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Bacillus amyloliquefaciens as a Biocontrol Agent Improves the Management of Charcoal Root Rot in Melon

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ABSTRACT

Seventy five bacterial strains were isolated from cucurbits rhizosphere and examined for antagonistic activities against *Macrophomina phaseolina* isolate 44, the causal agent of melon charcoal root rot disease. The results of screening strains including B2, B11, B12 and BKN showed high potential of antagonistic activities against the pathogen in laboratory experiments. Inhibition of mycelial growth varied from 54.3 to 62.22%, 39.43 to 54.82%, 78.52 to 100%, and 64.45 to 88.89% in dual culture, volatile metabolite, antibiotic production, and cell free culture tests, respectively. In greenhouse experiment, seed treatment with strain B2 significantly (P= 0.01) controlled the disease by increasing plant growth indices including height, shoot and root fresh weight, shoot and root dry weight by 37.98, 36.27, 32.97, 34.44, and 30.39%, respectively, as compared to the control. The biochemical and physiological tests as well as *gyr*A sequence confirmed these four strains as *Bacillus amyloliquefaciens*. Results indicated that strain B2 could be an important new biological control agent for charcoal root rot disease of melon.

Keywords: Antagonistic activity, Biological control, Cucurbits *Macrophomina phaseolina*, Rhizosphere.

INTRODUCTION

Melon (*Cucumis melo* L.) is one of the most popular cucurbits in the world. It is the third most important vegetable crop next to tomato and onion (Ghebretinsae *et al*., 2007). This crop is susceptible to a large number of disease agents such as seedling and root pathogens that cause serious damages to this plant. One of these soilborne pathogens is *Macrophomina phaseolina* (Tassi) Goid, the causal agent of charcoal root rot. This polyphagous pathogen attacks and infects more than 500 plant species in 100 families of monocots and dicots (Jana *et al*., 2003). It is a major disease in dry regions of Asia, Africa, the Americas, and Australia. Germination of sclerotia of *M. phaseolina* might be triggered by root exudates from melon seedlings (Smith, 1969). The germinated sclerotia can infect the primary root and cause seedling blight (Livingston, 1945). If infection occurs before the emergence of secondary roots, the plants die. Less severely infected seedlings, however, survive and establish secondary roots and grow to mature plants. *M. phaseolina* infection leads to rotting of roots followed by rotting of stalks, resulting in lodging of the plant at later stages (Partridge *et al*., 1984).

Protection of root from infection of the fungus with microbial inoculants is targeted for eco-safe management of this disease (Spadaro and Gullino, 2004; Droby *et al*., 2009). The use of microorganisms or their secondary metabolites to prevent plant diseases are environment friendly, safe, and may provide long-term protection to the crop (San Lang *et al*., 2002; Fernando *et al*.,

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2005). Many bacteria such as *Bacillus amyloliquefaciens* (Arguelles-Arias *et al*., 2009; Arrebola *et al*., 2010), *B. subtilis* (Obagwu and Korsten, 2003; Romero *et al*., 2007), *Pantoea agglomerans* (Usall *et al*., 2008; Yu *et al*., 2010), *Pseudomonas fluorescens* (Mikani *et al*., 2008) and *Serratia plymuthica* (Meziane *et al*., 2006) have been reported as effective biological agents against root rot pathogens of cucurbits. Among these, *Bacillus* spp. have been studied as one of the excellent biocontrol agents against plant pathogens. They produce a wide range of antimicrobial cyclic lipopeptides (iturins, fengycins and surfactins), enzymes (chitinase and ß-1,3-glucanase), and antifungal Volatile Organic Compounds (VOCs), which inhibit growth of plant pathogens (Arrebola *et al*., 2010).

In this study, we aimed to isolate rhizobacterial isolates belonging to genus Bacillus from cucurbits fields with the objective of obtaining the efficient strains representing high disease control performance against charcoal root rot in vitro and greenhouse conditions.

MATERIALS AND METHODS

Fungal Pathogen and Antagonistic Bacteria

Macrophomina phaseolina isolate 44 (Mph44) was obtained from Plant Pathology Department of Tarbiat Modares University, Tehran, Iran. This isolate had been proved to be highly virulent in previous pathogenicity test.

All the bacteria isolated from the rhizosphere of cucurbit fields from Tehran province (Moradabad and Palaein villages) in 2013. Serial dilutions from collected soil samples were prepared and spread on Nutrient Agar (NA) medium. Petri dishes were incubated at 27°C for 48 hours. All bacterial colonies were purified and maintained at 4°C.

Investigation of Antagonistic Mechanisms *In Vitro*

For selection of bacterial antagonists, antagonistic activity of bacterial strains was tested against Mph44 using preliminary dual culture procedure (Dennis and Webster, 1971). Four bacterial strains (10^9 CFU mL^{-2}) were placed on Petri dishes (9 cm diameter) at equal distances from each other and around the periphery of the mycelial plug (5 mm diameter). In control plates, only the pathogenic fungus was grown. When control plates were completely covered by the fungus, the experiment was stopped and the bacterial antagonists were selected based on their ability to produce inhibition zone against *M. phaseolina* using the Equation (1):

$$IG= [(C-T)/C]\times100 \quad (1)$$

Where, *IG:* Percent of Inhibition Growth; C: Growth in Control (radial growth of fungus in control); T: Growth in Treatment (radial growth in treatment or dual culture).

After that, volatile metabolites production test for antagonistic bacteria was performed following Fernando *et al*. (2005) procedure. One hundred-microliter suspension of each antagonistic bacterial strain (10^9 CFU mL^{-1}) was smeared on fresh Nutrient Agar (NA). Sterile distilled water was used as a control. A 3-day-old PDA plug (5 mm diameter) of *M. phaseolina* with mycelium and microsclerotia was placed in the center of PDA medium. Dishes containing fungal pathogen were placed inverted on NA medium dishes containing bacterial strains and were sealed with parafilm. Petri dishes were maintained in dark at 28°C for three days. Inhibition percent of fungal mycelial growth was calculated using the Equation (1).

Production of antibiotics by bacterial antagonists was determined using Kraus and Lopper (1990) method. One hundred-microliter suspension of each antagonistic bacterial strain (10^9 CFU mL^{-1}) was cultured on fresh PDA. The Petri dishes were incubated in dark at 26°C for 72 hours. After

three days of bacterial growth, the colonies were wiped off by sterile cotton swap and the plates were exposed to Chloroform vapor and UV light for two hours and air flow under sterile condition for 30 minutes, in sequence. Sterile distilled water was used as a control. Then, a 3-day-old PDA plug (5 mm diameter) of *M. phaseolina* with mycelium and microsclerotia was placed in the center of Petri dishes. The Petri dishes were incubated at 28°C for three days. The percentage of pathogen growth inhibition was calculated by Equation (1).

Bacterial cell-free supernatant assay (Singh and Deverall, 1984) was done to select the best effective antagonistic bacteria. To study the effects of the cell-free supernatant on radial growth of fungus, the cell-free supernatant was added to autoclaved PDA medium to a final concentration of 25% (v/v), just before pouring the plate. After the medium was set, a 5 mm diameter disc from an actively growing fungus was inoculated at the center of the plate. In the control plate, the cell-free supernatant was substituted with 25% (v/v) sterile Nutrient Broth (NB). The plates were incubated at 28°C. Radius of the fungal colony was measured after 5 days.

All the experiments were performed with three treatments each with three replications in a Completely Randomized Design (CRD).

Identification of Bacterial Antagonists

The isolates were characterized based on their morphological, physiological, and biochemical characteristics (Schaad *et al*., 2001). The four best bacterial isolates were identified in detail through *gyr*A analysis (Roberts *et al*., 1994).

The *gyr*A sequences of four isolates were used to elucidate the phylogenetic position of the strains by comparing these sequences with published sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree was constructed with MEGA software (v. 4.0).

In Vivo Assays

Seed Coating Formulation

Bacterial suspensions were prepared from cells grown in shaking incubator at 27°C and 160 rpm in Luria Bertani (LB) broth. The bacterial cells were harvested by centrifugation at 5,000 rpm for 5 minutes. The pellet was re-suspended in sterile Distilled Water (DW) to adjust the bacterial suspension to 10^9 cells mL^{-1} (Kim *et al*., 1997) and mixed with equal volume of 1% CarboxyMethylCellulose (CMC) and added to disinfested seed. Then, bacterized seeds were transferred to dry sterilized filter paper and allowed to dry in laminar flow for 8 hours. The initial population of bacterial cells on the seed was about 10^9-10^{10} colony per seed. Shadegan cultivar of melon was used in this experiment. Carboxin thiram was used as effective fungicide for chemical control of charcoal root rot by seed treatment at rate of 0.15% (w/w).

Greenhouse Experiments

In greenhouse examinations, the effectiveness of bacterial antagonists and carboxin thiram fungicide was evaluated on inhibition of pathogen and promotion of melon growth indices. Plastic pots were filled with pasteurized field soils, perlite, and peat moss (1:1:1 ratio). Field soil was sterilized by autoclaving two times. Inoculums of fungal pathogen were prepared by growing them on millet grains in 100 mL flasks as follow: the grains were soaked in distilled water, autoclaved twice (121°C for 45 minutes) and inoculated with three agar discs (5 mm diameter) of 5-day-old fungal culture. Flasks were incubated at 28°C in dark for 21 days. The inoculums were then mixed with soil (10 g kg^{-1} soil) (Etebarian *et al*., 2000). Four treated melon seeds were

planted to each pot and the pots were kept at 25-33°C and allowed to grow for 60 days. Pots were watered every other day until seedling emergence and daily thereafter. There were three groups of treatments with three replications for each treatment, arranged in a completely randomized design. Treatments included: (1) Pathogen (as control), (2) *B. amyloliquefaciens*+pathogen, (3) Carboxinthiram+pathogen. The experiment was carried out in a glasshouse at day/night temperatures of 32/25°C with natural day light without supplementary lighting from September to December 2013, in Tehran. The effect of tested antagonist on plant growth was measured in terms of plant height. Thereafter, at the end of experiment, the above ground parts of plants were cut into small pieces and shoot fresh weight was recorded, then, those parts were dried at 60ºC for three days and dry weight of shoot pieces was recorded. Moreover, Growth Promotion Efficacy (GPE) was calculated to clarify the relative effect of tested antagonist on plant biomass according to the following Equation (2):

$$GPE\ (\%) = [(G_T - Gc)/G_C] \times 100 \qquad (2)$$

Where, *GPE* refers to Growth Promotion Efficacy, G_T refers to Growth parameter in antagonist-Treated group, and G_C refers to Growth parameter in the Control group.

Antagonist Population Dynamics in Melon Rhizosphere

Bacillus^{+rif1}, the spontaneous mutant of *B. amyloliquefaciens* B2, was obtained by plating the cell suspension (1×10^9 CFU mL^{-1}) on NA medium containing different concentrations of rifampicin (1, 5, 10, 20, 35, 50, 100 and 150 µg mL^{-1}). The plates were incubated at 28°C for 72 hours and mutant colonies were picked up. The mutants were evaluated for stability of resistance by sub-culturing 10 times on NA+rifampicin (100 µg mL^{-1}). Population dynamics of B2 strain was examined on roots at 7, 14, 21, 28, 35, 42, 49 and 56 days after sowing. Root sampling was done by uprooting seedlings, and each root piece was suspended in water. The suspension was incubated at 30°C for 1 hour, on a rotary shaker at 150 rpm. After serial dilution, the suspension was plated on NA medium with rifampicin (100 µg mL^{-1}). Inoculated plates were incubated at 28°C for 48 hours. The observed colonies were counted and expressed as log_{10} CFU cm^{-1} root. All experiments were performed in three replications. Plants treated by sterile water were used as control.

Statistical Analysis

For statistical analysis, data were subjected to Analysis Of Variances (ANOVA) and the means were compared by Duncan's Multiple Range Test (DMRT) using SPSS statistical software (version 16.0). Level of significance in different treatments was determined at 1% probability (Little and Hills, 1978).

RESULTS

In Vitro Assays

Seventy five bacterial strains, isolated from soil samples, were purified. Among them, four isolates (B2, B11, B12 and BKN) showed more than 50% growth inhibition (54.3 to 62.22%) in dual culture test. The VOCs from the antagonists produced 39.43 to 54.82% inhibition of mycelial growth of Mph44. The inhibition rates of mycelial growth were 78.52 to 100% in antibiotic production test. The cell-free supernatant at 25% dilution inhibited mycelial growth of Mph44 from 64.45 to 88.89% (Figure 1, Table 1).

Identification of Bacterial Antagonists

The strains which were isolated from the rhizosphere soil of cucurbits were finally selected as promising candidates and

Table 1. Results of *in vitro* assays.

Isolats	Region	Inhibition of mycelial growth (%)			
		Dual culture	VOCs	Production of antibiotics	Supernatant assay
1. B2	Tehran province (Moradabad Village)	62.22	54.82	100	88.89
2. B11	Tehran province (Palaein Village)	58.89	39.43	100	79.26
3. B12	Tehran province (Palaein Village)	54.3	50.52	100	82.22
4. BKN	Tehran province (Palaein Village)	56.15	40	78.52	64.45

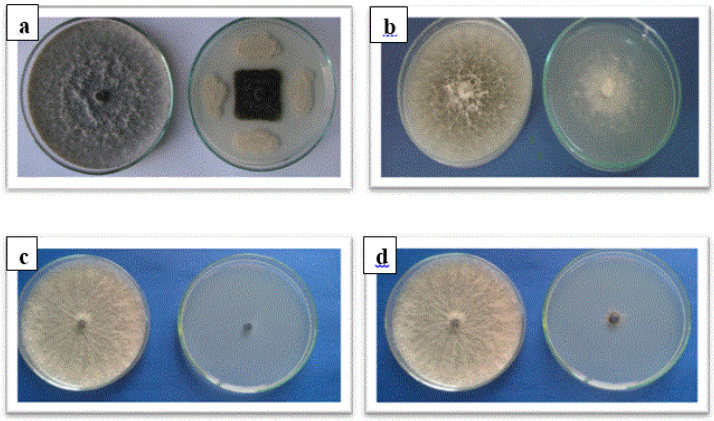

Figure 1. Dual culture (a), volatile compound (b), antibiotic production (c) and extra cellular liquid compound tests. In each picture, the left plate is control (*Macrophomina phaseolina* isolate 44) and the right plate is treatment (Mph44+*Bacillus amyloliquefaciens* B2).

identified as *Bacillus* sp. by results of morphological, physiological, and biochemical characteristics. They were gram positive, rod shaped, motile, aerobic growth and able to form spores. All strains gave positive reaction for catalase test and starch hydrolysis. Moreover, they were positive for utilization of lactic acid and *D*-sorbitol. The results of biochemical and physiological test were therefore guidable to discriminate the isolates from each other.

Partial *gyr*A nucleotide sequences of the test strains were deposited in Genbank database under accession numbers KJ136127, KJ829646, KJ829647, and KJ829648. The phylogenetic tree based on the Maximum Likelihood analysis of the *gyr*A sequences is given in Figure 2. Based on *gyr*A analysis, these strains (*Bacillus* B2, B11, B12 and BKN) were identified as *B. amyloliquefaciens* (Figure 2).

In Vivo Assays

Greenhouse Experiments

Analysis of greenhouse results showed that there was significant difference (at 1% level in Duncan test) among treatments in increasing melon growth indices and inhibition of charcoal root rot causal agent. In presence of pathogen, *B. amyloliquefaciens* B2 was the most effective antagonist against Mph44 and increased melon shoot and root fresh weights by 36.27 and 32.97%, shoot and root dry weight by 34.44 and 30.39%, respectively, in comparison with the control i.e. only

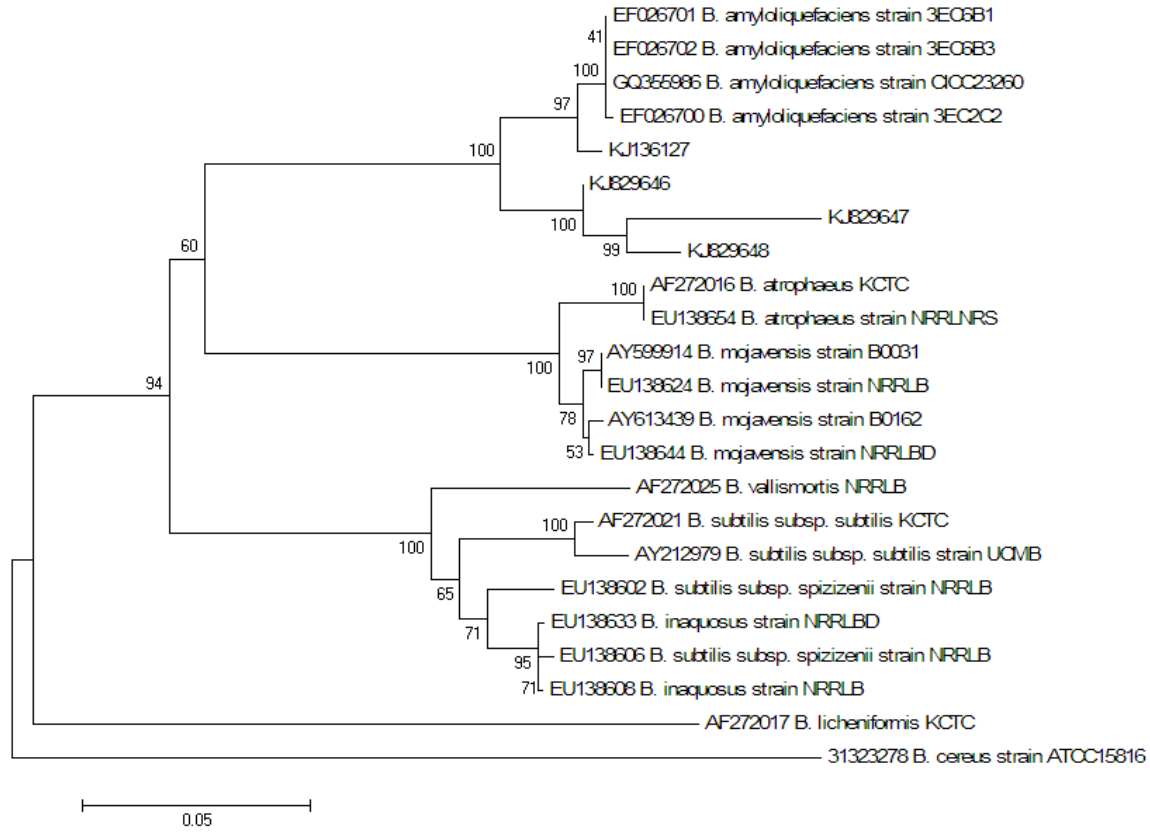

Figure 2. Maximum Likelihood phylogenetic tree of *Bacillus amyloliquefaciens* strain B2 (KJ136127), B11 (KJ829646), B12 (KJ829647) and BKN (KJ829648) based on *gyr*A sequences analysis. The bootstrap values (out of 1,000 repeats) are shown on clades.

pathogen was added. Carboxin thiram controlled the disease by increasing melon shoot and root fresh weights 89.55 and 91.26%, shoot and root dry weight 87.78 and 90.48%, respectively, in comparison with the control i.e. only pathogen was added (Table 2).

Antagonist Population Dynamics in Melon Rhizosphere

Survival of introduced biocontrol agents in the rhizosphere is of primary importance for biological control of plant diseases. Evaluation of the population dynamic of the *B. amyloliquefaciens* strain B2 using the rifampicin antibiotic resistance marker revealed that all the three isolates could proliferate successfully in the rhizosphere of melon, both at 30 and 60 days after seeding. The population of *Bacillus*$^{+rif}$ had increasing growth from 7 to 56 days after inoculation (Figure 3).

DISCUSSION

Soil-borne plant pathogens such as bacteria, fungi, and nematodes annually cause major economic losses on melon plants. *Macrophomina* charcoal root rot on melon is one of the most important diseases. The lack of chemical fungicides against *M. phaseolina* and the environmental contaminations by chemicals has encouraged researchers around the word to have an increasing interest in biocontrol agents. Rhizosphere microorganisms provide primary defense line against

Table 2. Effects of *Bacillus amyloliquefaciens* strains and carboxin thiram fungicide in combination with *Macrophomina phaseolina* isolate 44 on melon growth indices in pasteurized soil greenhouse 60 days after planting.[a]

Treatment	H (cm)	SFW (g)	RFW (g)	SDW (g)	RDW (g)
A(B2)+Seed+CMC+P	59.33 b	13.43 b	2.43 b	4.03 b	1.91 b
A(B11)+Seed+CMC+P	51.66 d	11.37 e	2.1 bc	3.45 d	1.66 e
A(B12)+Seed+CMC+P	55.33 c	12.33 c	2.28 b	3.67 c	1.80 c
A(BKN)+Seed+CMC+P	53.5 cd	11.88 d	2.15 b	3.60 cd	1.72 d
P+Seed+CMC (As control)	43 e	9.86 f	1.83 d	2.99 e	1.46 e
P+Seed	42.33 e	9.82 f	1.89 d	3.01 e	1.49 e
P+Fungicide[B]	79.33 a	18.69 a	4.30 a	6.63 a	3.80 a

[a] Means with different letters in each column are significantly different in Duncan test (P= 0.01). Data are the means of three replicates. H: Height; SFW: Shoot Fresh Weight; RFW: Root Fresh Weight; SDW: Shoot Dry Weight; RDW: Root Dry Weight; A: Antagonist; P: Pathogen; CMC: CarboxyMethylCellulose. [b] Results showed that 0.15% concentration of carboxin thiram fungicide inhibited the growth of *M. phaseolina* by 100% *in vitro*, so, this concentration of carboxinthiram was used in greenhouse experiment.

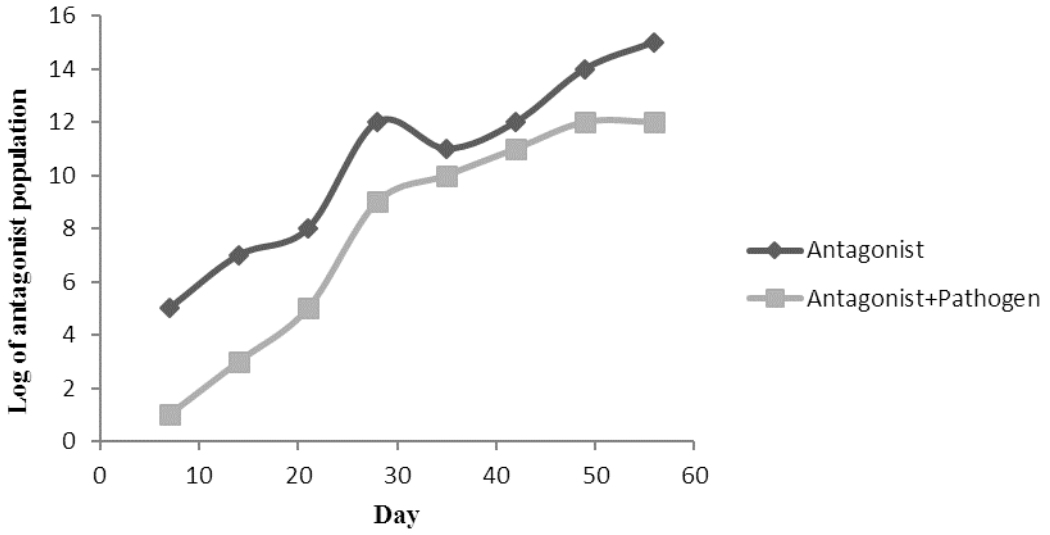

Figure 3. Increasing growth of *Bacillus amyloliquefaciens* $B2^{+rif}$ in melon rhizosphere.

pathogen attacks (Weller, 1988). The predominantly reported biocontrol agents for managing *M. phaseolina* include mycoparasitic fungi and bacteria such as *Trichoderma* spp., *Bacillus* spp., and *Pseudomonas* spp. (Gupta *et al*., 2002; Singh *et al*., 2008). In this study, we evaluated antagonistic effects of cucurbits rhizobacteria against *Macrophomina* charcoal rot on melon. High reduction of pathogen growth in *in vitro* tests was observed by all antagonists. We selected four potential biocontrol agents of *B. amyloliquefaciens* strains, namely, B2, B11, B12, and BKN to suppress the disease in greenhouse condition. Dual culture (Dennis and Webster, 1971), antibiotic production (Kraus and Loper, 1992), cell free culture (Weller *et al*., 1988) and volatile metabolite test (Fiddaman and Rossall, 1993) were used to observe the effect of *Bacillus* isolates on *Macrophomina phaseolina* isolate 44. These results were similar with previous studies reporting the antagonistic activity of *B. subtilis* (Pinchuk *et al*., 2002) and *B. amyloliquefaciens* (Liu *et al*., 2008; Yoshida

et al., 2001) against plant pathogenic bacteria and fungi. The inhibitory effects of antagonists' metabolites have been reported in various studies (Pal *et al*., 2001; Monte and Liobell, 2003). *B. amyloliquefaciens* has been reported to be effective in controlling plant pathogens by the production of antibiotics (Alvindia and Natsuaki, 2009; Chen *et al*., 2009) and VOCs (Koumoutsi *et al*., 2004; Romero *et al*., 2007; Arguelles-Arias *et al*., 2009). Arrebola *et al.* (2010) identified eight different VOCs from *B. amyloliquefaciens* PPCB004 and indicated that the *in vitro* radial mycelial growth of *Penicillium crustosum*, *P. digitatum* and *P. italicum* was inhibited by over 50, 30, and 25%, respectively, in the presence of VOCs of *B. amyloliquefaciens* PPCB004. Knowledge on the mechanisms of action involved in the biocontrol process could permit establishment of optimum conditions for the interaction between the pathogen and the biocontrol agent and is important for developing additional means and procedures in order to obtain better results from known antagonists. In addition, understanding the mechanism of biocontrol will help in selecting more effective and desirable strains of antagonists (Sharma *et al*., 2009).

In *in vivo* assays, heating of soils by autoclaving usually allows the pathogen to proliferate (Burgess *et al*., 1988). In greenhouse experiments, *B. amyloliquefaciens* B2 was the most effective antagonist in inhibition of *Macrophomina* charcoal rot and increased plant growth indices. Our greenhouse experiments showed that, in the presence of pathogen, all antagonists controlled the melon charcoal rot disease. It was determined that direct interaction of antagonist *B. amyloliquefaciens* isolate B2 with melon seeds increased height, shoot and root fresh weight, and shoot and root dry weight indices by 37.98, 36.27, 32.97, 34.44, and 30.39%, respectively. Ability of the four tested isolates to inhibit the growth of soil borne fungi means these isolates have several mechanisms to suppress plant pathogenic microorganisms, and have a high chance to keep their activity for protection of host plants in greenhouse. Hao *et al.* (2011) reported that at 1×10^8 CFU mL^{-1} of *B. amyloliquefaciens* HF-01, the incidence of green and blue mold and sour rot were 16.32, 23.64 and 31.65%, respectively, while the control fruit had more than 90% decay.

In conclusion, the results of this study indicated that use of *Bacillus* isolates as complementary biocontrol method could be integrated with other means of control e.g. fungicides. *B. amyloliquefaciens* B2 reduced disease severity in glasshouse condition, but further fundamental, multicomponent research into the interaction between host, pathogen, antagonist, and environment is required. Further studies are, however, needed to check the isolates for their ability to suppress the growth of other soil-borne plant pathogenic fungi to increase their chance by offering sufficient protection to the host plant against wide spectrum of soil-borne pathogens and, also, to investigate the mode of action of these strains in terms of inducing systemic resistance and enhancing their antibiosis activity against plant pathogens, as well as confirming the antagonistic ability of these strains in field trials at different locations.

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Bacillus amyloliquefaciens به عنوان عامل بیو کنترل در بهبود مدیریت پوسیدگی ذغالی خربزه

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چکیده

به منظور بررسی کنترل بیولوژیک بیماری پوسیدگی ذغالی خربزه، ناشی از قارچ بیمارگر *Macrophomina phaseolina*، اثر آنتاگونیستی ۷۵ استرین باکتری ناحیه ریزوسفر گیاهان خربزه روی جدایه ۴۴ قارچ بیمارگر *M. phaseolina* بررسی گردید. در شرایط آزمایشگاهی، استرین‌های B2، B11، B12و BKN اثر بازدارندگی بالایی علیه قارچ بیمارگر نشان دادند. در آزمون‌های کشت متقابل، ترکیبات فرار، تولید آنتی‌بیوتیک و ترکیبات مایع خارج سلولی این بازدارندگی‌ها به ترتیب از ۶۱/۲۲-۵۴/۳، ۵۴/۸۲-۳۹/۴۳، ۱۰۰-۷۸/۵۲ و ۸۸/۸۹-۴۵-۶۴ درصد متغییر بود. در آزمایش‌های گلخانه‌ای، تیمار بذری استرین B2 شاخص‌های رشدی گیاه خربزه شامل ارتفاع، وزن تر اندام هوایی و ریشه، وزن خشک اندام هوایی و ریشه را به ترتیب ۳۷/۹۸، ۳۶/۲۷، ۳۲/۹۷، ۳۴/۴۴ و ۳۰/۳۹ درصد در مقایسه با شاهد کنترل افزایش داد. آزمایش‌های بیوشیمیایی، فیزیولوژیکی و مولکولی با تعیین توالی ناحیه *gyrA* تأیید نمودند که تمامی این ۴ استرین به گونه *Bacillus amyloliquefaciens* تعلق دارند. با توجه به نتایج به دست آمده، جدایه B2 می‌تواند عامل بیوکنترل مهم جدیدی علیه قارچ عامل پوسیدگی ذغالی در نظر گرفته شود.