Effect of Root Colonizing Bacteria on Plant Growth and *Fusarium* Wilt in *Cucumis melo*

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ABSTRACT

During 2003-2004, a number of rhizospheric soil samples were collected of either healthy or symptomatic field grown melon plants infected with *Fusarium oxysporum* f. sp. *melonis* race1/2 (*Fom*). Twenty one bacterial strains capable of inhibiting *Fom* including *Burkholderia* sp., *Bacillus* sp., *Streptomyces* sp. and *Pseudomonas fluorescens* were isolated. The strains colonized roots of "long melon" cultivar of Mashhad and, within two weeks, resulted in increased fresh and dry weight, length of stem and root, and number and area of leaves, in the absence and presence of *Fom,* under greenhouse and growth chamber conditions. The growth inhibition of *Fom in vitro* was due to antagonism, siderophore and antibiotic production, and secretion of exogenous compounds. All antagonistic strains reduced infection of long melon seeds with *Fom* under controlled conditions.

Keywords: Antagonist bacteria, *Fusarium oxysporum* f. sp. *melonis*, Long melon, Rhizosphere, Root colonizer bacteria.

INTRODUCTION

Vascular wilt of melon caused by *Fusarium oxysporum* f. sp. *melonis* (*Fom*) was originally reported in USA, and its pathogenicity to *Cucumis melo* L. was confirmed by Leach (1933). The pathogen is not distributed worldwide, though it is reported from various parts of Iran (Banihashemi, 1982). Plant pathogenic *F. oxysporum* causes substantial yield losses in many economically important crops and is considered to be the most important soil borne pathogen (Chelkowski, 1989). The microorganisms capable of colonizing the rhizosphere and implementing their bio-control potential are a key issue in the use of bio-control inoculants for protection of crops against soil borne plant pathogens (El-Hassan and Gowen, 2005). A large group of bacteria of different species introduced as plant growth promoter and biocontrol agents, among their fluorescent pseudomonads are well-known (Weller, 1988). Other genera including *Bacillus* sp., *Burkholderia* sp. and *Streptomyces* sp. are also introduced as biological agents (Weller, 1988). Studies on action mechanisms of antagonistic bacteria have shown that production of antibiotic and siderophore or induced systemic resistance play an important role in the control of soil borne plant pathogens (Weller, 1988). In some research, the rhizobacterial strains such as *P. fluorescens, P. putidae* and *Bacillus* induced resistance against plant pathogens (Meena *et al*., 2000; Vidhyasekaran *et al*., 2000). Suppression of disease and promotion of plant growth by non-pathogenic rhizobacteria has been reported by Van Loon (2007). The efficacy of the biological control agents would largely depend on the types of formulation and delivery technology (Lumsden *et al.,* 1995). Studies have shown that seed treatment is ideal for introducing antagonists to control specific pathogens because it allows the antagonist to be placed where it is most needed and the antagonists growth can be supported by the

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plant exudates (Paulitz, 1992). Control of *Fusarium* wilt in tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* by some rhizobacteria have been reported (Akkopru and Demir 2005).

Soleimani *et al.*, (2005) have reported the biological control of stem and root-rot of wheat caused by *Biopolaris* spp by using fluorescent *Pseudomonas* and *Bacillus*. Also, antagonistic mechanism of wheat rhizosphere fluorescent pseudomonads and their inhibition on root pathogenic *Fusarium* species have been reported (Mostofizadah-Ghalamfarsa *et al.*, 2006)

The objectives of this study were isolation and identification of antagonistic bacteria from melon rhizosphere and their effect on growth promotion of *Cucumis melo* under greenhouse condition. Their antagonistic effect *in vitro* on *Fom* and their potential on biocontrol agents in growth chamber was also studied.

MATERIALS AND METHODS

Screening and Identification of Antagonistic Bacteria

During 2003-2004, rhizospheric soil samples of healthy and *Fom* infected field grown melon plants were collected and used as a source of bacteria. General procedure was used to isolate bacterial strains from rhizosphere soil or roots (Schaad *et al.*, 2001). The bacteria were isolated from soil sample and root surface of melon at their mature stage. Uninfested environments, including root and soil, which were pure and free of *Fom* were chosen for comparison.

154 strains of bacteria were tested for antagonistic activity *in vitro* against *Fom* according to the method of Hagedron *et al.* (1989). Bacterial suspension of each purified isolates were spotted close to the four edges of Petri plates containing nutrient agar medium (NA) and KingB medium (KB) for *Pseudomonas fluorescens* and were incubated at 25°C. After 48 hours, a 6 mm block of a five-day-old culture of *Fom* was placed in the center of each plate and incubated at 25°C. Inhibition zone of fungal growth was determined daily for 5-7 days. Strains with

maximam inhibition zone were identified based on standard bacteriological tests and were selected for further studies (Schaad *et al.,* 2001; Fahy and Persley, 1983).

Assessing of Colonizing Capacity of Bacterial Isolates on Roots

The prescreening experiment was performed by sand-soil tube method (Sher *et al.,* 1984). Bacterial suspension (10^8 CFU ml^{-1} (OD= 600 nm, abs= 1.5) was prepared and coated with 1% Arabic gum. Seeds of "Long melon" cultivar of Mashhad were surface sterilized using 0.5% sodium hypochlorite and soaked in the bacterial suspension for 15 minutes at 25°C and, then, air dried. Control seeds were soaked in 0.1M $MgSO_4$. Inoculum levels on seeds were determined by agitating three seeds from each treatment in 9 ml of 0.1M $MgSO_4$. Mean CFU per seed was determined by averaging the population from 6 replicates performed by serial dilution incubated at 25°C for 24 hours. Glass test tubes (24 mm×24 cm)

were filled with coarse sand to a depth of 5 cm (Sher *et al.,* 1984). Five ml DW was added to each tube and the sand was overlaid with 3 cm of either sterile or field soil. One bacterial-treated seed was added per tube and covered with another 2 cm layer of sterile or field soil. Tubes were sealed with parafilm and incubated at room temperature without further water addition. After two weeks, root segments were harvested and agitated in 9 ml of 0.1M $MgSo_4$ using a vortex, and mean CFU per gram of root was determined as previously described for seeds. Ten replications of the whole root system of one plant were used in 10 plates per treatment.

Antibiotic Production

To study antibiotic production, 1 ml of bacterial suspension (10^8 CFU ml^{-1}) was flooded on PDA plate and incubated at 25°C. After 72 hours the colonies were removed by sterile cotton swab and exposed to choloroform vapor for 30 minutes (Lindberg, 1981). Blocks (5 mm) of five-

day-old culture of *Fom* was placed in the center of plates and incubated at 25°C. The growth of *Fom* was monitored and the percentage of inhibition of mycelium growth was determined for 5 days (Kraus and Loper, 1990).

Siderophore Production

The isolates that exhibited fluorescent pigment on KB were streaked on KB medium containing 5, 50 and 100 mMol $FeCl_3$ as chelating agent and incubated at 25°C. After 48 hours, plates were sprayed with conidial suspension of *Geotrichum candidum* to detect $FeCl_3$ and incubated for 48 hours (Weller and Cook, 1983).

Secretion of Exogenous Cell Liquid

250 ml Erlenmyer flask containing potato dextrose broth (PDB) was inoculated with 1 ml of 24 hours old bacterial suspension (10^8 CFU ml^{-1}) and incubated at 25°C on constant rotary shaker at 70 rpm for 7 days. The biomass was collected on Whatman NO.1 filter paper and centrifuged at 8000 rpm for 20 min. The supernatant was filtered through 0.22 µml Millipore filter paper and 1, 3, and 5 ml of the filterate was added to 15, 17 and 19 ml of melted PDA (45°C) and added to the plate. After solidifying, 5 mm block of *Fom* was placed in the center of each plate. The hyphal growth was measured daily for 5 days using ruler and the percentage of growth inhibition were determined (Singh and Deverally, 1984).

Plant Growth Promoting Potential of Strains

The experiment was performed according to Suslow and Schorth (1981) as seed pelleting method. The seeds were first surface sterilized in 0.5% sodium hypochlorite. Two grams of long melon seeds were coated with a mixture of 2 ml of 1% carboxylmethyl cellulose and 2

ml of bacterial suspension (10^8 CFU ml^{-1}) for 2 hours and then coated with 0.5% talk powder and dried for 2 h under stream of sterile air at room temperature. Four seeds of long melon cultivar were sowed into 2-liter pot containing sand:clay soil mixture (1:2 v/v). After the first true leaf, they were thinned to one seedling per pot. To enhance bacterial colonization, roots were irrigated daily with 100 ml deionized water. After two weeks, 3 day irrigation intervals was practiced to enhance *Fom* colonization. After 3 months the strains were evaluated for their ability to increase plant growth under greenhouse condition by comparing shoot dry weight in each treated plant in respect to untreated control (Suslow and Schorth, 1981).

Inoculum Preparation

Chlamydospores of *Fom* were prepared according to Banihashemi and deZeeuw (1973). Two to three of 6 mm block of 4-day-old single spore culture of *Fom* race 1-2 (Banihashemi, 1982) was transferred into 250 ml flask containing 50 ml PDB and incubated at room temperature on a reciprocal shaker (60 strakes 1 Min) for 3 days. The conidia were centrifuged (3000 rpm for 10 minutes) and washed twice, then mixed with sterilized sand and incubated at 20°C for 1 month and, then, transferred to 4°C for 3 months. Before mixing the inocula with the soil, the population of *Fom* in sand was measured by serial dilution using semi selective medium (Banihashemi and deZeeuw, 1969). Sand inoculums were mixed with soil at a proportion to obtain *Fom* concentration at 200 CFU g^{-1} dry soils.

Effect of Antagonistic Bacteria on Disease Suppression and Plant Growth

The experiment was carried out in growth chamber under controlled conditions (24°C, 16 hours photoperiod, 70% humidity). Seeds of "Long melon" susceptible to *Fom* race1/2 were first surface sterilized in 0.5% sodium hypochlorite .and coated with bacterial

suspension (10^8 CFU ml^{-1}) (Akkopra and Demir, 2005). One-liter pots were filled to one-third with autoclaved soil infested with chlamydospores of *Fom* race 1-2 and 2/3 of the upper parts with a mixture of sterile sand-soil (1:2 v/v). Pots were watered daily with 100 ml deionized water for 3 weeks and, later, 3 days a week. Plant growth and infection were studied for 3 months as indicated earlier. The Experimental design was a completely randomized design. The data were analyzed using Proc GLM of the SAS Software. Comparison of the means was done based on Duncans Multiple Range Test. The number of replicates for the 22 treatments was 4 for each bacterial isolate

Statistical Analysis

The statistical analysis was done for five previous experiments. The Experimental design was a Completely Randomized Design. The data were analyzed using Proc GLM (Generalized Linear Models) of the SAS Software. Comparison of means has been done based on Duncans Multiple Range Test. The number of replicate for the 22 treatments were 4 for each bacterial isolate

RESULTS

A total of 154 bacterial strains were isolated on NA and soil extract media from rhizosphere of symptomatic and symptomless melon plants, of which 21 strains showed 100% inhibition to *Fom* in antagonistic assay *in vitro*. Based on standard bacteriological assay, the strains were identified as *Pseudomonas fluorescens*, *Streptomyces* sp., *Bacillus* sp., and *Burkholderia* sp., which were selected for further studies. Twenty one strains produced antibiotics *in vitro* and caused 100% inhibition of mycelial growth of *Fom*. Ten strains of *P. fluorescens* produced siderophore in the presence of 5, 50 and 100 mMol $FeCl_3$ and resulted in 100% growth

inhibition of *Fom* on KB medium after 48 hours at 25°C. One strain of *Bacillus* produced exogenous cell liquid that resulted in maximum inhibition of *Fom*.

Root and Seed Colonization Ability of Antagonistic Bacteria on Long Melon under Laboratory Condition

Twenty one of the bacterial strains showed good correlation between root and seed colonization of "Long melon" in the sand-soil tube method. Mean bacterial population densities on seeds assayed after 24 hours ranged from 0 to 80 CFU $seed^{-1}$. The most successful seed colonizers were strains 2, 7, 8 and 11 of *P. fluorescens* with 80.6±0.49, 78.6±0.61, 78.3±1.72 and 79.5±0.34 CFU $seed^{-1}$, respectively, compared to the untreated control with 0.0±0.0. In contrast, the other strains of *P. fluorescens* did not show significant differences in seed colonization with each other. *Burkholderia* strain18 was the most efficient strain with 78.1±1.83 CFU $seed^{-1}$. All strains of *Streptomyces* were the weakest seed colonizer. The values for the seed treatments differed significantly from the control value, as determined by an analysis of variance ($P< 0.001$) (Figure 1).

Mean bacterial root population densities was estimated and ranged from 0.0 to 665.1 CFU $root^{-1}$ weight. The best root colonizer was *Burkholderia* strain19 with 665.1±316.2 CFU $root^{-1}$ weight. *Streptomyces* and other strains of *Burkholderia* were the weakest colonizer. All *P. fluorescens* were better root colonizer than *Streptomyces* and most *Burkholderia* strains. The values for the root treatments differed significantly from the control value, as determined by an analysis of variance ($P< 0.001$) (Figure 2).

Effect of Antagonistic Bacteria on Plant Growth

All antagonistic bacteria including *P. fluorescens*, *Bacillus* sp., *Burkholderia* sp. and
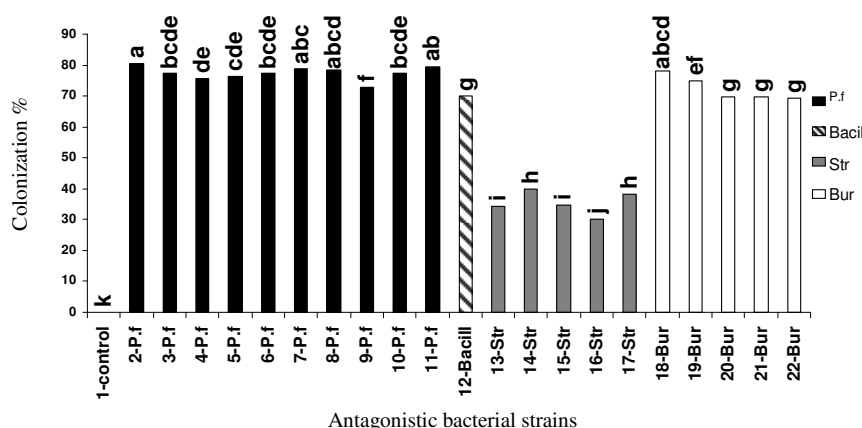
Figure 1. Seed colonization of long melon with antagonistic bacteria (concentration 10^4 CFU ml^{-1}). P.f (*Pseudomonas fluorescens*), Bacill (*Bacillus* sp.), Str (*Streptomyces* sp.), Bur (*Burkholderia* sp.).Different letters show significant differences between means of the treatments by the Duncan's test, 1% significance level.

Streptomyces sp. increased plant growth of "long melon" in the greenhouse.

All of the bacterial strains increased root length, but they were almost at the same level. The most efficient strain was *P. fluorescens* strain 5 with 12.62 cm±0.2 and the least one was *Streptomyces* strain 15 with 10.25cm±0.5 and *Burkholderia* strain 20 with 10.5±0.64 compared to the untreated control with 4.37 cm±0.2. The values for the root length treated with different strains of bacteria differed significantly from the control, as determined

by an analysis of variance (P<0.001) (Figure 3).

All bacteria increased dry root weights of the plants, compared to the untreated control. The most efficient strains were *P. fluorescens* strains and the least one was *Streptomyces*. Strains did not show significant differences among each other. The values for the root dry weight in treated plants differed significantly from the control value, as determined by an analysis of variance (P< 0.001) (Figure 4).
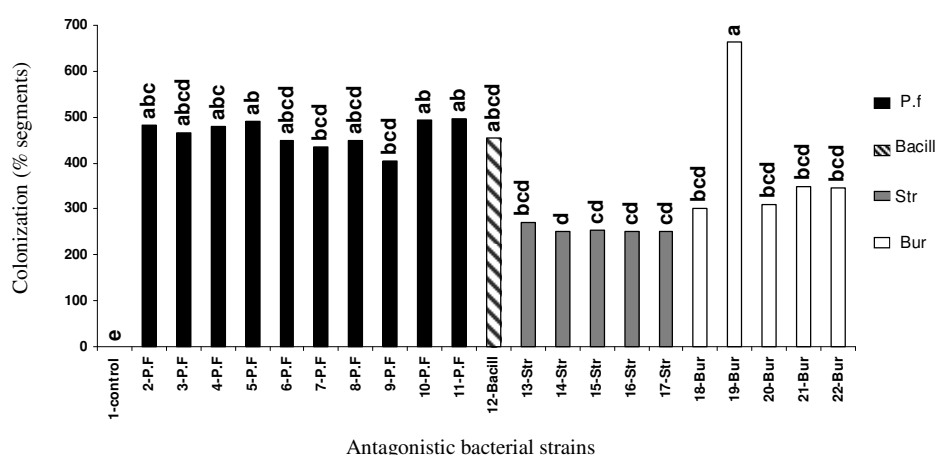

Figure 2. Root dry weight colonization of long melon with antagonistic bacteria (concentration 10^6 CFU ml^{-1}). P.f (*Pseudomonas fluorescens*), Bacill (*Bacillus* sp.), Str (*Streptomyces* sp.), Bur (*Burkholderia* sp.). Different letters show significant differences between means of the treatments by the Duncan's test, 1% significance level.
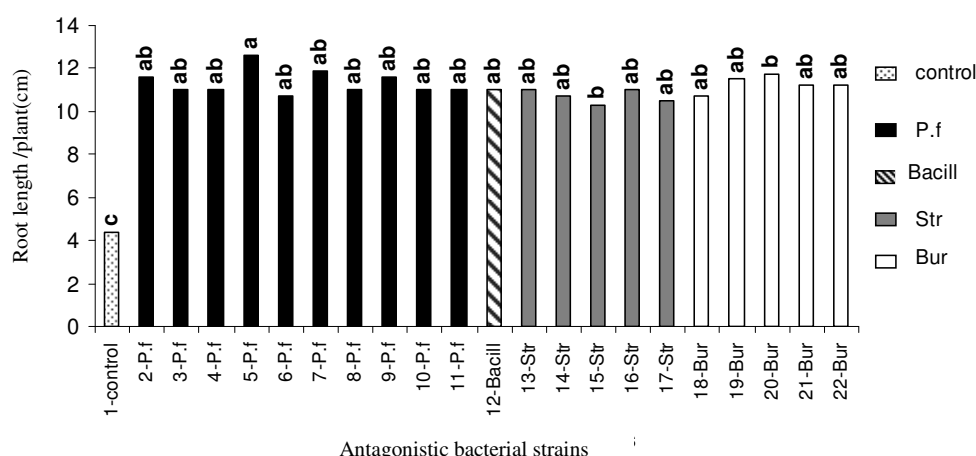
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Figure 3. Root length of long melon plants treated with antagonistic bacteria. Control (no inoculated), P.f (*P. fluorescens*), Bur (*Burkholderia*), Str (*Streptomyces*), Bacill (*Bacillus*). Different letters show significant differences between means of the treatments by the Duncan's test, 1% significance level.

Effect of Antagonistic Strains on Root Colonization of *C. melo* by *Fom*

Control plants in pathogen infested soil and in the absence of antagonists showed initial disease symptoms as brown necrotic spots on leaves, twenty days after planting. These spots gradually expanded and caused wilting, scorching and death, a week later. In the presence of bacterial strains, no disease symptoms were detected. Plants treated with *Burkholderia* strains 21 and 23 showed only mild chlorosis after 3 months and remained healthy. None of the bacterial inoculated plants in *Fom* infested soil died during the experiment. *Fom* could not be recovered from stem, crown and leaves of bacterial inoculated plants, except in *Burkholderia* strains 21 and 23. Root colonization of inoculated control and bacterial treated
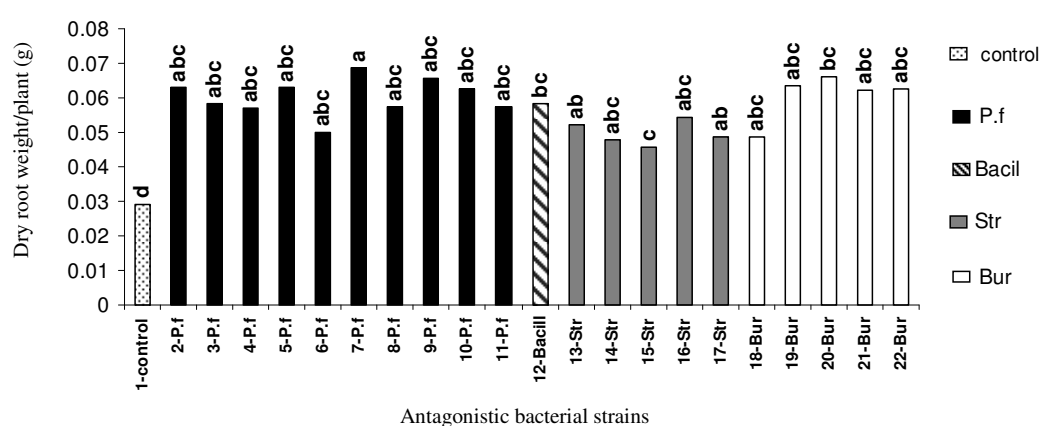

Figure 4. Root dry weight of long melon plants treated with antagonistic bacteria. Control (noninoculated), P.f (*P. fluorescens*), Bur (*Burkholderia*), Str (*Streptomyces*), Bacill (*Bacillus*). Different letters show significant differences between means of the treatments by the Duncan's test, 1% significance level.

plants showed that 97.25%±1.6 of root segments of the control plants were colonized by *Fom,* but bacterial treated plants had only 0 to16 % colonization. *Fom* did not colonize roots of plants treated with *P. fluorescens* strain 3 and *Streptomyces* strain 16 (Figure 5). The rate of *Fom* colonization in the presence of different strains varied (Figure 5). It was found that bacterial colonization in infested soil increased plant growth and dry weight more than the control inoculated plants (Figure 6). *P. fluorescens* strain 8 resulted in 1.32 g plant^{-1}±0 growth increase in infested soil compared to control in the presence of *Fom* 0.22 g±1.6 (Figure 6). Other strains showed different values of growth increasing.

DISCUSSION

Among 154 bacterial strains isolated from rhizosphere of symptomatic and symptomless melon plants 21 strains belonging to *Burkholderia* sp., *P. fluorescens, Streptomyces* sp. and *Bacillus* sp. were found to be antagonistic and capable to reduce infection of *Cucumis melo* by *F. oxysporum* f.sp. *melonis*. In *vitro P. fluorescens*, *Burkholderia, Streptomyces* and *Bacillus* were capable of antibiotic production, *P. fluorescens* also produced siderophore and *Bacillus* secreted exogenous cell liquids which were all capable to control disease under greenhouse and growth chamber condition. Production of antibiotic, siderophore and exogenous cell liquids among tested bacteria has been also reported by others (Singh and Deverally, 1984; Kraus and Loper, 1990; Taechowisan *et al.,* 2005). Root colonization of bacterial strains in the absence of pathogen varied among species. All bacterial strains increased growth in the absence and presence of *Fom*. The maximum growth increase was by *P. fluorescens* strain 8 and did not differ significantly from strain 5 (Figure 6). However there was a variation among species and strains on growth promoting ability. Growth increase was noticed two weeks after sowing the seeds. Growth promotion among bacterial strains has been reported on vascular plants such as sugar beet (Suslow and Schoroth, 1981), and radish (Kloepper and Schoroth, 1978). Growth promoting bacteria cause shift in rhizosphere microbial population (Klopper
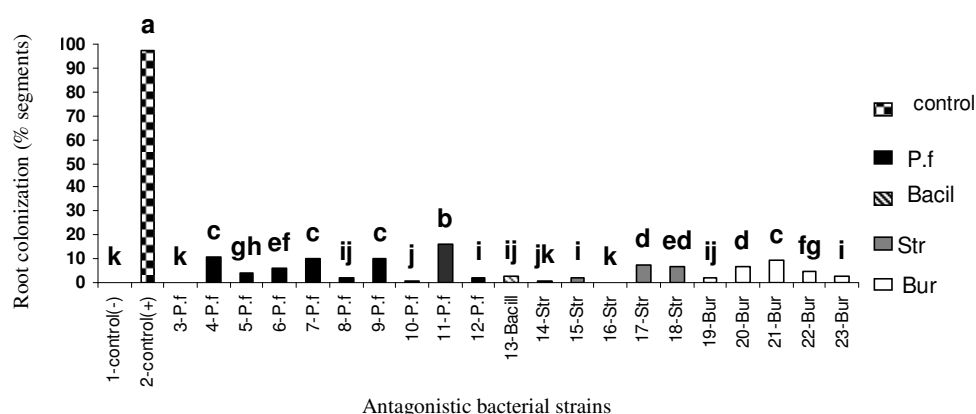

Figure 5. Root segments colonization of long melon plants by *Fusarium oxysporum* f.sp. *melonis* race1/2, treated with antagonistic bacteria and non treated control. P.f (*P. fluorescens*), Bur (*Burkholderia*), Str (*Streptomyces*), Bacill (*Bacillus*), control (-) (non inoculated by bacteria and *Fom*), control (+) (inoculated with *Fom*). Different letters show significant differences between means of the treatments by the Duncan's test, 1% significance level.

Figure 6. Shoot dry weight of long melon plants treated with antagonistic bacteria in soil infested with *Fusarium oxysporum* f.sp. *melonis* race 1/2 compare to control P.f (*P. fluorescens*), Bur (*Burkholderia*), Str (*Streptomyces*), Bacill (*Bacillus)* control (-) (non inoculated by bacteria and *Fom*), control (+) (inoculated with *Fom*). Different letters show significant differences between means of the treatments by the Duncan's test, 1% significance level.
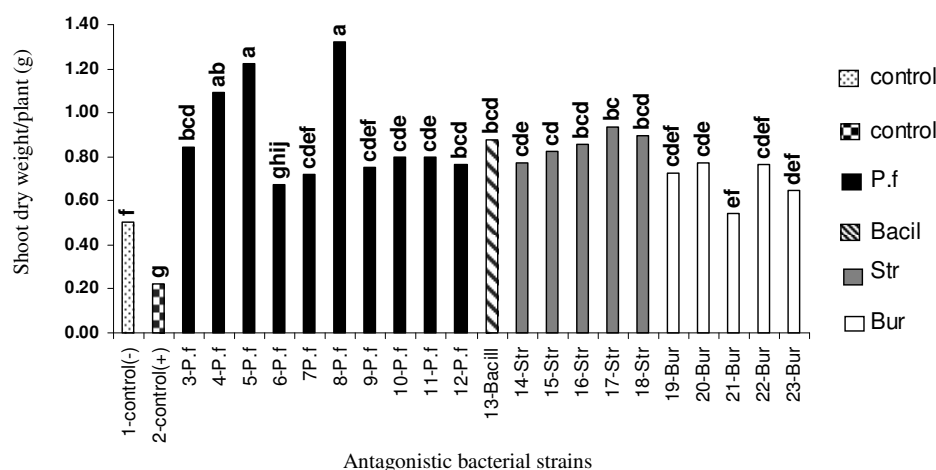
and Schoroth, 1978; Suslow and Schoroth, 1981. They regulate the translocation of some materials such as putrescine, spermine and spermidine and roots supply carbon source as muciliageneus substance to the bacteria (Kuiper *et al.,* 2001). *Burkholderia* strains 21 and 23, which showed lower antagonistic response, did not efficiently control the disease. After colonizing the root, growth promoting bacteria enter vascular system and spread in stem, leaf and other plant organs and produce inhibitor substances, resulting in induced resistance (Compant *et al.,* 2005). Strains of rhizobacteria have been shown to elevate resistance against plant pathogens (Meena *et al.*, 2000; Vidhyasekaran *et al.*, 2000 and 2001). Seed inoculation with antagonistic bacteria is an efficient method to control certain soil borne plant pathogens (Weller and Cook, 1983; Trapero-casas *et al.,* 1990; Parke *et al.,* 1991). Seed treatment of melon with *P. putidae* controlled *Fom* under field condition (Bora *et al.,* 2004). Disease reduction could be as

a result of several biological factors. Although no attempts were made to investigate the mechanism of *Fom* reduction in our strains, Elad and Baker (1985) reported siderophore production in cucumber by reducing chlamydospore germination in *F. oxysporum* f. sp. *cucumeriu.* Leeman *et al*. (1996) demonstrated that the siderophore of *P. fluorescens* can act as an elicitor of induced systemic resistance. Other investigators reported that siderophore production was responsible in reducing vascular wilt fusaria (Simeoni *et al.,* 1987; Scher *et al.,* 1984; Raajmakers *et al.,* 1995; Scher and Baker, 1982; Elad and Baker, 1985). Soleimani *et al*. (2005) reported that treating wheat seed with antagonistic rhizobacteria not only reduced the disease severity, but also showed positive influence on growth and yield of wheat cultivar. In our study, it was shown that the bacterial strains isolated from rhizospheric soils of melon are capable to promote growth of "long melon" and to reduce vascular wilt

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disease by producing either siderophore or other substances such as antibiotic and exogenous cell liquid. Use of bacterial antagonists under field conditions must be evaluated as a part of disease management.

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اثر باکتریهای فراریشه روی رشد گیاه خربزه و کنترل پژمردگی ناشی از فوزاریوم

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چکیده

طی سالهای ۱۳۸۲ تا ۱۳۸۳ از خاک فراریشه گیاهان خربزه بدون علائم و دارای علائم پژمردگی ناشی از *Fusarium oxysporum* f.sp. *melonis* (**Fom**) نمونه برداری شد. تعداد ۲۰ جدایه دارای توانایی جلوگیری از قارچ مذکور شامل گونه های , *Streptomyces sp., Bacillus sp.* و *Burkholderia sp.* , *Pseudomonus fluorescens* جدا سازی شد. جدایه های مذکور قادر به کلونیزه کردن ریشه خربزه و افزایش وزن تر و خشک ساقه و ریشه و تعداد برگ ها پس از دو هفته در حضور و عدم حضور قارچ مذکور در شرایط گلخانه و اتاقک رشد بودند. جلوگیری از رشد قارچ **Fom** در شرایط گلخانه بر اساس خاصیت آنتاگونیسم، تولید سیدروفور، آنتی بیوتیک و ترشح مایع برون یاخته‌ای بود. بذرهای خربزه تیمار شده با استرین های آنتاگونیست باعث کاهش آلودگی به قارچ **Fom** گردید.