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Production of Antimicrobial Metabolites by *Bacillus subtilis* and their Applications

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Abstract: The present investigation was carried out to produce of antimicrobial metabolites by *Bacillus subtilis* during two successive seasons 2011/2012. One bacterial strain was isolated from healthy leaves of infected cucumber plants with *Pseudoperonospora cubensis* or *Sphaerotheca fuliginea*. Spraying of cucumber plants was done with bioagent (10^8 CFU mL^{-1}) under greenhouse conditions as protective treatment comparing with fungicides. The obtained results exhibited that all treatments reduced the disease severity comparing with control. The microbial bioagent was identified by using 16S rDNA sequencing technique as *Bacillus subtilis*. The maximum accumulation of metabolites occurred at the stationary phase. Metabolites accumulation coincided with increase in the specific growth rate and reduction of disease severity. Maximum activity of metabolites was at pH 7 and 30°C. Mode of metabolites action on (sporangiophore and sporaniga) of *P. cubensis* and (conidiophore and conidia) of *S. fuliginea* by examination of Scanning Electron Microscope and their identification by GC-MS was investigated. It was observed that collapse in sporangiophore, sporangia and conidia. These compounds mostly included fatty acids known as Hexadecanoic acid, n-Hexadecanoic acid, Octadecanoic acid, 8-Octadecanoic acid, 9-Octadecanoic acid, Pentadecanoic acid, Heptadecanoic acid, dodecatrienoic acid, Nonanoic acid and Decanoic acid. Sprayed plants recorded best results for peroxidase and polyphenol oxidase enzymes activity compared with unsprayed one. Antibiotics exhibited also antagonistic activity against of *Staphylococcus aureus* by inhibition zone formation. In conclusion, bioagent can be use as an alternative and safe method to fungicides in controlling downy and powdery mildew diseases of cucumber and inhibition of *Staphylococcus aureus* as pathogenic bacteria.

Key words: *Bacillus subtilis*, downy and powdery mildew diseases, cucumber, fungicides, 16S rDNA gene

INTRODUCTION

Cucumber (*Cucumis sativus* L.) is one of the important economic vegetable crops grown under protected cultivations in Egypt, which belongs to family Cucurbitaceae (Kamel, 2003). Several foliar fungal diseases attack cucumber plants such as powdery mildew caused by *Sphaerotheca fuliginea* (Schlect. ex: Fr.) Pollacci and downy mildew caused by *Pseudoperonospora cubensis* (Berk. and Curt.) Rostow. The both diseases caused loss in yield and quality of the cucumber fruits (Dixon, 1981). These diseases are managed primarily with fungicides. Application of the fungicides is not desirable and this due to their adverse effects on the environment and ecosystem (Cook and Baker, 1983). Biological control of plant pathogens has been considered a more natural and environmentally acceptable alternative to the existing chemical treatment methods (Xi *et al*., 1996). Successful biological control of foliar diseases has been achieved by a number of researchers under greenhouses and field trials (Singh *et al*., 2000; Abd-El-Moneim, 2001; Kamel, 2003; Hussein *et al*., 2007). 16S rRNA was used as a molecular technique to identify of the bacteria (Woese *et al*., 1985; Tortoli, 2003; Woo *et al*., 2003). Biocontrol agents induced host resistance through increased peroxidase and polyphenol oxidase activity which playing a defense role against invading pathogens (Caruso *et al*., 2001; Nawar and Kuti, 2003). Antibiotic production by some bacteria plays a major role in inhibition of plant pathogens and other pathogenic bacteria. *Bacillus amyloliquefaciens* strain B94 and *B. subtilis* were used as a biocontrol agent to suppress *Rhizoctonia solani* and other fungal plant pathogens as well as other pathogenic bacteria (Yu *et al*., 2002; Awais *et al*., 2007). Therefore, the present investigation was designed to isolate of *Bacillus subtilis*, characterization of antimicrobial metabolites by *B. subtilis* and their applications.

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MATERIALS AND METHODS

Selected cucumber cultivar: Cucumber (*Cucumis sativus* L.) cultivar Hesham was used under greenhouse conditions to evaluate the efficacy of the tested treatments against downy mildew caused by *Pseudopernospora cubensis* and powdery mildew caused by *Podosphaera xanthii* (previously known as *Sphaerotheca fuliginea* and *Erysiphe cichoracearum*) during two successive seasons 2011/2012.

Antagonist: Healthy leaves of infested cucumber plants with downy and powdery mildew diseases were collected from different locations of cucumber commercial plantation at Elbehira Governorate, Egypt. Whole blades of healthy cucumber leaves were cutted into small pieces and then 10 gm were added to 90 mL sterilized distilled water. The samples were shaken at 30°C and 150 rpm for 30 min. Serial dilutions were prepared in glass tubes containing 9 mL sterilized distilled water up to 10^6 CFU mL^{-1}. One hundred micro litter from the three latter dilutions were spread over synthetic agar medium (Awais *et al*., 2010).

Inoculated plates were incubated at temperature 30°C until arising maximum number of separated colonies. The single colonies were picked and repurified.

Antagonists were applied as spray treatment. Plants were sprayed with bacterial suspension (10^8 CFU mL^{-1}). The treatment was carried out after 4 weeks from transplanting (Abd-El-Moneim, 2001) intervals 6 weeks, the plants were sprayed weekly. Cultures were amended with calculated aliquots of an adhesive surfactant (bio-film 1265, registered by ministry of Agric., Egypt) as recommended (30 ml 100 L^{-1} water) and sprayed onto the upper and the lower leaf surfaces of the plants until run off. Hesham cultivar was used and the treatments were randomizally distributed. Three replicates for each treatment and six plants in each replicate were used. The temperature was 21±2 at 80-86% humidity. Disease severity was calculated as was mentioned below. Plants sprayed with tap water only (likely amended with the adhesive surfactant served as check treatment (control). In addition, plants were sprayed with Equation-Pro 52.5% WG and sumi-8 as fungicides with recommended dose control of downy and powdery mildews, respectively. Plants were irrigated whenever needed and fertilized with calculated doses of the macro and micro nutrients.

Severity of powdery and downy mildews were assessed and scored using 0-9 rating scale based on the percentage of leaf area affected as described method (Warkentin *et al*., 1996), where: 0 = no infection, 1 = 1%, 2 = 1-5%, 3 = 5-10%, 4 = 10-20%, 5 = 20-40%, 6 = 40-60%, 7 = 60-80%, 8 = 80-90%, 9 => 90% of leaf area affected:

$$\text{Percentage of protection was expressed as} = \frac{\text{Check-treatment}}{\text{Check}} \times 100$$

16S rRNA sequence determination: The efficient selected bioagent was identified depending morphological and physiological characteristics according to Parry *et al*. (1983) and 16S rDNA sequencing as follow:

The amplified product of approximately 1254 bp (1,254 bp for nested PCR) was curried out according to the described method of Sacchi *et al*. (2002). Sequencing was performed using a Big Dye terminator cycle sequencing kit (Applied BioSystems, Foster City, CA). Sequencing products were purified by using Centri-Sep™ Columns (Princeton Separations, Adelphia, NJ) and were resolved on an Applied BioSystems model 3100 automated DNA sequencing system (Applied BioSystems).

The evolutionary history was inferred using the Minimum Evolution method (Rzhetsky and Nei, 1992). The optimal tree with the sum of branch length = 0.67162741 was shown. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method of Tamura *et al*. (2004) and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000) and a search level of the Neighbor-joining algorithm (Saitou and Nei, 1987). The analysis involved 43 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 765 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011).

Cultivation of *Bacillus subtilis* in nutrient liquid medium for metabolites (antibiotics) production: One hundred millilitter synthetic liquid medium were inoculated with 1 mL of a cell suspension of *Bacillus subtilis* E5 (synthetic medium, 10^7 CFU mL^{-1}, incubated at 30°C and 150 rpm for 3 days). The culture was incubated at 30°C and 150 rpm for 7 days. Cells number of the bacterial strain was determined by plating appropriate dilutions of liquid medium onto synthetic agar medium. The culture broth was passed through a sterile membrane filter (0.2 μm). Cells number of the bacterial strain was determined by plating appropriate dilutions of liquid medium onto synthetic agar medium. The production of metabolites

(antibiotic) was determined daily by determination of the disease severity of the pathogen after treating infected plants. On the other hand, cucumber plants were sprayed with water and these treatments were used as control. The experiments were performed in three replicates. The effect of metabolites on sporangiophore and sporangia of *P. cubensis*, conidiophore and conidia on *S. fuleginea* was examined by using Scanning Electronic Microscope (SEM) as follow: Samples of treated leaves of cucumber plants and leafs of untreated control were taken and immediately fixed in 3% glutaraldehyde in 0.2 m phosphate buffer (pH 7.2) for 24 h at 4°C, followed by exposure to osmium tetraoxide (1% OsO_4) for one hour at room temperature (Harley and Fergusen, 1990). The samples were dehydrated by putting their in ascending concentrations of acetone and then dried till the critical point and finally, the samples were sputter coated with gold. The examination and photographing were done through a Jeol Scanning Electron Microscope (T. 330 A) in the Central Laboratory of the Faculty of Agriculture, Ain Shams University, Egypt.

Effect of pH and temperature on growth of *B. subtilis*: One hundred mL of synthetic liquid medium was used to determine the effect of pH and temperature on growth of *B. subtilis*. The medium was inoculated by 1 mL (10^8 CFU mL^{-1}) of culture of *B. subtilis*. The experiments were carried out at pH 5, 6, 7, 8 and 9 and then the cultures were incubated on a rotary shaker at 30°C and 150 rpm for 5 days. To determine the optimum temperature, synthetic liquid medium at pH 7 was incubated at 20, 25, 30, 35 and 40°C and 150 rpm for 7 days. Cells number of the bacterial strain was determined by plating appropriate dilutions of liquid medium onto synthetic agar medium.

Extraction and identification of metabolites of *Bacillus subtilis* by GC-MS: Culture was centrifuged at 10.000 rpm for 15 min after cultivating of *Bacillus subtilis* on synthetic liquid medium at 30°C for 5 days. The produced supernatant was adjusted at pH 2.5 with 6 M HCl and centrifuged 15.000 rpm for 20 min. and thereafter, the precipitate was collected (Mckeen *et al*., 1986; Leelasuphakul *et al*., 2008). Precipitates were was discarded to have free supernatant with 80% methanol, pooled and dried by sodium sulphate anhydrous and it stored at 4°C until identification by GC-MS. The metabolites were extracted from supernatant with one volume of methanol 100% (Loba, India) (Bernal *et al*., 2002). The identification for metabolites was done by gas chromatography and mass spectrometry (GC-MS) at Plant Pathogenic Lab., Institute of Agricultural Research, Ministry of Agriculture, Egypt. Extract solution were injected into HP 6890N gas chromatograph equipped with HP5975 Mass detector and fused silica capillary column HP5 MS (5% phenyl methyl silicone, 30 mL length, 0.25 mm i.d.). The temperature was programmed from 30°C (1 min) to 230°C (20 min) at the rate of 4°C min^{-1}. Detector was heated at 250°C, injector at 230°C. Helium was used as a carrier gas at 5 Psi pressure. Mass spectra were obtained by electron ionization at 70 eV.

Effect of antibiotics (metabolites) on growth of *Staphylococcus aureus*: One hundred micro litter (72 h old culture) for *Staphylococcus aureus* (10^8 CFU mL^{-1}) were spreaded by using glass spreader on nutrient agar plates, respectively. After that, 50 µL from metabolites were putted in wells (5 mm in diameter) in nutrient agar medium and then the plates were incubated at 35°C and examined daily. The inhibition zone was recorded after 24 h as incubation periods. Three replicates were used.

Tested fungicides: Equation-Pro 52.5% WG [(Famoxadone: 3-aniline-5-methyl-50-(4-phenoxyphenyl)-2 4-oxazolidinedione) (Cymoxanil: 2-(2-cyano-2-methoxyimino-acetyl) -3-ethylurea)] and Sumi-8 (Sumitomo) IUPAC name (E)-(RS)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl) pent-1-en-3-ol) were used at the concentration of 30 g per 100 L against *P. cubensis* and *S. fuliginea*, respectively. Spraying was repeated weekly for 3 times. All treatments were applied as protective (before appearance of symptoms).

Control of the cucumber downy and powdery mildews using the *B. subtilis* and fungicides under greenhouse conditions: This study was carried out in greenhouse at Elbehira Governorate, Egypt and using randomized complete blocks design with three replicates for each treatment. Each greenhouse (6×40 m) was divided into equal experimental units (replicates). Each unit contained 12 plants. Cucumber seedlings (cv. Hesham) were transplanted at 50 cm apart at mid of November of 2010/2011 and 2011/2012. The treatment was carried out after 4 weeks from transplanting (Abd-El-Moneim, 2001) intervals 6 weeks, the plants were sprayed weekly. Equation-Pro 52.5% WG and Sumi-8 (Sumitomo) fungicides were used as described above. Disease severity and fungicides efficacy were calculated as was mentioned above. All treatments were compared with untreated plants as control.

Enzymes extraction and assay: Healthy and infected cucumber leaves were collected after 24 h from last

spraying with *B. subtilis* or fungicides and from untreated plants to assay peroxidase and polyphenol oxidase activity. Enzyme extract was obtained by grinding leaf tissue in 0.1 M sodium phosphate buffer at pH 7.1 (2 m g^{-1} leaf tissues) in a porcelain mortar. The extracted tissues were strained through four layers of cheese cloth. Filtrates were centrifuged at 3000 rpm for 20 min at 6°C. The clear supernatants were collected and considered as crude enzyme. Peroxidase (POX) activity was determined according to Allam and Hollis (1972) by measuring the oxidation of pyrogallol to pyrogalline in the presence of hydrogen peroxide. Peroxidase activity was measured following the changes in absorbance at 425 nm min^{-1} up to 4 min. Polyphenol Oxidase (PPO) was determined according to the described method of Maxwell and Bateman (1967). The changes in absorbance was following spectrophotometrically measured at 495 nm and recorded every 1 up to 4 min.

Statistical analysis: The completely randomized design was used for the laboratory and greenhouse experiments. Each experimental design has its previously mentioned replication. Data were transformed before subjection to analysis of variance using IRRI Stat Computer Program and zero values were replaced by minimum values before transforming the data. Means were compared using LSD method (Steel and Torrie, 1980) and multiple range tests according to Duncan (1955).

RESULTS AND DISCUSSION

The initial screening of more than 100 bacterial originated from different healthy leaves of infected cucumber plants, resulted in the isolation of one isolate exhibiting obvious reduction of disease severity of cucumber downy and powdery mildews disease under greenhouse conditions (data not shown). A preliminary classification based on the morphology of the isolate revealed that, this isolate belongs to the group of bacteria. This bacterial strain (E5) was identified according to morphological, physiological as well as using analysis of 16S rDNA (Fig. 1). Phylogenetic tree show the relationships of the organisms represented by the examined sequence and their closest relatives among fourteen bacterial isolates. The tree is based on the results of distance matrix analyses of all available 16S rRNA primary structures for *Bacillus* species. The topology of the tree was evaluated by performing maximum parsimony and maximum Close-Neighbor-Interchange analyses of the full data set and subsets, respectively. Only sequences that were at least 95% complete were used for treeing. Alignment positions at which less than 50% of sequences of the entire data set have the same residues were excluded from the calculations. The phylogenetic positions of organisms presented by partial sequences were roughly reconstructed by applying the parsimony criteria without changing the overall tree topology. Multifurcations indicate that a common branching order was not significantly supported by applying different treeing methods. The sequences of two almost identical clones, *Bacillus subtilis* E5 could be assigned to the rRNA sequences of *Bacillus subtilis* RONN1 and *Bacillus subtilis* ST 168, respectively. They had a similarity of 100 and 95% to the sequence of *Bacillus subtilis* RONN1 and *Bacillus subtilis* ST 168, respectively.

Characterization of the metabolites (antibiotic) system of *B. subtilis* and their efficacy on cucumber downy and powdery mildews suppression: A series of experiments were carried out to study the growth behaviour of *B. subtilis* in synthetic broth medium and

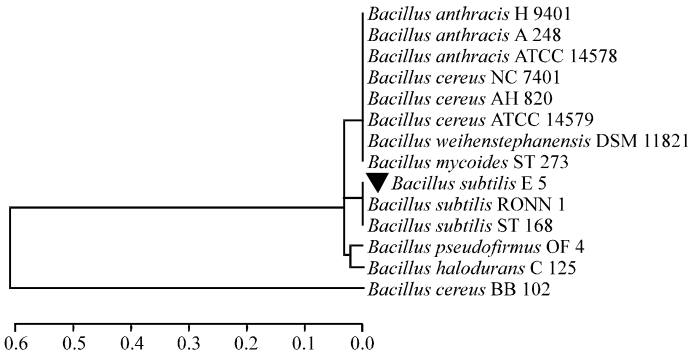

Fig. 1: Phylogenetic tree of bacterial 16S gene sequences from *Bacillus subtilis* (E5). The scale bar represents 0.6 substitutions per nucleotide position

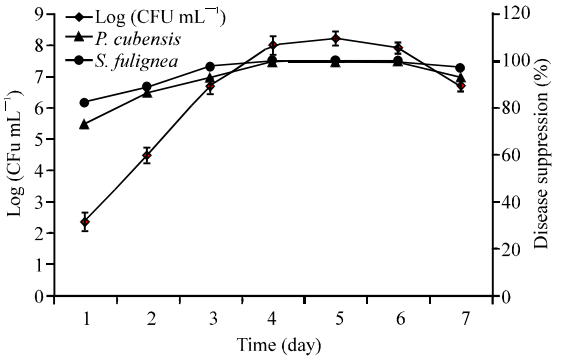

Fig. 2: Effect of incubation period on growth behaviour of *B. subtilis* on cucumber downy and powdery mildews diseases suppression

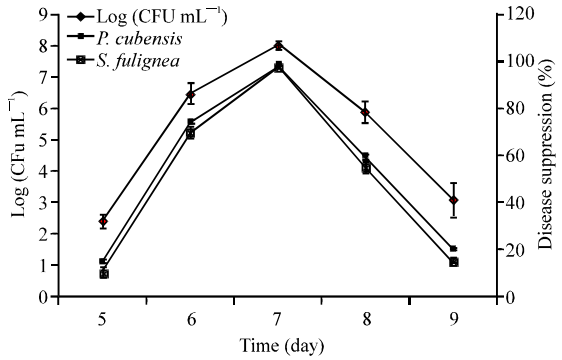

Fig. 3: Effect of pH on growth of *B. subtilis* and its metabolites (antibiotics) on cucumber downy and powdery mildews diseases suppression

its metabolites effect on suppression of cucumber downy and powdery mildews disease. Data presented in Fig. 2 shows that the growth was increased gradually till the fourth day. The metabolites (antibiotic) formation started when the strain grew on the medium. The highest accumulation of metabolites (antibiotic) exhibited in the fourth, fifth and sixth days of cultivation. The maximum accumulation of metabolites (antibiotic) occurred at end of the exponential growth phase and at the stationary phase. The accumulation of metabolites (antibiotic) decreased at end of the stationary growth phase. Metabolites (antibiotic) excretion coincided with increase in the specific growth rates and reduction of disease severity. It has been reported by Egorov *et al.* (1986) that the maximum efficiency of the bacitracin synthesis in case of *B. licheniformis* coincides with the end of the exponential growth phase and the onset of sporification. These findings are in agreement with the present study.

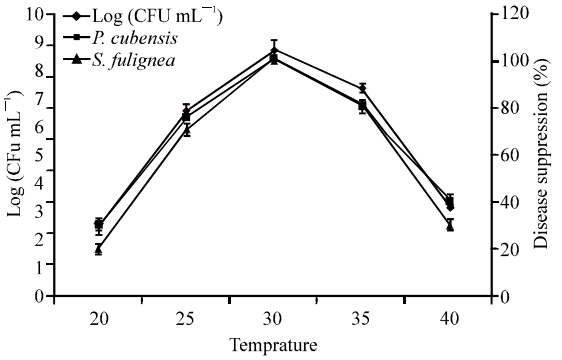

Fig. 4: Effect of temperature on growth of *B. subtilis* and its metabolites (antibiotics) on cucumber downy and powdery mildews diseases suppression

Effect of pH and temperature on growth of *B. subtilis* and cucumber downy and powdery mildews suppression: Data presented in Fig. 3 and 4 shows that the optimum pH and temperature for growth of *B. subtilis* in synthetic liquid medium. The optimum pH and temperature were 7 and 30°C, respectively. The highest production of metabolites was at 7 and 30°C. The obtained results indicated that the highest disease reduction of both cucumber downy (caused by *P. cubensis*) and powdery mildews (caused by *S. fuligenea*) was recorded at pH 7 and 30°C. The disease suppression of the both fungal pathogens increased with increasing growth of *B. subtilis*. Therefore, it can be deduced from the results that the pH is considered an important environmental factor on production of *B. subtilis* metabolites. Changes in external pH affect many cellular processes such as the regulation of the biosynthesis of secondary metabolites. Yousaf (1997) reported that optimum bacitracin yield from *B. licheniformis* was at pH of 7.0.

Mode of action efficacy of metabolites on cucumber downy and powdery mildews diseases suppression: Data presented in Fig. 5 and 6 shows the mode of action of *B. subtilis* metabolites on *P. cubensis* and *S. fuliginea* inhibition by examination of Scanning Electron Microscopy (SEM). It was observed that loss of turgor in sporangia and collapse in sporangiophore and hyphae of *P. cubensis* (Fig. 5) as a result of treating with *B. subtilis*. Microscopical examination of the treated lesions showed sporangia suffering from osmolysis. On the other hand, the treated lesions of powdery mildew showed dead hyphal remnants of the pathogen and have conidiophores without spore chains (Fig. 6).

The metabolites were identified by GC-MS. It was found that 10 compounds as metabolites excreted by

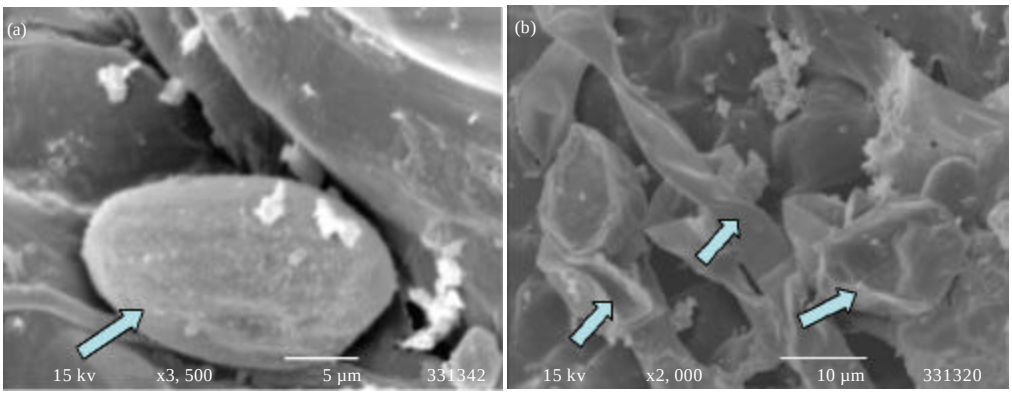

Fig. 5(a-b): Scanning electron microscopy (SEM) on cucumber leaves infested with *P. cubensis* sprayed with *B. subtilis* under protected cultivations (a) Control (untreated cucumber leaves) and (b) Sprayed with *B. subtilis*

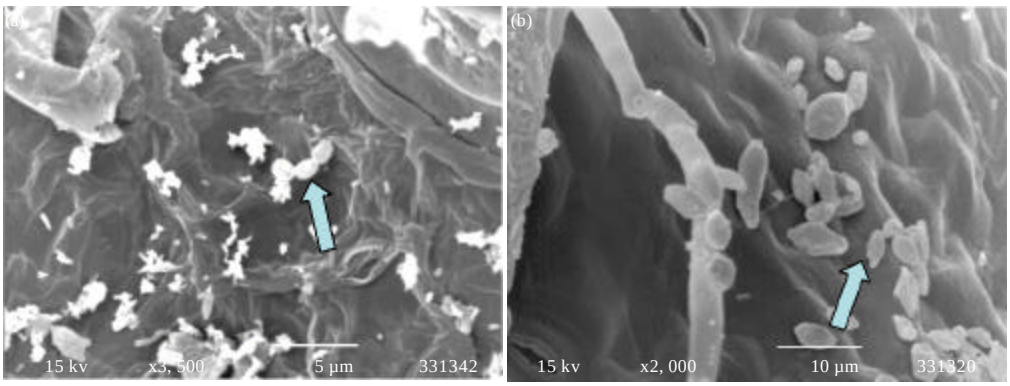

Fig. 6(a-b): Scanning electron microscopy (SEM) of cucumber leaves infested with *S. fuliginea* sprayed with *B. subtilis* under protected cultivations (a) Control (untreated cucumber leaves) and (b) Sprayed with *B. subtilis*

B. subtilis. These compounds mostly included fatty acids known as Hexadecanoic acid, n-Hexadecanoic acid, Octadecanoic acid, 8-Octadecanoic acid, 9-Octadecanoic acid, Pentadecanoic acid, Heptadecanoic acid, dodecatrienoic acid, Nonanoic acid and Decanoic acid.

Other organic compounds such as amines, amides, organic acid, esters, heterocyclic compound, alcohol and ketones are concluded. Cyclotetrasiloxane octamethyl is used as a surfactant in many chemical compounds such as certain pesticide products. Mass spectrum was obtained and the confirmation of compounds was done by comparison of the retention time and mass spectrum with those in the wiley 7n.1, NIST98.1 and Pest. 1 library.

Generally, numerous members of *Bacillus* species are known as producers of lipopeptides belonging to the surfactin, iturin and fengycin families (Zuber *et al*., 1993). Fengycin is an antifungal lipopeptide complex produced by *Bacillus subtilis* F-29-3 (Vanittanakom *et al*., 1986). It consists of two main components, fengycin A and fengycin B. The lipid moiety of both analogs is more variable, as fatty acids have been identified as anteiso-pentadecanoic acid (ai-C15), iso-hexadecanoic acid (i-C16), n-hexadecanoic acid (n-C16) and there is evidence for further saturated and unsaturated residues up to C18. In the present study, these components of fatty acids had been detected in supernatant of *B. subtilis* analysis using GC-MS. The antibiotic production by some bacteria plays a major role in plant pathogens. *B. amyloliquefaciens* strain B94 was used as a biocontrol agent to suppress *Rhizoctonia solani* and other fungal plant pathogens (Yu *et al*., 2002).

Effect of antibiotics (metabolites) on growth of *Staphylococcus aureus*: Results presented in Fig. 7 shows the effect of antibiotics (metabolites) on growth of *Staphylococcus aureus* as positive gram bacteria on nutrient agar plates by formation of inhibition zone.

The amount of antibiotics produced by bacilli was approaching 167, being 66 derived from *B. subtilis*, 23 from *B. brevis* and the remaining peptide antibiotics are produced by other species of genus *Bacillus*. The main antibiotic producers of this genus are *B. brevis* (e.g., gramicidin, tyrothricin), *B. cereus* (*e.g.*, cerexin, zwittermicin), *B. circulans* (e.g., circulin), *B. laterosporus* (e.g., laterosporin), *B. licheniformis* (e.g., bacitracin), *B. polymyxa* (e.g., polymyxin, colistin), *B. pumilus* (e.g., pumulin), *B. subtilis* (e.g., polymyxin, difficidin, subtilin, mycobacillin, bacitracin). As is generally assumed, these antibiotics are mainly polypeptides (Berdy, 1974; Daversa and Stern, 1997; Hancock and Chappel, 1999).

Most of the peptide antibiotics produced by *Bacillus* are active against Gram positive bacteria (Ming and Epperson, 2002). However, compounds such as polymyxin, colistin and circulin exhibit activity almost exclusively upon Gram-negative forms, whereas bacillomycin, mycobacillin and fungistatin are effective agents against molds and yeasts (Katz and Demain, 1977).

Biological control of cucumber downy and powdery mildews diseases by *B. subtilis* under greenhouse conditions: Data presented in Table 1 and 2 showed that all treatments were significantly decreased disease severity on cucumber plants (cv Hesham) under greenhouse conditions as protective treatment during two seasons 2010/2011 and 2011/2012. Results indicated that *B. subtilis* play outstanding role in controlling of cucumber downy and powdery mildews. It significantly decreased disease severity from 9.2-4.5 for downy mildew and from 9.5-2.5 for powdery mildew in season 2010/2011, respectively. It significantly also decreased disease severity from 9-3.8 for downy mildew and from 9.4-2.2 for powdery mildew in season 2011/2012, respectively. The mean of fungicide efficacy of Equagen pro on downy mildew was 4.3 and 3.5 during two seasons. The mean of fungicide efficacy of sum-8 on powdery mildew was 2.4 and 2.2 during two seasons, respectively. This effect

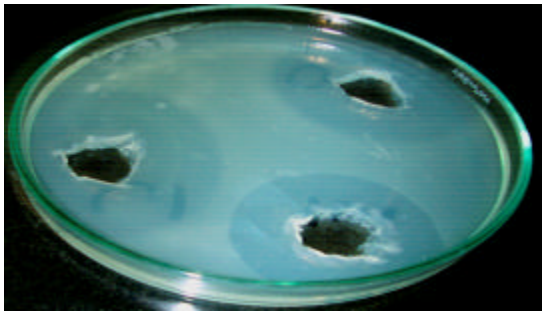

Fig. 7: Antimicrobial activity of *B. subtilis* on growth of *Staphylococcus aureus*

Table 1: Biological control of cucumber downy mildew disease caused by *P. cubensis* with *B. subtilis*

	Downy mildew						
	Season 2010-2011			Season 2011-2012			
Treatments	Application start	End experiment	Disease Inhibition (%)	Application start	End experiment	Disease Inhibition (%)	Protection (%)
Disease severity							
Control (untreated)	0	9.2^{b}	-	0	9.0^{b}	-	-
B. subtilis E5+*P. cubensis*	0	4.5^{a}	51.1	0	3.8^{a}	57.8	54.5
Equation Pro+*P. cubensis*	0	4.3^{a}	53.3	0	3.5^{a}	61.1	57.2

Mean followed by the same letter are not significantly different according to Duncan's multiple range test (DMRT) at 0.5% level

Table 2: Biological control of cucumber powdery mildew disease caused by *S. fuliginea* with *B. subtilis* under greenhouse conditions during two seasons 2011 and 2012

	Powdery mildew						
	Season 2010-2011			Season 2011-2012			
Treatments	Application start	End experiment	Disease Inhibition (%)	Application start	End experiment	Disease Inhibition (%)	Protection (%)
Disease severity							
Control (untreated)	0	9.5^{b}	-	0	9.4^{b}		-
B. subtilis E5+*S. fuliginea*	0	2.5^{a}	73.7	0	2.2^{a}	76.9	75.3
Sumi-8+*S. fuliginea*	0	2.4^{a}	74.7	0	2.2^{a}	76.9	75.8

Means followed by the same letter are not significantly different according to Duncan's multiple range test (DMRT) at 0.5% level

Table 3: Effect of *B. subtilis* on activity peroxidase and polyphynoloxidase

	Enzyme activity (Δ_{abs}/time)	
Treatments	Peroxidase	Polyphenoloxidase
Control (un treated)	0.742[d]	0.432[d]
B. subtilis	1.546[c]	1.702[c]
Equation pro	1.460[c]	1.607[c]
Sumi-8	1.505[c]	1.663[c]
B. subtilis+*P. cubensis*	1.321[b]	1.401[b]
B. subtilis+*S. fuliginea*	1.313[b]	1.392[b]
Equation pro+*P. cubensis*	1.191[a]	1.160[a]
Sumi-8 *S. fuliginea*	1.130[a]	1.150[a]

Values followed by the same letter are not significantly differences at p<0.5 level

of the both fungicides and *B. s ubtilis* was similar during the two seasons. Trankner (1992) reported that, *Bacillus subtilis* grew on the treated surfaces of leaves and utilize available nutrient substances and prevent *P. cubensis* spores to establish, germinate and invade healthy tissues. *Bacillus* sp. also grows very fast and occupies the court of infection and preventing pathogen spores to reach susceptible tissues in competition for spaces (Wolk and Sarkar, 1994). All treatments were compared with untreated plants (control). Our results are in agreement with previous finding by many investigators (Abd-El-Moity *et al*., 2003; Xing *et al*., 2003).

Effect of spraying cucumber plants with *B. subtilis* on peroxidase and polyphenol oxidase activity: Data presented in Table 3 show that, the effect of spraying cucumber plants with *B. subtilis* on peroxidase and poly phenol oxidase activity. The highest activity of peroxidase and polyphenol oxidase was observed when *B. subtilis* was sprayed on cucumber plants followed by fungicides (Equation pro and Sum-8) compared with control. Spraying of cucumber plants with *B. subtilis* gave an increment of peroxidase and polyphenol oxidase enzymes activity and this indicate a positive relationship between increasing in peroxidase and polyphenol oxidase enzymes activity and reduction in disease severity of cucumber downy and powdery mildews. Many investigators supported this work since they stated that there are positive relationships between peroxidase enzyme and resistance developed in plants (Nawar and Kuti, 2003).

Protection resulting from Induced Systemic Resistance (ISR) elicited by *Bacillus* spp. has been reported against leaf-spotting fungal and bacterial pathogens (Choudhary and Johri, 2009). In the present work, the estimated activities of both peroxidase and polyphynoloxidase enzymes in specimens sampled from plants pretreated with the tested bioagent revealed significant increasing in both enzyme compared with the untreated plants. Many investigators reported that peroxidase is known to be involved in the oxidation of polymerization of hydroxycinnamyl alcohols to yield lignin and cross-linking isodityrosine bridges in cell wall. Ride (1983) and Tarrad *et al.* (1993) stated that increase in peroxidase activity enhances lignifications in response to infection with pathogens which may restrict fungal penetration. Peroxidase also produces free radicals and hydrogen peroxide which are toxic to many microorganisms (Vance *et al*., 1980; Peng and Kuc, 1992). The most reviewed indicators for induced resistance in plants are peroxidase and polyphynoloxidase enzymes (Li *et al*., 1991). Lignin biosynthesis is mediated by the peroxidase-H_2O_2 system. Cell wall-bound peroxidase is probably involved in the generation of hydrogen peroxide, which in return is necessary for lignification (Goldberg *et al*., 1987). In addition, peroxidase generating hydrogen peroxide may function as an antifungal agent in disease resistance. Hydrogen peroxide inhibits pathogens directly and or it may generate other active free radicals that are antimicrobial (Peng and Kuc, 1992). On the other hand, polyphenoloxidases have been described to play an important role in physiological functions in plant growth and in plant defense against pathogens (Li and Steffens, 2002). The active quinines produced by polyphenoloxidase may possess direct antibiotic and cytotoxic activities against pathogens (Mayer and Harel, 1979; Peter, 1989). In addition, polyphenoloxidase is also involved in the lignification of plant cells that contributes to the formation of defense barriers against pathogens (Nicholson and Hammerschmidt, 1992). Polyphenoloxidase activity correlates with resistance to downy mildews *P. cubensis* in cucumber (Li *et al*., 1991).

CONCLUSION

From the foregoing results, it can be concluded that the obtained results of this study suggest that metabolites (antibiotics) *B. subtilis* proved to be an effective under the optimum growth conditions in controlling the tested pathogenic fungi (*P. cubensis* and *S. fuliginea*) and could be considered an alternative to existing chemical products and hence it can reduce the environmental pollution resulting of using fungicide in controlling plant disease. Antibiotics of *B. subtilis* have also a wide spectrum efficacy against positive gram bacteria such as *Staphylococcus aureus* by recording inhibition zone.

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