings were transplanted in pots containing infested soil (infected control). Plants were irrigated when needed and fertilized as usual (Superphosphate was added at rate of 121 kg P_2O_5/hectar plus supplementary N-fertilizer, in urea form, at a rate of 215 kg N/hectar). 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).

Diseases severity assessments. Disease severity (DS) was estimated 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.

$DS\%=\Sigma(1A+2B+3C+4D)/4T\times100$ 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.

For each treatment 10 plants were used (two plants per pot) to determine DS. The experiment was repeated twice. Reduction % was calculated using the formula of Guo et al.

(2004) as following: reduction %=([disease incidence of control–disease incidence of treatment group]/disease incidence of control)×100%.

Field Experiment. Field experiment was conducted at the Experimental Farm of Faculty of Agriculture, Assiut University, Assiut, Egypt in 2007 and 2008 growing seasons. Prichard cultivar and FOL were used in this study during the winter growing seasons. Before the transplanting, roots of transplants were dipped into broth culture of each PGPR or PGPY 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. A field plots (3×3.5 m) each comprised of 2 rows and 4 holes/row were used in complete randomized block design. Three plots were used as replicates for each treatment as well as for untreated control treatment. Disease was recorded after 8 weeks from planting as the total percentage of plants showing any wilt symptoms. For each treatment 10 plants were used as replicates. The experiment was repeated twice. At harvest time, the average accumulated yield was calculated for all applied treatments and control as well. All plants from each replicate were pulled for assessment the total yield of each treatment (ton) per hectar.

Statistical analysis. All experiments were performed twice. Analyses of variance were carried out using MSTATC computer programme. Analysis showed no significant between the two tests seasons for any 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≤0.05 according to Gomez and Gomez (1984).

Effect of inoculation with PGPY and PGPR 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. While, yeast strains, *S. cerevisiae*, *C. sake* and *P. membranifaciens* scored non-significant increases in number of branches and total N in shoot compared to healthy control.

Enhancement of growth parameters imposed by *A. brasilense* inoculation may due to N_2-fixation (Shabaev et al., 1991). In addition, the increase in root mass induced by the inoculated strain *A. brasilense* is quite obvious, indicating direct hormonal effects (Romero et al., 2003). The reports of other investigators on *Azospirillum* had attributed the improvements in growth and yield to the production of growth promoting substances such as indole acetic acid (IAA), gibberellins, possibly other plant growth regulators (PGRs) (Bahan and De-Bashan, 2003), and 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 had more leaves, taller and had higher shoot and root dry weight, than

Table 1. Effect of inoculation with plant growth-promoting yeasts (PGPY) and rhizobacteria (PGPR) on growth of tomato plant and total nitrogen in shoot of plants in pots

Treatments[a]	Plant height (cm)	Shoot weight (g/plant)		Root weight (g/plant)		Number of branches (per Plant)	Total N in shoot (mg/plant)
		fresh	dry	fresh	dry		
Healthy control	45.55 a	40.15 bc	14.01 b	4.63 b	0.83 b	6.5 c	462.2 b
Infected control	41.12 b	36.03 d	12.61 c	3.64 c	0.64 d	5.0 e	404.6 c
A. brasilense	45.25 a	45.08 a	15.86 a	5.71 a	1.02 a	8.0 a	572.1 a
B. subtilis	45.47 a	44.47 a	15.25 a	4.67 b	0.83 b	7.8 a	541.1 a
S. cerevisiae	40.45 bc	41.31 c	14.55 b	3.79 c	0.67 d	7.3 b	454.2 b
C. sake	40.95 bc	43.27 a	15.00 a	4.19 b	0.74 c	6.2 c	492.9 b
P. membranifaciens	42.17 b	42.18 b	14.81 b	3.74 c	0.68 d	5.8 d	478.8 b

[a]Tomato transplants (cv. Prichard) were treated by dipping the root into broth culture of PGPY or PGPR for one hour. Control plants (not infected and infected control) were treated with tap water. At 8 weeks after transplanting, plants of five replicates from each treatment were used for determination of plant height, fresh and dry weights of shoot and roots. Values in the column followed by different letters indicate significant differences among treatments according to least significant difference test (P=0.05).

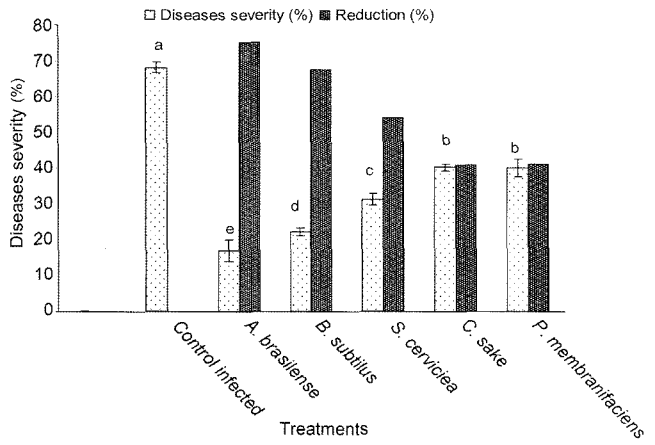

Fig. 1. Effect of inoculation with plant growth-promoting yeasts (PGPY) and rhizobacteria (PGPR) on development of Fusarium wilt in tomato plants cultivar cv. Prichard under greenhouse conditions. Tomato transplants were treated by dipping the root into broth culture of PGPY or PGPR for one hour. Control plants (not infected and infected control) were treated with tap water. Disease severity was estimated at 8 weeks after transplanting, as a wilting percent (% wilt). Different letters indicate significant differences among treatments according to least significant difference test (LSD) (P=0.05). Bars indicate the standard deviation.

non bacterized plants. Also, *B. subtilis* increased fresh and dry weight of shoots and roots than infected control. Such results may be due to the increased of plant hormone. Arkhipova et al. (2005) observed a general increase in the hormone content of lettuce roots inoculated with a cytokinin-producing *B. subtilis* strain. They suggested that the change in hormone content was a result of the indirect effect of the bacterium on the ability of plants themselves to produce cytokinins and other phytohormones, such as IAA and abscisic acid (ABA) (Schmidt et al., 2004). *B. subtilis* 101 could produce cytokinin-like molecules or other compounds through a similar mechanism (Felici et al., 2008).

Effect of PGPY and PGPR on developments of Fusarium wilt of tomato plants under greenhouse conditions. Results presented in Fig. 1 show that seedling treatment with any of the tested bioagents significantly reduced wilt percent in tomato plants. 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 *B. subtilis* and *A. brasilense* compared to infected control may be due to simulative 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. Thanaa, Ibrahim (1990) reported that the antagonistic effect of *B. 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 β-1,3-glucanase (Masih and Paul, 2002) and mycoparasitism (Wisniewski et al., 1991).

Table 2. Effect of inoculation with plant growth-promoting yeasts (PGPY) and rhizobacteria (PGPR) on development of Fusarium wilt and yield in tomato plants under the field conditions

Treatments[a]	Disease severity (%)[b]	Disease reduction (%)	Amount of yield (ton/hectar)[c]	Increase of yield (%)
Control healthy	–	–	47.0 cb	–
Control infected	75.0 a	0.0	24.7 d	–
A. brasilense	35.6 e	52.5	64.2 a	160
B. subtilis	43.2 d	42.4	54.3 b	120
S. cerevisiae	53.0 c	29.3	54.3 b	120
C. sake	65.6 b	12.5	49.4 cb	100
P. membranifaciens	66.2 b	11.7	51.9 b	110

[a]Tomato plants (cv. Prichard) were treated by dipping the root into broth culture of PGPY or PGPR for one hour. Control plants (not infected and infected control) were treated with tap water.
[b]Disease severity % was recorded at 8 weeks after planting.
[c]At harvest time, the average accumulated yield was calculated. Values in the column followed by different letters indicate significant differences among treatments according to LSD test at 0.05.

Field conditions. The results presented in Table 2 indicate that all inoculation treatments significantly reduced the disease severity and increased tomato yield relative to control infected with *F. oxysporum* f.sp. *lycopersici*. The treatments inoculated with *A. brasilense* or *B. subtilis* resulted in greater 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 2), but these two treatments resulted in 100 and 110% increases in fruits yield.

Our study showed that tomato plants treated with PGPR and PGPY caused higher reduction in disease severity and 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). *B. pumilus* induces callose and pectin in close association with phenolic compounds in newly formed wall appositions in pea roots in response to attack by *F. oxysporum* (Benhamou et al., 1996). However, treatment with *B. pumilus*, induced a rapid lignification in cucumber plants in response to ingress of *Colletotrichum orbiculare*, and total peroxidase and superoxide dismutase activities 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 on the pathogens. Also, our results agree with those reported by Hassan and Abd El-Rehim (2002), they reported that dipping onion seedlings before transplanting and foliar spray by *Saccahromyces cerviciea*, after 45 days from transplanting, were significantly influenced the exportable, total and culls onion bulb yield as well as incidence of neck rot disease. From the present experiments it may be concluded that application of PGPY and PGPR provide a reasonable level of protection against FOL especially in organic farming system, where plant nutrition and disease control are the main limiting factor.

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