بررسی فعالیت آنتاگونیستی و افزایش رشد گیاهی در باکتری های اندوفیت گوجه فرنگی در مواجه با *Verticillium dahliae* در شرایط آزمایشگاهی و گلخانه

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چکیده

مقدمه: در سال‌های اخیر علاقه زیادی به استفاده از دست آوردهای بیولوژیکی بعنوان جایگزینی برای آفتکش‌ها و کودهای شیمیایی برای مدیدیت بیمارگرهای گیاهی و بهبود تولید محصول به وجود آمده است. به دلیل وجود پتانسیل‌های کارآمد کنترل زیستی در باکتری‌های اندوفیت، در سال‌های اخیرگرایش به استفاده از این باکتری‌ها بعنوان افزایش دهنده رشد گیاه و عوامل کنترل زیستی در حال افزایش است. هدف از این تحقیق ارزیابی توانایی کنترل زیستی و افزایش رشد گیاه توسط باکتری‌های اندوفیت در مواجه با قارچ *Verticillium dahliae*در شرایط آزمایشگاه و گلخانه بود.

مواد و روش‌ها: باکتری‌های اندوفیت جداسازی شد و کنترل زیستی آن‌ها براساس روش کشت متقابل انجام گرفت. ویژگی‌های ضد قارچی و افزایش دهندگی رشد گیاه مانند تولید ترکیبات فرار، آنتی‌بیوتیک، پروتئاز، کتیناز، سیانید هیدروژن، سیدرفور، ایندول استیک اسید و انحلال فسفات ارزیابی شدند. اثرات آن‌ها بر جوانه زنی و فاکتورهای رشدی گیاهچه‌ها در آزمایشگاه و اثرات آن‌ها بر کنترل بیماری و رشد گوجه‌فرنگی در گلخانه بررسی شدند.

نتایج: طبق نتایج آزمون کشت متقابل ایزوله‌های FS67، FS167، FS300 و FS339 فعالیت ضد قارچی معنی داری را نشان دادند که به ترتیب بعنوان *Pseudomonas mosselli*، *P. fuorescence*، *Stenotrophomonas maltophilia* و *Acinetobacter calcoaceticus*شناسایی شدند. همه ایزوله‌های آنتاگونیست بیش از یک نوع ازترکیبات ضد قارچی و افزایش دهنده رشد گیاه را در شرایط آزمایشگاه تولید کردند و قادر به افزایش جوانه زنی بذر و فاکتورهای رشدی گیاهچه‌ها بودند. آن‌ها باعث کاهش بیماری و بهبود رشد گیاهان در مواجه با *V. dahliae* در گلخانه شدند.

بحث و نتیجه‌گیری: مطالعه حاضر نشان داد که باکتری‌های اندوفیت گوجه فرنگی دارای پتانسیل کنترل زیستی و کود زیستی هستند و عوامل مناسبی برای جایگزینی مواد شیمیایی در مدیریت *V. dahliae* می‌باشند. نتایج نشان می‌دهد که آن‌ها با مکانسیم‌های مختلفی ممکن است باعث افزایش رشد و سلامتی گیاه گوجه فرنگی شوند و احتمالاً اغلب آن‌ها بیش از یک مکانسیم را به کار می‌برند.

واژه‌های کلیدی: کنترل زیستی، پژمردگی ورتسیلیومی،باکتری‌های افزایش دهنده رشد گیاه

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Assessment of antagonistic and plant growth promoting activities of tomato endophytic bacteria in challenging with *Verticillium dahliae* under *in-vitro* and *in- vivo* conditions

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Abstract

Introduction: In recent years, there has been a considerable interest in the use of biological approaches, as an alternative to chemical fertilizers and pesticides to management of plant pathogens and improvement of crop productivity. Recently, endophytic bacteria have gained attention due to their efficient bio-control and plant growth promoting potentials. The objective of this study was to evaluate bio control and plant growth promoting ability of endophytic bacteria in challenging with *Verticillium dahliae* under i*n-vitro* and greenhouse conditions.

Materials and methods**:** Endophytic bacteria were isolated from tomato plants and their bio-control activity was screened based on dual culture method**.** Antifungal and their plant growth promoting traits such as production of volatile compounds, antibiotics, proteases, chitinases, hydrogen cyanide, siderophore, indole acetic acid and phosphate solubilizing were evaluated. Their effects on seed germination and growth parameters of seedlings under *in-vitro* condition and on the control of disease and tomato growth were evaluated in greenhouse.

Results: In dual culture tests, FS67, FS167, FS300 and FS339 isolates showed significant antifungal activity and they were identified as *Pseudomonas mosselli*, *P. fuorescence*, *Stenotrophomonas maltophilia* and *Acinetobacter calcoaceticus*, respectively. All strains produced several kinds of antifungal and growth promoting agents under *in-vitro* conditions. They increased seed germination and growth parameters of seedlings. They also reduced the disease and improved the growth parameters of the plants in challenging with *V. dahliae* in greenhouse.

Discussion and conclusion: The present study has shown that these endophytic bacteria have the bio-control and bio-fertilizer potentials, which make them suitable candidates as an alternative tool of chemicals in management of *V. dahliae*. Results indicated they might enhance tomato plant growth and health via various mechanisms and most of them probably employ more than one of these mechanisms.

Key words: Bio-control, *Verticillium* wilt, plant growth promoting bacteria

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Introduction

Verticillium wilt, caused by the soil born fungus *Verticillium dahlia* Kleb, is one of the most important plant diseases worldwide. It is a devastating plant disease, which can affect both annual crops as well as woody perennials, hence inducing major food losses (1). *V. dahliae* invades roots and causes wilt diseases through colonization in xylem tissue of host plants. Synthetic chemical fungicides have been used in reducing the plant diseases for many years (2). However, because of the ecological and economical reasons, the management of *Verticillium* wilt by conventional chemical methods is raising concerns. It seems appropriate to search for an alternative or supplement control strategy (3). In recent years, there has been a considerable interest in the use of biological approaches, as an alternative to chemical fertilizers and pesticides to improve crop productivity (4,5). Using microbial antagonists such as endophytic bacteria to control phytopathogens are now of growing interest (6). Bacterial endophytes are microorganisms that colonize living internal tissues of plants without causing damage (7). They colonize a large number of plants, including monocots and dicots. Varieties of endophytes have been isolated from various species of plants (hosts) and are not in generally organ specific. Thus, they may be isolated from roots, leaves, and stems, and a few from inflorescences and fruits (8). They can act both as growth promoters and as bio-control agents. Entophytic bacteria suppress disease caused by soil born pathogen such as *V. dahliae* (9,10, 11), and have beneficial effects on plant growth and yield (7). The beneficial effects of bacterial endophytes on their host plant occur via variety of mechanisms (12) including antibiosis (antibioticproduction), growth promotion, inducing host defenses (systemic resistance), parasitism, competition and signal interference (quorum sensing) (7,13). Considering many beneficial features of bacterial endophytes, there is an increasing interest over the last few years in using them as bio-fertilizers and biological control agents (12). *V. dahliae* is one of the important pathogen of tomato (*Solanum lycopersicum* L.) in Iran. In the present study, we discuss potential antifungal mechanisms and plant growth promoting traits of these bacteria and demonstrate their effects on disease development in greenhouse experiments, which can be further developed as bio-control agents against *V. dahliae* for tomato crop.

Materials and methods

Sample collection and isolation of bacteria: Tomato plants including stem, leaf and root were collected from different tomato fields of 14 sites in Hamedan province, Iran**.** Endophytic bacteria were isolated from the internal tissues of roots, leaves and stems according to Hung and Annapurna (14).

Fungal pathogen: The fungus *V. dahliae* used in this study was obtained from Agricultural Research Centre of Hamadan province.

Screening of bacterial isolates for antagonism against *V. dahlia*: The total number of 80 bacterial isolates was evaluated for their antagonistic activity against *V. dahlia* using a dual culture technique (15). A 5 mm agar disc of a ten-day old culture of fungal pathogen was placed in the center of potato carrot agar (PCA) plates. Bacterial suspension (2×10^8 cfu ml^{-1}) was streaked parallely on each side of the fungal disc at a distance of 2 cm. The plates with only fungal disc, without bacterial streaks, were considered as the control. The inoculated plates were incubated at 28 ± 2°C. Colony diameter of the fungal pathogen was measured and compared with the control. The Inhibition percentage of the pathogen by the antagonistic bacteria over the control

was calculated by using the formula as follows (16):

$$I = (\frac{C - T}{C}) \times 100$$

Where, I= Inhibition rate of mycelium growth; C= (a control value) represents the radial growth of the fungus in control sets without bacteria, T= radial growth of the fungus in sets inoculated with the bacterium. The experiments were conducted in triplicate in a completely randomized design.

Identification of potential antagonistic bacteria: The selected antagonists were identified based on their reactions to standard biochemical and phenotypic tests from *Bergey's Manual of Systematic Bacteriology* (17) and Schaad *et al.* (18). Furthermore, the molecular characterization was done through partial sequencing of their 16S r-DNA.

Assessments of antagonistic and plant growth promoting mechanism(s) of antagonist bacteria: Bacterial isolates showing significant antagonistic activity against *V. dahliae*, were further examined for explanation of the possible mechanism(s) underlying their antagonistic behavior.

Screening for the production of fungal cell wall-degrading activity: Protease activity (casein degradation) was determined by the formation of a clear zone around the bacterial growth, in skimmed milk agar (19), which indicated a positive proteolytic activity. Chitinolytic activity was screened by plating on colloidal chitin agar medium. Clearance halos indicating the enzymatic degradation was measured after 5 days of incubation at 28 ± 2°C (20). Cellulase production was screened in medium containing 1 g of K_2HPO_4, 0.5 g of $NaNO_3$, 0.5 g of KCl, 0.01 g of $FeSO_4$ and 1000 ml water. A piece of 9 × 1-cm wathman filter paper was placed in a tube containing 9 ml of the cellulose solution and after inoculation with one loopful of each bacteria start culture were incubated at 28±2°C for 3 week (21).

Production of diffusible antifungal metabolites: To determine the production of diffusible antifungal metabolites by antagonistic bacteria, Montealegre *et al.* (22) methods were used (with some modifications). Overnight activated antagonistic bacterial suspension (2×10^8 cfu ml^{-1}) were stab inoculated in the center of the plates covered by a cellophane membrane and incubated at 28 ± 2°C for 72 hours. Afterwards, the membrane with the bacterial growth was removed from the petri plate and used two drops of chloroform in petri plate lids, kept upside down for 20 minutes. Afterward they were inoculated with a 5 mmplug of the pathogen in the center of the plate and the control PCA plate inoculated with sterile distilled water in place of bacteria and further inoculated with the pathogen at 28 ± 2°C. When fungal pathogen was grown completely in control petri plate, the colony diameter of treatments was measured and compared with the control. Inhibition percentage of fungal growth was calculated as mentioned before. Three replicates of each treatment were performed in a completely randomized design.

Production of volatile antifungal metabolites: The production of volatile metabolites was tested by the paired plate technique of Fiddaman and Rossall (23), with some modifications. A petri plate containing nutrient agar (NA) medium was streak inoculated with 500 microliter of antagonistic bacterial suspension (2×10^8 cfu ml^{-1}). A second petri plate containing PCA was inoculated with a 5 mm plug of the activated pathogen at the center of the plate. Both half plates were sealed together and the paired plates were incubated at 28 ± 2°C. Control paired plates were designed with only the test fungus on PCA half plate inverted over unstreaked NA half plate. After incubation period (when tested

fungus was grown completely in the control plates), colony diameter of the fungus was measured and compared with the control set. Inhibition percentage of the radial growth of the fungus was calculated as mentioned before. Three replicates of each treatment were performed in a completely randomized design.

Siderophore production: Siderophore production was determined by the chrome azurol S agar assay based on change in the medium color from blue to orange after 3 days (24).

Hydrogen cyanide (HCN) production: HCN was estimated qualitatively by the sulfocyanate colorimetric method (25). The bacteria were grown in NA amended with glycine (4.4 g L^{-1}). One sheet of whatman filter paper No.1 (7 cm diameter) was soaked in 1% picric acid (in 10% sodium carbonate; filter paper and picric acid was sterilized separately) for a minute and placed inside petri dish lids. The plates were covered with cellophane membrane and were incubated at 28 ± 2° C for five days. Degree of HCN production was evaluated according to the color change on the filter paper, ranging from yellow to reddish brown.

Indole Acetic Acid (IAA) Production: IAA production by bacteria was carried out according to Gordon and Weber (26) by Salkowsky reagent. Quantification of IAA was done by measuring the absorbance in a spectrophotometer at 530 nm. A standard curve was plotted to quantify the IAA (µg ml^{-1}) present in the culture filtrate.

Phosphate Solubilizing: To detect the phosphate solubilizing bacteria, the strains were streaked onto Pikovskaya's agar medium, pH 6.8 (27). Strains that induced clear zone around the colonies after 3 days were considered as positive.

Evaluation of the effect of endophytic bacteria on seed germination and seedlings growth by seed priming: This test was carried out using seed bacterization method (28), with some modification. Tomato seeds cultivar "CALL j N 3" were surface sterilized with 1% sodium hypochlorite for 1 min then, they were rinsed 3 times in sterile water and dried on sterile tissue paper and then were soaked for 12 h under shaking (150 rpm) in the antagonists suspension (10^8 cfu/ml) and carboxymethyl cellulose (CMC) then the seeds were dried. Seeds treated with distilled water were used as control treatments. The seeds were placed in glass flasks with 200 mL of water agar (1%) medium and then placed in a growth chamber with a photoperiod of 16 h light/8 h dark and a light intensity of 200 $molm^2s^{-1}$ at 22°C. Growth parameters (length of the stem and main root, fresh and dry weight of biomass) were recorded two weeks after sowing. Germination rate of seed and vigor index of seedling were calculated using the following formulas (29).

$$\text{germination}\% = \frac{\text{number of seeds that germinated}}{\text{number of seeds on the tray}} \times 100$$

$$\text{seedling vigor index} = \text{germination (\%)} \times \text{seedling length}$$

Evaluation of the effect of volatile organic compounds (VOCs) of endophytic bacteria on growth of seedlings: We evaluated the plant growth promotion by VOCs emission from antagonist strains, according to the Orozco-Mosqueda *et al*. (28) with some modification and as mentioned above, except for bacteria (approximately 10^6 cfu/ml) were inoculated in plates with NA medium. Each plate was placed within the flasks containing five tomato seeds without any direct bacteria-plant interaction. Control experiments did not contain bacterial inoculum. Four flasks with five tomato seeds were prepared for each treatment. Growth parameters were recorded after two weeks as mentioned above.

Greenhouse tests: Three potential antagonistic bacterial isolates (FS67, FS167, FS300 and FS339) were evaluated in a greenhouse for their antagonistic

potential against *V. dahliae*. For the preparation of the fungal inoculum, a mixture containing 100 g of quartz sand, 6 g maize meal and 25 ml distilled water was used. The medium was inoculated with agar plugs of the fungus and incubated for 2 weeks at a temperature of 25 °C (30). Pot mixture (1000 g) was prepared by mixing red soil, sand and farm yard manure at 3:2:2 (autoclaved) and filled in plastic pots followed by inoculation with *V. dahliae* inoculum (20% of the pot weight). Inoculum was mixed thoroughly with the pot mixture. Prior to planting , the roots of twenty day-old seedlings cultivar "CALL j N 3" were dipped in a suspension of antagonistic bacteria (2×10^9 cfu ml^{-1}) for 20 minutes. The control plants were dipped in distilled water and planted in infested soil and negative control was inoculated with only *V. dahliae*. Three replicates of each treatment were performed in a completely randomized block design. The experiments were conducted under greenhouse conditions (18 h light periods, 100 mEm^{-2} s^{-1} 25 ± 2°C) in six-week period. The symptoms were rated one month after inoculation on a 0 to 4 scale according to Tjamos *et al.* (31) with some modifications. A scale 0–4 was used according to the percentage of plant tissue affected by chlorosis and necrosis (0= absence of symptoms, 1= light chlorosis in 1–9% of plant canopy, 2= moderate chlorosis and necrosis (10–25%), 3= severe chlorosis and moderate necrosis (26– 50%) and 4= plant death). The percent disease index (PDI) was calculated as follows (32):
Disease index %= (∑(rating×number of plants rated) / Total number of plants × highest rating) × 100

In addition, growth parameters including stem and root length and fresh and dryweight of tomato plants were recorded.

Statistical analysis: The data obtained in this study was subjected to the analysis of variance (ANOVA), using the SAS 9.1.3 statistical software, for a completely randomized design and completely randomized block design. The means were separated by Duncan's multiple range tests with $P< 0.05$ being accepted as significant.

Results

Screening of bacterial isolates for antagonism against *V. dahlia*: Among a total number of 80 isolates, obtained from internal tissues of tomato, FS67, FS167, FS300 and FS339 strains (isolated from root) showed *in-vitro* antifungal activity against *V. dahlia* (Figure 1). Inhibition was clearly discerned by limited growth of fungal mycelium in the inhibition zone surrounding a bacterial colony. Average inhibition percentage of the pathogen was varied significantly between the isolates ($P< 0.05$) and strain FS300 had the highest inhibition percentage of radial growth (96.6 %) of *V. dahliae* (Table 1).

Identification of antagonistic bacteria: According to the morphological and biochemical characteristics and molecular identification FS67, FS167, FS300 and FS339 isolates identified as *Pseudomonas mosselii, P.fluorescens, Stenotrophomonasmaltophilia* and *Acinetobactercalcoaceticus,* respectively.

Table 1- *In-vitro* average inhibition percentage of *Verticillium dahliae* by antagonistic bacteria isolated from tomato root, based on dual culture technique on agar plates.

Isolate	Inhibition percentageof radial growth
FS300	96.6 ± 0.7 [a] *
FS339	90.3 ± 0.4 [b]
FS167	83.3 ± 1.3 [c]
FS67	63.9 ± 1.3 [d]
Control	0
CV%	2

*The values given are mean (n= 3) with standard deviation. Values with different letter indicate significant differences ($P < 0.05$) according to Duncan's multiple range tests. FS67 (*Pseudomonas mosselii)*, FS167 (*P. fluorescens*), FS300 (*Stenotrophomonas maltophilia)* and FS339 (*Acinetobacter calcoaceticus*)

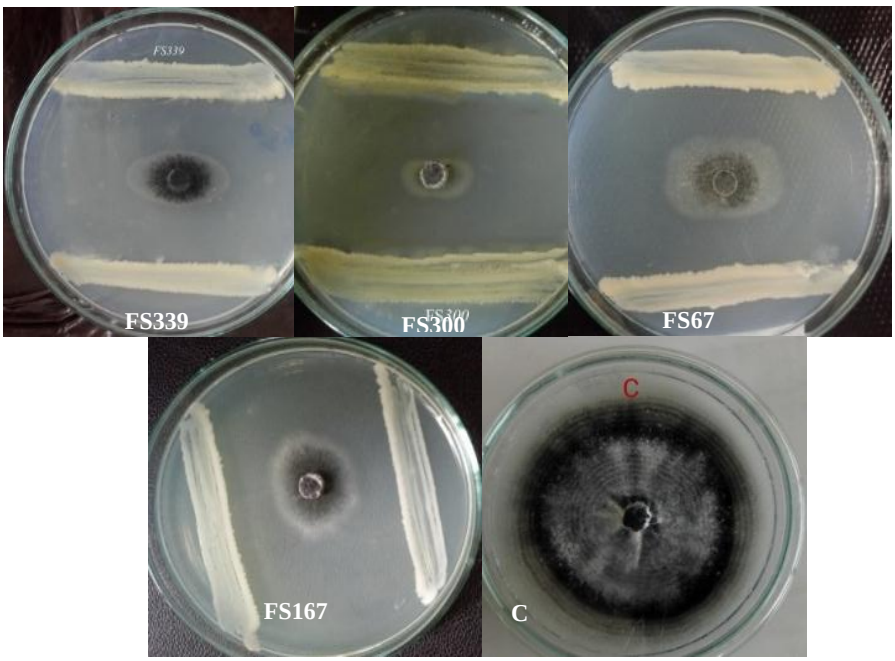

Fig. 1- *In-vitro* inhibition of *Verticillium dahliae* by antagonistic bacteria; FS67 (*Pseudomonas mosselii)*, FS167 (*P. fluorescens*), FS300 (*Stenotrophomonas maltophilia)* and FS339(*Acinetobacter calcoaceticus*) on the basis of dual culture technique. (C) control plate

Assessments of antagonistic and plant growth promoting mechanism(s) of antagonist bacteria: All of four bacterial isolates were able to produced more than one kind of antifungal compounds and plant growth promoting traits under *in-vitro* conditions but these traits were varied among isolates. All bacterial isolates were able to produce chitinase, its production was higher in *P.fluorescens.* Protease and HCN production were observed in all isolates except *P. mosselii* and *A. calcoaceticus*, respectively. According to our results from phosphate solubilizing ability of studied bacteria, all of them could solubilize phosphate but there were no significant differences among isolates. There was significant difference ($P< 0.05$) in IAA production by isolates and FS167 had highest amount of IAA (Figure 2). None of the isolates produced cellulase. The results have been shown in table 2.

Table 2- Production of various antifungal compounds by antagonistic bacteria isolated from tomato root against *Verticillium dahliae*.

Antagonist isolates				
Character	FS67	FS167	FS300	FS339
Siderophore production	+*	++	+	+
Protease production	-	+	+	+
Chitinase production	+	++	+	+
Cellulase production	-	-	-	-
HCN production	+	++	++	-
IAA production	++	+++	+	++
Phosphate production	+	+	+	+

*-: Nil; +: Low production; ++: Medium production, +++: High production. Antagonist isolates: FS67 (*Pseudomonas mosselii)*, FS167 (*P. fluorescens*), FS300 (*Stenotrophomonas maltophilia)* and FS339 (*Acinetobacter calcoaceticus*)

Volatile and diffusible antifungal metabolites: All the antagonistic isolates produced diffusible and volatile antifungal metabolites and the level of inhibition of mycelial growth of *V. dahliae* was varied significantly among them (P <0.05). Isolates *A. calcoaceticus* and *P. mosselii* showed maximum and minimum inhibition of 64% and 57 %, respectively due to diffusible antifungal metabolites. In volatile antifungal metabolite tests, isolates of *A. calcoaceticus* and S. *maltophilia* gave maximum and minimum inhibition of 73.5% and 55.8%, respectively (Table 3).

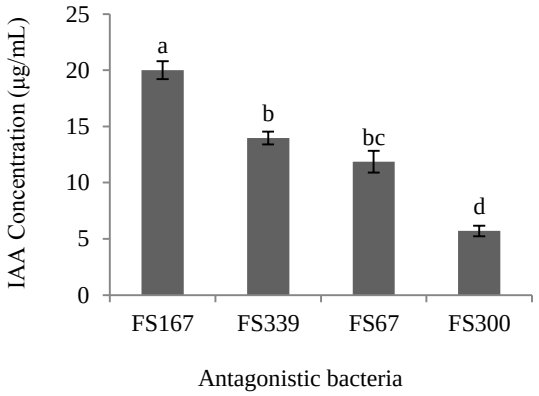

Fig. 2- Production of indole acetic acid (IAA) by antagonist isolates; FS67 (*Pseudomonas mosselii*), FS167 (*P. fluorescens*), FS300 (*Stenotrophomonas maltophilia)* and FS339 (*Acinetobacter calcoaceticus*, value (mean =3) with same letter indicate no significant differences (P< 0.05) according to Duncan's multiple range tests.

Table 3- Inhibition of *Verticillium dahlia* by diffusible and volatile antifungal metabolites produced by antagonistic bacteria isolated from tomato root.

Percentage inhibition of radial growth		
Antagonist isolates	Diffusible antifungal metabolites	Volatile antifungal metabolites
FS339	64 ± 0.7^{a}	73.5 ± 0.46^{a}
FS167	62.8 ± 1^{ab}	61.6 ± 0.8^{b}
FS300	60 ± 0.8^{b}	55.8 ± 1.3^{c}
FS67	57 ± 0.4^{c}	58.9 ± 0.8^{cb}
CV%	2.6	2.7

*The values given are mean (n= 3) with standard deviation. Values with different letter indicate significant differences according to Duncan's multiple range tests (P< 0.05). Antagonist isolates; FS67 (*Pseudomonas mosselii*), FS167 (*P. fluorescens*), FS300 (*Stenotrophomonas maltophilia)* and FS339 (*Acinetobacter calcoaceticus*)

Evaluation of the effect of endophytic bacteria on seed germination and growth of seedlings by seed priming: We tested capacity of the bacterial strains for inducing the growth of seedlings by seed bacterization in vitro directly (Figure 3). Tomato seeds priming with antagonistic bacteria, increased seed germination and all growth parameters (stem and root length, fresh and dry weight and vigor index). The plant growth promoting efficiency of antagonist isolates monitored by measuring seedlings biomass and results showed variation among seedlings treated with antagonists and the untreated control. Average fresh and dry weight and vigor index of tomato seedlings were significantly higher (P< 0.05) in seedlings treated with FS167 (*P. fluorescens*), FS300 (*S. maltophilia*) and FS67 (*Pseudomonas mosselii*) compared with the control (Table 4). However fresh and dry weight and vigor index of seedlings were higher in FS339 (*A. calcoaceticus*) treatment than control, there were no significant differences according to Duncan's multiple range tests (P< 0.05), between them. The highest effect on all growth parameters were obtained from the seedlings inoculated with *P. fluorescens* isolate.

Evaluation of plant growth promotion by VOCs emission of endophytic bacteria: With regard to the effect of VOCs on plant growth promotion, all strains except FS67 (*P. mosslii)*, improved seed germination and increased tomato seedling biomass in comparison with the control, but only FS167 (*P. fluorescens*) increased significantly vigor index, fresh and dry weight of seedlings (Table 5 and Figure 3).

Greenhouse evaluation of antagonist isolates for *Verticillium*-wilt disease control: In greenhouse evaluation, all four antagonist isolates reduced significantly *Verticillium*-wilt with compared to pathogen inoculated treatment. Disease index was 40.90, 53.5, 61.3, 63.9 and 86.1 with antagonist FS167 (*P. fluorescens*), FS339 (*A. calcoaceticus*), FS67 (*P. mosselii)*, FS300 (*S. maltophilia*) and pathogen treatment, respectively. FS167 (*P. fluorescens*) isolate had highest disease inhabitation (Figure 4-A). The plant growth promoting efficiency of antagonist isolates was monitored by measuring plant biomass. Average fresh and dry weight of tomato plants were significantly higher ($P< 0.05$) for plants treated with antagonist isolates compared with the pathogen-inoculated control (Figure 4-B).

Table 4- Effects of antagonist strains FS67, FS167, FS300 and FS339 isolated from tomato root, on growth parameters of tomato seedlings in seed priming evaluation.

Antagonist isolates						
Character	FS67	FS167	FS300	FS339	C	CV%
ES(%)	95 ± 5^{a}*	100 ± 0^{a}	100 ± 10^{a}	95 ± 10^{a}	80 ± 8^{b}	6.9
RL(cm)	4.3 ± 1^{b}	5.7 ± 0.4^{a}	4 ± 0.5^{b}	3.3 ± 0.5^{b}	2.9 ± 0.7^{b}	13.7
SL(cm)	6 ± 0.9^{bc}	9 ± 1^{a}	6.2 ± 1^{bc}	7.2 ± 1^{ab}	3.1 ± 0.6^{c}	10.6
VI	405.3 ± 16.5^{b}	583.2 ± 16^{a}	430.4 ± 10^{b}	329.39 ± 19^{cd}	303.7 ± 13^{d}	14.2
FW(mg)	30.2 ± 1.6^{b}	44.1 ± 1.3^{a}	28.8 ± 1.7^{b}	23.5 ± 1.7^{c}	16.6 ± 2.4^{C}	10
DW(mg)	3.1 ± 0.3^{b}	4.9 ± 0.5^{a}	3 ± 0.5^{b}	3 ± 0.3^{bc}	1.8 ± 0.6^{c}	17.3

*Values (n=20) with the different letter(s) indicate that means differ significantly by Duncan's multiple range test ($P< 0.05$).C: (un-inoculated control), Character; ES: mean emergence of seedling (%), RL: mean root length, SL: mean shoot length, FW: mean fresh weight, DW: mean dry weight and VI: mean vigor index (was determined (mean root length + mean shoot length) × % germination). Antagonist isolates; FS67 (*Pseudomonas mosselii)*, FS167 (*P. fluorescens*), FS300 (*Stenotrophomonas maltophilia)* and FS339 (*Acinetobacter calcoaceticus*).

Table 5- Effects of antagonist strains FS67, FS167, FS300 and FS339 isolated from tomato root on growth parameters of tomato seedlings by volatile organic compounds produced by antagonist *in-vitro* evaluation.

Antagonist isolate						
Character	FS67	FS167	FS300	FS339	C	CV%
ES(%)	90 ± 1^{ab}*	100 ± 0.8^{a}	95 ± 1.7^{a}	95 ± 0.7^{a}	80 ± 0.8^{b}	9.9
RL(cm)	3.7 ± 0.6^{a}	4.3 ± 0.4^{a}	3.5 ± 0.6^{a}	3.6 ± 0.6^{a}	2.9 ± 0.7^{a}	15.9
SL(cm)	4.2 ± 0.8^{b}	7.7 ± 0.8^{a}	5.3 ± 0.9^{b}	4.9 ± 0.7^{b}	3.1 ± 0.6^{a}	16.1
VI	347.1 ± 11.3^{b}	496.5 ± 17^{a}	331.4 ± 15.2^{b}	316.5 ± 15.2^{b}	303.7 ± 13^{b}	16.8
FW(mg)	16.4 ± 2.6^{b}	32.6 ± 1^{a}	22.6 ± 0.7^{b}	21.6 ± 1.7^{b}	16.6 ± 2.4^{b}	13
DW(mg)	1.9 ± 0.5^{ab}	3.3267 ± 0.4^{a}	2.9 ± 0.6^{ab}	2.7 ± 1^{ab}	$1.8\pm0.6\pm0.4^{b}$	19.2

*Values (n=20) with the different letter(s) indicate that means differ significantly by Duncan's multiple range test ($P< 0.05$).C: (un-inoculated control), Character; ES: mean emergence of seedling (%), RL: mean root length, SL: mean shoot length, FW: mean fresh weight, DW: mean dry weight and VI: mean vigor index (was determined (mean root length+mean shoot length)×% germination). Antagonist isolates; FS67 (*Pseudomonas mosselii)*, FS167 (*P. fluorescens*), FS300 (*Stenotrophomonas maltophilia)* and FS339 (*Acinetobacter calcoaceticus*).

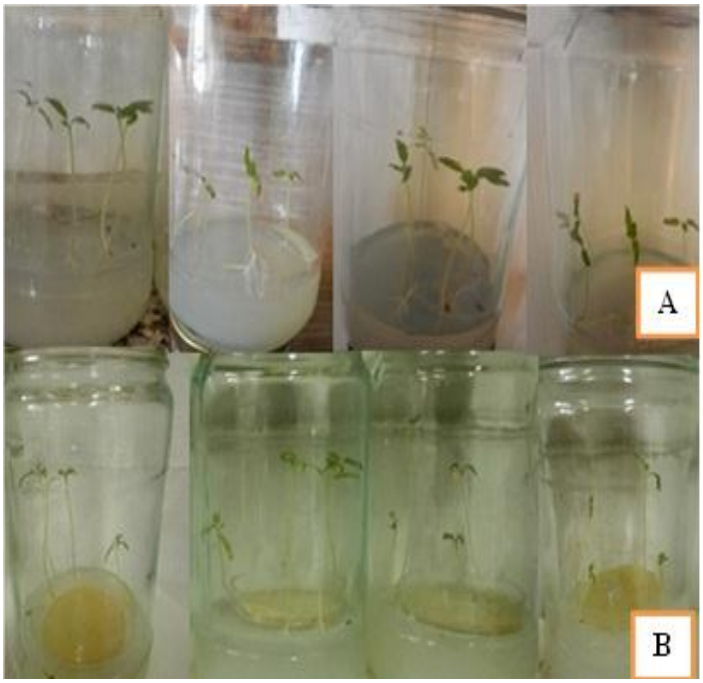

Fig.3- General view of the tomato seedlings after two weeks of interaction with antagonist strains FS67 (*Pseudomonas mosselii)*, FS167 (*P. fluorescens*), FS300 (*Stenotrophomonas maltophilia)* and FS339 (*Acinetobacter calcoaceticus*) to evaluating of their effect on seedlings growth. A: Seed priming test (direct interaction) and B: effect of volatile organic compounds test.

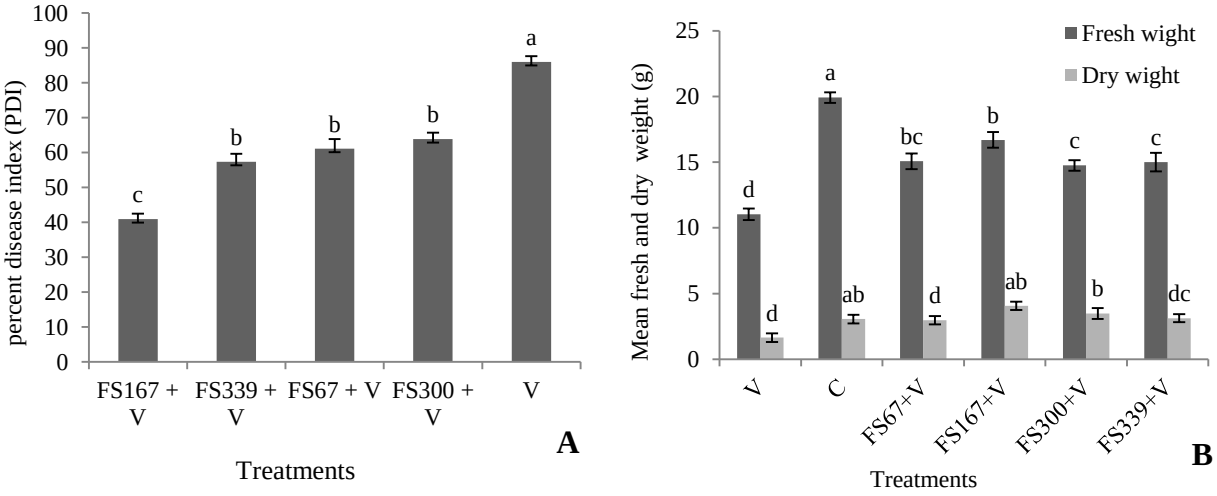

Fig. 4- A: Antagonistic effects of endophytic bacteria on tomato *Verticillium*-wilt disease in greenhouse. B: Plant growth promoting effect of antagonist strains on fresh and dry weight of tomato plants in challenging with *Verticillium dahliae* in greenhouse conditions. Bars represent the mean ± standard error values. Values with different letter indicate significant differences ($P< 0.05$) according to Duncan's multiple range tests. Treatments: FS67 (*Pseudomonas mosselii)*, FS167 (*P. fluorescens*), FS300 (*Stenotrophomonas maltophilia*) and FS339 (*Acinetobacter calcoaceticus*) in combination with pathogen. V: *Verticillium dahliae* inoculated control.

Discussion and conclusions.

Nowadays, sustainable agriculture is an important subject in crop production and being attempted to find alternative ways to reduce the use of chemicals in agriculture. For this purpose, plant growth-promoting bacteria as well as endophytic bacteria are one of the promising tools. Bacterial endophytes are able to lessen or prevent the deleterious effects of certain pathogenic organisms. There are similar reports of antagonistic activity of endophytic bacteria against *V. dahliae* (3; 33,34).

In most plants, roots have the higher numbers of endophytes compared with aboveground tissues (35). In this study, we reported antifungal properties of endorhizoplane bacteria against *V. dahliae*, an important soil born fungus. *P. mosselii*, *P. fluorescens*, *S. maltophilia* and *A. calcoaceticus* strains inhibited *V. dahliae* growth *in vitro* and in greenhouse conditions. There are several reports about the plant growth promoting and antifungal activity of *P. mosselii* (36, 37) *P. fluorescens* (38) *S. maltophilia* (39) and *A. calcoaceticus* (40) strains in many crops.

The diversity of endophytic bacteria might reflect the large number of probable mechanisms of action to disease suppression. (12). Antagonism is known to be mediated by a variety of compounds of microbial origin, e.g., bacteriocins, enzymes, toxic substances, volatiles, and indirectly by antagonizing pathogenic fungi by the production of siderophores, chitinase, antibiotics, fluorescent pigments and cyanide (41). In this study, bacterial strains produced at least one of the antagonistic agents such as HCN, siderophores, chitinase and protease that are involved in their bio-control activities. Production of chitinase, (a hydrolytic enzyme capable of degrading fungal cell wall components) is one of the bio-control mechanisms in many bio-control agents (42). Furthermore, in the present study applications of chemical pesticides and fertilizers have caused many environmental problems, since most research is these strains produced antibiotics and volatile compounds that significantly inhibited fungal mycelia growth. Volatiles produced by some bacterial strains trigger growth promotion and induce systemic resistance in plants (43, 44).

These metabolites make chemical communication between plant and bacteria resulted to induce the growth of plants and decreased disease severity. In addition, they enhance root colonization by these bacteria and have effect on primary root growth and development (44). Seed priming results showed all endophytic bacteria significantly increased seed germination and growth of seedlings. Although, in VOCs emission test, only *P. fluorescens* significantly increased seed germination and growth of seedling as well as seed priming. These results, suggested that volatile components of *P. fluorescens* are capable of increasing seedling growth, but in other strains production of diffusible and volatile compounds and their co-stimulation effects might be the efficient mechanism to promote seedlings growth.

Seed vigor is mainly determined based on the seedling length. Vigor index reflects the seedlings health, establishment and the state of final productivity of the plant (46). According to the results obtained here, since the seedling length was significantly increased by bacterial inoculations, the seed vigor enhancement was anticipated, as it was. This has been approved by other researchers who said the effective plant growth promoting bacteria must be able to establish themselves and colonize plant to reach at an appropriate density sufficient to produce beneficial effects (47).

Vegetative growth is an important growth phase in many crops as it determines the amount of biomass production. There is evidence that bacterial

influence on the plant growth is also an important determinant in bio-control (48). In the present study, all antagonistic bacterial strains increased growth parameters of tomato and reduced disease in greenhouse evaluation. Therefore, the increase of tomato biomass, following inoculation by *P. mosselii, P. fluorescens, S. maltophilia* and *A. calcoaceticus* might be one of their bio-control mechanisms. Plant growth enhancement mechanisms induced by plant growth promoting bacteria (PGPB) include the production of phytohormones such as indole-3-acetic acid (IAA), nitrogen fixation, phosphate solubilization and iron sequestration by bacterial siderophores (41). The beneficial effects of PGPBon growth of many plants can be partly explained by their ability to produce phytohormons. Production of IAA from all treated bacteria can explain the different effectiveness of beneficial bacteria on tomato growth. There are reports that showed IAA produced by endophytic bacteria *Pseudomonas, Acinetobacter* and *stenotrophomonas* involved in root growth regulation and protecting plants against adverse conditions (49, 50). In addition, solubilizing of phosphate can be another reason for growth enhancement of tomato by these bacteria. Phosphorus is one of the major nutrient requirements for plant growth (51). According to our result from phosphate solubilizing ability of studied bacteria, all of them could solubilize phosphate but there was not a significant difference between them.

Siderophores are involved in both plant growth promotion and plant protection (52). Most microorganisms are competing to acquire available resources. Some evidences are available to show that bacterial siderophores do play a role in competition in rhizosphere which indirectly becomes beneficial for plant growth by endophytes (53). Most microorganisms are competing to acquirt various limiting nutrients in the plant rhizosphere, one of them is iron. Siderophore production by PGPB, sequester most of the available Fe3+ in the rhizosphere, force the pathogens for iron starvation, and caused pathogen suppression (54). In addition, siderophores are also involved in the induction of plants defense against pathogens and improved plants health and growth indirectly (55).

It is assumed that endophytic organisms are better bio-control agents compared to rhizospheric bacteria based on the following reasons:

(A) They do not compete for nutrition and/or niche in the apoplast and are also more adapted to environmental changes (56); (B) bacterial endophytes are able to colonize an ecological niche similar to that of vascular wilt pathogens favors them as potential bio-control agents against wilt diseases. (57); (C) The endophytic niche offers a unique habitat to control pathogens, since the endophyte is not subject to influence the environment directly and bacterium is within a stable environment (7).

In the present investigation, functional characteristics of *P. mosselii, P. fluorescens, S. maltophilia* and *A. calcoaceticus* have been determined. All strains could control the *Verticillium*-wilt, due to the plant growth promoting and antifungal activity and they may be considered as inoculants for plant growth and plant protection against tomato *Verticillium*-wilt disease. Several possible disease suppression mechanisms of beneficial bacteria were proposed. The optimization and improvement of the strategies employed in the endophytic research can help finding effective and competent bio-control bacterial endophytes. Additionally, using genomic technologies in investigating the bio-control potential of bacterial endophytes can deepen our knowledge of their mode of action and understanding their potential in agro-ecosystem as biological control agents.

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References

(1) Eljounaidi K., Lee SK., Bae H. Bacterial endophytes as potential bi ocontrol agents of vascular wilt diseases – review and future prospects, Biological Control 2016;103: 62–68.

(2) Tjamos E., Rowe RC., Heale JB., Fravel DR. Advances in Verticillium research and disease management. Minnesota: American Phytopathology Society.St.Paul; 2000.

(3) Berg G., Fritze A., RosKot N., Smalla K. Evaluation of potential bio-control rhizobacteria from different host plants of Verticillium dahliae Kleb. Journal of Microbiology 2001; 91:963–971.

(4) Rosas SB., Avanzini G., Carlier E., Pasluosta C., Pastor N., Rovera M. Root colonization and growth promotion of wheat and Maize by Pseudomonas Aurantiaca SR1. Soil Biology and Biochemistry 2009; 41: 1802-1806.

(5) Dastager SG., Deepa CK., Pandey A. Plant growth promoting potential of Pontibacter Niistensis in cowpea (Vigna Unguiculata (L) Walp)Applied Soil Ecology 2011;49:250-255.

(6) Leifert C. Challenges for endophyte research: the need to focus on food security. In: Schneider C., Leifert C., Feldmann F., editors. Endophytes for plant protection: the state of the art. Proceeding of the 5th international symposium on plant protection and plant health in Europe. Berlin, Germany; 26-29 May 2013: 2-32.

(7) Bacon CW., Hinton DM. Bacterial endophytes: the endophytic niche, its occupants, and its utility. In: Gnanamanickam SS., editor. Plant-Associated Bacteria. Netherlands: Spinger; 2006: 155-194.

(8) Kobayashi DY., Palumbo JD. Bacterial endophytes and their effects on plants and uses inagriculture. In: Bacon CW., White JF., editors. Microbial entophytes.New York, Marcel Dekker, Inc; 2000:199-233.

(9) Erdogan O., Benlioglu K. Biological control of Verticillium wilt on cotton by the use of fluorescent Pseudomonas spp. under field conditions. Biological Control 2010; 53: 39–45.

(10) Cabanás CGL., Schilirò E., Valverde-Corredor A., Mercado-Blanco J. The bio control endophytic bacterium Pseudomonas fluorescens PICF7 induces systemic defense responses in aerial tissues upon colonization of olive roots. Frontiers in Microbiology 2014; 5: 427. 431.

(11) Cao P., Liu C., Sun P., Fu X., Wang S., Wu F., Wang X. An endophytic Streptomyces sp. strain DHV3-2 from diseased root as a potential bio control agent against Verticillium dahliae and growth elicitor in tomato (Solanumlycopersicum). Antonie van Leeuwenhoek 2016; 109:1573–1582.

(12) Ryan RP., Germaine K., Franks A., Ryan DJ., Dowling DN. Bacterial endophytes: recent developments and applications. FEMS Microbiological Letter 2008; 278: 1–9.

(13) Compant S., Clément C., Sessitsch A. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biology and Biochemistry 20104; 2(5): 669–678.

(14) Hung P.Q., Annapurna K. Isolation and characterization of endophytic bacteria in soybean (Glycine sp.).Omonric 2004; 12: 92-101.

(15) Narayanasamy P. Detection and identification of bacterial biological control. In: Narayanasamy, P., editor. Biological management of diseases of crops, Volume 1 Characteristics of Biological Control Agents. New York: Springer; 2013:236-237.

(16) Vincent JM. Distortion of fungal hyphae in the presence of certain inhibitors. Nature1947; 150: 160.

(17) Holt JG., NR., Krieg PHA., Sneath JT., Staley ST., Williams. Bergey's Manual of Determinative Bacteriology, 9th ed. NewpYork: Baltimore; 1994.

(18) Schaad NW., Jones JB., Chun W. Laboratory Guide for Identification of Plant Pathogenic Bacteria. Minnesota: American Phytopathology Society; 2001.

(19) Nielsen P., Sùrensen J. Multi-target and medium independent fungal antagonism by hydrolytic enzymes in Paenibacillus polymyxa and Bacillus pumilus isolates from barley rhizosphere. FEMS Microbiology Ecology 1997; 22: 183-92.

(20) Nisa RM., Irni M., Amaryllis A., Sugeng S., Iman R. Chitinolytic bacteria isolated from chilirhizosphere: chitinase characterization and its application as bio control for whitefly (Bemisia tabaci Genn). American Journal of Agricultural and Biological Sciences 2010;5 (4): 430–435.

(21) Ahmadzadeh M., Sharifi Tehrani A. Evaluation of fluorescent pseudomonads for plant growth promotion, antifungal activity against Rhizoctonia solani on common bean, and bio-controlpotential. Biological Control 2009; 48: 101–107.

(22) Montealegre JR., Reyes R., Pérez LM., Herrera R., Silva P., Besoain X. Selection of antagonistic bacteria to be used in biological control of Rhizoctonia solani in tomato. Electronic Journal of Biotechnology 2003; 6(2): 115- 127.

(23) Fiddaman PJ., Rossall S. The production of antifungal volatiles by Bacillus subtilis. Journal of Applied Bacteriology 1993; 74: 119-126.

(24) Schwyn B., Neilands J.B. Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry 1987; 160: 47–56.

(25) Alstrom S., Burns R.G. Cyanid production by rhizobacteria as a possible mechanism of plant growth inhibition. Biology and Fertility of Soil. 1989;7: 232a-235.

(26) Gordon SA., Weber RP. Colorimetric estimation of indole acetic acid. Plant Physiology 1951; 26: 192-197.

(27) Pikovskaya RE. Mobilization of phosphorus in soil in connection with vital activity of some microbial species. Microbiology 1948; 17: 362–370.

(28) Orozco-Mosqueda Ma. del C., Velazquez-Becerra C., Macias-Rodriguez L.I., Santoyo G., Flores-Cortez I., Alfaro-Cuevas R., et al. Arthrobacter agilis UMCV2 induces iron acquisition in Medicago truncatula (strategy I plant) in vitro via dimethyl hexadecylamine emission. Plant and Soil 2013a; 362:51–66.

(29) Abdul Baki AA., Anderson J.D. Vigor determination in soybean seed by multiple criteria. Crop Science 1973; 13: 630–633.

(30) Zeise K. The potential of Talaromyces flavus (Kloekner) Stolk and Samson in controlling Verticillium dahliae. Proceedings of the "Seventh International Verticillium Symposium";6-10 October 1997; Greece, Athens: APS Press; 2000: 61-67.

(31) Tjamos EC., Biris DA., Paplomatas EJ. Recovery of olive trees with Verticillium wilt after individual application of soil solarization in established olive orchards. Plant Disease 1991; 75: 557–562.

(32) Shanmugam V., Kanoujia N. Biological management of vascular wilt of tomato caused by Fusarium oxysporum f. sp. lycospersici by plant growth-promoting rhizobacterial mixture. BiologicalControl 2011; 57: 85–93.

(33) Tjamos EC., Tsitsigiannis DI., Tjamos SE., Antoniou PP., Katinakis P. Selection and screening of endorhizosphere bacteria from solarized soils as bio control agents against Verticillium dahliae of solanaceous hosts. European Journal of Plant Pathology 2004;110:35–44.

(34) Uppal AK., El Hadrami A, Adam LR., Tenuta M, Daayf F. Biological control of potato Verticillium wilt under controlled and field conditions using selected bacterial antagonists and plant extracts. Biological Control 2008; (44): 90–100.

(35) Rosenblueth M., Martínez-Romero E. Bacterial endophytes and their interactions with hosts. Molecular Plant Microbe Interaction 2006; 19: 827-837.

(36) Marcano I.E., Díaz-Alc_antara CA., Urbano B., Gonzalez-Andres F. Assessment of bacterial populations associated with banana tree roots and development of successful plant probiotics for banana crop. Soil Biology and Biochemistry2016; 99: 1-20.

(37) Jha BK., Pragash MG., Cletus J., Raman G., Sakthivel N. Simultaneous phosphate solubilization potential and antifungal activity of new fluorescent Pseudomonad strains, P. aeruginosa, P. pelcoglossicida and P. mosselii. World Journal of Microbiology and Biotechnology 2009; 25: 573-581.

(38) Mansoori M., Heydari A., Hassanzadeh N., Rezaee S., Naraghi L. Evaluation of Pseudomonas and Bacillus bacterial antagonists for biological control of cotton *Verticillium* Wilt Disease. Journal of Plant Protection Research 2013; 53: 11–14.

(39) Lugtemberg B.,Kamilova F. Plant-growth-promoting rhizobacteria. Annual Review of Microbiology 2009; 63: 541-556.

(40) Vessey JK. Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil 2003; 255:571–586.

(41) Narayanasamy P. Mechanisms of action of bacterial biological control agents. In: Narayanasamy P., editor. Biological Management of Diseases of Crops Volume 1 Characteristics of Biological Control Agents. New York: Springer; 2013: 295-429.

(42) Fernando WGD., Nakkeeran S., Zhang Y., Sarchuk S. Biological control of Sclerotinia sclerotiorum (Lib.) de Bary by Pseudomonas and Bacillus species on canola petals. Crop Protection 2007;26:100–107.

(43) Schnider-Keel U., Seematter A., Maurhofer M., Blumer C., Duffy BK. Gigot-Bonnefoy, C. et al.. Autoinduction of 2,4-diacetylphoroglucinol biosynthesis in the bio-control agent Pseudomonas fluorescens CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. Journal of Bacteriology 2000;182:1215-1225.

(44) Ryu CM., FaragMA., Hu CH., Reddy MS., Kloepper JW.,Paré PW. Bacterial volatiles induce systemic resistance in Arabidopsis . Plant Physiology 2004; 134:1017–1026.

(45) Gutie´rrez-Luna FM., Lo´pez-Bucio J., Altamirano-Herna´ndez J, Valencia-Cantero E, de la Cruz HR, Macı´as-Rodrı´guez L. Plant growth-promoting rhizobacteria modulate rootsystem architecture in Arabidopsis thaliana through volatile organic compound emission. Symbiosis 2010; 51:75–83.

(46) Mcdonald MB., Copeland LO. Seed production: principles and practices. London: Chapman & hall; 1997.

(47) Haddad N.; Krimi Z.; Raio A., Endophytic bacteria from weeds promote growth of tomato plants in vitro and in greenhouse. In: Schneider C., Leifert C., Feldmann F., editors. Endophytes for plant protection: the state of the art, proceeding of the 5th international symposium on plant protection and plant health in Europe. Berlin, Germany; 26-29 May 2013: 27-32.

(48) Xiao K., Samac D.A., Kinkel L.L. Biological control of Phytophthora root rots on alfalfa and soybean with Streptomyces. Biological Control 2002; 23:285–95.

(49) Naik PR., Sahoo N., Goswami D., Ayyadurai N., Sakthivel N., Genetic and functional diversity among fluorescent pseudomonads isolated from the rhizosphere of banana Microbial Ecology 2008; 56:492–504.

(50) Khan Z., Doty SL. Characterization of bacterial endophytes of sweet potato plants. Plant and soil 2009; 322: 197-207.

(51) Tao G.C., Tian S.J., Cai M.Y., Xie H. Phosphate-solubilizing and -mineralizing abilities ofbacteria isolated from soils. Pedosphere 2008; 18:515–523.

(52) Robin A., Vansuyt G., Hinsinger P., Meyer JM., Briat JF., Lemanceau P. Iron dynamics in the rhizosphere: consequences for plant health and nutrition. Advancesin Agronmy2008; 99:183–225.

(53) Ozaktan H., Gul A., Caklr B., Yolageldi L., Akkopuru A., Fakhraei D. Isolation optimization of bacterial endophytes from cucumber plants and evaluation of their effects on growth promotion and bio control. In: Schneider C., Leifert C., Feldmann F., editors. Endophytes for plant protection: the state of the art. Proceeding of the 5th international symposium on plant protection and plant health in Europe. Berlin, Germany; 26-29 May 2013:262-268.

(54) Beattie GA., plant-associated bacteria: survey, molecular phylogeny, genomics and recent advances, In: Gnanamanickam SS. editor. Plant-Associated Bacteria. Dordrecht, Netherlands: Springer; 2006:1-56

(55) Patel S., SayyedRZ., SarafM.bacterial determinants and plant defense induction: Their role as bio control agents in sustainable agriculture In: Hakeem KR. Akhtar MS. editors. Plant, Soil and Microbes. Switzerland: springer; 2016: 187-204

(56) Compant S.; Reiter B.; Sessitsch A.; Nowak J.; Clement C.; Barka E. Endophytic colonization of Vitis vinifera L. by plant growth promoting bacterium Burkholderia sp. strain PsJN. Applied and Environmental Microbiology 2005; 71: 1685-1693.

(57) Ramamoorthy V., Viswanathan R., Raguchander T., Prakasam V., Samiyappan R. Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. Crop Protection 2001; 20: 1–11.