View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Repository of the Academy's Library

Acta Biologica Hungarica 66(4), pp. 436–448 (2015)
DOI: 10.1556/018.66.2015.4.8

CHITINASE PRODUCTION BY *BACILLUS SUBTILIS* ATCC 11774 AND ITS EFFECT ON BIOCONTROL OF *RHIZOCTONIA* DISEASES OF POTATO

Wesam I. A. Saber,[1] Khalid M. Ghoneem,[2] Abdulaziz A. Al-Askar,[3] Younes M. Rashad,[4,5]* Abeer A. Ali[6] and Ehsan M. Rashad[1]

[1]Microbial Activity Unit, Microbiology Department, Soils, Water and Environment Research Institute, Agricultural Research Center, Giza, Egypt
[2]Seed Pathology Research Department, Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt
[3]Botany and Microbiology Department, Faculty of Science, King Saud University, Riyadh, Saudi Arabia
[4]Science Department, Teachers College, King Saud University, Riyadh, Saudi Arabia
[5]Plant Protection and Biomolecular Diagnosis Department, Arid Lands Cultivation Research Institute, City of Scientific Research and Technology Applications, Alexandria, Egypt
[6]Mycology and Plant Diseases Survey Department, Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt

(Received: January 7, 2015; accepted: April 2, 2015)

Stem canker and black scurf of potato, caused by *Rhizoctonia solani*, can be serious diseases causing an economically significant damage. Biocontrol activity of *Bacillus subtilis* ATCC 11774 against the *Rhizoctonia* diseases of potato was investigated in this study. Chitinase enzyme was optimally produced by *B. subtilis* under batch fermentation conditions similar to those of the potato-growing soil. The maximum chitinase was obtained at initial pH 8 and 30 °C. *In vitro*, the lytic action of the *B. subtilis* chitinase was detected releasing 355 µg GlcNAc ml^{-1} from the cell wall extract of *R. solani* and suggesting the presence of various chitinase enzymes in the bacterial filtrate. In dual culture test, the antagonistic behavior of *B. subtilis* resulted in the inhibition of the radial growth of *R. solani* by 48.1% after 4 days. Moreover, the extracted *B. subtilis* chitinase reduced the growth of *R. solani* by 42.3% when incorporated with the PDA plates. Under greenhouse conditions, application of a bacterial suspension of *B. subtilis* at 10^9 cell mL^{-1} significantly reduced the disease incidence of stem canker and black scurf to 22.3 and 30%, respectively. In addition, it significantly improved some biochemical parameters, growth and tubers yield. Our findings indicate two points; firstly, *B. subtilis* possesses a good biocontrol activity against *Rhizoctonia* diseases of potato, secondly, the harmonization and suitability of the soil conditions to the growth and activity of *B. subtilis* guaranteed a high controlling capacity against the target pathogen.

Keywords: Antagonism – antifungal – biological control – black scurf – stem canker disease

INTRODUCTION

Chitin is being present in most fungal cell walls, representing the principal structural component of most biological systems. It consists of a β-1,4-linked homopolymers of N-acetylglucosamine (GlcNAc). This component and its derivatives are of interest because they have varied biological functions to the organisms [7].

*Corresponding author; e-mail address: younesrashad@yahoo.com

Chitinases, which are responsible for chitin degradation, have been divided into two main categories. The first is endochitinases (E.C. 3.2.1.14), which randomly split chitin at internal sites, thereby forming the dimer diacetylchitobiose and soluble low molecular mass multimers of GlcNAc such as chitotriose, and chitotetraose. The second is exochitinases, which further divided into two subcategories, i.e. 1) chitobiosidases (E.C. 3.2.1.29) that are involved in catalyzing the progressive release of diacetylchitobiose starting at the non-reducing end of the chitin microfibril, and 2) 1-4-β-glucosaminidases (E.C. 3.2.1.30) that cleaving the oligomeric products of endochitinases and chitobiosidases, thereby generating monomers of GlcNAc [7, 21]. Chitinases are considered important in the biological control because of their ability to be induced by- and degrade the chitin of the fungal cell wall [2], to yield GlcNAc that is consumed by the bioagent for growth. Among several microbes, certain strains of *Bacillus* spp. have been shown to secret highest amount of chitinase [7]. Their secondary metabolites have antimicrobial activity against phytopathogens, also acting as plant growth-promoting substances, as well as promoting the nutrients uptake and self-defense of the plants [2].

Potato is a staple crop in 130 countries worldwide, ranking fourth in production after rice, maize and wheat. In Egypt, the area under potato cultivation in 2013 was 178,000 hectare yielding 4.8 million tons of tubers. It is important for local consumption and exportation, representing approximately 5.9% of agricultural exports [3]. Stem canker and black scurf are a serious diseases commonly observed in most potato-producing areas of the world. The disease is caused by *Rhizoctonia solani* AG3 and AG4 (teleomorph *Thanatephorus cucumeris* [Frank] Donk). The fungus is capable to survive in soil as sclerotia and mycelium in plant debris for long periods, and environmental conditions of low soil temperature and high soil moisture [4, 18].

Chemical and cultural control of stem canker and black scurf that extensively studied in many potato-producing countries revealed that such techniques are not completely effective, and *Rhizoctonia* diseases have been remained a persistent problem [10]. Alternatively, biological control has increased considerably in the last few decades, to provide control of diseases that cannot or partly managed by other control strategies. Biological control of *Rhizoctonia* diseases using different microbial antagonists has been studied by many researchers on several host crops [1, 2, 12]. Particularly, *B. subtilis* has been shown to be effective for the control of many phytopathogens including *R. solani* [12, 13]. Brewer and Larkin [1] recorded that, among 28 tested potential biocontrol organisms, treatment with *B. subtilis* was most effective in reducing stem canker severity on potato (40–49% reduction). The antagonistic activity of *B. subtilis* may be attributed to the production of bioactive compounds and/or extracellular hydrolytic enzymes [2, 8, 11]. The main idea is to decrease the inoculum or the disease producing activity of a pathogen through one or more mechanisms [1]. Most of inoculants perform better under laboratory conditions, but fail to work efficiently when moved to the outdoor application. The reason is the different nature between both environmental conditions. Each environmental condition has its own microbial community. Therefore, it is important to choose a biocontrol agent that previously adapted or at least already have growth conditions similar to the environ-

ment, where it will be applied. Chitinase as important enzyme in various fields, including biological control, is suitable candidate to evaluate such hypothesis.

The aim of the present work was to a) optimally produce chitinase enzyme from *B. subtilis* under simulated soil conditions, b) detect the lytic action of the *B. subtilis* chitinase, c) investigate the biocontrol activity of *B. subtilis* and its chitinase against *R. solani in vitro*, d) evaluate the efficacy of application of bacterial cell suspension of *B. subtilis* for control of stem canker and black scurf diseases of potato under greenhouse conditions, and e) investigate some biochemical, growth and yield parameters that may be affected as a response of the treatment with the biocontrol agent.

MATERIALS AND METHODS

Bacterial, fungal and plant material

B. subtilis ATCC®11774™ was obtained from American Type Culture Collection (Illinois, USA). The stock culture of the bacterial strain was stored in 30% glycerol at –7 °C. Prior to each experiment, it was subcultured from the frozen stocks onto nutrient agar medium. *R. solani* AG3, isolated from severely infected potato tubers, was kindly supplied by the Vegetable Diseases Research Department, Plant Pathology Research Institute, Agricultural Research Center (Giza, Egypt). Potato tubers (40–50 mm in diameter) of cultivar Spunta (susceptible for black scurf and stem canker infection) were used in this study.

Batch fermentation for chitinase biosynthesis and its assay

Inoculum of *B. subtilis* was prepared by scraping 24 h aged cultures, using sterile distilled water. The final inoculum concentration was adjusted to 10^8 cfu ml^{-1}. The medium used for the production of chitinase, contained (gL^{-1}); chitin from crab shell (Sigma-Aldrich, MO, USA) (1.0), yeast extract (0.5), $(NH_4)_2SO_4$ (1.0), $MgSO_4 \cdot 7H_2O$ (0.3) and KH_2PO_4 (1.36). The incubation temperature and pH were investigated at various ranges. The general full factorial design was applied, where the first factor has 4 levels (20, 25, 30 and 35 °C) and the second factor has 5 levels (pH 6.5, 7.0, 7.5 8.0 and 8.5). Fifty ml portions of the fermentation medium were dispensed in 250 Erlenmeyer flasks and sterilized. The medium was inoculated with 1% cell suspension. After incubation for 3 days on an orbital shaker (100 rpm) the flasks were filtered and centrifuged at 8000 rpm for 15 min at 4 °C. The clear supernatants were collected as crude chitinase. Chitinase activity was determined spectrophotometrically according to the method of Miller [17]. Colloidal chitin was prepared by the method of Hsu and Lockwood [9].

Evaluation of biological activity of B. subtilis *against* R. solani

Degradation of R. solani *cell wall*

The fungal cell wall was prepared by growing *R. solani* on 50 ml potato dextrose broth (PDB) and incubation at 30 °C for 4 days under shaking condition (100 rpm). The mycelium was harvested by filtration and thoroughly washed with distilled water, then homogenized for 3 min in a tissue phosphate homogenizer. The homogenate was further subjected to ultrasonic disintegration in ice bath for 6 min in 12 cycles each of 30 s in MSE Soniprep 150 ultrasonic disintegrator (MSE Scientific Instruments, Sussex, UK). The pellet obtained by centrifugation at 5000 rpm for 25 min at 4 °C in a refrigerated centrifuge was washed four times with distilled water and dried. Equal portions of the obtained cell wall (100 mg), suspended in 10 ml of 0.05 M phosphate buffer (pH 7.0), were incubated with 10 ml (≈140 U) of the crude chitinase preparation, after incubation for different periods (2 to 12 h) at 30 °C with reciprocal shaking (60 strokes/min), the reaction mixture was centrifuged at 4000 rpm, then the released monomers was determined by the method of Miller [17].

Dual culture assay

In vitro antagonistic assay was performed according to the dual culture method on potato dextrose agar (PDA) medium (Difco, USA). A disk (5 mm) of one-day-old culture of *R. solani* was disposed at the center of Petri dishes and *B. subtilis* was streaked in a square form around the agar disk at 2 cm distance. The antagonistic activity of *B. subtilis* was estimated by the inhibition of the fungal growth in comparison to a solely cultivated fungus. The reduction in fungal growth was monitored by measuring the diameter in centimeter of the colony until 4 days at 30 °C and 16 h photophase.

Measuring antagonistic activity of B. subtilis *chitinase*

To determine the activity of chitinase against *R. solani*, 1.0 ml (≈14 U) of crude chitinase preparation sterilized using Millipore filter was mixed with 9 ml PDA before solidification. Plates were then inoculated at the center with 5 mm disk of *R. solani* and incubated at 30 °C for 4 days. The diameter of the fungus colonies were then determined and compared with control plates that did not contain chitinase.

Greenhouse experiment

The inoculum of *B. subtilis* was prepared on chitinase production medium. The obtained bacterial cells were re-suspended in sterile 0.85% NaCl and centrifuged at

5000 rpm for 25 min at 4 °C. The supernatant was discarded and the washed bacterial cells were re-suspended in sterile distilled water. The concentration of cells in the suspension was adjusted to 10^9 cell mL^{-1}. Inoculum of *R. solani* was prepared by culturing on PDA plate and incubation at (25 ± 2 °C) for 3 days. Mycelium plugs were transferred to sterilized medium of sorghum : coarse sand : water (2 : 1 : 2, v/v) and incubated at room temperature for 10 days.

Plastic bags (50 cm in diameter) were filled with 10 kg sterilized soil, clay : sand (2 : 1, v/v). Healthy potato tubers were surface sterilized in 1% sodium hypochloride, and then washed several times with sterilized water. The chemical treatment was carried out by soaking in Rizolex-T (50% of tolclofos methyl-thiram, Sumitomo Chemical Co., Japan) at 3 g kg^{-1} tuber for 2 h and then dried using sterilized paper. Some bags were singly infested with the previously prepared pathogen inoculum at the rate of 0.4% (w/w). The soil was mixed thoroughly with *R. solani* and regularly watered to near field capacity with sterilized water and left for one week to ensure even distribution of the pathogen. *B. subtilis* was applied at planting by adding 200 ml of the bacterial suspension as soil drench. One week after inoculation with *R. solani,* two tubers were planted in each bag. Bags were regularly watered to near field capacity with tap water. Three treatments were applied; Rhizolex + *R. solani*, *R. solani* and *R. solani* + *B. subtilis*. Another set of bags of disinfected soil and untreated tubers was used as a control. The experiment was arranged in a completely randomized block design. Nine replicates were included per treatment. Plants were grown in a greenhouse for 100 days at temperature ranged between 20–30 °C, soil pH was 7.9.

After six weeks from planting, three plants from each treatment were selected, washed and air dried to evaluate the severity of lesions developed on the infected plants, based on scale described by Brewer and Larkin [1]. When two or more shoots emerged from one seed piece, the average rating of all shoots was recorded. Incidence of stem canker was measured as the percentage of plants with a severity rating of two or greater. After harvest, newly formed sclerotia were visible on the seed pieces, therefore, black scurf was assessed according to Brewer and Larkin [1]. Incidence of black scurf was calculated as the percentage of tubers with a severity rating of one or greater. After 40 days from sowing, the total phenol content in potato plant was determined according to Malik and Singh [15], as well as assay of polyphenoloxidase [16] and peroxidase [6]. After 70 days of sowing, photosynthetic pigments were measured according to Mackinney [14]. The growth was evaluated in terms of height and fresh and dry weight of shoot, number of leaves and stolons. At harvest, number and weight of tubers were recorded.

Statistical analysis

The design and analysis of fermentation conditions (temperature and pH) of *B. subtilis* growth medium were carried out based on the general full factorial design using Minitab software (version 17). The data of the greenhouse experiment were analyzed

using CoStat software (version 6.4). The analysis of variance was applied and comparison among different means was carried out according to Duncan multiple range test, at probability (*P*) level ≤0.01 or 0.05.

RESULTS

Optimization of temperature and pH for chitinase biosynthesis

The environmental conditions (temperature and pH) for *B. subtilis* chitinase biosynthesis were investigated using the full factorial design (Fig. 1). The maximum chitinase secretion was obtained at an initial pH 8. This means that the acidic conditions affect negatively more than the alkaline one on the biosynthesis of this enzyme. Anyhow, any increase or decrease in the pH 8 resulted in variable reduction in enzyme productivity. Within this pH, the optimum incubation temperature for chitinase secretion was observed at 30 °C. Chitinase production reached 13.9 U at pH 8 and incubation temperature of 30 °C as the optimum environmental fermentation conditions. Out of these optimum points chitinase biosynthesis was reduced, but the bacterium was still able to secret the enzyme, since there was significant activity of chitinase within all the tested ranges of initial pH (6.5–8.5) and incubation temperature (20–35 °C).

Based on the analysis of variance, the contribution and significance of both tested factors are summarized in Table 1. The contribution of the overall model reached 100% indicating the aptness of this model for chitinase biosynthesis. The temperature is responsible for 31.5%, whereas the initial culture pH is sharing by 62.6%. This indicates that both factors are important since they individually recorded significant effect in the production process. However, the initial pH is more important than the temperature. The interaction of both tested variables recorded lower contribution, being 5.9%, in spite of the low contribution of the interaction; it also, reached to the level of significance. This means that both factors as well as their interaction are principals and important during the biosynthesis of such enzyme as indicated by the high F-value and very low *P*-value.

Table 1

Statistical contribution and significance of incubation temperature and initial culture pH on chitinase biosynthesis by *Bacillus subtilis*

Statistical parameter		The overall model	The individual linear model		The interaction model (temperature × initial pH)
			temperature	initial pH	
Contribution (%)		100	31.5	62.6	5.9
Significance	F-value	751.3	1655.9	2469.3	77.6
	P-value	0.00*	0.00*	0.00*	0.00*

Presented values are based on analysis of variance.
*Significant at $P \leq 0.01$.

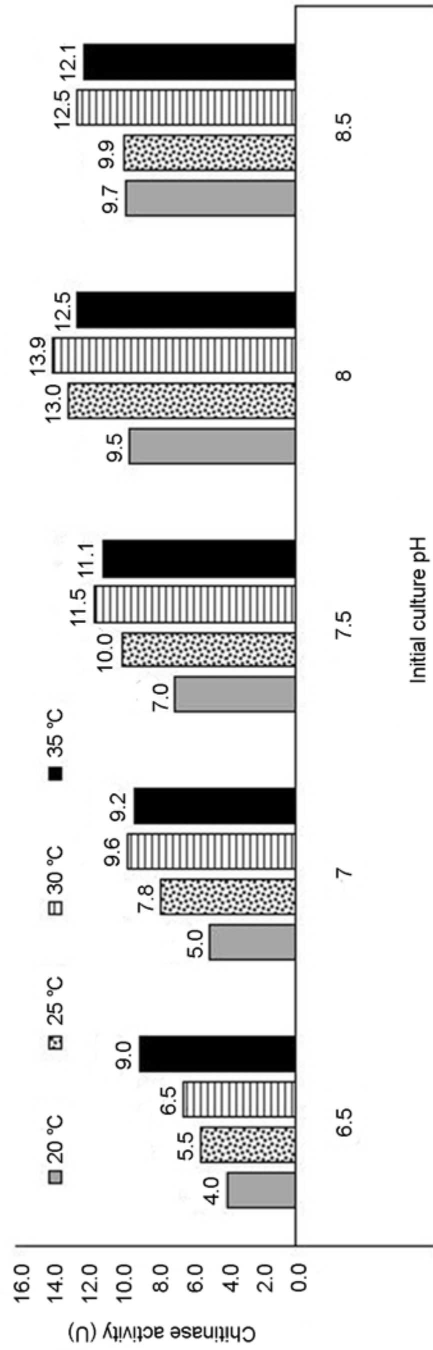

Fig. 1. The interaction influence of initial culture pH and incubation temperature on chitinase biosynthesis by *Bacillus subtilis*

Bioactivity of B. subtilis *and its chitinase against* R. solani

Hydrolysis of cell wall of *R. solani* was markedly observed over the incubation period (2–12 h) (Fig. 2). *B. subtilis* chitinase as a lytic enzyme exerted steadily catalytic activity against the isolated cell wall as a chitin containing substance, reaching its maximum at the end of incubation period (12 h), releasing 355 μg GlcNAc ml^{-1} from the cell wall isolated from *R. solani*. There was strong linear positive relationship, represented by simple correlation (r = 0.98) and coefficient of determination (R^2 = 0.97), between the reaction time and degree of cell wall hydrolysis.

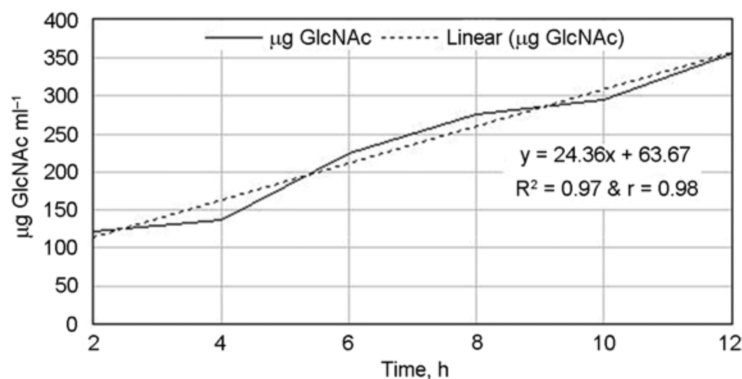

Fig. 2. Lytic activity of *Bacillus subtilis* chitinase on *Rhizoctonia solani* cell wall during 12-hour incubation period

Dual culturing test between *R. solani* and *B. subtilis*, and the antagonism test between *R. solani* and the bacterial chitinase itself were applied. Both assays showed marked retardation of fungal growth. However, the dual culture test was more effective than the addition of chitinase preparation to PDA growth medium, and this reduction was more pronounced with prolongation of time. The dual culture assay effectively reduced the growth of *R. solani* by 20.9 and 48.1% after 2 and 4 days, respectively, compared with 19.5 and 42.3% reduction with the bacterial chitinase alone. However, chitinase is still responsible for the majority of the reduction in growth of *R. solani*, and only 5.8% of the reduction back to factors other than chitinase.

Greenhouse evaluation of B. subtilis *as a bioagent*

Data obtained from greenhouse experiment (Table 2) show that, treatment with *B. subtilis* significantly reduced the disease incidence and severity of stem canker (22.3 and 1%, respectively) and black scurf diseases (39.9, 1%, respectively) compared with treatment of *R. solani* alone. In spite of the fact that the treatment of the infected plants with *B. subtilis* significantly lowered both diseases incidence and

Table 2
Influence of the treatment with *Bacillus subtilis* strain on the development of stem canker and black scurf of potato under greenhouse conditions

Treatments	Stem canker (%)		Black scurf severity (%)	
	Incidence	Severity	Incidence	Severity
Control	0.0d	0.00d	0.0d	0.00d
Rhizolex + *R. solani*	15.0c	0.67c	25.2c	0.47c
R. solani	100.0a	3.00a	83.7a	3.40a
R. solani + *B. subtilis*	22.3b	1.00d	39.9b	1.00b

Means within each column followed by different letter significantly differ ($P \leq 0.05$) according to Duncan multiple range test.

severity, treatment with the chemical fungicide was most significantly effective in diseases reduction (Table 2). The severity of stem canker and black scurf reduced to 0.67 and 0.47%, respectively, in case of the treatment with Rhizolex+*R. solani* compared with 3 and 3.4% in the case of infection by *R. solani* alone.

Infection with *R. solani* excreted stress on potato plants and led to accumulation of some defense related bioactive compounds. Data presented in Table 3 showed that contents of total phenol, polyphenoloxidase and peroxidase were significantly induced by the application of *R. solani*+*B. subtilis*, being 1.574 g^{-1} fresh wt., 2.167 U and 1.133 U, respectively, comparing with the other treatments. On the other hand,

Table 3
Biochemical activities and growth characteristics of potato as affected by the inoculation by *Bacillus subtilis* strain under greenhouse conditions

Parameter		Control	Rhizolex + *R. solani*	*R. solani*	*R. solani* + *B. subtilis*
Bioactive compound (g^{-1} fresh wt.)	Total phenol (mg catechol)	0.514c	0.779b	0.618c	1.574a
	Polyphenoloxidase (Unit min^{-1})	1.233b	1.300b	0.883b	2.167a
	Peroxidase (Unit min^{-1})	0.800b	0.900b	0.433c	1.133a
Photosynthetic pigment (mg g^{-1} fresh wt.)	Chlorophyll a	1.450a	1.248b	0.919d	1.082c
	Chlorophyll b	0.738a	0.589ab	0.324c	0.466bc
	Total chlorophyll	2.188a	1.887b	1.243d	1.515c
	Carotenoids	0.094b	0.099b	0.291a	0.145b
Potato growth ($plant^{-1}$)	Shoot height (cm)	36.0a	33.0b	22.0d	31.0c
	Shoot fresh weight (g)	47.0a	46.1a	33.5b	44.6a
	Shoot dry weight (g)	10.0a	9.1b	7.4c	9.0b
	No. of leaves	18.7a	17.0b	13.7c	16.7b
	No. of stolons	4.0a	3.0ab	1.7b	3.3a

Means within each row followed by different letter(s) significantly differ ($P \leq 0.05$).

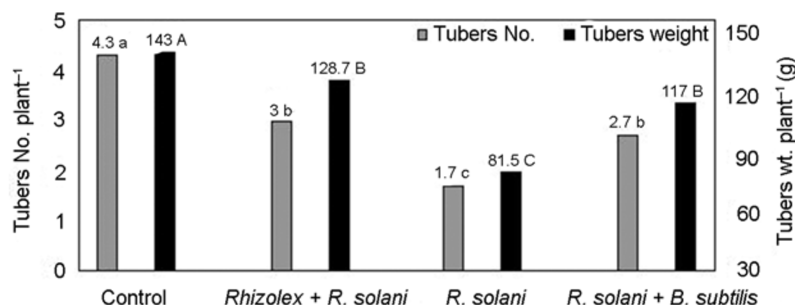

Fig. 3. Effect of treatment with *Bacillus subtilis* on tubers number and weight of potato plants infected with *Rhizoctonia solani* under greenhouse conditions. Columns (of the same type) superscripted with the same letter are not significantly different at $P \leq 0.05$

all the photosynthetic pigments (chlorophylls a, b and total) significantly increased in the control plants over the other treated plants, carotenoids was the only exception, which reached the maximum (0.291 mg g^{-1} fresh wt.) in plants developed under stress of *R. solani* alone. In addition, the control treatment recorded the highest values of the all tested growth parameters of the potato compared with the infection with *R. solani* alone. On the other hand, inoculation with *B. subtilis*, even in the presence of *R. solani*, showed improvements in both shoot fresh weight (44.6 g $plant^{-1}$) and number of stolons (3.3), however, that later is critical criterion in the development of tubers in the late stage of plant life. Comparing to the control treatment, considerable yield losses were observed in infected plants, especially with the absence of *B. subtilis* inoculation (Fig. 3). However, treatment of the infected plants with *B. subtilis* led to a significant increase in the potato weight (117 g $plant^{-1}$) and number (2.7 $plant^{-1}$) in comparison with only infected treatment.

DISCUSSION

Temperature and pH are among the main factors that affect the microbial growth and activity in soil. So, it was decided to optimize and simulate these conditions in laboratory to ensure the ability of chitinlytic bacterium to work as a bioagent under similar soil conditions, where it will be applied. In this connection, our results indicated that the maximum chitinase secretion was obtained at an initial pH 8 and incubation temperature 30 °C. Such conditions represent suitable support for the growth and activity of the tested bacterium under Egyptian soil conditions during the growing season of potato (pH 7.9 and temperature range 15–30 °C). These findings are in agreement with that of Ghafil [5] who recorded the maximum specific activity of chitinase produced by *B. subtilis* (A3) at 30 °C and pH 8. Change in pH affects the ionization state of the amino acids in the enzyme leading to protein alteration and accordingly enzyme inactivation. On the other hand, change in the incubation temperature affects the protein nature of the enzyme leading to protein denaturation and so enzyme inactivation.

Bioactivity of B. subtilis *and its chitinase against* R. solani

Hydrolysis of cell wall of *R. solani* by chitinase extract reflects the antagonistic nature and the mode of action of the bioagent *B. subtilis*. The prolongation of chitinase over a long period of catalytic action reflects its resistance and stability in addition to the continual activity as long as the substrate is available. This is may be due to the high specificity and affinity between the enzyme and the substrate (cell wall). This means that this enzyme preparation could effectively be used in suppression of the cell wall formation of *R. solani*, so considered an effective tool for biological control of many plant pathogens, as well. The cell wall acts as protective agent to the fungus, one possible explanation for the inhibition of fungal growth is the action of chitinase on chitin present in the cell wall [2]. The hydrolysis of cell wall and release of GlcNAc units from the reaction mixture of bacterial chitinase and fungal call wall may be explained based on the occurrence of complementary chitinase system in the bacterial preparation [21].

The superiority of dual culture over chitinase alone in reducing the growth of *R. solani* may be back to the presence of other mechanisms, such as the competition between the bacterium and the fungus for nutrients and space. Additionally, the presence of *B. subtilis* guarantees continuous supplementation of various bioactive materials in the growth medium, which restrict the fungal growth. These results are in consistence with that of Kumar et al. [12]. In general, the hydrolysis of fungal cell wall, affecting greatly on fungal growth. Several strains of the genus *Bacillus* have been found to show antibacterial or antifungal activity against different phytopathogens, beside the fast growth and forming resistant spores with high thermal tolerance to overcome the adverse conditions [13, 21]. All these properties make this bacterium suitable candidate as a biocontrol agent. This enzyme may play an important role in the defence against the parasitic *R. solani* on higher plants, which was evaluated in next trial.

Greenhouse evaluation of B. subtilis *as a bioagent*

Our findings on the control of *Rhizoctonia* diseases of potato under greenhouse conditions by *B. subtilis* comply with that reported by Jeger et al. [10]. *Bacillus* spp. produce extracellular chitinase and other enzymes as well as ribosomally synthesized peptides, and other unusual antibiotic peptides such as the rhizocticin and L-amino acid ligase, which led to the remarked reduction in the development of the disease [11]. The extracellular chitinase degrades the cell wall, due to their physical interactions that suppressing the growth of soil borne plant pathogens [13]. This mode of action may be the proposed mechanism in the present study. Another proposed mechanism by *Bacillus* strains is the advantage of being able to form endospores (that confer the bacterial strain high stability as a bio-fungicide or bio-fertilizer) and antibiotics that manage the stem canker and black scurf diseases of potato [12].

Induction of some defense related bioactive compounds in the plant (total phenol, polyphenoloxidase and peroxidase) by *B. subtilis* forms another aspect of disease control. Plant phenolics and their oxidation products such as quinones are highly toxic to invading fungi. The rapid accumulation of phenols at the infection site is prerequisite for plant defense mechanism that prevents the pathogen from being developed; they act as antioxidant, antimicrobial, and photoreceptor [20]. Polyphenoloxidase enzyme is involved in the oxidation of polyphenols into quinones (antimicrobial compounds) and lignification of plant cells during microbial invasion, and also may participate in the responding defense reaction and hypersensitivity by inducing plant resistance against fungi. Peroxidase is a component of an early response of plants to pathogen infection and plays a major role in the biosynthesis and polymerization of proteins and lignin or suberin cell wall constructing a physical barrier that could prevent pathogen penetration of cell walls or movement through vessels [19, 20]. On the other hand, variation in photosynthetic pigments may occur by enhancing the efficacy of photosynthetic apparatus with a better potential for disease resistance and decrease in photophosphorylation rate usually occurring after infection [19]. It could be said that the presence of *B. subtilis* alongside *R. solani* reduced its harmful impact on plants. These results are in consistence with that of Kumar et al. [12] obtained under greenhouse conditions and in the field. However, the disease severity is not always associated with yield reduction, but formation of tuber-borne sclerotia downgrades tuber quality with the development of malformation and size alteration of the target tubers [10].

In conclusion, chitinase was secreted by *B subtilis* under conditions similar to those present in the soil. Therefore, the bacterium is already adapted to work under such soil conditions. That is why *B. subtilis* and its chitinase demonstrated antifungal activity against *R. solani* in laboratory, as well as exhibited reduction in stem canker and black scurf diseases under greenhouse conditions. Based on the test of our hypothesis, at selection of a bioagent, it is important to emphasize on the similarity between the growth nature in laboratory and at site of application. This may guarantee the success of the bioagent in the greenhouse application.

ACKNOWLEDGEMENT

The authors would like to express their sincere appreciation to the Deanship of Scientific Research at King Saud University for the funding of this research through the Research Group Project No. RGP-VPP-327.

REFERENCES

1. Brewer, M. T., Larkin, R. P. (2005) Efficacy of several potential biocontrol organisms against *Rhizoctonia solani* on potato. *Crop Prot. 24*, 939–950.
2. Compant, S., Duffy, B., Nowak, J., Clement, C., Barka, E. A. (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol. 71,* 4951–4959.

3. FAOSTAT (2014) FAOSTAT databases. (http://faostat3.fao.org)
4. Fox, R. (2006) *Rhizoctonia* stem and stolon canker of potato. *Mycologist 20,* 116–117.
5. Ghafil, J. A. (2013) Extraction and purification of chitinase from *Bacillus subtilis*. *World J. Exp. Biosci. 1,* 5–9.
6. Galeazzi, M. A. M., Sgarbieri, V. C., Constantides, S. M. (1981) Isolation, purification and physico-chemical of polyphenol oxidase (PPO) from a dwarf variety of banana. *J. Food Sci. 46,* 150–155.
7. Hamid, R., Khan, M. A., Ahmad, M., Ahmad, M. M., Abdin, M. Z., Musarrat, J., Javed, S. (2013) Chitinases: An update. *J. Pharm. Bioall. Sci. 5,* 21–29.
8. Hiraoka, H., Asaka, O., Ano, T., Shoda, M. (1992) Characteristics of *Bacillus subtilis* RB14, coproducer of peptide antibiotics iturin A and surfactin. *J. Gen. Appl. Microbiol. 38,* 635–640.
9. Hsu, S. C., Lockwood, J. L. (1975) Powdered chitin agar as a selective medium for enumeration of Actinomycetes in water and soil. *Appl. Microbiol. 29,* 422–426.
10. Jeger, M. J., Hide, G. A., Van Den Boogert, P. H. J. F., Termorshuizen, A. J., Van Baarlen, P. (1996) Pathology and control of soil-borne fungal pathogens of potato. *Potato Res. 39,* 437–469.
11. Kino, K., Kotanaka, Y., Arai, T., Yagasaki, M. (2009) A novel L-amino acid ligase from *Bacillus subtilis* NBRC3134, a microorganism producing peptide-antibiotic rhizocticin. *Biosci. Biotech. Bioch. 73,* 901–907.
12. Kumar, S. S., Rao, R. K. M., Kumar, R. D., Sachin, P., Prasad, C. S. (2013) Biocontrol by plant growth promoting rhizobacteria against black scurf and stem canker disease of potato caused by *Rhizoctonia solani*. *Arch. Phytopathol. Plant Protec. 46,* 487–502.
13. Kumsingkaew, S., Akarapisan, A. (2014) Efficiency of *Bacillus subtilis* EPB14 as biocontrol to control bacterial leaf blight of anthurium. *J. Agric. Technol. 10,* 755–766.
14. Mackinney, G. (1941) Absorbtion of light by chlorophyll solution. *J. Bio. Chem. 140,* 315–322.
15. Malik, C. P., Singh, M. B. (1980) Estimation of total phenols in plant enzymology and histoenzymology. In: Malik, C. P., Singh, M. B. (eds) *Plant enzymology and histoenzymology: A text manual.* Kalyani Publishers. New Delhi.
16. Maxwell, D. P., Bateman, D. F. (1967) Changes in the activities of some oxidases in extracts of *Rhizoctonia*-infected bean hypocotyl in relation to lesion maturation. *Phytopathol. 57,* 132.
17. Miller, G. L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem. 31,* 426–428.
18. Rashad, Y. M., Abdel-Fattah, G. M., Hafez, E. E., El-Haddad, S. A. (2012) Diversity among some Egyptian isolates of *Rhizoctonia solani* based on anastomosis grouping, molecular identification and virulence on common bean. *Afr. J. Microbiol. Res. 6,* 6661–6667.
19. Saber, W. I. A., Abd El-Hai, K. M., Ghoneem, K. M. (2009) Synergistic effect of *Trichoderma* and *Rhizobium* on both biocontrol of chocolate spot disease and induction of nodulation, physiological activities and productivity of *Vicia faba*. *Res. J. Microbiol. 4,* 286–300.
20. Selvaraj, T., Ambalavanan, S. (2013) Induction of defense-related enzymes in anthurium by application of fungal and bacterial biocontrol agents against *Colletotrichum gloeosporioides*. *Int. J. Curr. Microbiol. App. Sci. 2,* 661–670.
21. Thiagarajan, V., Revathi, R., Aparanjini, K., Sivamani, P., Girilal, M., Priya, C. S., Kalaichelvan, P. T. (2011) Extra cellular chitinase production by *Streptomyces* sp. PTK19 in submerged fermentation and its lytic activity on *Fusarium oxysporum* PTK2 cell wall. *Int. J. Curr. Sci. 1,* 30–44.