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Hellenic Plant Protection Journal **14:** 1-13, 2021
DOI 10.2478/hppj-2021-0001

Tomato bacterial endophytes in controlling Verticillium wilt and their advantage on biostimulation

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Summary A total of 688 bacterial endophytes were isolated from both greenhouse and field-grown tomatoes in the Tehran and Alborz provinces, Iran in order to obtain effective bacterial endophytes against the fungus *Verticillium dahliae*. 128 bacterial isolates, with respect to their different phenotypic characteristics were further analysed. All bacteria with positive hypersensitivity reaction on tobacco and geranium leaves and potato soft rot were eliminated, and totally 39 isolates were selected for in vitro antagonism and greenhouse tests. The potential biocontrol isolates were evaluated using seed treatment and soil drench methods on two tomato cultivars. The results indicated that seven bacterial isolates had a high potential for the control of the fungus and reduced the severity disease to 95-98%. This reduction was coincided with an increase in some growth factors like plant dry weight, root dry weight, plant height, root length, root fresh weight and plant fresh weight ranged between 92-98%. The seven antagonists' preliminary identification was confirmed using 16SrRNA gene sequencing analysis. The BLAST analysis was performed, and the bacteria were also identified as *Bacillus pumilus* (two isolates)*, Bacillus subtilis, Bacillus safensis, Enterobacter ludwigi, Serratia marcesens* and *Pseudomonas beatica*. Biocontrol mechanisms examination indicated that protease production was positive for all isolates and differentiated isolates *E. ludwigii* and *P. beatica* as higher producers with protease levels up to 65%. The three bacteriocins producing isolates inhibited the phytopathogenic mycelium up to 70% in dual culture assay. Also, five of the isolates produced siderophores and *P. baetica, S. marcesens* and *E. ludwigii* produced remarkable amount of auxin hormone.

Additional keywords: biocontrol, endophytic bacteria, tomato, Verticillium wilt

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is considered as one of the most important commercial vegetable crops in Iran and all over the world. Iran ranks as the sixth largest producer of tomato in the world (http://www.fao.org/faostat/en/#rankings/countries_by_commodity). However, one of the main problems in tomato production is fungal diseases causing damping-off, wilts and blights. Verticillium wilt, in particular, caused by the soil-borne fungus *Verticillium dahliae,* is considered as an economically important disease that limits the production of a broad range of agricultural crops, including tomato (Gayoso *et al*., 2007). The tomato yield losses due to this disease are estimated by 50% (Jabnoun *et al*., 2009). Several methods have been proposed and implemented for managing the Verticillium wilt.

Host plant resistance is generally the most favorable control method for plant disease management, but resistant cultivars to Verticillium wilt are not available for many crops and therefore the management of the disease is notoriously difficult (Rekanovic *et al*., 2007; Song and Thomma, 2016). Synthetic agrochemicals are applied as conventional and common method to control plant diseases. However, the use of chemical pesticides adversely affects the environment and human health (Prabhat *et al*., 2013). The use of biological control agents has increasingly become popular in plant protection as a more environmentally friendly alternative to chemical pesticides (Martin and Bull, 2002; Naraghi *et al*., 2006; Naraghi *et al*.,

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2008; Heydari and Pessarakli, 2010). This can include the soil inoculants and foliar sprays of beneficial microorganisms, while the increased knowledge about microbial ecology in the phytosphere has stimulated new thoughts for biocontrol approaches (Rabiey *et al*., 2019).

Application of bacterial endophytes to tomato plants and their effects on disease control along with plant growth promotion are considered as a novel method in tomato Verticillium wilt management (Latha *et al*., 2019). Endophytic bacteria usually colonize the whole tomato plant, obtain the essential nutrients from plant tissues and protect the host plants against biotic and abiotic stresses (Botta *et al*., 2013). According to Amaresan *et al.* (2012), the most identified endogenous bacteria with promoting plant growth capability in tomato belong to *Bacillus* and *Serratia* genera. They have the ability to control phytopathogens such as *Fusarium oxysporum, Colletotrichum capsici, Sclerotinia rolfsii* and *Pythium* sp. and are also able to produce siderophore, indole acetic acid and solubilize phosphorus. Several other reports recommend bacterial endophytes as an appropriate alternative to chemical fungicides. Some of these bacteria like *Bacilli* and *Streptomyces* isolates have been commercialized (Kwak and Weller, 2013; Lagzin *et al*., 2013). The latter can induce systemic resistance in their host plants to different diseases and abiotic stresses through the production of diverse antimicrobial compounds, enzymes and siderophores (Botta *et al.,* 2013; Kandel *et al.,* 2017). In addition, they promote plant growth and yield in many agricultural and horticultural crops (Rosenblueth and Martínez-Romero, 2006). Therefore, endophytic bacteria are considered as biocontrol agents which protect their host under unfavorable conditions better than soil, rhizoplane and phylloplane microbes and they can be transmitted through gene transfer between generations in plants, an attribute that also differentiates them from rhizosphere bacteria (Nejad and Johnson, 2000).

Since, host resistance against Verticillium wilts is not available for many plant species, and combating with this disease is notoriously difficult (Song and Thomma, 2016), host-induced gene silencing (HIGS) has been identified as a promising strategy for improving the plant resistance against these pathogens (Song and Thomma, 2016). Specifically, HIGS by employing RNA silencing mechanisms to silence the targets of invading pathogens has been successfully applied in crop disease prevention (Qi *et al*., 2019).

The objective of this study was to isolate and identify effective bacterial endophytes from tomato plants in order to control the tomato Verticillium wilt and promote the growth parameters.

Materials and methods

Evaluation of biocontrol action of endophytic bacteria against *V. dahliae* in tomato plants

Plant sampling. Tomato plants sampling was carried out from 20 greenhouses and 10 farms free of fungal disease during spring and summer of 2017 in Tehran and Alborz provinces. Ten healthy plants were selected from each greenhouse and field.

Isolation of endophytic bacteria. The root, stem, leaf and fruit samples of each greenhouse and field were mixed for isolation of endophytic bacteria. The samples were rinsed with tap water for 10 min., then sterilized with 75% ethanol for 40 sec. and in 5% sodium hypochlorite for 5 min., and disinfected in 70% ethanol for 30 sec. Afterwards, the samples were rinsed with sterile distilled water at least three times and the specimens' surface was scraped with sterile scalpel and immersed in distilled water for 30 min. All samples were crushed in sterile mortar, and a loopful from each suspension was cultured on nutrient agar medium (NA, Merck, Germany). All plates were incubated for 3-4 days at 37°C. The distinguished colonies were selected and purified. The representative strains were maintained in nutrient broth medium at -20°C (NB, Difco, USA) containing 15% (w/v) glycerol (Atugala and Deshappriya, 2015).

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Grouping of the endophytic bacteria on the basis of phenotypic characteristics. Thirty seven (37) representative strains were characterized initially, with respect to certain phenotypic tests including Gram staining, mobility, aerobic or anaerobic growth, fluorescent pigment production on King's B medium, levan, potato soft rot, oxidase, arginine dihydrolyase as well as gelatin and starch hydrolyses and also nitrate reduction test (Schaad *et al*., 2001). Hypersensitivity reaction (HR) was also examined on tobacco and geranium leaves.

Pathogenic isolate. Verticillium dahliae strain VD-Co-P-G-22, isolated from wilted tomato, was obtained from the Iranian Research Institute of Plant Protection, Tehran, Iran and used in all experiments of this study.

In vitro screening of endophytic bacteria antagonistic activity against V. dahliae. The antifungal activity of 128 endophytic bacteria was evaluated in vitro by dual culture assay on PDA medium with three replications. Sterile distilled water was used as a control. An agar disc with *Verticillium dahliae* mycelium was placed 5 cm away from the bacterial cultures, and the plates were incubated at 25°C. The percent of inhibition was calculated using the following formula: Percent Growth Inhibition= [(R-r) ×100]/R, where R is the fungal growth radius in the control, and r is the fungal growth radius in the antagonist presence. (Munif *et al*., 2012).

Effect of endophytic bacteria on seed germination and seedling growth under laboratory and greenhouse conditions. For bacterization of seeds surface of two tomato cultivars, "Falat" and "Super amber", the seeds were immersed for 40 min. in the individual bacterial suspension with a concentration of 1×108 cfu/ml. The treated seeds were then placed on filter paper for seed germination. The germinated seeds were sown in sterile soil and growth rates were monitored daily for one month. All tests were conducted in a completely randomized design with three replications (Vichova and Kozova, 2004; Jabnoun et al., 2009).

Evaluation of biocontrol capability of selected endophytes under greenhouse conditions. Seeds of the cv. Falat were disinfected with sodium hypochlorite and ethanol, thoroughly washed with sterilized distilled water and planted in a plastic tray (Atugala and Deshappriya, 2015). Three weeks later, the roots were individually placed in bacterial suspension of 37 endophytic bacteria isolates (3×108 cfu/ml). Phosphate buffer (PBS) was used as a negative control. The treated seedlings were transferred to some pots containing infested soil with *V. dahliae* (Nejad *et al*., 2000). After three weeks, tomato roots and stems were crushed and the extracts were streaked on nutrient agar (NA) medium for determining the bacterial colonization. The disease severity was measured after 90 days of cultivation using the following scale: 0 = no symptoms, 1= infection symptoms on the root, 2 = infection symptoms on the root and crown, 3 = infection symptoms on the stem base, and 4 = infection in the stem middle and end (Jabnoun *et al*., 2009). The experimental design was a completely randomized design with three replicates. ANOVA for a factorial design (factors: endophytic bacteria, *V. dahliae*) was conducted, using the SAS program. The treatments means was compared by Duncan's multiple range test.

Measuring plant growth parameters. Different growth parameters including shoot and root fresh and dry weight, and growth lengths were measured. Ten tomato plants at the age of 1-month, treated with 37 bacterial isolates were assigned to measure the parameters by random (Atugala and Deshappriya, 2015).

Identification of the most efficient bacteria. A combination of phenotypic characteristics and 16S rDNA sequences was applied in order to identify the endophytic bacteria (Kumar *et al*., 2016).

For DNA extraction and PCR reaction, the selected bacterial isolates were cultured in NA medium and after 24 hours, a single colony was selected and mixed in the sterile microtubes containing 500 µl of NaOH and 10 µl of SDS. The samples were placed in boiling water for 5-10 min. after vortexing for 3 min. (gram-negative bacteria for 5

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min. and gram-positive for 10 min.). This was followed by ice-cold shock treatment for 3 min. Then, the samples were centrifuged at 13000 rpm at 25°C. 100 µl of the upper phase containing DNA was transferred into the 1.5 ml Eppendorf tubes and was preserved at -20°C (Elboutahiri *et al.*, 2009). The primers pairs that were applied were P1 and P6 with the sequences as followings: (5'ATC CAGAGTTTGATCCTGGTCAGAACGAACGCT -3') and (5'-CGGGATCCTACGGCTACCTTGT-TACGACTTCACCCC-3'), respectively (Kumar *et al.*, 2016).

For the PCR product electrophoresis and sequencing, the PCR mixture was prepared in a 25µl final volume containing 2 µl of extracted DNA (10 ng/µl), 1 µl of $MgCl_2$ (50 mM), 0.6 µl of dNTPs (10 mM), 1 µl of each primer (10 ng/µl), 0.1 µl of Taq polymerase, 3 µl of PCR buffer (10X) and 16.3 µl of SDW. The thermal cycling conditions for initial denaturation were for 2 min. at 94°C, followed by 25 cycles for 30 S at 94°C (denaturation), for 30 S at 52°C (annealing), for 2 min at 72°C (extension), and a final extension step for 20 min at 72°C. Mixtures without DNA templates (water) were used as negative control. 4 µl of each PCR product was mixed with a 1 µl staining marker (GeneRuller 100bp) and run in 1% agarose in 1x TAE buffer. 1 µl of 100 bp DNA ladder SMO323 (Thermo Scientific) was applied. The gel was photographed using Gel Document Uvitec/DM 500. The PCR products with sharp bands were sent to the two Microsynth (Switzerland) and Bioneer (South Korea) companies for sequencing.

Detection of antifungal metabolites produced by representative endophytic bacteria

Detection of antifungal metabolites was examined in five bacterial isolates, *E. ludwigii* NZ101, *P. baetica* NZ108, *B. subtilis* NZ104 and NZ103, *B. pumilus* NZ106, namely through the production of protease, hydrogen cyanide (HCN), inhibitory extracellular metabolites, volatile antifungal metabolites, indole-3-acetic acid (IAA), siderophore and bacteriocins.

Protease production. The bacterial isolates were spot inoculated on SCA medium (Simmons' citrate agar) and the Perti plates were kept for 48 hours at 25°C. The colorless halo formation around the bacterial colonies indicated proteolytic proteins activity (Gull and Hafeez, 2012).

HCN production. Bacteria were heavily inoculated on SCA growth medium (Simmons' citrate agar) and a filter paper strip of 1×1 cm containing 5 ml of copper ethyl acetoacetate, 5 mg of 4,4' methylene bis (N,N'-dimethyl) aniline and 2 ml of chloroform was placed inside the lids upside-down. All Petri plates were incubated for 48 hours at 28°C. Any change in the filter paper color (to blue color) was considered as a positive result of hydrogen cyanide production (Gull and Hafeez, 2012).

Production of inhibitory extracellular metabolites. Bacterial isolates were prepared at a concentration of 1×10^7 cells/ml, cultured on PDA medium and were incubated at 28 °C for three consecutive days. After that, DW washed the bacteria from the surface of the culture medium, and a sterilized cotton swab soaked in chloroform was placed on lids. After 30 min, a 15-day fungal growth was cultured in the middle of each plate. The inhibitory zones were measured after incubation of plates at 28°C for 10 days using the formula proposed by Raut and Hamed (2016).

Production of volatile antifungal metabolites. All bacterial suspensions were prepared at a concentration of 1×10^7 cells/ml, cultured on nutrient agar + 2% glucose (NGA) and were incubated for 24 hours at 25°C. Meantime a 15day fungal culture was placed in the middle of the PDA medium and the two plates were sealed together with Parafilm M®. The control sets did not contain the bacteria. The inhibitory zones were measured after incubation of plates at 28°C for 10 days as mentioned above.

Production of IAA. The bacterial isolates were grown in tubes containing 100 ml of KB medium for 16 h at 30°C. Tubes containing 100 ml of KB medium were inoculated with bacteria and incubated for at least 16 hours at a temperature of 30°C. The 0.6-0.7 absorbance value was determined at 600 nm.

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The suspension was centrifuged at 8,000 rpm for 10 min and the supernatants were mixed with Salkowski reagent in a 2:1 ratio and ethanol, chloroform and concentrated sulfuric acid were mixed in a ratio of 2: 2: 2. The tubes were placed in a dark box for 30 minutes. The pink color developed due to the production of IAA was measured by spectrophotometry at 530 nm. The concentration of IAA in each culture was calculated with respect to the standard curve (Merck, Germany) (Gull and Hafeez, 2012).

Production of siderophore. The KB and PDA culture media containing FeCl3 in the final concentrations of 25, 20, 15, 10 µg/L and control without iron, were prepared, respectively, and a 15-day Verticillium culture was inoculated and incubated at 28°C for 5 days in darkness (Raut and Hamed, 2016; Gull and Hafeez, 2012).

Bacteriocin production and their antibacterial activity. Antagonistic isolates were cultured on a NB medium for 24 hours at 25°C. The supernatants that were obtained by centrifugation for 20 min. at 8000 rpm were adjusted to pH 6.5 with 1M NaOH. Trypsin (1 mg ml^{-1}) was added to each tube and isolates were incubated for 12 hours at 37°C. Two wells with the depth of 5 mm were made on each NA medium, and each one of the bacterial isolates was added to each well, up to the time of completely absorbing by medium. Finally, the Petri plates surface were sprayed with *Escherichia coli* indicator (isolate code: PTCC1330) and incubated for 1 week. Any inhibitory effect was considered as a result of bacteriocins (Tagg and McGiven, 1971).

Results

Biocontrol action of endophytic bacteria against *V. dahliae* in tomato plants

Altogether, 688 endophytic bacterial isolates were obtained from the root, stem, leaf, and fruit of tomato plants. 128 isolates were selected in initial screening based on phenotypic traits. Finally, 37 isolates which did not cause hypersensitivity reactions and soft rot in potato slices were selected for further studies.

All 37 representative isolates were examined for their inhibitory effects against *V. dahliae* using the agar diffusion method on PDA medium. The results indicated that there was a significant difference in the fungal growth inhibitory effect. The maximum inhibitory effect was produced by *P. beatica* with 3.80 mm, *E. ludwigii* with 3.70 mm and *B. subtilis* with 3.60 mm zone diameters, respectively.

The results from the tests on the effect of endophytic bacteria on germination of tomato seeds and seedling growth (viability and vigor) under laboratory and greenhouse conditions indicated that the seeds viability in cv. Falat and super amber cultivars was 99% and 50%, respectively. As a result, all experiments were carried out on tomato cv. Falat.

By analyzing the data attained from the greenhouse test, it was demonstrated that there was a significant difference ($P < 0.0001$) between tomato plants treated with endophytic bacteria and *V. dahliae* in comparison with positive (inoculated with pathogen) and negative controls (non-inoculated) (Fig. 1). Moreover, five distinct groups of a, b, c, d and e were obtained as indicated in Table 1. In group e, the non-inoculated controls along with seven isolates were recognized as high potential biological control agents reducing the disease severity to 95-



Figure 1. Seed treatment and reduction of disease symptoms and growth promoting effect of the endophytic bacterium *Pseudomonas beatica* NZ-101 (middle) compared to healthy control (right) and *Verticillium dahliae* infected tomato plant (left).

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Table 1. Disease incidence and control disease against *Verticillium dahliae*, and means of fresh and dry weight of seedlings, seedling length, root fresh and dry weight, and root length of tomato seedlings, cv. Falat, inoculated with endophytic bacteria.

Treatment	Disease incidence	Disease Control (%)	Seedling wet weight (g)	Seedling dry weight (g)	Seedling height (cm)	Root wet weight (g)	Root dry weight (g)	Root length (cm)
Inoculated Control	94.5 ± 0.28 a	4.50	19.15 ± 0.005 h	4.67 ± 0.005 i	13.83 ± 0.005ef	6.11 ± 0.005i	1.98 ± 0.005 m	4.83 ± 0.005 j
RA^{3}_{1}	64.50 ± 0.28 b	45	26.40 ± 0.005 de	9.65 ± 0.005 e	14.16 ± 0.005ef	11.48 ± 0.005 ef	5.27 ± 0.005 g	7.66 ± 0.005ef
RH^{1}_{1}	45.50 ± 0.28 c	65	49.71 ± 0.005 c	18.20 ± 0.005 d	24.00 ± 0.005 c	31.66 ± 0.005 bc	12.62 ± 0.005 d	16.50 ± 0.005 d
NZ 104	**2.50 ± 0.28 e**	**98**	**70.07 ± 0.005 a**	**30.73 ± 0.57 a**	**35.66 ± 0.57 a**	**42.31 ± 0.005 a**	**17.23 ± 0.57a**	**25.50 ± 0.57 a**
RE^{1}_{1}	20.20 ± 0.11 d	82.5	49.71 ± 0.58 c	18.20 ± 0.57 d	24.00 ± 0.57c	31.66 ± 0.58bc	12.62 ± 0.58 d	16.50 ± 0.57 d
RA^{2}_{1}	50.50 ± 0.28 c	46.5	24.62 ± 0.57 defg	7.89 ± 0.83 gh	9.66 ± 0.56 gh	9.84 ± 0.01 h	3.20 ± 0.57 l	5.66 ± 0.56 ij
MA^{1}_{2}	49.50 ± 0.28 c	52.5	26.40 ± 0.57 de	9.65 ± 0.58 e	14.16 ± 0.58ef	11.48 ± 0.57ef	4.94 ± 0.01 gh	7.66 ± 0.01 ef
SB^{1}_{6}	89.5 ± 0.28 a	8.5	26.40 ± 0.57 de	9.65 ± 0.58 e	14.16 ± 0.58 ef	11.48 ± 0.58 ef	5.27 ± 0.58 g	7.83 ± 0.56 e
MA^{3}_{3}	55.50 ± 0.28 c	45.5	25.50 ± 0.57 def	9.65 ± 0.58 e	13.00 ± 0.57 gef	10.22 ± 0.01 ghf	4.20 ± 0.57 ij	7.83 ± 0.56 e
RC^{1}_{3}	50.50 ± 0.28 c	52.50	49.71 ± 0.005 c	18.20 ± 0.58d	24.00 ± 0.57 c	31.66 ± 0.58 bc	12.62 ± 0.01 d	16.50 ± 0.57 d
NZ 101	**2.50 ± 0.28 e**	**98**	**70.07 ± 0.005 a**	**30.73 ± 0.57 a**	**35.66 ± 0.57 a**	**42.31 ± 0.005 a**	**17.23 ± 0.57a**	**25.50 ± 0.57 a**
NZ 102	**4.50 ± 0.28 e**	**95**	**69.74 ± 0.01 a**	**29.13 ± 0.57 b**	**33.83 ± 0.01 a**	**41.21 ± 0.58 a**	**16.95 ± 0.01 a**	**23.66 ± 0.01b**
RB^{13}_{3}	70.50 ± 0.28 b	35.5	26.40 ± 0.57 de	9.65 ± 0.57 e	14.16 ± 0.01 ef	11.48 ± 0.57ef	5.27 ± 0.57g	7.66 ± 0.01ef
SC^{7}_{6}	92.50 ± 0.28 a	6.5	21.73 ± 0.01 gh	6.99 ± 0.57 h	10.00 ± 0.57 gh	10.11 ± 0.005 gh	3.25 ± 0.57 i	6.00 ± 0.57 hi
BC^{1}_{3}	55.50 ± 0.28 c	48.50	49.71 ± 0.005 c	18.20 ± 0.57 d	24.00 ± 0.57 c	31.66 ± 0.58 bc	12.62 ± 0.01 d	16.50 ± 0.57 d
RD^{7}_{6}	25.50 ± 0.28 d	77.50	27.40 ± 0.57 d	8.65 ± 0.02 gf	14.66 ± 0.001 e	12.48 ± 0.57 e	4.27 ± 0.005 ij	6.66 ± 0.001 gh
NZ 103	**3.50 ± 0.28 e**	**96**	**69.74 ± 0.01 a**	**29.13 ± 0.57 b**	**33.83 ± 0.01 a**	**41.21 ± 0.58 a**	**16.95 ± 0.01 a**	**23.66 ± 0.01b**
NZ 106	**4.50 ± 0.28 e**	**95**	**69.74 ± 0.01 a**	**29.13 ± 0.57 b**	**33.83 ± 0.01 a**	**41.21 ± 0.58 a**	**16.95 ± 0.01 a**	**23.66 ± 0.01b**
BB^{1}_{6}	30.20 ± 0.11 d	65.80	25.40 ± 0.11def	8.65 ± 0.11gf	12.00 ± 0.57 gehf	11.28 ± 0.01 gef	3.90 ± 0.57 kj	5.00 ± 0.57 j
BA^{1}_{3}	32.50 ± 0.28 d	64.50	47.71 ± 0.005 c	17.47 ± 0.01 d	18.50 ± 0.57 d	29.83 ± 0.01 d	11.07 ± 0.04 f	16.33 ± 0.01 d

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SB^{1}_{6}	94.50 ± 0.28 a	4.50	23.79 ± 0.01 efg	9.05 ± 0.02 ef	11.66 ± 0.01 gehf	9.15 ± 0.02 h	4.04 ± 0.01 kij	5.66 ± 0.01 ji
SD^{1}_{6}	65.50 ± 0.28 b	35.50	24.03 ± 0.01 defg	7.22 ± 0.01 h	12.00 ± 0.57 gehf	10.15 ± 0.02 gh	4.38 ± 0.01 ij	7.00 ± 0.57 gef
MA^{1}_{4}	59.50 ± 0.28c	38.50	26.06 ± 0.03ed	8.68 ± 0.01 gef	13.16 ± 0.01 gef	11.48 ± 0.01 ef	5.48 ± 0.01 g	7.16 ± 0.01gef
MA^{1}_{2}	25.50 ± 0.28 d	72.50	24.20 ± 0.01defg	8.20 ± 0.11 gf	9.16 ± 0.01 h	10.22 ± 0.01 ghf	3.34 ± 0.02 i	7.00 ± 0.57 gef
BA^{1}_{6}	30.50 ± 0.28d	68.50	25.40 ± 0.11 def	8.59 ± 0.005 gf	14.16 ± 0.01 ef	11.15 ± 0.02 gf	4.54 ± 0.02 ih	6.83 ± 0.01 ghf
MB^{5}_{6}	55.50 ± 0.28 c	40.50	19.15 ± 0.02 h	4.67 ± 0.01 i	9.00 ± 0.57h	6.11 ± 0.005 i	1.98 ± 0.005 m	3.83 ± 0.01 k
BC^{2}_{4}	52.50 ± 0.28c	45.50	25.79 ± 0.005 de	9.05 ± 0.02 ef	14.00 ± 0.57ef	11.48 ± 0.005 ef	5.04 ± 0.02 gh	7.33 ± 0.01gef
NZ 108	**2.50 ± 0.28 e**	**98**	**70.07 ± 0.005 a**	**30.73 ± 0.57 a**	**35.66 ± 0.57 a**	**42.31 ± 0.005 a**	**17.23 ± 0.57a**	**25.50 ± 0.57 a**
BA^{4}_{6}	25.50 ± 0.28 d	72.5	22.20 ± 0.11 fgh	7.20 ± 0.11 h	10.66 ± 0.01 ghf	9.85 ± 0.02 h	3.62 ± 0.01 kl	6.66 ± 0.01gh
SA^{1}_{6}	94.50 ± 0.28 a	4.50	6.39 ± 0.005 i	1.66 ± 0.01 j	3.00 ± 0.57 i	2.06 ± 0.01 j	0.67±0.01 n	1.33 ± 0.01 l
MA^{2}_{6}	45.50 ± 0.28 c	54.5	47.71 ± 0.005 c	17.47 ± 0.01 d	18.50 ± 0.18 d	29.83 ± 0.01 d	11.07 ± 0.01 f	16.33 ± 0.01 d
ME^{3}_{6}	46.50 ± 0.28 c	52.5	49.71 ± 0.005 c	18.20 ± 0.02 d	24.00 ± 0.57 c	31.66 ± 0.01bc	12.62 ± 0.01 d	16.50 ± 0.01d
SA^{2}_{6}	94.50 ± 0.28a	4.50	6.39 ± 0.005 i	1.60 ± 0.02 j	3.00 ± 0.57 i	2.06 ± 0.01 j	0.67 ± 0.01 n	1.33 ± 0.01 l
MA^{5}_{6}	48.50 ± 0.28 c	54.50	49.71 ± 0.005 c	18.20 ± 0.11 d	24.00 ± 0.57 c	31.66 ± 0.01 bc	12.62 ± 0.01 d	16.50 ± 0.57 d
MD^{3}_{6}	65.50 ± 0.28b	35.50	48.71 ± 0.005 c	17.47 ± 0.01 dc	22.16 ± 0.01 c	30.50 ± 0.15 dc	11.74 ± 0.18 ec	16.16 ± 0.01dc
MA^{5}_{6}	56.50 ± 0.28 c	42.50	26.40 ± 0.23 de	9.65 ± 0.02 e	14.16 ± 0.01 ef	11.48 ± 0.01 ef	5.27 ± 0.01 g	7.66 ± 0.01 ef
MD^{5}_{6}	55.50 ± 0.28 c	42.5	49.71 ± 0.005 c	18.20 ± 0.11 d	24.00 ± 0.57 c	31.66 ± 0.01 bc	12.62 ± 0.01 d	16.50 ± 0.57 d
NZ 107	**3.50 ± 0.28 e**	**96**	**69.74 ± 0.01 a**	**29.13 ± 0.57 b**	**33.83 ± 0.01 a**	**41.21 ± 0.58 a**	**16.95 ± 0.01 a**	**23.66 ± 0.01b**
SD^{6}_{6}	65.50 ± 0.28 b	42.5	48.71 ± 0.005 c	17.47 ± 0.01 d	22.16 ± 0.01 c	30.50 ± 0.15 cd	11.74 ± 0.18 e	16.16 ± 0.01 a

RA^{3}_{1}= *Pseudomonas* sp., RH^{1}_{1} = *Bacillus* sp., **NZ 104= *Bacillus subtilis*,** RE^{1}_{1}=*Pseudomonas* sp., RA^{2}_{1}=*Bacillus* sp., MA^{1}_{2}= *Bacillus* sp., SB^{1}_{6}= *Enterobacter* sp., MA^{3}_{3}= *Bacillus* sp., RC^{1}_{3}= *Bacillus* sp., **NZ 101 = *Pseudomonas beatica*, NZ 102= *Serratia marcesens*,** RB^{13}_{3}= *Enterobacter* sp., SC^{7}_{6}= *Serratia* sp., BC^{1}_{3}= *Bacillus* sp., RD^{7}_{6}= *Pseudomonas* sp**., NZ 103= *Bacillus pumilus*, NZ 106= *Bacillus pumilus*,** BB^{1}_{6}=*Bacillus* sp., BA^{1}_{3}= *Bacillus* sp., SB^{1}_{6}= *Enterobacter* sp., SD^{1}_{6}= *Serratia* sp., MA^{1}_{4}= *Bacillus* sp., MA^{1}_{2}= *Enterobacter* sp., BA^{1}_{6}= *Pseudomonas* sp., MB^{5}_{6}= *Bacillus* sp., BC^{2}_{4}= *Pseudomonas* sp., **NZ 108= *Enterobacter ludwigii*,** BA^{4}_{6}= *Pseudomonas* sp., SA^{1}_{6}= *Serratia* sp., MA^{2}_{6}= *Bacillus* sp., ME^{3}_{6}=*Enterobacter* sp., SA^{2}_{6}= *Bacillus* sp., MA^{5}_{6}= *Bacillus* sp., MD^{3}_{6}= *Enterobacter* sp., MA^{5}_{6}= *Bacillus* sp., MD^{5}_{6}= *Pseudomonas* sp., **NZ 107= *Bacillus safensis*,** SD^{6}_{6}= *Bacillus* sp.

Means followed the same letters are not significantly different ($P < 0.0001$). Separation of means using Duncan's Multiple Range Test.

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98%. In group d with seven representative isolates, the disease severity has a reduction between 70-90%. The group c containing fifteen isolates had intermediate control effects (45-70%). The two lowest groups were as followings: the group b with five isolates and 25-45% disease reduction effects and the group a, with four bacterial strains that could lower the disease by 5-20%.

The statistical analyses on the dry weight, root dry weight, seedlings length, root length, root fresh weight and fresh weight of the seedlings treated with representative isolates, showed a significant difference amongst the treatments. The seven isolates of *P. baetica* NZ101, *B. subtilis* NZ104 and *E. ludwigii* NZ108, *S. marcesens* NZ102, *B. pumilus* NZ103, *B. safensis* NZ107 and *B. pumilus* NZ106 have increased the growth factors by 92-98%. Other isolates had a modest effect in comparison with the control (Table 1).

BLAST analyses demonstrated that the isolates NZ101, NZ102, NZ104, NZ106, NZ108 belong to the species *P. baetica, S. marcesens, B. subtilis, B. safensis* and *E. ludwigii*, respectively. Both NZ103 and NZ106 belong to the species *B. pumilus*.

Detection of antifungal metabolites produced by isolates of endophytic bacteria

Six isolates including *P. baetica* NZ101, *S. marcesens* NZ102, *B. pumilus* NZ103 and NZ106, *B. subtilis* NZ104 and *B. safensis* NZ107 could significantly inhibit the *V. dahliae* growth by production mechanism of the protease enzyme at P <0.0001 (Fig. 2). *E. ludwigi* NZ108 failed to produce this enzyme completely, whereas *P. baetica* NZ101 showed the highest protease activity by 65% (Table 2).

S. marcesens NZ102 and *B. pumilus* strains NZ103 and NZ106 produced hydrogen cyanide in the medium containing glycine (Table 2).

Regarding the production of agar-permeable extracellular inhibitor metabolites, a significant decrease (P <0.0001) in *V. dahliae* mycelial growth was observed. The highest growth inhibition with 2.76 mm in diameter was associated to *P. baetica* NZ101, *E. ludwigi* NZ108 and *B. subtilis* NZ104 (Table 2).

A significant reduction by 68% (P <0.0001) was observed in the *V. dahliae* mycelial growth compared to the control, due to volatile antifungal metabolites production by the isolates *P. baetica* NZ101, *E. ludwigi* NZ108 and *B. subtilis* NZ104 (Table 2).

Three representative isolates, *P. baetica* NZ101, *S. marcesens* NZ102 and *E. ludwigii* NZ108, produced the auxin hormone IAA, in concentrations calculated with respect to the standard curve (Merck, Germany) (Table 2).

Siderophore production by almost all bacteria had a significant effect on the fungal mycelium growth of *V. dahliae* at concentrations of 5, 10, 25 and 50 ug/l (P <0.0001). The results indicated that the isolates *E. ludwigii* NZ101, *P. baetica* NZ108, *B. subtilis* NZ104 and NZ103, *B. pumilus* NZ106 reduced or inhibited the *V. dahliae* mycelial growth, at the media emended with 10 and 5 µg/l of FeCl3 (Table 2).

The mean value of the inhibition zone caused by bacteriocin producing strains indicated that *P. baetica* NZ101, *E. ludwigii* NZ108, NZ104 and *B. subtilis* NZ104 could decrease *V. dahliae* growth up to 70% compared to the control (Table 2).

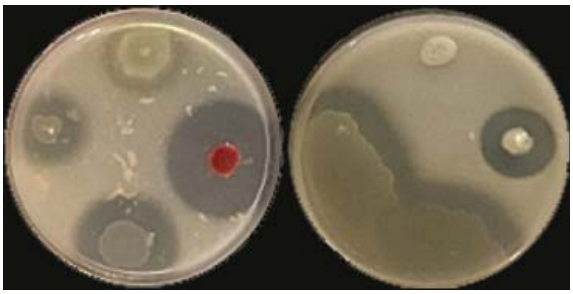


Figure 2. Protease enzyme production by different bacteria on SMA medium. Clockwise from top, at the left plate: *Pseudomonas baetica, Serratia marcesens, Bacillus pumilus* and *Bacillus subtilis*, and at the right plate: *Bacillus safensis, Bacillus pumilus* and *Enterocater ludwigii*.

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Table 2. Effect of protease, bacteriocin, extracellular and volatile antifungal metabolites, and siderophore on inhibitory growth of *Verticilliun dahliae* in tomato plants, cv. Falat, inoculated with endophytic bacteria. Production of IAA and hydrogen cyanid by the endophytic bacteria.

Treatment	Protease (mm)	Bacteriocin (mm)	Extracellular metabolites (mm)	Volatile antifungal metabolites (mm)	Siderophore (10-µl ferric chloride)	Concentration of IAA (µg/ ml)	Optical density at 530 nm	Hydrogen cyanid
P. baetica NZ101	1.33 ± 0.01 a	1.23 ± 0.01 e	2.76 ± 0.01 c	1.50 ± 0.05 e	1.33 ± 0.01 a	25	0.059	–
S. marcesens NZ102	0.23 ± 0.01 e	1.89 ± 0.05 c	3.69 ± 0.03 b	3.69 ± 0.03 b	0.80 ± 0.005 c	25	0.059	+
B. pumilus NZ103	0.63 ± 0.01 c	2.89 ± 0.001 d	3.29 ± 0.02 b	2.73 ± 0.001 c	1.20 ± 0.01 ab	3.12	0.004	+
B. subtilis NZ104	0.83 ± 0.01 b	1.23 ± 0.01 e	2.76 ± 0.01 c	1.65 ± 0.05 e	1.20 ± 0.01 ab	12.5	0.025	-
B. pumilus NZ106	0.23 ± 0.01 e	2.06 ± 0.01 b	3.29 ± 0.02 b	2.50 ± 0.01 d	1.20 ± 0.01 ab	3.12	0.004	+
B. safensis NZ107	0.63 ± 0.01 c	2.16 ± 0.01 ab	2.76 ± 0.01 c	1.65 ± 0.05 e	0.80 ± 0.005 c	12.5	0.025	-
E. ludwigii NZ108	0.00 ± 0.00 f	1.23 ± 0.01 e	2.76 ± 0.01 c	1.65 ± 0.05 e	1.33 ± 0.01 a	25	0.059	-

Means followed the same letters are not significantly different ($P < 0.0001$). Separation of means using Duncan's Multiple Range Test.

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Discussion

One of the most applicable methods for tomato wilt disease management caused by *V. dahliae* is the use of biological control agents, such as fungal and bacterial antagonists, both *in vitro* and *in vivo* conditions. Furhermore, disease control along with plant growth promotion traits has been considered as a novel method in tomato Verticillium wilt disease management all over the world (Martin and Bull, 2002; Munif *et al*., 2012; Thomas and Upreti, 2015). In this study, 688 endophytes bacteria were isolated from roots, stems, leaves and fruit of tomato plants and their antagonistic activities against *V. dahliae* were evaluated. Among them, 39 bacterial isolates with negative hypersensitivity reaction (HR) on tobacco and geranium and failure to decay potato slices were selected for greenhouse investigations.

Seven endophytic bacterial isolates, *B. pumilus* (NZ103 and NZ106)*, B. subtilis* NZ104*, S. marcesens* NZ102*, E. ludwigii* NZ108, *B. safensis* and *P. baetica* NZ108*,* were found to be highly potent in controlling *V. dahliae* and reducing the severity of the symptoms by 98%. In addition, the endophytes could accelerate the growth of tomato plants, based on several growth characteristics, in greenhouse conditions. These findings are in agreement with the results of Martin and Bull (2002) and Naraghi *et al*. (2008), supporting the fungal and bacterial antagonists and bacterial endophytes application against Verticillium wilts in other crops. Munif *et al*. (2012) identified 32 genera of endophytic bacteria in tomatoes enable to control fungal pathogens with dominant genera *Bacillus* and *Pseudomonas* against *Fusarium* and *Rhizoctonia*.

Tomato seeds infested with *V. dahliae* and treated with individual endophytic bacteria of the seven isolates demonstrated that some bacterial metabolites play a significant role in disease symptoms reduction in comparison with the controls i.e. with respect to the mechanisms investigated, it was established that *P. baetica* NZ101 could produce the highest protease and auxin IAA level, whereas, the isolates of *S. marcesens* NZ102 and *E. ludwigii* NZ108 could only produce auxin IAA in order to modulate plant growth. These results are consistent with the Gull and Hafeez (2012) findings, indicating that 8 isolates of *Pseudomonas fluorescens* could increase plant growth and also reduce root decay disease by *R. solani* in wheat plants. The results are also consistent with the research work presenting that bacteria isolated from canola roots and shoots not only improved the seed germination, seedling length and rapeseed and tomato growth, but also lowered the disease symptoms of *V. dahliae* and *F. oxysporum* (Nejad and Johnson, 2000). In respect to hydrogen cyanide production, three isolates of *S. marcesens* NZ102 and *B. pumilus* NZ103 and 106 were able to produce hydrogen cyanide in glycine containing medium. The results were in agreement with the results of Gall and Hafeez (2012).

The production of secondary metabolites in producing various antibiotics, enzymes and biofilm is well documented (Morikawa, 2006). The endophytic bacteria *E. ludwigii* NZ108, *P. baetica* NZ108*, B. subtilis* NZ104 and *B. pumilus* (NZ103 and NZ106) produced siderophore on PDA medium containing 10-µl ferric chloride iron to reduce or inhibit *V. dahiae* growth. The three first species produced bacteriocin that could reduce *V. dahiae* growth by 70% as well as volatile antifungal metabolites and diffusible inhibitors in agar media to decrease the normal mycelial growth by 68%. Henis and Inbar (1986) report that extracellular and volatile antifungal compounds production by *B. subtilis* strains were effective in reducing plant diseases. According to Ongena *et al.* (2008), *B. subtilis* can induce resistance to pathogens by producing secondary metabolites.

In conclusion, this study isolated, identified and evaluated the endophytic bacteria diversity in different tomato plant organs from tomato plants sampled in Iran in relation to their effect on Verticillium wilt disease control and plant growth. Four endo-

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phytic bacterial isolates, *P. baetica* NZ101, *B. subtilis* NZ104, *E. ludwigii* NZ108 and *B. safensis* NZ107 inhibited fungal growth and indirectly stimulated plant growth by producing different types of antimicrobial metabolites. Since no similar research has been performed in Iran before, the results are notable for future application of these most promising bacterial endophytes in biocontrol of the destructive Verticillium wilt in greenhouse tomato.

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Received: 24 August 2019; Accepted: 5 April 2020

Ενδοφυτικά βακτήρια της τομάτας στην αντιμετώπιση της αδρομύκωσης και το πλεονέκτημά τους στη βιοδιέγερση

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Στη μελέτη αυτή απομονώθηκαν 688 ενδοφυτικά βακτηριακά στελέχη από φυτά θερμοκηπιακής και υπαίθριας καλλιέργειας τομάτας, τα οποία συλλέχθηκαν από τις επαρχίες της Τεχεράνης και του Alborz, Ιράν και ελέγχθηκαν ως πιθανοί ανταγωνιστικοί παράγοντες έναντι του μύκητα *Verticillium dahliae*. Ακολούθησε περαιτέρω επιλογή 128 αντιπροσωπευτικών απομονώσεων βάσει της διαφορετικότητας των φαινοτυπικών χαρακτηριστικών τους. Στη συνέχεια μετά από δοκιμή υπερευαισθησίας αποκλείστηκαν τα στελέχη που εμφάνισαν θετική αντίδραση σε φύλλα καπνού, γερανίου και στην υγρή σήψη της πατάτας. Βάσει αυτών επιλέχθηκαν συνολικά 39 απομονώσεις για περαιτέρω δοκιμές ανταγωνισμού *in vitro* και σε συνθήκες θερμοκηπίου. Από αυτά, επτά ενδοφυτικά ανταγωνιστά στελέχη με τα πλέον επωφελή χαρακτηριστικά ταυτοποιήθηκαν βάσει της ανάλυσης του γονιδίου 16S rRNA ως *Bacillus pumilus* (δύο στελέχη), *Bacillus subtilis, Bacillus safensis, Enterobacter ludwigi, Serratia*

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marcesens και *Pseudomonas beatica*. Αξιολογήθηκε η βιολογική δράση των απομονώσεων με τη χρήση μεθόδων επεξεργασίας σπόρων και διαβροχής εδάφους σε δύο ποικιλίες τομάτας. Τα αποτελέσματα έδειξαν ότι και οι επτά βακτηριακές απομονώσεις είχαν υψηλό δυναμικό για τον έλεγχο του μύκητα και μείωσαν τη δριμύτητα της ασθένειας κατά 95-98%. Αυτή η ανταγωνιστική δράση συνδυάστηκε με την αύξηση ορισμένων παραμέτρων ανάπτυξης όπως το ξηρό βάρος φυτού, το ξηρό βάρος ρίζας, το ύψος φυτού, το μήκος ρίζας, το νωπό βάρος ρίζας και το νωπό βάρος φυτού, κατά 92-98%. Η διερεύνηση των μηχανισμών βιολογικής δράσης τους έδειξε παραγωγή πρωτεάσης από όλες τις απομονώσεις, με υψηλότερα επίπεδα, έως 65%, από τα στελέχη *E. ludwigii* και *P. beatica*. Τρεις από τις απομονώσεις που παράγουν βακτηριοσίνες ανέστειλαν την ανάπτυξη του μυκηλίου του φυτοπαθογόνου μύκητα έως και 70% σε δοκιμή διπλής καλλιέργειας. Επίσης, πέντε από τις απομονώσεις παράγουν σιδηροφόρα, ενώ τα ενδοφυτικά βακτήρια *P. baetica, S. marcesens* και *E. ludwigii* παρήγαγαν αξιοσημείωτη ποσότητα αυξίνης.

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