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The Use of Bacillus Amyloliquefaciens to Control of Sclerotinia Stem Rot (Sclerotinia Sclerotiorum) of Cucumber

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ABSTRACT: Sclerotinia sclerotiorum (Lib.) de Bary is a soil-borne pathogen capable of infecting more than 400 host plants worldwide. Antagonistic rhizobacteria, more specifically certain species of Bacillus, are known to control of fungal root diseases of agronomic crops (Ashwini and Srividya, 2013) . In this study, 182 bacteria were isolated of cucumber rhizosphere and screened as potential biological control agents against S. sclerotiorum in vitro condition. Morphology, physiological and biochemical tests and 16S rDNA analysis demonstrated that Bacillus amyloliquefaciens significantly reduce the mycelial growth of S. sclerotiorum by 72.04% . Non-volatile compounds of 3 isolates showed noticeable inhibition zone (>36%) against S.sclerotiorum, whereas volatile compounds of 7 isolates could prevent more than 30% of the mycelial growth of the fungus. All strains except of FE1 promoted significantly plant growth in comparision to non-treated control. The population density on roots of plants treated with PE and AE reached to 8.40 and 7.81 $\log^{10}$ CFU g^{-1} root respectively. The use of bacteria as a soil treatment (PE 69.6%) was more effective to suppress the disease than those as a seed treatment (PE 62.3%). These results indicate that bacterial species used in this study could be used in the control of S. sclerotiorum.

***Keywords*:** Bacteria, Biological control, growth promotion, Sclerotinia sclerotiorum

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a cosmopolitan, homothallic and necrotrophic pathogen that attacks a wide range (64 families, 225 genera and 361 species) (Purdy, 1979) of plants including cucumber, (Boland and Hall, 1994) and wild plant species (Dilantha Fernando et al., 2004) on which it causes fruit and stem rots (Onaran and Yanar, 2011).

Cucumbers are among the most widely grown vegetable crops the world over. (Paris et al., 2011).Cucumbers are the third most widely cultivated vegetables in the world and 3.6% of total annual production of crops in Iran is allocated to the cucumber cultivation.

The increasing demand for a steady food supply to the growing world population will require controlling of plant diseases that reduced crop yield subsequently. In order to control the plant diseases, biological control is gaining greater attention due to low cost and eco-friendly application. Biocontrol of plant pathogens using antagonistic fungi and bacteria plays a significant role (Abhiniti et al., 2011). Among rhizobacteria, Bacillus spp. can play a vital role in the management of plant diseases to increase crop productivity via various mechanisms (Satpute, 2008). B. amyloliquefaciens strain S499 has shown the ability to protect plants against several.

phytopathogenic fungi (Puopolo et al., 2013) The broad-spectrum antagonistic activities are executed by secretion of a number of metabolites including antibiotics, volatile compound HCN, siderophores (Arrebola et al., 2010).

Biocontrol of S. sclerotiorum via antagonists such as Epicoccum purpurascens (Zhou & Reeleder, 1989) and Bacillus spp. (Huang et al., 1993; Onaran and Yanar, 2011) was reported previously. Plant growth-promoting bacteria (PGPR) are associated with many, if not all, plant species and are commonly present in many environments (Compant et al., 2005).

The aim of this study is therefore to isolate bacteria from the cucumber rhizosphere and screen them against S. sclerotiorum, one of the major causes of Sclerotinia disease of cucumber in Iran.

MATERIALS AND METHODS

Preparation of Pathogen and pathogenicity test

S. sclerotiorum isolates were obtained from cucumber plants grown in greenhouses in Varamin region, Iran. For isolation, a single sclerotia or infected host tissue, surface-sterilized by dipping in 2% sodium hypochlorite solution for 2 min, and then rinsed three times with sterile distilled water, using dry out blotter for 3 min, was aseptically transferred into potato dextrose agar (PDA) plates. The plates were incubated at 25 ± 2°C. Mycellial discs (diameter of 5 mm) taken from the edge of the actively growing colonies were transferred to the Petri dishes containing PDA to obtain pure cultures of the S. sclerotiorum (Onaran and Yanar, 2011).

To find the pathogenicity of the fungi, 3 week-old cucumbers were inoculated with a 5 mm diameter colonized PDA disk by S. sclerotiorum isolates which were placed in the wounds made in the basal stem with a sterile scalpel. Control plants were treated with the agar disk that did not contain mycelium. Then, plants were covered with a plastic bag for 48 h to maintain high humidity. After that, the bags were removed and the plants were maintained in a growth chamber at 25°C with a 12 h photoperiod and 75% relative humidity. Pathogenicity tests were repeated three times (Baharlouei et al., 2011).

Isolation of bacteria

Bacteria were isolated of healthy and diseased cucumber plants grown in greenhouse that are known to be infected by *S. sclerotiorum*. Plant samples were collected and then subjected to surface sterilization as described earlier. Then, they were placed on nutrient agar (NA) and incubated at 28 ± 2°C for 4 days. Pure bacterial cultures were obtained from them. 182 bacterial strains were isolated and 6 of them showed antagonistic activity against *S. sclerotiorum*. These antagonistic strains were identified and classified by PCR (Onaran and Yanar, 2011).

In vitro antagonistic activity

Inhibition of mycelia growth of S. sclerotiorum by non-volatile compounds of the bacterial isolates was tested by using the dual culture technique as described by (Ahmed Idris et al., 2007). Briefly, 20 µl drops from the 10^8 cfu/ml suspension were equidistantly placed on the margins of potato dextrose agar (PDA) plates and incubated at 28 ^{0}C for 24 h. A 6 mm agar disc from fresh cultures of S. sclerotiorum was placed at the centre of the PDA plate for each bacterial isolate and incubated at 27 ± 1^{0}C for seven days. The radii of the fungal colony towards and away from the bacterial colony were measured. The percentage growth inhibition was calculated using the following formula (1):

$$(1) \quad \%\text{Inhibition} = \frac{(R - r)}{R} \times 100$$

Where, r is the radius of the fungal colony opposite the bacterial colony and, R is the maximum radius of the fungal colony away from the bacterial colony (control without bacterial colony).

Antifungal activity of volatile compounds produced by bacteria were detected in a split plate experiment (Kraus and loper, 1992). Radial growth of *S. sclerotiorum* on the fungal side was measured after 5 day of incubation at 27ºC and the percentage of growth inhibition was calculated.

Identification of bacterial isolates

The physiological and biochemical characters of antagonistic bacteria were performed as described by (Schaad et al., 2001).

Were further characterized by sequencing the 16S rDNA gene. For this, genomic DNA was extracted as described previously (de Souza et al., 2003).The primers used for PCR amplification of the 16S rRNA gene were (5′AGAGTTTGATCCTGGCTCAG3′), (5′TACCTTGTTACGACTT3′) (Esikova et al., 2002). All DNA sequence analysis Bacteria were identified from nucleotide sequence resulting from sequencing a portion of the 16S rRNA gene.

Plant growth promotion and root colonization by rhizobacteria

Isolates which showed antagonistic activity in the dual culture assays, were tested for their ability to promotion of plant growth and root colonization. Surface sterilized (1%NaOCl, 5 min) cucumber seeds of Hibrid cultivar were pregerminated in moist chambers at 22°C for 36 h. Bacteria were grown on NB for 16 h and then centrifuged and resuspend in physiological salt solution (0.85%). At the next stage, standard 24-well microplates were filled with 1 ml of water agar (1.2%, pH 6.8). In each well, one seed was placed on the agar surface and inoculated with 20 µl of bacterial suspension (10^5 cfu per ml physiological salt solution (0.85%). Inoculated micro plates were transferred to a phytochamber for two weeks (22/16oC, 16/8 h day/night, and artificial light) and the length and dry weight of stems and roots were measured to determine the effects of bacterial treatments on plant growth. Moreover one gram of root from each treatment was aseptically excised and placed in 25-ml flasks containing 5 ml of sterile 0.85% NaCl, and vigorously shaken at 300 rpm for 30 min. The resulting suspensions were serially diluted and plated on NA, then incubated at 28oC for colony counts. The level of root colonizing- bacteria was calculated as colony forming units/ g root (cfu/g root).

Statistical analyses

Statistical analyses were carried out using the software package SAS (V. 9.1). A mean comparison was performed using the Duncan's multiple range test. Correlations between volatile and non-volatile antifungal activities and root colonization ability with disease suppression were done by using the software SPSS (V. 10.1).

RESULTS AND DISCUSSION

Isolation and screening of antagonistic isolates

In this study, effects of antagonistic bacterial strains on *S. sclerotiorum* were tested in *in vivo* and *in vitro* conditions.

There were significant differences among the bacterial strains ($P < 0.05$) for reducing the fungal growth in vitro condition (Table 1). According to the (Table 1), among of the 182 bacterial isolates, 7 isolates exhibited a more than 30% inhibition of mycelia growth of S. sclerotiorum by non-volatile inhibitors. The maximum inhibition achieved by isolates PE, SH and AE (>36%). Furthermore, volatile metabolites of three isolates ME, LE and AE inhibited the mycelial growth of S. sclerotiorum considerably. Growth inhibition of S. sclerotiorum by PE, SH and AE was significantly greater than others (Figure1 & Table 1), while strain FE2 had less effect on the growth of the pathogen (Table 1). In current study we isolated and screened the rhizobacteria from cucumber plants with antagonistic activity against S,sclerotiurom the causal agent of cucumber stem rot. The approach provided an opportunity to select effective biocontrol strains capable of antagonizing soil-borne pathogens in thesame environment where they will be used commercially (Maleki et al., 2011).

The mechanisms by which they protect plants against pathogens are diverse and include competition, antibiosis, degradation of fungal pathogenicity factors, and induced systemic resistance (Fallahzadeh *et al.,* 2009; Haas & Deffago, 2005). In this study indicate that disease incidence is significantly correlated with the inhibition activity by non-volatile compounds.

Table 1. Inhibition of *Sclerotinia sclerotiorum* mycelial growth on potato dextrose agar by rhizobacterial isolates

Bacterial isolates	Mycelial inhibition* (%)		%Inhibition zone
	Non-volatile	Volatile	
PE	41.89*a**	19.25 a	72.04a
SH	40.476a	18.37 a	55.74b
AE	36.40 b	23.00 a	52.45c
ME	18.03 c	26.70 a	40.69d
FE2	17.69 c	19.25 a	24.16f
FE1	16.57 cd	13.75 a	34.78e
LE	14.16 d	23.37 a	35.83e
Control	0 e	0 b	0g

*Mycelial inhibition (%) was calculated as (R−r)/R × 100, where R is mycelial growth away from the bacterial colony (the maximum growth of the fungal mycelia), r is mycelial growth towards the bacteria

**Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test

Identification of antagonistic bacteria

According to morphology, physiological and biochemical characteristics of promising isolates, isolates PE, SH, AE and ME identified as *B. amyloliquefaciens* (data not shown).

The 16S rDNA sequences (1500 bp (Figure 2) of the 16S rDNA gene from each of these strains) were obtained by PCR with the established primers. The alignment analysis showed that the 16S rDNA sequence of all isolates were nearly identified to *B. amyloliquefaciens*.

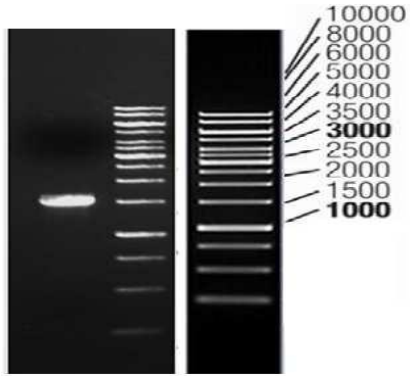

Figure 2. PCR products amplified whit primers (27F and 1492R) separated by agarose gel electerophooresis to identifty B. amyloliquefaciens

Plant growth promotion and root colonization in phytochamber

The effect of bacterial isolates on the growth of cucumber seedlings was monitored by measuring of length and dry weight of the roots and stems (Table 2). All strains except of FE1 promoted significantly plant growth in comparison to nontreated control. Strains PE, SH.AE and ME caused to increase the length of stem and root, considerably. In addition, Strains PE, SH and AE showed the most effect on enhancing the foliar weight of cucumber seedling. However, both strains, PE and AE, exhibited the greatest effect to enhance root weight of seedling.

Study of root colonization *in vitro* demonstrated that some of the strains have more ability to root colonization than others. The population density on roots of plants treated with PE and AE reached to 8.40 and 7.81 $\log^{10}$ CFU g^{-1} root respectively. Strains FE1 and FE2 were not effective root- colonizers as their cell count was less than 5 $\log^{10}$ CFU g^{-1} root. It seems there is a correlation between root colonization talent of selected strains and their growth promoting ability, as in the most cases the strains with a high efficacy of root colonization resulted in

increasing growth of plants. In this study, the strains with high ability of root colonization were more effective in suppressing of *Sclerotinia* stem rot on cucumber plants. In the most cases, strains with a higher level of root colonization caused to higher growth of plants. Growth promotion of plants can increase their resistance to disease (Weller, 1988).

Table 2. In vitro growth promotion and root colonization of cucumber plants by rhizobacterial strains

Bacterial isolates	Stem length	Root length	Foliage weight	Root weight
PE	12.68a*	9.41a	4.69a	4.12a
SH	12.38a	9. 20a	3.126b	2.99b
AE	12.13a	9.14a	4.16a	3.89a
ME	11.76b	7b	3.91ab	2.98b
LE	9.11c	6.21c	3.12b	2.82b
FE1	6.58de	5.30d	2.6c	1.59c
FE2	7.10d	6.10c	2.4c	1.58c
Control	7.03d	5.52d	1.88d	1.6cd

*Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test

Greenhouse experiment

According to the results (Table 3), the use of bacteria as a soil treatment was more effective to suppress the disease than those as a seed treatment. The percentage of healthy plants in the presence of strains (all of them) as soil treatments and Sclerotinia inoculums was significantly more than those of infected control plants.

without bacterial inoculums). The results of this study have shown that biological control of S. sclerotiorum plant and growth promotion ability with soil isolated bacteria is possible.

Table 3. Suppression of *S. sclerotiorum* by selected strains in greenhouse. Isolates was applied as soil and seed treatments, percent of healthy plants was determined after 3 weeks

Bacterial isolates	Seed treatment	Soil treatment
PE	62.3*ab**	69.6d
AE	47.2ab	60.52a
SH	59.51c	55.5c
ME	41.98c	44.11e
Control	100b	100b
Benomyl	39.31a	42.53a

**Values followed by the same letter were not significantly different at 5%, *as determined by variance analysis followed by Duncan's test

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