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Elucidation of the antagonistic effect of *Bacillus* species against white mold fungus *Sclerotinia sclerotiorum*

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Abstract

Sclerotinia sclerotiorum is one of the most devastating and cosmopolitan soil-borne fungus and causes white mold diseases on several economically important crops, such as oilseed rape, soybean, cotton and numerous vegetable crops. The disease has been controlled primarily through the use of several chemicals; however, the fungicides are often ineffective and not environmentally safe. The use of Bacillus species has attracted much attention because of their biocontrol potential for combating plant fungal diseases. The aims of this study were therefore to evaluate the antifungal activity of *Bacillus* species and to elucidate the underlying inhibitory mechanism against *S. sclerotiorum*. The inhibitory effect of *Bacillus* species on *S. sclerotiorum* was screened by agar diffusion assay. In dual culture agar diffusion experiment, strong antifungal activity was observed against mycelial growth of *S. sclerotiorum* by four isolates of *Bacillus,* while *Bacillus amyloliquefaciens* isolate A1 showed highest antifungal activity (67%). Moreover, cell free culture filtrate of isolate A1 exhibited strong mycelial growth inhibition of fungus (77.5%). Microscopic studies revealed the morphological alteration of fungal hyphae after treated with isolate A1. The abilities of biofilm, siderophore and lip peptides production were also investigated to clarify the underlying inhibitory mechanism of the most effective strains. Results indicated that isolate A1 showed prolific production of biofilm (0.2129 of OD_{570}) and strong siderophore activity (1.69 cm) as compared to other *Bacillus* isolates. Moreover, three metabolites groups namely iturin, fengycin and surfactin were detected in the single cell of isolate A1 using MALDI-TOF-MS spectrum analysis. Taken together, these results demonstrated that *B. amyloliquefaciens* isolate A1 may have potential as an effective biocontrol agent for fighting against white mold fungus *S. sclerotiorum*.

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Introduction

Sclerotinia sclerotiorum is the most damaging causal agent of white mold diseases on more than 400 plant species including oilseed, soybean and many important vegetables namely broccoli, cabbage, peppers, potatoes(Gerlagh *et al.*, 1999). Particularly, this pathogen causes a severe damage of potato production in the potato producing countries worldwide (Dutta *et al.*, 2009; Ojaghian *et al.*, 2016). At first, the disease symptoms appeared as water-soaked lesions on plant portion which is in contact with soil after that infected portion quickly enclosed with white cottony growth that can extend rapidly nearby stem and leaves if moisture retain for several hours. Control of this pathogen is complicated due to a wide host range and the persistence of survival structures (sclerotia) in the soil for long periods(Nelson, 1998). These factors necessitate the use of fungicides, which have been known to have detrimental effects on non-target organisms (McGrath, 2001; Heckel, 2012). In addition, as most inoculum is ascosporic, ascospores could travel long distance from neighboring fields while a few germinating sclerotia can lead to significant infection levels (Ojaghian, 2009). Therefore, there is a need for research into biocontrol of *S. sclerotiorum* as an alternative of fungicides. As this is a narrow window to protect the plant from infection, biological control may work well in controlling the germination of ascospores on plant parts.

Rhizospheric and endophytic *Bacillus* has been shown to inhibit many fungal and bacterial pathogens. For example, *Bacillus subtilis, Bacillus amyloliquefaciens*, *Bacillus subtilis* strain UK 9 and *Bacillus methylotrophicus* were successfully used against fungal pathogen *Curvularia gloeosporioides, Fusarium oxysporum*, *Alternaria brassicae* and *Fusarium graminiarum* (Sharma and Sharma, 2008; Ashwini and Srividya, 2014; Sotoyama *et al.*, 2016). However, little is known about *Bacillus* with the potential to suppress *S. sclerotiorum*. Most of the *Bacillus* species produce lip peptides as secondary metabolites which have strong antimicrobial activity(Yang *et al.*, 2015). Depending on amino acid sequence lip peptides are classified into three groups namely iturin, fengycin and surfactin, among them iturin and fengycin attribute mostly to control soil borne fungus (Romero *et al.*, 2007; Arrebola *et al.*, 2010; Perez-Garcia *et al.*, 2011). In addition, biofilm formation of *Bacillus* spp. may allow them to colonize the root surface and to the secretion of liopopetide antibiotic surfactin(Bais *et al.*, 2004). Many *Bacillus* species also show the ability to produce siderophore which are low molecular weight compound and this can bind the firm complex with ferric ion (Santos *et al.*, 2014; Spadaro and Droby, 2016). Iron plays an important role for survival of fungus so by creating an iron limited condition, siderophore producing *Bacillus* could play an important role for biocontrol(Saravanakumar *et al.*, 2008).

The objectives of the present study were to evaluate the inhibitory effect of several *Bacillus* spp. against the white mold pathogen *S. sclerotiorum* and also explored the various antimicrobial related mechanisms of the most effective *Bacillus* strain.

Materials and methods

Microorganisms

Six endophytic strains (*Bacillus amyloliquefaciens* A1, A3, A13, *Bacillus methylotrophicus* A2, Am, and *Bacillus subtilis* A15) and three rhizospherial strains (*Bacillus methylotrophicus* D29, H8, and *Bacillus subtilis* D16) collected from the Institute of Biotechnology at Zhejiang University, which were used as a biocontrol agent in this experiment.

All antagonistic bacteria were grown on nutrient agar (NA) by streaking the bacteria on the plate after that incubate in NA broth at 200 rpm for overnight maintaining the temperature 30°C. After centrifuging the bacteria at 8000 rpm for 5 min, suspend the pellet with sterile saline water to obtain the final concentration 10^8 colony forming unit (CFU)(El-shakh *et al.*, 2015). A white mould fungus *Sclerotinia sclerotiorum* was previously isolated from infected potato plants (Ojaghian *et al.*, 2016). Potato Dextrose Agar (PDA) media used to grow the fungi. All micro-organisms are stored at -80°C on glycerol.

In vitro growth inhibition of Sclerotinia sclerotiorum by different Bacillus

To observe the antifungal activity of *Bacillus* spp. against *S. sclerotiorum*, agar diffusion assay was performed on PDA plate. A five mm diameter inoculums disc was taken from 5 days old actively growing fungal plate and placed near the edge of the plate containing PDA media. A bacterial streak of each *Bacillus* strains suspension which contains 10^8 CFU/ml was placed at the opposite side of fungal disc. The PDA plate with fungal disc without bacterial inoculation was served as control. The experiment was repeated three times with three replications. All plates were incubated for 30°C and colony diameter of test fungus was observed after 24 h interval until the mycelium cover the control plate (Ramzan *et al.*, 2014). The assessment of the inhibitory effect was measured as percentage by using the following formula (Kheiri *et al.*, 2016).

$$\%\text{inhibition rate} = \frac{R1 - R2}{R1} \times 100$$

R1= Radial mycelia growth of *S. sclerotiorum* in the control plate

R2= Radial mycelia growth of *S. sclerotiorum* antagonistic bacteria treated plate. The experiment was repeated three times independently for each treatment.

Inhibition of Sclerotinia sclerotiorum by culture filtrates of antagonistic bacteria

Four effective *Bacillus* strains based on *in vitro* screening were cultured in NA broth in rotary shaker at 180 rpm maintain the temperature 30°C. Culture filtrates were obtained by centrifugation of liquid culture at 16000 rpm for 10 min at 4°C after 48 h of incubation. The collected supernatant was sterilized by filtration through 0.22µM filter. To confirm that no cell was left in the culture filtrate, 100 µl of culture filtrate was covered on agar plate and waited for 24 h (Almoneafy *et al.*, 2014). Antagonism of this culture filtrate was tested by agar well diffusion technique (Raton *et al.*, 2012). Plate was previously inoculated by placing 5 mm pathogen disc in the center and three holes were prepared equidistant of pathogen disc. After that wholes were filled with 50 µl culture filtrate and incubate at 30°C for 5 days. The plate inoculated with fungal disc and without filled with culture filtrate served as control. Inhibition zone was calculated by the following formula (Ji *et al.*, 2013).

$$\text{Inhibition percentage (\%)} = \frac{A1 - A2}{A1}$$

A1= Radial growth of *S. sclerotiorum* without culture filtrate

A2= Radial growth of *S. sclerotiorum* with culture filtrate. The same experiment was conducted three times independently for each treatment.

Alteration of Sclerotinia sclerotiorum mycelium by the effective antagonistic Bacillus strain A1

After antagonism, the fungal hyphal morphology was observed to check the abnormalities. Hypha from the border of the fungal colony nearest to the growth inhibition zone were remove gently using sterile needle and placed on a microscopic slides with glycerol and examine under a microscope (Nikkon phase contrast microscope, eclips Ci). Mycelium from the control plate was used to compare with treated mycelium.

Biofilm assay of effective antagonistic isolate A1

The Biofilm production capacity of the most effective four *Bacillus* strains was examined using the solid-surface-associated biofilm formation with the crystal violate staining method (Elshakh *et al.*, 2016). Bacterial strains were grown in NA broth. After 24 hr the concentration of each strain were adjusted OD_{600}=0.3 which is approximately 10^7 CFU/ml. Then 5µl of bacterial suspension was added to 195 µl pure NA broths in a 96-well plastic microtitre plate. Six well filled with pure NA broth was measured as negative control. The plate was reserved at 30°C for 24 h. After that, the culture were removed cautiously from the each well and softly rinsed two times with double-distilled water (ddH_2O). Residual cells and matrices were stained with 150 µl of 1% crystal violate solution (CV) for 25-30 min at room temperature. Following two time washing with distilled water, the CV attached to the biofilm was solubilized in 150 ml of 33% acetic acid. The absorbance was measured at 570 nm by using Thermo Multiskan EX microplate

photo meter (Thermo Fisher Scientific inc., Waltman MA). The experiment was repeated three times six replications.

Siderophore detection of effective antagonistic isolate A1

Siderophore production capacity of four effective antagonistic isolate were tested in Chromeazurol S (CAS) complex (Schwyn and Neilands, 1987).Single colony of selected *Bacillus* strains were grown in CAS plate and observed after three days incubation in room temperature. Siderophore production in confirmed by change the blue colour to orange-yellow hallow in the region around the colonies.

The diameter of the hallow was measured to compare the ability of *Bacillus* for siderophore production. The experiment was repeated three times with three replications.

Detection of antimicrobial related lip peptides biosynthetic gene

The total genomic DNA of most effective *Bacillus amyloliquefaciens* A1 was extracted using the TIANamp bacteria DNA kit (spin coloum) (Tiangen Biotech (Beijing) Co., Litd., Beijing, China) following the manufacture instructions. The concentration and purity of DNA was measured using the Nano drop 2000 spectrometer (Thermo Fisher, Inc, Waltham, MA, USA). To detect the presence of lipopetide biosynthesis gene an investigation was done using the five primers for *B. amyloliquefaciens* A1(Mora *et al.*, 2011). PCR was performed using PCR assay kit (TaKaRa, Dalian, China) according to manufacturer's instructions. 20 µl of reaction mixture was used containing mix 2 (10 µl), primers (0.5 µl each) templates (1 µl each) and sterile distilled water (8 µl). The cyclic conditions for the amplification of target genes were as follows: 95°C for 4 min, 40 cycles of 94°C for 1 min, primer annealing at 58°C for 1 min and 70°C for 1 min.

The final extension step at 70°C for 5 min was followed by a soak. The amplification products were analyzed in a 2% agarose gel in 1x tris-borate-EDTA (TEB, p^H 8.0). Size comparisons were made with DL 2000 DNA Marker (TaKaRa, Shanghai, China) and gel images were captured with and imaging UV light system.

Identification of Lip opeptides by MALDI TOF mass spectra analysis

Lip opeptides from single cell of *B. amyloliquefaciens* A1 were identified by their mass-to-charge ratio (m/z) which were measured using MALDI-TOF-MS(Ben Ayed *et al.*, 2014). To get the single colony *B. amyloliquefaciens* (A1) was grown on NA plate and incubates for 48h at 30°C. After smearing the single colony on to the target mixed with matrix solution (α-Cyano-4-hydroxy cinnamic acid), it was dried naturally and then measured by Bruker ultra flextreme instrument equipped with a 337-nm nitrogen laser for desorption and ionization.

Statistical analysis

The mean and standard deviations of the inhibitions of radial growth were calculated. All the data were subjected to analysis of variance (ANOVA) using SPSS 16.0. Significant differences ($P \leq 0.05$) among the means were determined by Duncan's multiple rage tests.

Results

In vitro growth inhibition of Sclerotinia sclerotiorum by different Bacillus spp.

In vitro growth inhibition of *S. sclerotinia* by nine *Bacillus* spp. was assayed in agar diffusion plate and the inhibition rate were demonstrated in Fig. 1.

Most of the *Bacillus* strains showed potential inhibition against the fungus. Highest rate of inhibition (67.10%) was observed by *B. amyloliquefaciens* A1 (Fig. 2) followed by *B. amyloliquefaciens* A13 (60%), *B. methylotrophicus* H8 (53.73%), *B. subtilis* D16 (54.12%), while there was no significant difference between D16 and H8, *B. methylotrophicus* A2 and *B. subtili*A15, *B. amyloliquefaciens* A3 and *B. amyloliquefaciens* Am. Lowest rate of inhibition (47.06%) was observed by *B. amyloliquefaciens* D29.

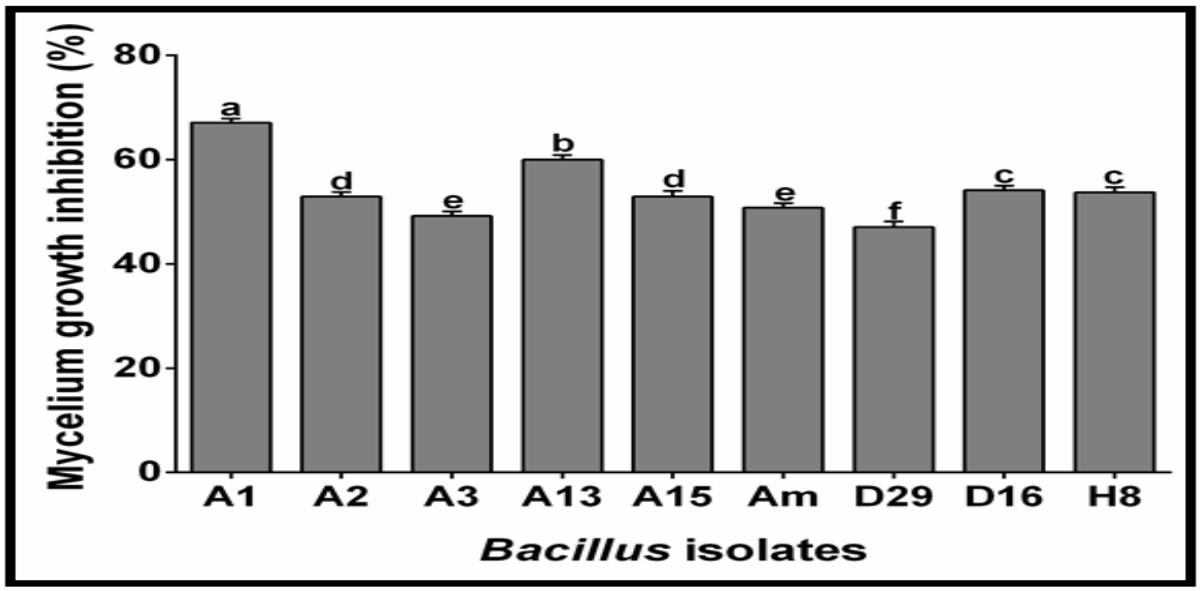

Fig. 1. *In vitro* radial mycelium growth inhibition percentage of *S. sclerotiorum* by different *Bacillus* isolates. Same letter indicate no significant differences between treatments at (P<0.05) using the LSD test.

Effect of culture filtrate on growth inhibition of Sclerotinia sclerotiorum

Antifungal activity of culture filtrate of four *Bacillus* strains H8, D16, A13, A1 was also assayed against the *S. sclerotiorum* (Fig. 3 and 4). The *Bacillus* strains were selected on the basis of their antifungal activity by bacterial cell in previous experiment. After 5 days a clear inhibition zone was observed near the culture filtrate whereas fungus mycelium covered the control plate. Highest inhibition rate was observed by A1 (77.5%) followed by A13 (72.5%), D16 (68.75%), H8 (65%) (Fig. 3). However, there was no statistical difference between A13 and D16.

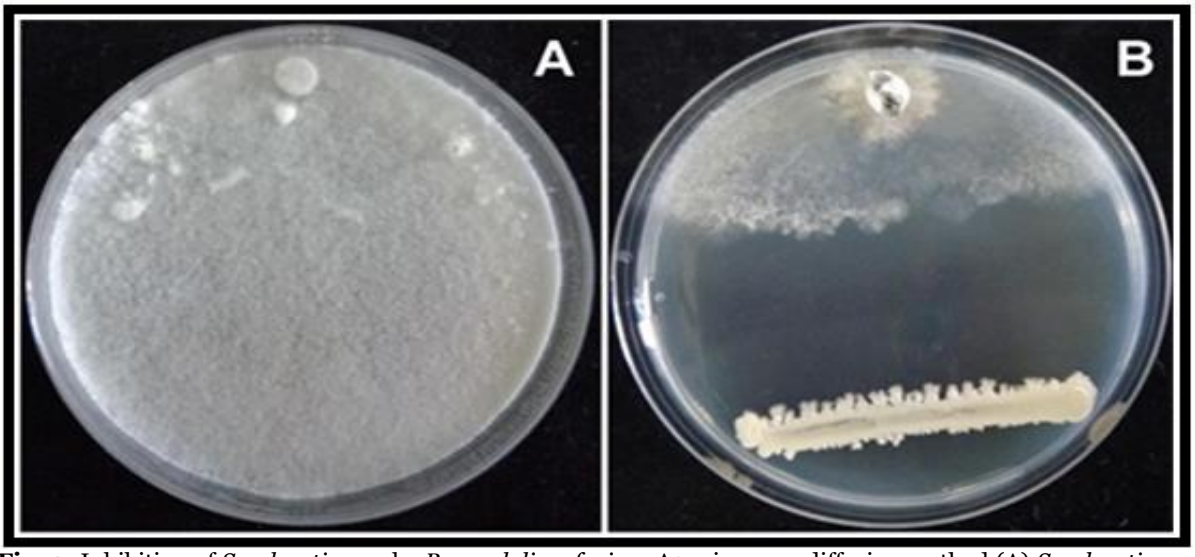

Fig. 2. Inhibition of *S. sclerotiorum* by *B. amyloliquefaciens* A1 using agar diffusion method (A) *S. sclerotiorum* and (B) Dual culture of *B. amyloliquefaciens* A1 and *S. sclerotiorum*.

Alteration of mycelium of Sclerotinia sclerotiorum after interaction of Bacillus

The alteration of mycelia of *S. sclerotiorum* after exposing to *Bacillus* was observed after 5 days of incubation. To compare the alteration of fungal structure, mycelium near the inhibition zone and from untreated growth plate were observed under microscope. Swelling of hypha, bulb like structure

was found in treated hyphae with *Bacillus*, where as thin, filamentous hyphae was found in untreated hyphae of fungus (Fig. 5).

Biofilm formation

The biofilm formation capacity of four effective *Bacillus* strains H8, D16, A13, A1 was assayed after 24 h incubate at 30°C without shaking. All strains produced high amount of biofilm. Among them *B. amyloliquefaciens* A1 produced highest amount of biofilm (0.2129 of OD_{570}), while *B. methylotrophicus* H8 produced lowest amount (0.1362 of OD_{570}) of biofilm which was statistically similar with *B. subtilis* D16 (0.140 of OD_{570}) (Fig. 6).

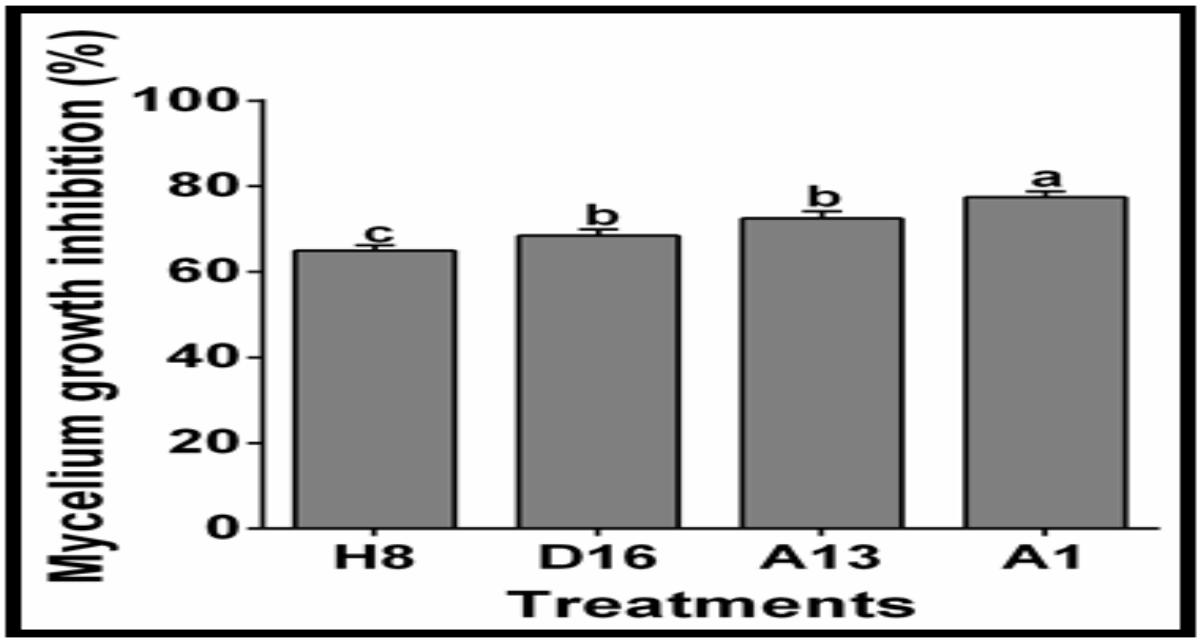

Fig. 3. Mycelium growth inhibition percentage of *S. sclerotiorum* by culture filtrate of selected four *Bacillus* isolates. Different letters indicate significant differences between treatments at ($P<0.05$) using the LSD test.

Siderophore detection

By producing orange-yellow halo near the single colony of bacteria in blue CAS plate, each selected four *Bacillus* strains confirmed that they were capable to produce siderophore whereas no distinct color change in control plate. Siderophore production ability was compared by measuring the diameter of the halo that they produced. Highest diameter of halo was observed in A1 (1.69cm) followed by A13 (1.58cm), D16 (1.41cm), H8 (1.39cm) (Fig. 7).

Lip opeptides biosynthetic gene detection

DNA from the *B. amyloliquefaciens* A1 was subjected to screen the presence of the Lip opeptides biosynthetic genes *ituC*, *fenD*, *srfAA*, *bmyB* and *bacA* using specific primer. The PCR results showed the bands for the specific liopopeptide gene ranging from 100 bp to 500 bp (Fig. 8) indicated that that *Bacillus* strain A1 harbor these biosynthesis genes in its genome.

Identification of lip peptides by MALDI TOF mass spectra analysis

Lip opeptides from the *B. amyloliquefaciens* A1 were identified using MALDI TOF MS spectrum analysis. The results showed cluster of peaks with different m/z, which were attributed to three Lip opeptides family. Lip opeptides fengycin (m/z 1449.679, 1463.707, 1477.519, 1485.489, 1491.532 and 1501.470), iturin (m/z 1074.599), surfactin (m/z 1030.591, 1044.625, 1058.635 and 1060.581,) were detected in the mass spectra of *B. amyloliquefaciens* A1 (Fig. 9).

Discussion

The present study revealed that *S. sclerotiorum* was successfully inhibited by *Bacillus* species in *in-vitro*

dual culture as well as by their cell free culture filtrates. Some species from *Bacillus* such as *B. amyloliquefaciens, B. subtilis*, *B. cerus* have been known as a biocontrol agents in solanaceae and oil seed crop (Asaka and Shoda, 1996; Fernando *et al.*, 2007; Rahman *et al.*, 2007; Tendulkar *et al.*, 2007).

In agreement with previous studies, our results exhibited that *B. amyloliquefaciens* cells and their cell free culture filtrates inhibited *in vitro* growth of *S. sclerotiorum* (Fernando *et al.*, 2007). Results also showed that antagonistic *Bacillus* spp. caused the hyphal malformation that was examined under the microscope. Hyphal swelling, vaculations and thickening of pathogenic mycelia were observed due to interaction with *Bacillus*, which were consistent to the results in many investigations(Rahman *et al.*, 2007; Tendulkar *et al.*, 2007).

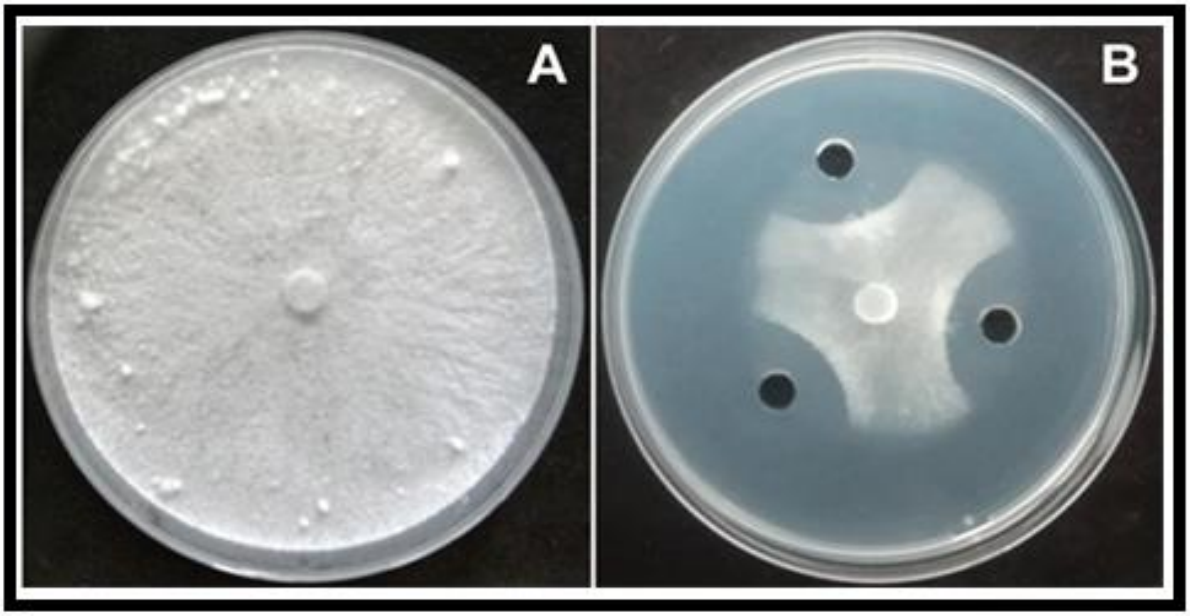

Fig. 4. Effect of culture filtrate of *B. amyloliquefaciens* A1on *S. sclerotiorum* agar well diffusion method. (A) *S. sclerotiorum* and (B) clear zone produced by culture filtrate of *B. amyloliquefaciens* A1.

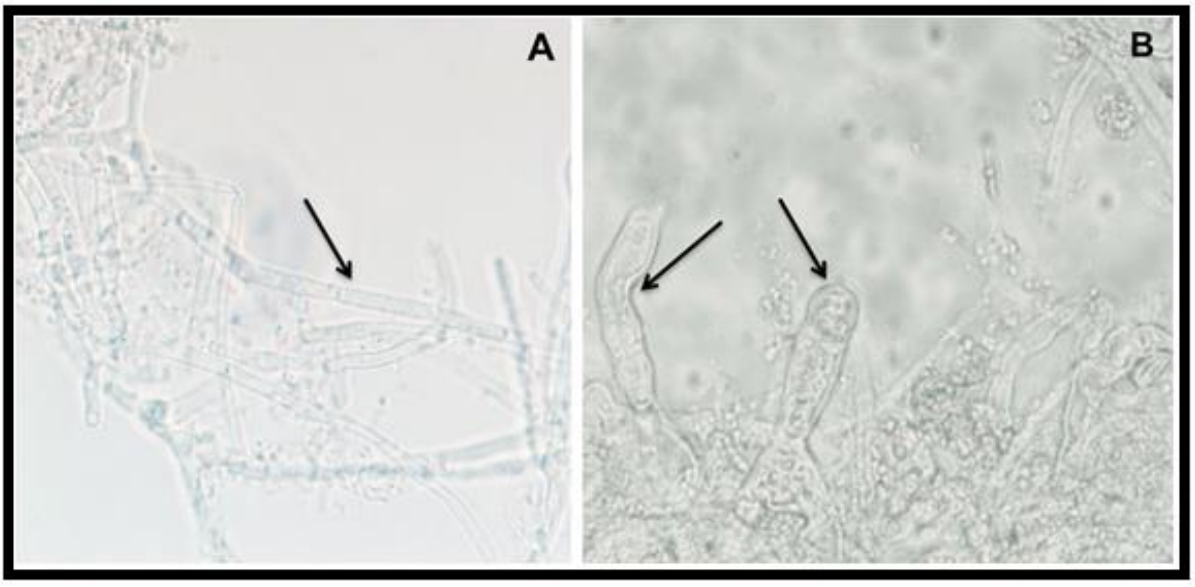

Fig. 5. Hyphal morphology of *S. sclerotiorum* affected by *B. amyloliquefaciens* A1.**(A)** Normal mycelia, arrow indicate the thin filamentous mycelia **(B)** Mycelia co-inoculated with A1, arrows indicate swollen, thick and bulb like structure in mycelia.

It is speculated that the synthesis of antimicrobial Lip opeptides in a non-ribosomal mode by *Bacillus* spp. is one of the possible means to use their antimicrobial action. We performed the conventional PCR assay in the current study to confirm the presence of biosynthetic peptide genes in the genome structure of tested isolate A1 and their production were confirmed MALDI-TOF-MS, which might be at least partly contributed to antifungal action.

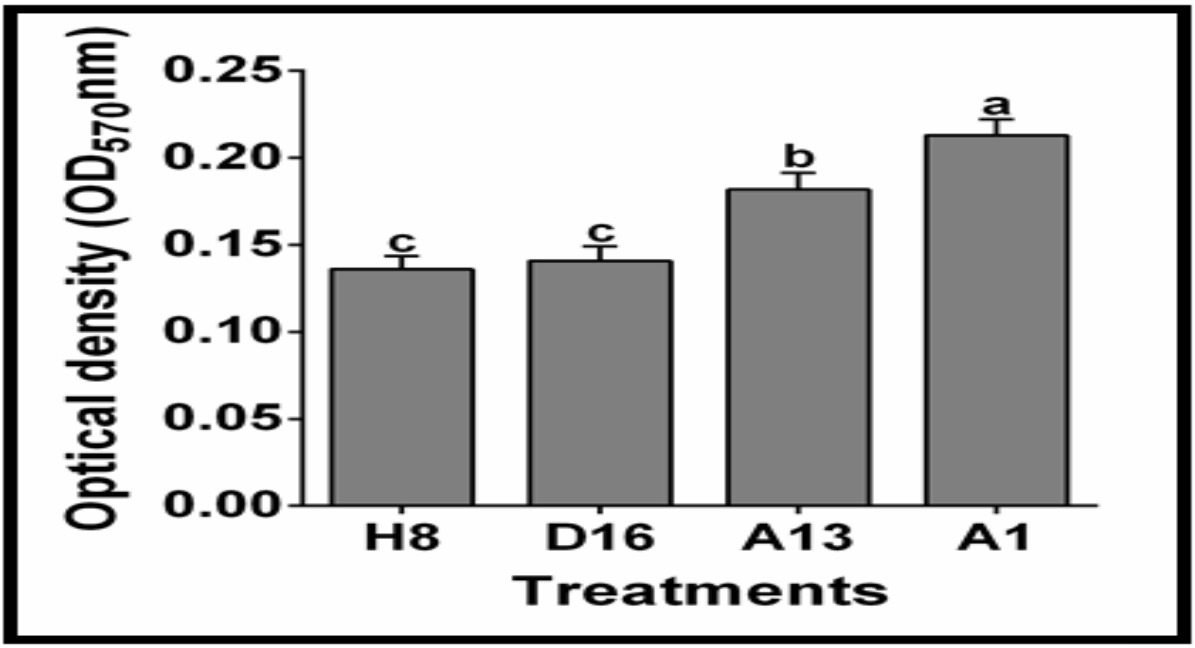

Fig. 6. Biofilm production ability of A1, A13, D16 and H8 in NA broth. Average values of six replicates with standard error were showed. Different letter indicate significant differences between treatments at (P<0.05) using the LSD test.

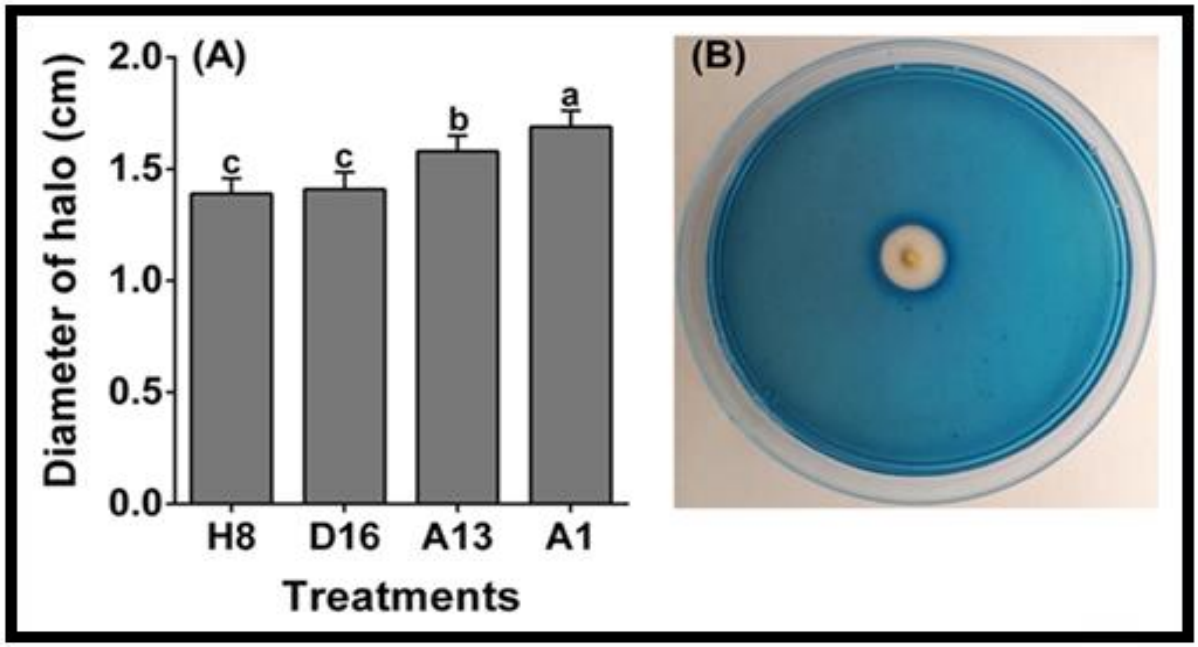

Fig. 7. (A) Detection of siderophore production capacity of A1, A13, D16 and H8 in CAS media. Bar indicate the standard error of average values of six replicates. Same letter indicate no significant differences between treatments at (P<0.05) using the LSD test. (B) Color change from blue to orange-yellow by A1 isolate in CAS plate.

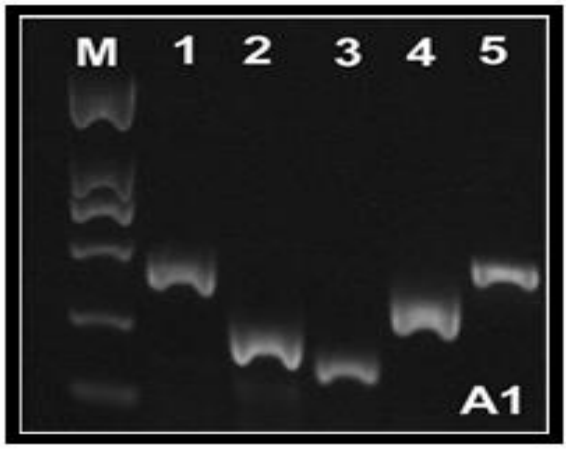

Fig. 8. Confirmation of PCR amplifications using specific primers for biosynthetic genes in *B. amyloliquefaciens* A1, Lane M: 2000 bp marker (TaKaRa), Lane 1, 2, 3, 4, and 5 indicate*ituC*, *fenD*, *srfAA*, *bmyB,* and *bacA,* respectively.

Other studies have also been reported that enzyme and Lip opeptides of *Bacillus* spp. such as iturin, surfactin can alter the morphology of fungus and restrain the further growth of *M. grisea* (Tendulkar *et al.*, 2007; Perez-Garcia *et al.*, 2011).

Moreover, iturin A and fengycin from *B. subtilis* can strongly inhibit the conidial germination of *Podosphara fusca* which cause powdery mildew of cucurbit in Spain and also responsible of controlling damping off of tomato caused by *Rhizoctonia solani* (Asaka and Shoda, 1996; Mizumoto *et al.*, 2006; Romero *et al.*, 2007; Romero *et al.*, 2007; Zohora *et al.*, 2016). These results are at least partly supported the antifungal efficacy of *B. amyloliquefaciens* against *S. sclerotiorum*.

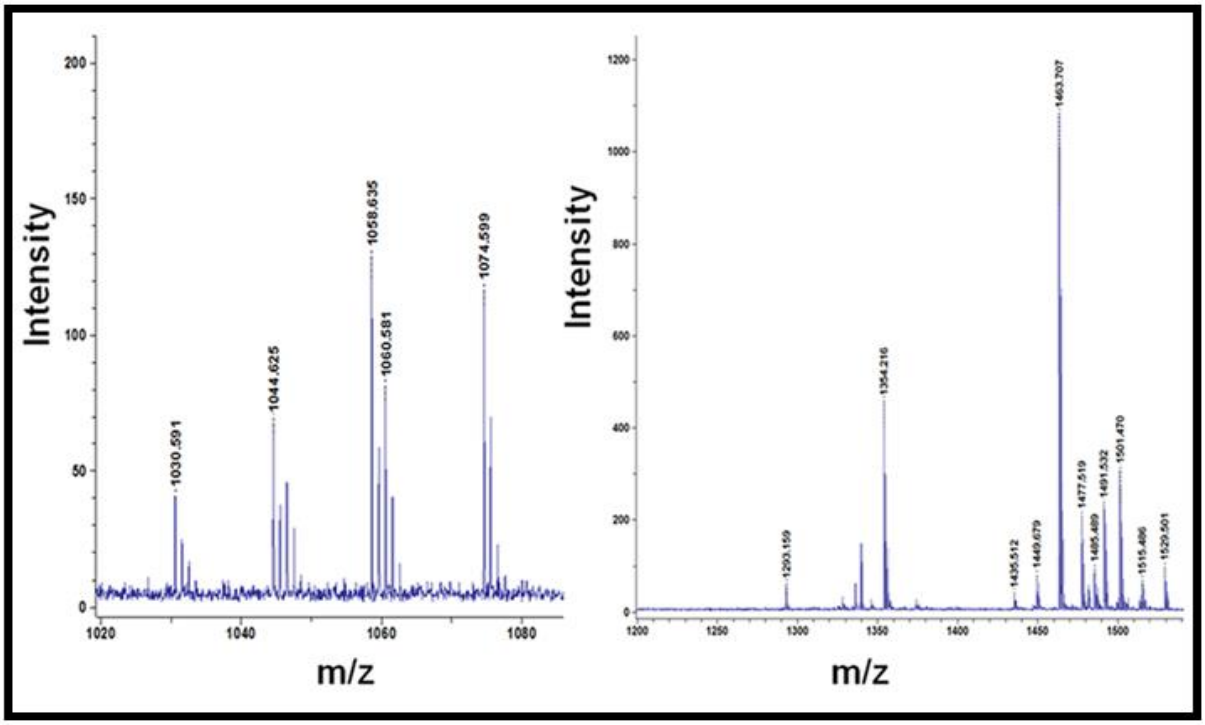

Fig. 9. Identification of fengycin, iturin, surfactin Lip opeptides from single cell of *B. amyloliquefaciens* A1 using Mass spectroscopy (MALDI-TOF-MS) spectra.

Another noticeable finding of this study is that four most effective *Bacillus* strains can produce profuse biofilm. A number of studies have also been reported that the ability of biofilm production of *Bacillus* or *Paenibacillus* species play an important role to control phytopathogens (Davey and O'Toole, 2000; Timmusk *et al.*, 2005). Besides, biofilm formation of *Bacillus subtilis* give supports itself to colonize the root, and thereby facilitate them to control root infection by *Pseudomonas syringae* in *Arabidopsis* plant(Yu *et al.*, 2011). Moreover, the capability of siderophore production by efficient *Bacillus* strains in this study were also justified by the results of de Boer (de Boer *et al.*, 2003) who reported that siderophore production act as an important antagonistic trait because it can bind the iron firmly and make

unavailable this iron for pathogenic microorganism. In the agreement of the present results, other studies showed that siderophore producing antagonistic bacteria *Bacillus subtilis* CAS 15, *Pseudomonas* sp. can combat fusarium wilt of pepper, bacterial wilt of tomato, fusarium wilt of radish(Jagadeesh *et al.*, 2001; Yu *et al.*, 2011). Therefore, biofilm formation and siderophore production by antagonistic *Bacillus* spp. may play a great role *in vitro* growth inhibition of *S. sclerotiorum*. In conclusion, most of the *Bacillus* strains showed inhibitory effect against the pathogenic fungi *S. sclerotiorum* but *B. amyloliquefaciens* A1 was found as most effective strain due to have the highest biofilm and siderophore formation capacity and harboring the most important Lip opeptides biosynthetic genes as well as the production of the antifungal related Lip opeptides.

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References

Almoneafy AA, Kakar KU, Nawaz Z, Li B, Saand MA, Chun-lan Y, Xie GL. 2014. "Tomato plant growth promotion and antibacterial related-mechanisms of four rhizobacterial Bacillus strains against Ralstonia solanacearum." Symbiosis **63,** 59-70.
http://dx.doi.org/10.1007/s13199-014-0288-9

Arrebola E, Jacobs R, Korsten L. 2010. "Iturin A is the principal inhibitor in the biocontrol activity of Bacillus amyloliquefaciens PPCB004 against postharvest fungal pathogens." Journal of Applied Microbiology **108,** 386-395.
http://dx.doi.org/10.1111/j.1365-2672.2009.04438.x

Asaka O, Shoda M. 1996. "Biocontrol of Rhizoctonia solani damping-off of tomato with Bacillus subtilis RB14." Appllied Environmental Microbiology **62,** 4081-4085.

Ashwini N, Srividya S. 2014. "Potentiality of Bacillus subtilis as biocontrol agent for management of anthracnose disease of chilli caused by Colletotrichum gloeosporioides OGC1." 3 Biotech **4,** 127-136.
http://dx.doi.org/10.1007/s13205-013-0134-4

Bais HP, Fall R, Vivanco JM. 2004. "Biocontrol of Bacillus subtilis against infection of Arabidopsis roots by Pseudomonas syringae is facilitated by biofilm formation and surfactin production." Plant Physiology **134,** 307-319.
http://dx.doi.org/10.1104/pp.103.028712

Ben Ayed H, Hmidet N, Bechet M, Chollet M, Chataigne G, Leclere V, Jacques P, Nasri M. 2014. "Identification and biochemical characteristics of Lip opeptides from Bacillus mojavensis A21." Process Biochemestry **49,** 1699-1707.
http://dx.doi.org/10.1016/j.procbio.2014.07.001

Davey ME, O'Toole GA. 2000. "Microbial biofilms: from ecology to molecular genetics." Microbiology and Molecular Biology Reviews **64,** 847-+.
http://dx.doi.org/10.1128/mmbr.64.4.847-867.2000

de Boer M, Born P, Kindt F, Keurentjes JJB, Van der Sluis I, Van Loon LC, Bakker P. 2003. "Control of Fusarium wilt of radish by combining Pseudomonas putida strains that have different disease-suppressive mechanisms." Phytopathology **93,** 626-632.
http://dx.doi.org/10.1094/phyto.2003.93.5.626

Dutta S, Ghosh PP, Kuiry SP. 2009. "Stem rot, a new disease of potato in West Bengal, India." Australian Plant Disease Notes **4,** 80-81.
http://dx.doi.org/10.1071/dn09034

El-shakh ASA, Kakar KU, Wang X, Almoneafy AA, Ojaghian MR, Li B, Anjum SI, Xie GL. 2015. "Controlling bacterial leaf blight of rice and enhancing the plant growth with endophytic and rhizobacterial Bacillus strains." Toxicologicl Environmental Chemistry **97,** 766-785.

http://dx.doi.org/10.1080/02772248.2015.1066176

Elshakh ASA, Anjum SI, Qiu W, Almoneafy AA, Li W, Yang Z, Cui ZQ, Li B. 2016. "Controlling and Defence-related Mechanisms of Bacillus Strains Against Bacterial Leaf Blight of Rice." Journal of Phytopathology **164,** 534-546.
http://dx.doi.org/10.1111/jph.12479

Fernando WGD, Nakkeeran S, Zhang Y, Savchuk S. 2007. "Biological control of Sclerotinia sclerotiorum (Lib.) de Bary by Pseudomonas and Bacillus species on canola petals." Crop Protection **26,** 100-107.
http://dx.doi.org/10.1016/j.cropro.2006.04.007

Gerlagh M, Goossen-Van de Geijn HM, Fokkema NJ, Vereijken PFG. 1999. "Long-term biosanitation by application of Coniothyrium minitans on Sclerotinia sclerotiorum infected crops." Phytopathology **89,** 141-147.
http://dx.doi.org/10.1094/phyto.1999.89.2.141

Heckel DG. 2012. "Insecticide Resistance After Silent Spring." Science **337,** 1613-1614.
http://dx.doi.org/10.1126/science.1226994

Jagadeesh KS, Kulkarni JH, Krishnaraj PU. 2001. "Evaluation of the role of fluorescent siderophore in the biological control of bacterial wilt in tomato using Tn(5) mutants of fluorescent Pseudomonas sp." Current Science **81,** 882-883.

Ji SH, Paul NC, Deng JX, Kim YS, Yun BS, Yu SH. 2013. "Biocontrol Activity of Bacillus amyloliquefaciens CNU114001 against Fungal Plant Diseases." Mycobiology **41,** 234-242.
http://dx.doi.org/10.5941/myco.2013.41.4.234

Kheiri A, Jorf SAM, Malihipour A, Saremi H, Nikkhah M. 2016. "Application of chitosan and chitosan nanoparticles for the control of Fusarium head blight of wheat (Fusarium graminearum) in vitro and greenhouse." International Journal of Biological Macromolecules **93,** 1261-1272.
http://dx.doi.org/10.1016/j.ijbiomac.2016.09.072

McGrath MT. 2001. "Fungicide resistance in cucurbit powdery mildew: Experiences and challenges." Plant Disease **85,** 236-245.
http://dx.doi.org/10.1094/pdis.2001.85.3.236

Mizumoto S, Hirai M, Shoda M. 2006. "Production of lipopeptide antibiotic iturin A using soybean curd residue cultivated with Bacillus subtilis in solid-state fermentation." Applied Microbiology and Biotechnology **72,** 869-875.
http://dx.doi.org/10.1007/s00253-006-0389-3

Mora I, Cabrefiga J, Montesinos E. 2011. "Antimicrobial peptide genes in Bacillus strains from plant environments." International Microbiology **14,** 213-223.
http://dx.doi.org/10.2436/20.1501.01.151

Nelson B. 1998. "Biology of Sclerotinia." Proceedings of the 10th International Sclerotinia workshop, Fargo, ND, USA, North Dakota State University Department of Plant Pathology, Fargo, ND (1998), 1-5.
http://dx.doi.org/10.1080/21691401.2016.1241793

Ojaghian MR. 2009. "First report of Sclerotinia sclerotiorum on potato plants in Iran." Australian Plant Disease Notes **4,** 39-41.
http://dx.doi.org/10.1071/dn09016

Ojaghian MR, Zhang JZ, Zhang F, Qiu W, Li X. N, Xie GL, Zhu SJ. 2016. "Early detection of white mold caused by Sclerotinia sclerotiorum in potato fields using real-time PCR." Mycological Progress **15,** 959-965.
http://dx.doi.org/10.1007/s11557-016-1222-8

Perez-Garcia A, Romero D, de Vicente A. 2011. "Plant protection and growth stimulation by microorganisms: biotechnological applications of Bacilli in agriculture." Current Opinion in Biotechnology **22,** 187-193.
http://dx.doi.org/10.1016/j.copbio.2010.12.003

Rahman MA, Kadir J, Mahmud TMM, Rahman RA, Begum MM. 2007. "Screening of antagonistic bacteria for biocontrol activities on Colletotrichum gloeosporioides in papaya." Asian Journal of Plant Sciences **6,** 12-20.

Ramzan N, Noreen N, Shahzad S. 2014. "Inhibition of in vitro growth of soil-borne pathogen by compost-inhabiting indigenous bacteria and fungi." Pakistan Journal Botany **46,** 1093-1099.

Raton TO, Giro ZG, Diaz MS, Perez SR. 2012. "In vitro growth inhibition of Curvularia gudauskasii by Bacillus subtilis." Annual Microbiology **62,** 545-551.
http://dx.doi.org/10.1007/s13213-011-0290-x

Romero D, de Vicente A, Olmos JL, Davila J. C, Perez-Garcia A. 2007. "Effect of Lip opeptides of antagonistic strains of Bacillus subtilis on the morphology and ultrastructure of the cucurbit fungal pathogen Podosphaera fusca." Journal of Applied Microbiology **103,** 969-976.
http://dx.doi.org/10.1111/j.1365-2672.2007.03323.x

Romero D, de Vicente A, Rakotoaly RH, Dufour SE, Veening JW, Arrebola E, Cazorla FM, Kuipers OP. 2007. "The iturin and fengycin families of Lip opeptides are key factors in antagonism of Bacillus subtilis toward Podosphaera fusca." Molecular Plant-Microbe Interactions **20,** 430-440. http://dx.doi.org/10.1094/mpmi-20-4-0430

Santos S, Neto IFF, Machado MD, Soares H. MVM, Soares V. 2014. "Siderophore Production by Bacillus megaterium: Effect of Growth Phase and Cultural Conditions." Applied Biochemestry Biotechnology **172,** 549-560.
http://dx.doi.org/10.1007/s12010-013-0562-y

Saravanakumar D, Clavorella A, Spadaro D, Garibaldi A, Gullino ML. 2008. "Metschnikowia pulcherrima strain MACH1 outcompetes Botrytis cinerea, Alternaria alternata and Penicillium expansum in apples through iron depletion." Postharvest Biology Tecnology **49,** 121-128.
http://dx.doi.org/10.1016/j.postharvbio.2007.11.006

Schwyn B, Neilands JB. 1987. "Universal chemical-assay for the detection and determination of siderophores." Analytical Biochemistry **160,** 47-56.
http://dx.doi.org/10.1016/0003-2697(87)90612-9

Sharma N, Sharma S. 2008. "Control of foliar diseases of mustard by Bacillus from reclaimed soil." Microbiological Research **163,** 408-413.
http://dx.doi.org/10.1016/j.micres.2006.06.011

Sotoyama K, Akutsu K, Nakajima M. 2016. "Biological control of Fusarium wilt by Bacillus amyloliquefaciens IUMC7 isolated from mushroom compost." Journal of General Plant Pathology **82,** 105-109.
http://dx.doi.org/10.1007/s10327-015-0641-8

Spadaro D, Droby S. 2016. "Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists." Trends in Food Sciencs andTechnology **47,** 39-49.
http://dx.doi.org/10.1016/j.tifs.2015.11.003

Tendulkar SR, Saikumari YK, Patel V, Raghotama S, Munshi TK, Balaram P, Chattoo BB. 2007. "Isolation, purification and characterization of an antifungal molecule produced by Bacillus licheniformis BC98, and its effect on phytopathogen Magnaporthe grisea." Journal of Applied Microbiology **103,** 2331-2339.
http://dx.doi.org/10.1111/j.1365-2672.2007.03501.x

Timmusk S, Grantcharova N, Wagner EGH. 2005. "Paenibacillus polymyxa invades plant roots and forms biofilms." Applied and Environmental Microbiology **71,** 7292-7300.
http://dx.doi.org/10.1128/aem.71.11.7292-7300.2005

Yang H, Li X, Li X, Yu H, Shen Z. 2015. "Identification of lipopeptide isoforms by MALDI-

TOF-MS/MS based on the simultaneous purification of iturin, fengycin, and surfactin by RP-HPLC." Analytcal and Bioanalytical Chemistry **407,** 2529-2542.
http://dx.doi.org/10.1007/s00216-015-8486-8

Yu XM, Ai CX, Xin L, Zhou GF. 2011. "The siderophore-producing bacterium, Bacillus subtilis CAS15, has a biocontrol effect on Fusarium wilt and promotes the growth of pepper." European Journal of Soil Biology **47,** 138-145.
http://dx.doi.org/10.1016/j.ejsobi.2010.11.001

Zohora US, Ano T, Rahman MS. 2016. "Biocontrol of Rhizoctonia solani K1 by Iturin A Producer Bacillus subtilis RB14 Seed Treatment in Tomato Plants." Advances in Microbiology **6,** 424-431.
http://dx.doi.org/10.4236/aim.2016.66042