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Evaluation of biocontrol potential of Rhizosphere Antagonist Bacterial strains on *Fusarium* Wilt and Plant Growth in Muskmelon Plants

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ABSTRACT

Fusarium oxysporum f. sp. *melonis* (Fom) can causes *Fusarium* wilt in melon plants. This pathogen is distributed in various parts of Iran. Some genera including fluorescent *pseudomonads*, *Streptomyces* sp., *Bacillus* sp. and *Burkholderia* sp. are reported as biological agents to control plant pathogens. The aims of this research were isolation and identification of antagonistic bacteria from melon rhizosphere and estimate their effects 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. During 2015-2016, a number of soil samples from rhizospheric area were gathered of both healthy and symptomatic farmland grown melon plants infected with *Fusarium oxysporum* f. sp. *melonis*. Seventeen bacterial strains able of inhibiting *Fom* including *Pseudomonas fluorescens*, *Streptomyces* sp., *Bacillus* sp. and *Burkholderia* sp. were isolated. The Bacterial strains colonized roots of seeds of cultivar of Mashhadi melon and, within 16 days, leaded to enhance dry and fresh weight, root and stem length, and area and number of leaves, in the presence and absence of pathogen, under growth chamber and greenhouse conditions. The inhibition of *Fom* growth *in vitro* was because of siderophore and antibiotic production, antagonism and secretion of exogenous compounds. In this study, all of antagonistic Bacterial strains could reduce infection of Mashhadi melon along with *Fom* under controlled conditions.

KEY WORDS
Biocontrol potential, *Fusarium* Wilt, *Fusarium oxysporum* f. sp. *melonis*, Mashhadi melon, Rhizosphere Antagonist Bacterial strains.

INTRODUCTION

Fusarium oxysporum f. sp. *melonis* (Fom) can causes *Fusarium* wilt in melon plants that the first is originally reported from USA. This pathogen is distributed in various parts of Iran [3]. *F. oxysporum* causes some major yield losses in several important crops such as cantaloupe, cucumber, wax gourd, muskmelon, and watermelon is considered as the most important soil borne pathogen [21,8, 24].

Those microorganisms that are able to colonize the rhizosphere area around the plants roots and causing their bio-control potential are a natural factor to protect farming products against fungus that are soil borne [7,10, 25]. Different species of bacteria are reported as promoter for plant growth and biological agents which are able to control plant pathogens that the most well-known of them are fluorescent *pseudomonads* [45, 26]. Some genera including *Streptomyces* sp. *Bacillus* sp. and *Burkholderia* sp. are also reported as biological agents to control plant pathogens [22,14,3]. Several studies have been proved that production of siderophore and antibiotic, competition for nutrition and space, inducing resistance, inactivation of pathogen's enzymes and enhancement of root and plant development or induced systemic resistance play a major role in the biocontrol of soil borne pathogens [13, 30]. Some rhizobacterial strains such as *Pseudomonas fluorescens*, *P. putidae* and

Bacillus could induce resistance against plant pathogens [44,27] Also, Van Loon [40] has been reported that non-pathogenic rhizobacteria are capable to disease suppression in plants and promotion in plant growth.

The effect of the biological control agents are depend on the kinds of formulation and technology in delivery [19,35]. Several studies have illustrate that seed treatment is best way to introduce new antagonists to control pathogens as it permits the antagonist to be stood where that is most useful and the growth of antagonists can be aided by the plant exudates [24, 46]. Soleimani et al., [34] have shown that using fluorescent Pseudomonas and Bacillus are biological agents to control of stem and root-rot of wheat caused by *Biopolaris* spp. Use of biological control agents, such as plant growth promoting rhizobacteria (PGPR), can be a suitable approach in control of disease [31,28, 47]. The aims of this research were isolation and identification of antagonistic bacteria from melon rhizosphere and estimate their effects 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 2015-2016, all of soil and root samples from rhizospheric area of healthy and *Fom* infected plants were gathered and stored as a source of bacterial samples. The method and procedure was done to isolate strains of bacterial from rhizosphere soil and roots was general [9,29]. All of samples were separated from soil and root surface when the melon plants were at their final stage of fruiting. Uninfested soil and root of rhizospheric area, which were without any (free) of pathogen agent were chosen as control sample. According to the procedure of Hagedron et al. [11] all of samples (225 strains) were assessed for amount of antagonistic activity in vitro against pathogen. Petri dishes were contained KingB medium (KB) and nutrient agar medium (NA) for Pseudomonas *fluorescens* and incubated at 27°C in darkness. After 60 hours, a 5 mm slice of a six-day-old culture of pathogens was located in the middle of each Petri plates and was incubated at 27°C. Daily, the amount of inhibition region of fungal growth was specified for 6-7 days. Sample strains with highest inhibition region were distinguished based on method of Schaad et al. [29] and were elected for future studies.

Assessing of Colonizing Capacity:

In order to prescreening experiment the method of Scher et al. [30] that's mean sand-soil tube was used. Arabic gum (1%) was used to prepare (coating) suspension of bacterial (OD= 600 nm) 10^9 CFU ml^{-1}). In this experiment seeds of cultivar of Mashhadi melon were tested and using 0.8% sodium hypochlorite their surfaces were sterilized and soaked in the suspension of bacterial for 12 minutes at 27°C and after that air dried. Seeds that were used to control were soaked in 0.1M MgSO4. With motivating 5 seeds from each our treatment in 8 ml of 0.1M MgSO4, amounts of inoculum on seeds were calculated. Glass test tubes (25 mm×25 cm) were stowed with sand to a depth of 4 cm (Scher et al., 1982). By population averaging from 5 replication of treatments carried out with serial dilution which incubated at 27°C for 20 hours, Mean of CFU per seed was calculated. Three cm of either of both sterile and field soil was covered with 5 ml DW that was subjoined to all tubes and sand. Next, one seed that was treated by bacterial was subjoined per tube and veiled with 3 cm soil (sterile or field). With parafilm tapes tubes were closed to incubate at room temperature and, after 12 days, some part of roots were picked up and motivated in 8 ml of 0.1M MgSo4 by a vortex, and like we described earlier for seeds, mean of CFU per gram of root was calculated. For all root system of a single plant in this experiment 10 replications were used in 10 plates per treatment.

Antibiotic Production in samples:

One ml of suspension of bacterial (10^9 CFU ml^{-1}) was overflowed on PDA Petri dishes and incubated at 27°C to assess antibiotic production. The colonies were taken after 72 hours and exposed to choloroform vapor for 40 minutes [26]. Slices (5 mm) of six day-old culture of pathogen was located in the middle of Petri dishes and incubated at 27°C. The growth of pathogen was checked and the mycelium growth inhibition was calculated for 6 days in percentage [26].

Siderophore Production:

Those isolates that showed pigment of fluorescent on KB culture as chelating agent were transferred on KB which was medium and were contained of 5, 50 and 100 mMol FeCl3 and incubated at 27°C. In regard to detect FeCl3 Petri dishes after 48 hours were sprayed with suspension of conidial of *Geotrichum candidum* and incubated for 60 hours [26].

Secretion of Exogenous Cell Liquid:

200 ml Erlenmyer flask containing potato dextrose broth was inoculated with 1 ml of 30 hours suspension of old bacterial (10^9 CFU ml^{-1}) and incubated at 27°C on a constant rotary shaker at 90 rpm for 5 days. The

biomass was gathered on filter paper (Advantec NO.1) and centrifuged at 7000 rpm for 15 min. The supernatant was refined through a filter paper (0.22 µml Millipore) and 1, 2, and 4 ml of the filterate was subjoined to 14, 18 and 21 ml of melted PDA (45°C) and then were added to the Petri dishes. After tightening, 5 mm slice of pathogen was located in the middle of each Petri dish. The percentage of growth inhibition estimated with measuring the hyphal growth for 5 days using a Linear Encoder [33].

Plant Growth Promoting Potential of Strains:

The experiment was carried out pursuant to seed pelleting that is reported by Suslow and Schorth [36]. After surface sterilized, 3 grams of seeds were covered with a combination of carboxylmethyl cellulose (3 ml of 1%) and suspension of bacterial [3 ml (10^9 CFU ml^{-1})] for 1.5 hours and after that covered with talk powder (0.5%) and dried for 45 min at room temperature. Four seeds were planted into 2-liter pot containing sand:clay soil mixture (1:2 v/v). Once the first true leaf emerged, they were removed to one seedling per pot. We used 100 ml deionized water to increase bacterial colonization by irrigating plants daily. After two weeks, 4 day irrigation distances was done to increase pathogen colonization. According to method of Suslow and Schorth [36], after 3 months the bacterial strains were estimated for their capability to enhance plant growth under greenhouse condition by comparing shoot dry weight in each treatment in respect to control sample.

Inoculum Preparation:

Preparation of fungal inoculum was done on a mixture of 5 gr corn flour mixed with 95 gr sand and 50 ml sterile distilled water that were sterilized for 2 hrs at 120˚C. *Fusarium* spore suspesion was prepared from purified *Fusarium* isolate and the concentration adjusted to 4.2×106 spore/ml. 5 ml of spore suspension, was added to the flasks containing 100 g mixture of corn flour and sand. Control flasks contained mixture of corn flour and sand which 5ml sterile water was added to them. These flasks were incubated for 20 days at 27±2oC [21]. The *Fom* population in sand was estimated with serial dilution with method of Banihashemi and deZeeuw [2] before combination the soil with the inocula. Inoculums of sand were combined with the soil at a amount to reach concentration of *Fom* at 270 CFU g^{-1} in dry soils.

Effect of Antagonistic Bacteria on Disease Suppression and Plant Growth:

This experiment was done in growth chamber (27°C, 12 hours photoperiod, 65% humidity). Susceptible melon seeds to *Fom* race1/2 were sterilized by HCl (0.5%) and covered with suspension of bacterial (10^9 CFU ml^{-1}) [1]. Four-kilo pots were loaded to 1/3 of all with sterilized soil which was infested by chlamydospores of *Fom* (race 1-2) in top sections with a combination of sterile sand-soil (1:2 v/v). Plants were irrigated with 120 ml sterilized water for 25 days daily and, after a while, 2 days in a week.

Statistical Analysis:

The amount of growth in plants and the proportion of infection in them were recorded for 80 days. The data were analysed using Proc GLM of the SAS Software. The statistical analysis was performed for all previous experiments. 4replicates were considered for each bacterial isolate for the 18 treatments. Comparison of the means was performed based on Duncans Multiple Range Test. The Experimental design was a completely randomized design.

Results:

Seventeen strains illustrated 100% inhibition for *Fom in vitro* amongst of all 225 strains of bacterial genera. The strains of bacterial genera were identified as *Streptomyces* sp., *Bacillus* sp., and *Burkholderia* sp., *Pseudomonas fluorescens* based on standard bacteriological assay that were elected for future studies. Six strains of *P. fluorescens* generated siderophore when 5, 50 and 100 mMol FeCl3 was in their environment and after 48 hours at 25°C could inhibit 100% growth of *Fom*. Also, it is noticeable that 2 strain of *Bacillus* generated exogenous cell liquid which can be inhibited growth of *Fom* in maximum.

Ability of Antagonistic Bacteria in Root and Seed Colonization on Melon Seeds under Laboratory Condition:

Seventeen of our strains showed acceptable correlation between seed and root colonization of the susceptible melon cultivar in the method of sand-soil tube. The average population of bacterial densities on seeds assessed after 24 hours that was located between 0 to 83 CFU $seed^{-1}$. The most successful seed colonizers were strains 4, 5, and 7 of *P. fluorescens* with 83+0.59, 79.3+0.51, and 80.5+0.62 CFU $seed^{-1}$, respectively, in comparison with the untreated control with 0.0+0.0.

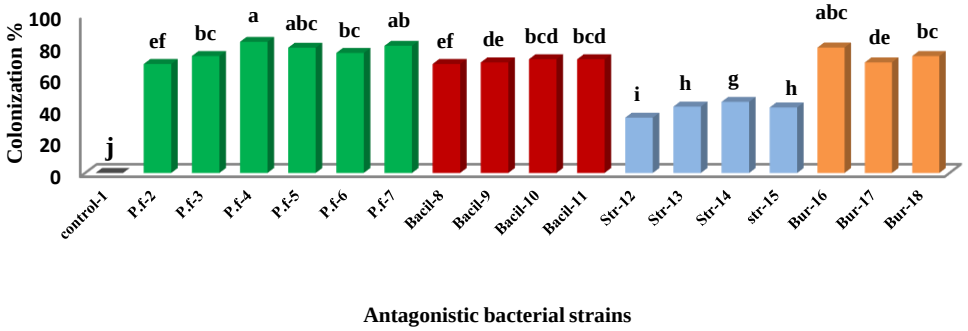

Fig. 1: Seed colonization of Mashhadi melon with antagonistic bacteria (concentration 10^5 CFU ml^{-1})**.** Bacill (*Bacillus* sp.**)**, P.f (*Pseudomonas fluorescens*), Bur (*Burkholderia* sp.), Str (*Streptomyces* sp.).Different letters show significant differences between means of the treatments by the Duncan's test, 1% significance level.

On the other hand, the other *P. fluorescens* strains did not show remarkable difference in colonization of seed with each other. *Burkholderia* (strain15) was the most impressive strain with 79.4+1.09 CFU $seed^{-1}$. The weakest colonizer of seeds among all strains was related to *Streptomyces*. The figures of seed treatments varied considerably from the control figure, as distinguished with an analysis of variance (P< 0.001) (Figure 1). On average population densities of bacterial root was evaluated and ranged from 0.0 to 615.1 CFU $root^{-1}$ weight. *Burkholderia* (strain16) was the best root colonizer with 615.1+232.2 CFU $root^{-1}$ weight. On contrast, the weakest root colonizer was *Streptomyces* and other strains of *Burkholderia*. All of *P. fluorescens* strains were better root colonizer than most *Burkholderia* and *Streptomyces* strains. The figures of root treatments varied considerably from the control figure, as distinguished with an analysis of variance (P< 0.001) (Figure 2).

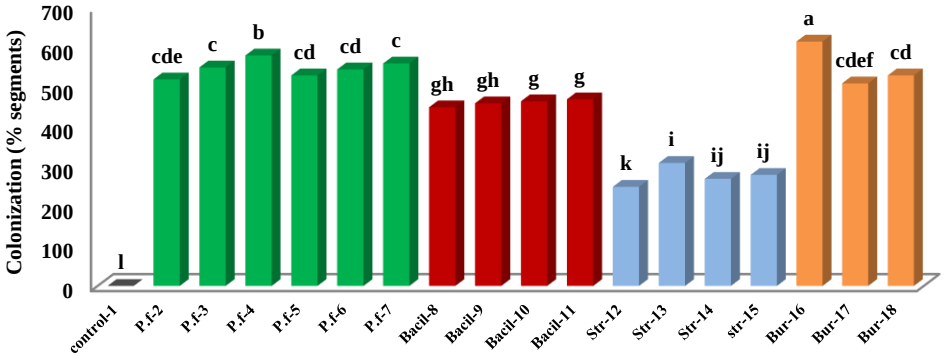

Fig. 2: Root dry weight colonization of Mashhadi melon with antagonistic bacteria (concentration 10^5 CFU ml^{-1}). Bacill (*Bacillus* sp.), P.f (*Pseudomonas fluorescens*), Bur (*Burkholderia* sp.), Str (*Streptomyces* sp.). Different letters show significant differences between means of the treatments by the Duncan's test, 1% significance level.

Assessing Antagonistic Bacteria efficacy on Plant Growth:

All of antagonistic strains including *Bacillus* sp., *P. fluorescens*, *Streptomyces* sp. and *Burkholderia* sp. elevated plant growth in the greenhouse. All of the bacterial strains promoted root length; however they were approximately at the similar level. *P. fluorescens* (strain 3) with

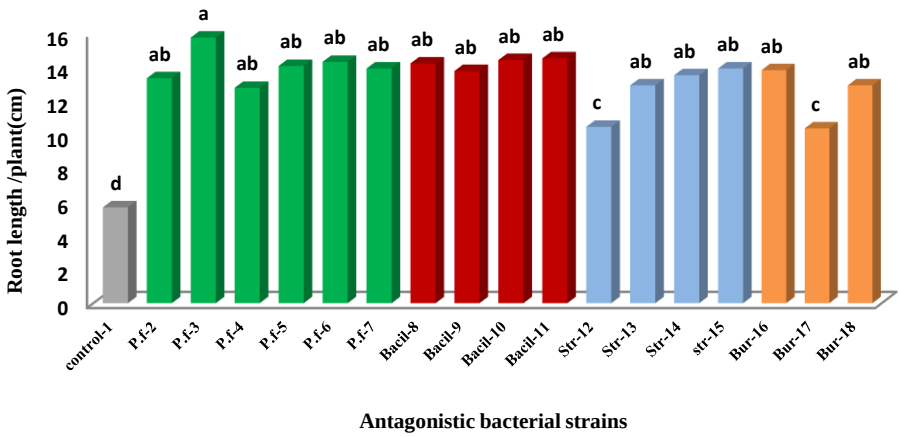

Fig. 3: Root length of Mashhadi melon plants treated with antagonistic bacteria. Control (no inoculated), Bacill (*Bacillus* sp.), P.f (*Pseudomonas fluorescens*), Bur (*Burkholderia* sp.), Str (*Streptomyces* sp.). Different letters show significant differences between means of the treatments by the Duncan's test, 1% significance level.

15.73 cm+0.3 was the most efficient strain and *Streptomyces* (strain 12) with 10.44cm+0.2 and *Burkholderia* (strain 17) with 10.35+0.32 were the least one in comparison with the untreated control with 5.67 cm+0.2. The figures of root length treated with different strains of bacteria varied considerably from the control plants, as distinguished with an analysis of variance (P<0.001) (Figure 3). All of bacteria elevated dry root weights in all plants in comparison with the untreated control plants. Strains did not show significant differences among each other. The most efficient strains were *P. fluorescens* strains and the least one was *Streptomyces*. The figures of root dry weight varied considerably from the control figure, as distinguished with an analysis of variance (P< 0.001) (Figure 4).

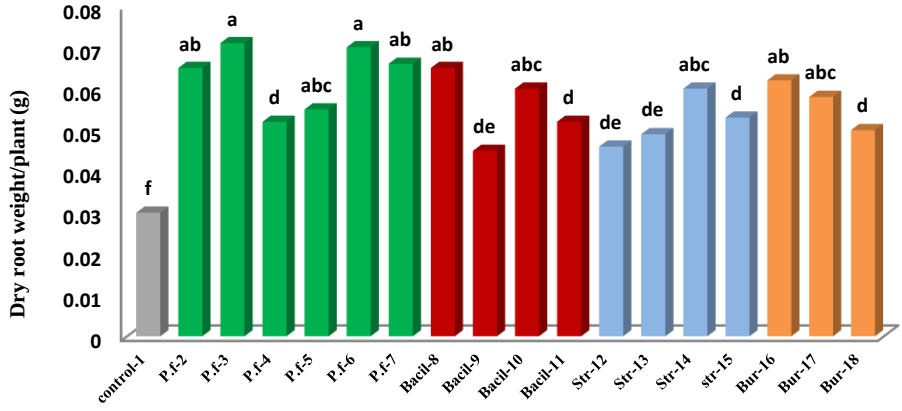

Fig. 4: Root dry weight of Mashhadi melon plants treated with antagonistic bacteria. Control (non-inoculated), Bacill (*Bacillus* sp.), P.f (*Pseudomonas fluorescens*), Bur (*Burkholderia* sp.), Str (*Streptomyces* sp.). Different letters show significant differences between means of the treatments by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

Assessing Antagonistic Strains efficacy on Root Colonization of C. melo by Fom:

Twenty days after planting, control plants in the absence of antagonists and in infested soil by pathogen shown primary disease symptoms as yellowing and wilting. A week later, these signs gradually expanded and caused scorching and death. There were not any disease symptoms in the presence of bacterial strains. Plants treated with *Burkholderia* strains 18 and 19 indicated just moderate chlorosis after 80 days and stood healthy and pathogen did not be retrieved from leaves, crown and stem of plants which were bacterial inoculated, except in *Burkholderia* strains 21 and 23.There were not any deaths in the bacterial inoculated plants in infested soil by *Fom*

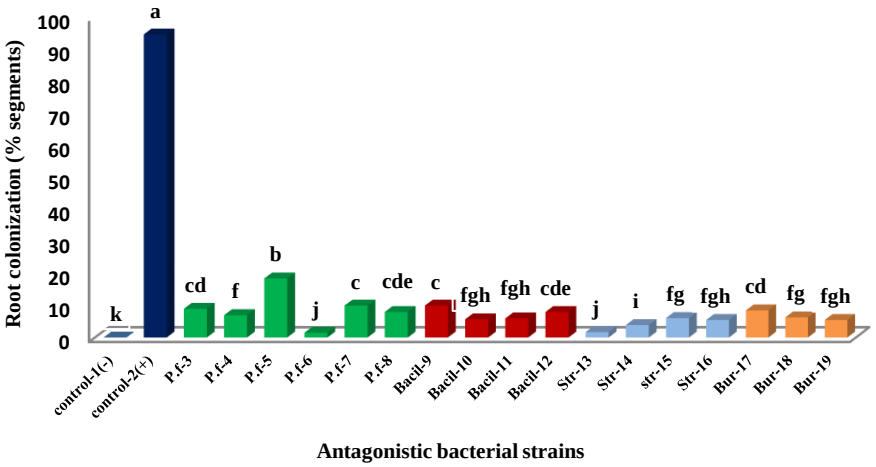

Fig. 5: Root segments colonization of Mashhadi melon plants by *Fom* race1/2, treated with antagonistic bacteria and non-treated control. Bacill (*Bacillus* sp.), P.f (*Pseudomonas fluorescens*), Bur (*Burkholderia* sp.), Str (*Streptomyces* sp.), control (-) (non inoculated by bacteria and ***Fom***),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

during the experiment. Root colonization of inoculated control and bacterial treated plants indicated that root sections of the control plants were colonized 94.65%+1.1 of by *Fom*, however, in contrast, colonization in plants were treated by bacterial was only 0 to18.5 %. *Fom* could not colonize plants roots treated with *P. fluorescens* strain 6 and *Streptomyces* strain 13 like other treatments (Figure 5). The amount of colonization of *Fom* in the presence of various strains differed (Figure 5). It was detected that colonization of bacterial in infested soil promoted growth of plant and dry weight more than the plants control that were inoculated (Figure 6). *P. fluorescens* strain 6 increased plant growths in infested soil to 1.53 g plant^{-1}+0 compared to control in the presence of pathogen 0.29 g+1.6 (Figure 6). Other strains depicted various figures of growth increasing.

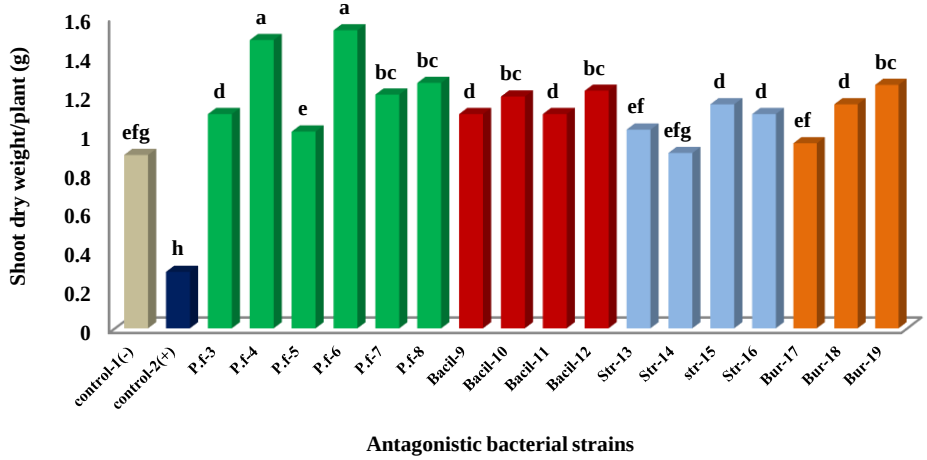

Fig. 6: Shoot dry weight of Mashhadi melon plants treated with antagonistic bacteria in soil infested with *Fom* race1/2 compare to control Bacill (*Bacillus* sp.), P.f (*Pseudomonas fluorescens*), Bur (*Burkholderia* sp.), Str (*Streptomyces* sp.) control (-) (non inoculated by bacteria and ***Fom***), control (+) (inoculated with ***Fom***). Different letters show significant differences between means of the treatments by the Duncan's test, 1% significance level.

Discussion:

Among 225 strains of bacterial isolated from rhizosphere of melon plants (symptomatic and symptomless) 17 strains including *P. fluorescens*, *Burkholderia* sp., *Bacillus* sp. and *Streptomyces* sp. were detected as antagonistic agents and capable to reduce infection of *Cucumis melo* by *F. oxysporum* f.sp. *melonis*. Under laboratory condition *Burkholderia*, *Bacillus*, *P. fluorescens*, and *Streptomyces* were able to produce antibiotic, in addition *P. fluorescens* could produce siderophore and *Bacillus* secreted exogenous cell liquids that all were able to control disease under glasshouse and growth chamber condition. Antibiotic Production, exogenous cell liquids and siderophore amongst tested bacteria has been reported by other researchers, too [37,16,33]. The amount of colonization of root by bacterial strains in the absence of pathogen differed. All bacterial strains

elevated growth in the absence and presence of pathogen. The highest growth promotion was by *P. fluorescens* strain 6 and could not differ considerably from strain 4 (Figure 6). But, there was an alteration amongst strains and species on ability to growth promoting. Growth promotion was observed two weeks after planting the seeds. Several researchers have been reported growth promotion on vascular plants amongst bacterial strains such as radish [15,12] and sugar beet [36,39]. Moreover, in rhizosphere area, growth promoting bacteria cause shift in microbial population [36,15].

They adjust the materials translocation such as spermine, putrescine and spermidine and carbon source of roots supply as muciliageneus substance to the bacteria [17]. *Burkholderia* strains 18 and 19 that shown lower antagonistic response, could not control the disease efficiently. After root colonizing, growth promoting bacteria enter vascular systems and spread in leaf, stem and other plant organs and produce inhibitor materials which can be induced resistance in plant [5,41]. Seed treatment of melon with *P. putidae* controlled *Fom* under field condition [4]. Seed inoculation with antagonistic bacteria is a useful method to manage certain plant pathogens that are soil borne [38]. Strains of rhizobacteria have been illustrated to elevate resistance against plant pathogens [44,23,42].

Disease reduction could be as a result of several biological factors. Leeman *et al*. [18] indicated that the siderophore of *P. fluorescens* can act as an elicitor of induced systemic resistance. Though no efforts were performed to study the *Fom* reduction mechanism in our bacterial strains, Elad and Baker [6] introduced siderophore production in cucumber with reducing chlamydospore germination in *F. oxysporum* f. sp. *cucumeriu.* Other researchers demonstrated that production of siderophore was reducing agent in vascular wilt fusaria [32,25,6,43]. In 2005, Soleimani *et al*. reported that wheat seed treated with antagonistic rhizobacteria not only decreased the disease severity, even they showed affirmative efficacy on yield and growth of wheat cultivar.

In our study, it was depicted that the bacterial strains isolated from rhizospheric soils of melon are able to increase growth of "Mashhadi melon" and reduction of vascular wilt disease by producing siderophore or other substances such as exogenous cell liquid and antibiotic. Use of bacterial antagonists under field conditions can be assessed as a section of disease management.

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