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Research Article

Biocontrol of *Fusarium oxysporum* in cucumber by some antagonist bacteria under drought stress

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Abstract: Fusarium crown and root rot of cucumber caused by *Fusarium oxysporum* is one of the most important diseases in cucumber. Although various methods have been recommended to manage this disease, biological control is considered as an environmentally friendly method. In the present study, antagonistic effects of six *Pseudomonas* and *Bacillus* genera strains were investigated against *F. oxysporum*, where *in vitro* and *in vivo* assays were performed under drought stress. All of the strains were capable to inhibit the growth of *F. oxysporum*. The results of drought stress also indicated that the bacterial strains were able to tolerate different levels of drought stress. In general, *Pseudomonas fluorescens* VUPF5 caused the best inhibitory effect in all of the assays *in vitro* and under greenhouse conditions.

Keywords: Antagonist, Cucumber, *Fusarium oxysporum*, *Pseudomonas* and *Bacillus*

Introduction

The use of fungicides for controlling plant diseases as well as irregular usage of chemical fertilizers for reducing the effects of environmental stress has negatively influenced the human health due to high residues of poisonous chemicals in food and in the environment. Therefore, usage of disease biological control factors is a good strategy to reduce the effects of poisonous chemical compounds (Dubey *et al.*, 2007).

The root and stem rot of cucumber plant is one of the important diseases which is induced by *Fusarium oxysporum*. Recently, root and stem rot has reduced the yield quality and quantity of cucumber plant especially in controlled environments. Although there are various control methods against *Fusarium* root and stem rot, but biological control using antagonistic bacteria has been known as an alternative and more suitable strategy when compared to the other control methods (Vatchev and Maneva, 2012).

Abiotic stresses such as drought and salinity are the most important climate changes causing loss of soil organic matter and soil degradation and negatively affecting plant growth and yield (Ahmad *et al*., 2015). The use of beneficial microorganisms improves plant growth, increases tolerance to environmental stress, and protects plants against plant diseases (Egamberdieva *et al*., 2016). Biological disease control and the use of antagonist bacteria can enhance plant tolerance to stress condition by inducing several defense mechanisms (Marulanda *et al*., 2007). *Pesudomonas* and *Bacillus* are two main groups of beneficial bacteria which have been used to control diseases induced by soil-borne pathogens (Weller, 1988; Susilowati *et al*., 2010).

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Biocontrol activity of these bacteria such as production of several antibiotics, siderophore, and competition for space and food are some mechanisms utilized against pathogens (Handelsman and Stabb, 1996). These bacteria are also known as plant growth-promoting rhizobacteria (PGPR) by colonizing plant roots and promoting plant growth. They also synthesize phytohormones and plant growth stimulators (Parray *et al*., 2016), increase nutrients uptake (Berg *et al*., 2013), produce of fungi-toxic compounds (Landa *et al*., 2004), and induce defense enzymes in plants, thereby enhancing the plant tolerance to abiotic and biotic stress (Glick, 2012). Strains of *Pesudomonas* and *Bacillus* may have different responses to stress conditions and some strains are far more tolerant than others. Accordingly, the aim of the present study was to investigate the effect of some strains of *Pseudomonas fluorescens* and *Bacillus subtilis* on suppression effect on *F. oxysporum* under drought stress condition by evaluation of volatile compounds and productions of Protease, Hydrogen cyanide (HCN), and Siderophore.

Materials and Methods

Drought Tolerance

In order to investigate the growth potential of bacterial strains under drought stress, NB medium was modified with different levels of PEG 6000 including 0, 203.362, 298.587, 438.40, 548.838 g PEG 6000 per 1 Kg of media creating osmotic pressures of 0, -5, -10, -20 and -30 Mpa, respectively. The growth of bacterial strains was determined by measuring the OD at 600 nm (Michel and Kaufman, 1973).

Antagonistic activity in vitro

Dual culture assay

The ability of six strains of bacteria to produce antibiotic compounds against *F. oxysporum* was assayed as described by Keel *et al*. (1996). The diameter of the inhibition zone was measured by a ruler. The percentage of growth inhibition was calculated as follows:

$$\textit{Inhibition}\,(\%)=(1-\frac{\textit{Treatment growth}}{\textit{Control growth}})\times 100$$

Antifungal activity assay of volatile compounds

The antifungal activity of bacterial strains was determined according to Fiddaman and Rossall (1993). For this purpose, 200 µl of bacteria suspension was inoculated on nutrient agar medium. Then, 5mm plug from the margin of 3-day fungal culture was placed in the center of the plate, and the lids of the plates (both antagonist and pathogen) were removed. The pathogen colony plate was inversely placed on the plate containing the bacterial antagonist colony. The plates were sealed by parafilm and were incubated at 25 °C in darkness for 7 days. The control plates were cultured only with *F. oxysporum* plug. The percentage of fungal growth inhibition was calculated as mentioned above.

Protease production

Protease production was determined using the method of Maurhofer *et al*. (1995). The bacterial strains were spotted on skim milk agar medium and incubated at 28 °C for 48h. Protease activity can be characterized by formation of halo zones around the spot. The medium containing the halo zone had a positive response to protease production.

Hydrogen cyanide (HCN) production

The production of hydrogen cyanide was performed according to the method described by Alstrom and Burns (1989). For this purpose, 100µl of the bacterial inoculum was inoculated on King's B medium amended with glycine (4/4gl-1). Then, Whatman No. 1 filter papers were soaked in a picric acid 0.5% + Na_2CO_3 2% solution and fixed beneath the petri dishes. To prevent volatilization, the plates were sealed with parafilm and incubated at 28 °C for 4 days. HCN production was represented by change in the color of the filter paper. Bacterial strains that showed yellow, cream, light brown, and reddish-brown colors received 0, 1, 2, and 3 scores, respectively. The scores of 0, 1, 2 and 3

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represented no ability or low, medium, high, and very high ability for HCN production, respectively.

Siderophore production

Determination of siderophore production was performed according to the method of Meyer and Abdallah (1978). For this purpose, 100µl of bacteria suspension was inoculated in succinate medium at 27 °C on a rotary shaker (120rpm). After 40 h, the bacterial cells were removed by centrifugation, and siderophore isolated from the supernatants was measured using absorption maximum at 400nm.

Greenhouse assay

Plant growth condition

Seeds of cucumber (*Cucumis sativus* Cv. Yazd) were soaked in sodium hypochlorite 0.5% solution for 5 min as surface sterilized and then rinsed with sterile water. The seeds were placed into wetted sterile bags for germination at 28 °C for 24 h. Then, four germinated seeds were sown in 1kg pots containing sand and soil, at pH 7.6 and EC 1.2 ds. m. After 20 days, the seedlings were exposed to two drought stress treatments: 70% field capacity (well-watered) and 30% field capacity (severe stress), with seedlings simultaneously inoculated with pathogen and antagonist bacteria.

Bacterial inoculation

Pseudomonas fluorescens strains (VUPF5, CHA0, T17-4) and *B. subtilis* strains (Bs96, BsVRU, BsVRU1) were prepared from the Plant Protection Collection Center of Vali-e-Asr University of Rafsanjan, Iran (these strains were selected based on previous studies which showed suitable biocontrol ability and plant growth promotion (Lagzian *et al*., 2013; Baradar *et al*., 2015). The bacterial suspensions were diluted with distilled water and the concentration was adjusted to 10^{10} CFU/mL (OD 0.5 at 540 nm = 10^{10}) using a spectrophotometer (U-2000, Hitachi Instruments, Tokyo, Japan). Finally, 10 ml of these suspensions were added to each pot and used for soil drenching.

Inoculation of pathogen

For inoculation of *F*. oxysporum (which was obtained from the Collection of Plant Protection Group of Vali-e-Asr University of Rafsanjan, Iran.), flasks containing 250 g of autoclaved wheat seeds were inoculated with four plugs of fungal medium. The flasks were incubated at 25 °C for 3-4 weeks and were shaken every day to achieve uniform fungal colonization of all wheat seeds. Then, the colonized seeds were mixed up with soil in the pot at a rate of 0.5% (5 g per 1000 g soil).

Disease assessment

Evaluation of disease severity was calculated based on a scale of 0-3; 0: no symptoms, 1: light or moderate rot on taproot, secondary roots and crown, light vascular discoloration in the stem, 2: severe rot on taproot, secondary roots and crown, with or without wilting and stunting, vascular discoloration in the stem and 3: dead seedling (Vakalounakis *et al*., 1996).

The disease severity was calculated using the following equation:

$$\text{The disease index percentage (DI\%)} = \frac{(0 \times a) + (1 \times b) + (2 \times c) + (3 \times d)}{3 \times n} \times 100$$

Where a, b, c and d is number of plants with indices of 0, 1, 2 and 3, respectively.

N = total number of plants.

Statistical analysis

Data analysis was conducted using the SAS 9.1 version and comparison of the data mean was performed using Duncan's multiple range test at 1% ($p \leq 0.01$) level.

Results

Dual culture assay

The results of the dual culture revealed that all bacterial strains had an inhibitory effect on the pathogenic fungi. The highest and lowest inhibition effects were about 66.66% and

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41.66% as recorded by BsVRU1 and CHA0, respectively (Fig. 1).

Volatile compounds

In this study, we investigated the effect of volatile compounds on the hyphal growth of *F. oxysporum*. We found that bacterial strains were capable of producing antifungal VOCs on NA medium. Among them, *P. fluorescens* VUPF5 had the greatest inhibitory effect (53.33%) on fungal growth (Fig. 2).

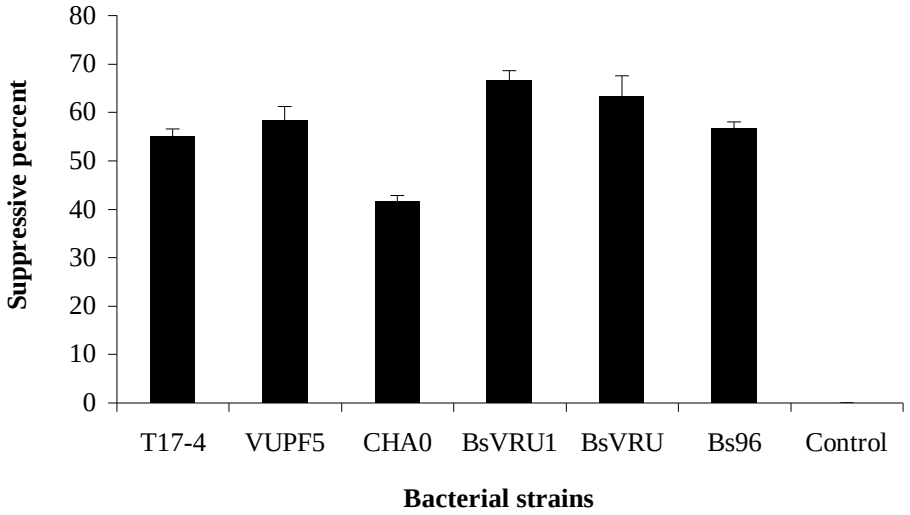

Figure 1 The inhibitory effect of bacterial antagonists on the hyphal growth of *Fusarium oxysporum* in a dual culture assay.

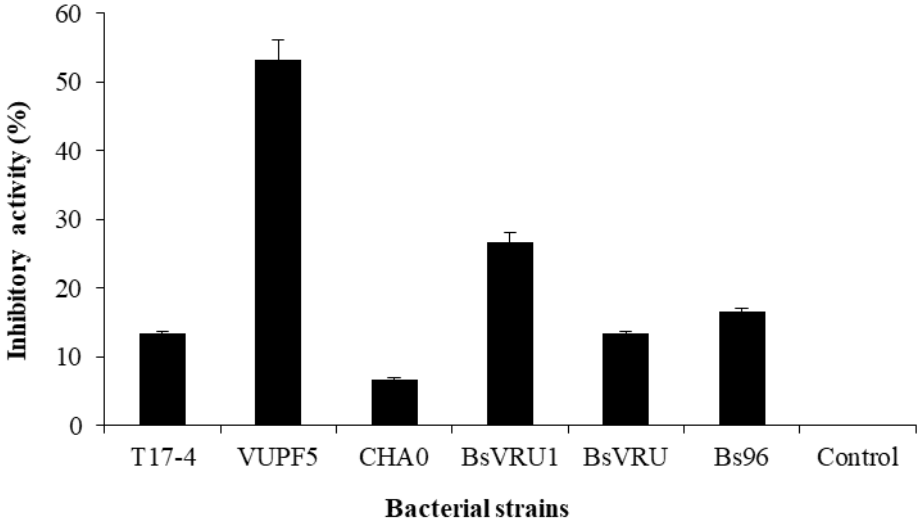

Figure 2 The effect of bacterial volatile compounds on the hyphal growth of *Fusarium oxysporum.*

Production of antifungal metabolites

As presented in Table 1, all bacterial strains had the ability of HCN production. The VUPF5 strain indicated the greatest ability of HCN production (Table 1). The results also showed that VUPF5 and BsVRU1 strains had the

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highest ability of protease production, while CHA0 demonstrated the lowest extent of protease production (Table 1).

Table 1 Production of metabolites by bacterial strains.

Bacterial strains	HCN[1]	Protease[2]
T17-4	++	10.0
VUPf5	+++	12.0
CHA0	+	6.5
$BsVRU_1$	+	12.0
BsVRU	+	8.5
Bs96	+	11.0

1. HCN production - negative, + weak, ++ moderate, +++ strong.
2. Diameter of halo zone around the bacterial colonies (mm).

The results of siderophore production (pyoverdin) indicated a high level of siderophore production observed in *P. fluorescens* VUPF5 (Fig. 3).

Drought stress

In this study, the effect of PEG600 on six bacterial strains was evaluated *In vitro* (Fig. 4). The bacterial strains' growth declined by increasing the drought stress intensity induced by PEG. The minimum growth reduction (9.55%) was observed in VUPF5 and CHA0, while the greatest reduction was observed in BsVRU1 (50.64%) compared to the control.

In vivo efficacy of bacterial strains against *F. oxysporum*

Twenty days after the infection of cucumber seedlings with *F. oxysporum* under drought stress, incidence of disease with different severities was seen. The results showed that the use of bacterial strains significantly decreased the effect *F. oxysporum* on cucumber plants, but the bacterial strains could not completely control the pathogen. Under all conditions, the maximum reduction of disease severity was recorded in plants inoculated with VUPF5 (about 70%), while the minimum reduction of the disease severity was found in the plant inoculated with BsVRU (about 25%). The disease of cucumber seedlings under drought stress was more severe compared to the seedlings' growth under non-stress conditions. As shown in Fig. 5, among all strains T17-4, VUPF5, and BsVRU1 exhibited the highest tolerance compared to the untreated control at both levels (30% and 70%) (Fig. 5).

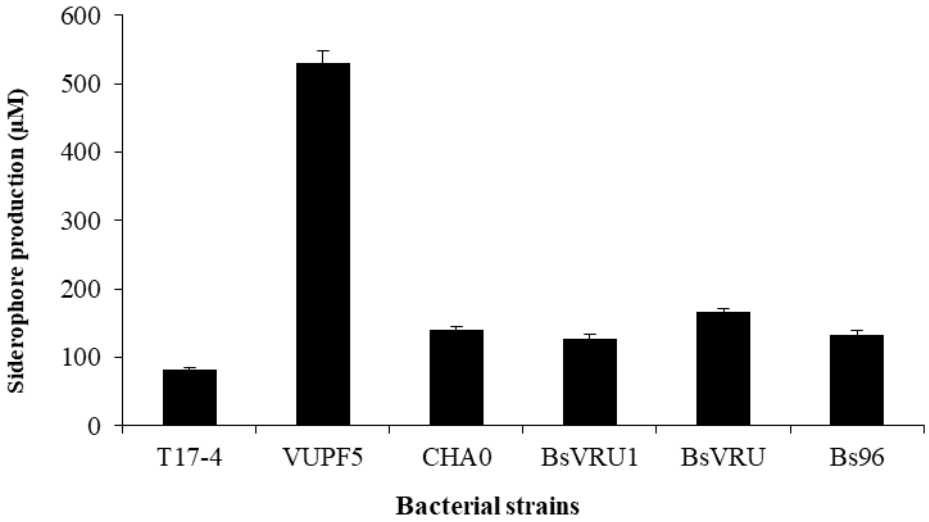

Figure 3 Production of siderophore by bacterial strains in succinate medium.

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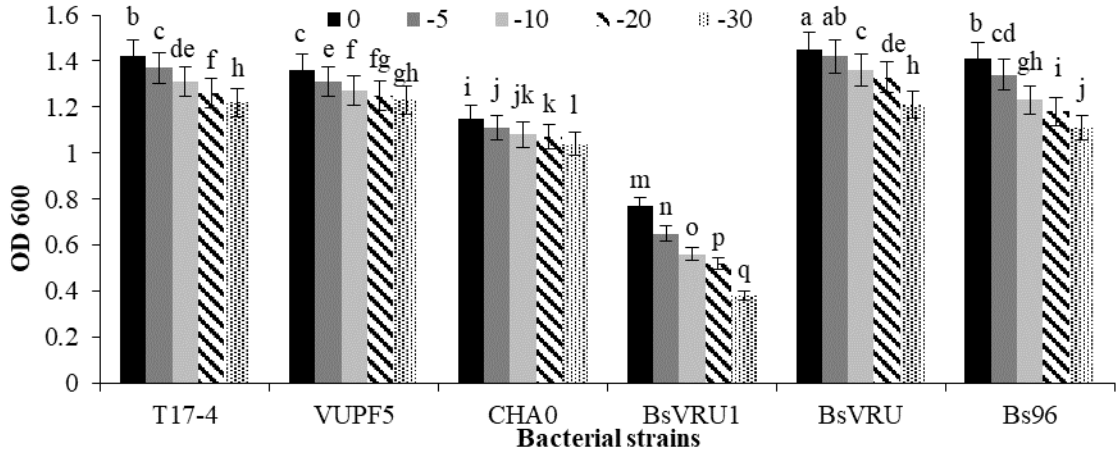

Figure 4 Effect of polyethylene glycol (PEG) levels on bacterial growth. Means with the same letters are not significantly different (Duncan's multiple range test, $p \leq 0.01$).

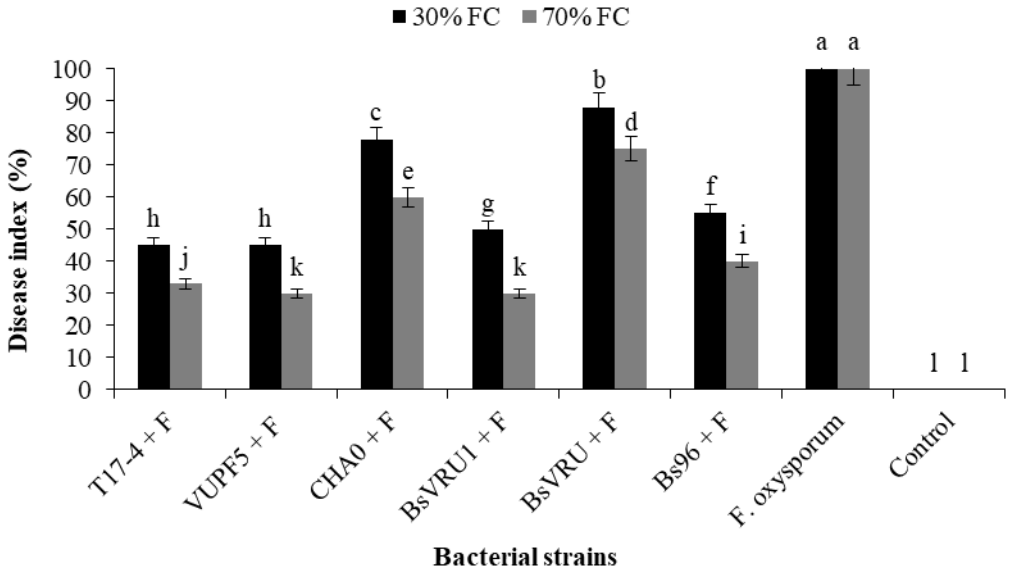

Figure 5 Effects of bacterial strains on disease severity of cucumber seedlings under drought stress. Means with the same letters are not significantly different (Duncan's multiple range test, $p \leq 0.01$).

Discussion

In recent years, many studies have been conducted on the biological control of plant diseases. Plant growth-promoting rhizobacteria (PGPR) are a group of beneficial bacteria with the ability of root colonization, plant growth promotion, and suppression of phytopathogens (Beattie, 2006). Several mechanisms are involved in the biocontrol ability of bacteria such as production of antifungal metabolites including antibiotics, enzymes, siderophores, hydrogen cyanide (HCN), ethylene, and phytohormones. These bacteria introduce their metabolites into the environment as secretion of extracellular and volatile compounds (Kamilova *et al*., 2005).

In this study, we investigated the antagonistic and inhibitory effects of six strains

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of *Pseudomonas* and *Bacillus in vitro*, as well as *in vivo* suppressive effects on the disease under drought stress conditions. Many researchers have reported that *in vitro* assays can be used as a quick method for the primary selection of antagonists for controlling plant disease (Weller, 1998; Hulme and Trevethick, 2010). For this purpose, the antagonistic potential of six bacterial strains was evaluated based on an *in vitro* dual culture assay against *F. oxysporum.* The results of dual culture revealed that all strains were capable of inhibiting the growth of pathogen. Although the inhibition zone assay is often used to evaluate and select bacterial strains with bio-control abilities and efficacy, it should be noted that the potential inhibitory effect of strains *in vitro* is different from that of *in vivo* conditions. In other words, it can only give us general information about their antagonistic potential under a special condition (Fravel, 1988). Similar results have also reported that the use of dual culture to investigate the potential inhibitory effect of strains *in vitro* is different from that under *in vivo* conditions (Kunova *et al*., 2016).

Volatile compounds are a series of metabolites with low molecular weight, low polarity, and high vapor pressure (Vespermann *et al*., 2007). Several biological effects including inter-species and species communication and growth inhibition of nematodes, plants, and fungi are attributed to bacterial VOCs (Insam and Seewald, 2010). Antifungal VOCs produced by bacteria could inhibit mycelial growth, enzyme activity, and spore germination of pathogenic fungi (Fiddaman and Rossall, 1993). Among the examined strains, *P. fluorescens* VUPF5 produced the highest amount of VOCs.

Hydrogen cyanide is a secondary metabolite of bacteria which is directly made from proline, glycine, and cyanogenic glycoside, which has also toxic effects on fungi. Hydrogen cyanide can cause formation of hairy root by bacteria (Schippers *et al*., 1987). According to the results, bacterial strains have a different ability to produce hydrogen cyanide (HCN). The maximum amount of HCN production was observed in *P. fluorescens* VUPF5.

In the investigation on protease enzyme production, all strains produced colorless halo around their colonies, where *P. fluorescens* VUPF5 and *B. subtilis* BsVRU1 showed a high enzyme activity. The production of protease enzyme by bacteria is an effective mechanism for biological control of the plant (Keel and Defago, 1997).

Soil microorganisms form a stable complex with Fe^{3+} by secretion of siderophore under iron deficiency conditions. The formation of Fe (III)–siderophore complexes scavenge iron from mineral phases and make it available for plants (Leoni *et al*., 2002). Siderophore-producing bacteria limit the absorption of iron by the pathogen and enhance the plant growth (O'Sullivan and O'Gara, 1992). All strains in this study were able to produce siderophore among which *P. fluorescens* VUPF5 offered the maximum production.

Fusarium wilt of cucumber is one of the most important diseases causing a great deal of damage every year. Furthermore, drought stress is another major environmental factor limiting the crops of this plant. In the present study, we investigated the effects of bacterial strains on *F. oxysporum* under drought stress. Biased on our results, the inhibitory effect under *in vitro* was not the same as *in vivo* condition, but in general the inhibitory effect of strains was satisfactory. The strains VUPF5, T17-4 and BsVRU1 presented better results than strains CHA0, Bs96, and BsVRU under greenhouse conditions. However, the results of *in vitro* drought stress had a low correlation with greenhouse results. *In vitro* condition strain $BsVRU_1$ revealed the minimum tolerance to drought stress compared to BsVRU, Bs96, and CHA0, but in the greenhouse condition $BsVRU_1$ was better than these three strains. In addition to its antagonistic activity, this bacterial strain could stimulate the host resistance against pathogen (Kloepper *et al*. 2004). Among the tested bacteria, *Pesudomonas* strains were more successful compared to *Bacillus* strains. In general, in this

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study, the strains *P. fluorescens* VUPF5, *P. fluorescens* T17-4, and *B. subtilis* VRU1 had suppressive effects on *F. oxysporum.*

In general, *Pesudomonas* and *Bacillus* are successful biocontrol agents of soil-borne pathogens *P. fluorescens* strains often lie in plants' rhizosphere and are among the important groups in soil-borne disease biocontrol (Ellis *et al*., 2000). There are many studies suggesting the inhibitory effect of different species of *Bacillus* and *Pseudomonas* on a wide range of pathogens (Lartey, 2003; Ongena *et al*., 2007; Raupach and Kloepper, 2000; Zhang *et al*., 2004). In our study, *P. fluorescens* VUPF5 demonstrated the greatest inhibition rate on *F. oxysporum* across all of the assays.

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کنترل زیستی *Fusarium oxysporum* در خیار توسط برخی باکتری‌های آنتاگونیست تحت تنش خشکی

روح الله صابری ریسه* و فریبا فتحی

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چکیده: یکی از مهم‌ترین بیماری‌های خیار، پوسیدگی طوقه و ریشه خیار می‌باشد که توسط *Fusarium oxysporum* ایجاد می‌شود. اگرچه روش‌های زیادی برای این بیماری توصیه شده است، اما کنترل زیستی به‌عنوان یک روش سازگار با محیط‌زیست مورد توجه قرار گرفته است. در مطالعه حاضر اثرات آنتاگونیستی شش استرین از دو جنس *Pseudomonas* و *Bacillus* در برابر قارچ *F. oxysporum* در شرایط آزمایشگاه و گلخانه تحت شرایط تنش خشکی مورد ارزیابی قرار گرفت. همه استرین‌ها قادر به بازدارندگی از رشد قارچ بودند. نتایج حاصل از تنش خشکی نشان داد که این استرین‌ها قادر به تحمل سطوح خشکی می‌باشند. به‌طور کلی باکتری *Pseudomonas fluorescens* استرین VUPF5 بهترین اثر بازدارندگی را در تمامی آزمایش‌ها در شرایط آزمایشگاه و در شرایط گلخانه نشان داد.

واژگان کلیدی: آنتاگونیست، خیار، *Fusarium oxysporum*، سودوموناس و باسیلوس

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