J. Basic. Appl. Sci. Res., 3(1)670-682, 2013
© 2013, TextRoad Publication
ISSN 2090-4304
Journal of Basic and Applied Scientific Research
www.textroad.com

Extracellular Metabolites Produced by a Novel Strain, *Bacillus alvei* NRC-14: 5. Multiple Plant-Growth Promoting Properties

Shadia M. Abdel-Aziz

Microbial Chemistry Dept., Genetic Engineering and Biotechnology Division, National Research Center, Dokki, Cairo, Egypt

ABSTRACT

Plant growth promoting rhizobacteria (PGPR) are known to enhance plant growth and healthy by direct or indirect mechanisms. Plant healthy could be increased by controlling a range of plant pathogens, including bacteria, fungi, and nematodes. The use of PGPR, recently named plant-probiotics, to control plant-pathogens is receiving increasing attention, as they may represent an alternative approach to chemical pesticides. Tomato root rot, caused by *Fusarium oxysporum*, is an economically important disease. In the present study, the strain *Bacillus alvei* NRC-14 potentially suppressed fungal growth and prevented root-rot of tomato plants caused by *Fusarium oxysporum* and enhanced plant growth and healthy. Both *in vitro* and *in vivo* experiments confirmed the efficacy of this strain as an excellent biocontrol agent. When it is applied as soil drench, it significantly reduced wilt incidence by 94% with a plant-growth promotion and biocontrol efficiency of 180 and 151%, respectively. In general, application of the strain or its extracellular metabolites increased plant growth parameters. The strain produced mycolytic enzymes *viz*. chitinase, chitosanase, ß-1,3glucanase as well as cellulaces, proteases and potential bioactive compound(s). These results suggest that the strain may has potential to be considered as a potent biocontrol agent, effective against several plant diseases, pest insects, and plant parasitic nematodes due to its antagonistic characteristics and multiple plant-growth promoting properties.

KEYWORDS: lytic enzymes, *Bacillus*, plant pathogens, plant probiotic, *Fusarium oxysporum*, nematodes.

INTRODUCTION

The root surface and close rhizosphere are habitats for microorganisms, where microbial activity is maximal due to several modification of soil environment such as release of bioactive compounds and emergence of elicitors, soil aggregate formation, roots respiration, *etc*. In fact, rhizospheric plant-growth-promoting prokaryotes and eukaryotes (now called: plant-probiotic microorganisms) do positively and directly enhance plant growth through several mechanisms, such as biological nitrogen fixation, solubilization of organic nitrogen, phosphorous, iron and oligoelements, synthesis of plant hormones and regulators (Picard and Bosco, 2008). The term biological control has been used in different fields of biology as well as in plant pathology. The organism (or its natural extracts) that suppresses pests or plant pathogen is referred to as a biocontrol agent. Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria which have the ability to colonize the roots and promote plant growth. Beneficial microorganisms can: 1) produce antimicrobial compounds which can inhibit the action of phytopathogens, pests, and nematodes; 2) induce systemic resistance (ISR) by triggering the defense mechanism of the plant; and 3) compete with pathogens for nutrients and for occupying of niches on the root surface.

Strains with PGPR activity, belonging to genera *Azoarcus*, *Azospirillum*, *Azotobacter*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Gluconacetobacter*, *Pseudomonas*, *Serratia*, and *Trichoderma*, have been reported (Hurek and Reinhold-Hurek 2003). These bacteria competitively colonize the roots of plant and can act as bio-fertilizers (i.e. increasing mineral and nutrient supply to the plants); as biocontrol (bio-pesticides, i.e. protect plants from phytopathogens, insects, and weeds) or simultaneously both (Ramyasmruthi *et al*. 2012). In such mechanisms, plant root colonization is crucial as the delivery system.

Many species of *Bacillus* are known to promote plant growth and contribute to crop productivity directly or indirectly. The main features of bacilli as attractive PGPR are their wide prevalence, the capacity for rapid growth, formation of heat- and desiccation- resistance endospore, relatively safety for humans and animals, as well as the production of a broad spectrum of biologically active compounds (Aktuganov*et al*. 2007).

***Corresponding Author:** Shadia M. Abdel-Aziz, Microbial Chemistry Dept., Genetic Engineering and Biotechnology Division, National Research Center, Dokki, Cairo, Egypt

Bacillus species express antagonistic activities by suppressing the pathogens and enhance plant growth by root-colonizing, and numerous reports both under *in vitro* and *in vivo* conditions are available (Arrebola *et al.*2010; Chen *et al*. 2009). Induced systemic resistance in plants, a process by which PGPR stimulates the defense mechanisms of host plants without causing harm to the host, are extensively studied. Induction of ISR in plant has been reviewed by *Bacillus* spp., for protection of plants such as tomato and soy bean. Various species of bacilli such as *B.brevis, B. amyloliquefaciens, B.subtilis, B.pasteurii, B.cereus, B.pumilus, B.mycoides, B.sphaericus,* and *B.coagulans,* are known as potential elicitors of ISR and exhibit significant reduction in the incidence of various diseases (Choudhary and Johri 2009, Kloepper *et al*. 2004, Wang *et al.* 2012). In the present study, *Bacillus alvei* NRC-14 is introduced for the first time as a potent PGPR in biological control against phytopathogenic fungi. This strain has never been reported previously in literatures despite of its: 1) efficiency for production of carbohydrate-active enzymes using a minimal medium; 2) capability for production of bioactive compounds; 3) capability for adaptation with various abiotic stress conditions as well as secretion of a variety of extracellular metabolites and lytic enzymes.

Therefore, the objectives of this study were: 1) to optimize culture conditions for production of the metabolite(s) by the strain; 2) to study some properties of these metabolite(s); and 3) to evaluate the antifungal activity of the strain against some phytopathogens in *in vitro* with special reference to *F. oxysporum* under the *in vivo* system.

MATERIALS AND METHODS

Growth conditions and enzymes extract

Bacillus alvei NRC-14, used in this study, was isolated from soil and identified as described previously (Abdel-Aziz *et al*. 1999), maintained on nutrient agar slants, and kept at 4^{o}C. This strain was found to produce a variety of mycolytic enzymes and bioactive compound(s) and was thought to be introduced as an antagonist and efficient biocontrol agent because it is confirmed to possess a potential antifungal effect (Abdel-Aziz *et al*. 2012 *a, b).* For preparation of enzyme extract, cells of the fungus *Aspergillus niger* were used as carbon source. A pre-culture (24 hrs old) of the strain was grown with fungal spores and incubated at 28^{o}C (130 rpm) for 48 hrs, after which cell free culture supernatant was obtained by centrifugation of culture broth at 3000 x g for15 min. The activity of enzymes in the crude supernatant was measured using dinitrosalicylic acid solution (Miller 1956). Total protein in the crude supernatant was detected by the method of Lowry *et al*. (1951) with bovine serum albumin as the protein standard.

Estimation of enzymes

Activity of ß-1,3glucanase was estimated by the method of Pan *et al.* (1991) using laminarin as substrate. The enzyme activity was defined as micromoles equivalents of glucose released /ml of culture/min. Activity of cellulase and ß-1,4glucanase was estimated using carboxymethyl cellulose as the substrate (Diby *et al.* 2005). Chitinase and chitosanase activity was determined as described previous (Abdel-Aziz *et al*. 2012 *a*). Protease activity was estimated as reported previously (Abdel-Aziz *et al*. 2004).

In vitro assessment for antagonism

Antagonistic activity, *in vitro,* was determined using conical flask (250-ml) containing potato dextrose broth as described previously (Hamed *et al*. 2011). Control flasks not inoculated with bacteria were also prepared. Each treatment was performed twice. Percentage of fungal growth inhibition was calculated as follows:

Inhibition % = [{fungal growth in control-fungal growth with the antagonist}/fungal growth in control] x100.

Biocontrol efficacy against nematodes

Efficacy of whole cells as well as the culture metabolites produced by the strain in biocontrol of egg mass and larvae of nematode (*Meloidogyne incognita*) was detected as described by Sahebani and Hadavi (2008). In the present work, strain NRC-14 was grown on nutrient broth in which nematode egg or larvae was used as carbon source.

Thermal stability of bioactive metabolite(s)

Different temperatures were used in this assay to evaluate the thermal stability of the metabolites produced by the strain. Samples of the culture supernatant were incubated in a water-bath with temperatures varying from 50ºC to 100ºC for 30, 60 and 90 min. After incubation, 50µL of each

sample were poured into wells, previously prepared in plates with PDA medium containing fungal spore suspension of *F. oxysporum*. Culture supernatant without heat treatment was used as control. Plates were incubated at 28ºC for 5 days and observed for inhibition zones (Carissimi *et al*. 2009).

The pH stability of bioactive metabolite(s)

The pH-stability of bioactive metabolites was determined using different buffer solutions for pH 4-11. In glass tubes, a mixture with 4ml of each buffer, 0.9 mL of the culture metabolite, and 0.1mL of spore suspension (10^7/mL) was prepared and incubated at 28ºC for 5 days. The culture metabolite was replaced by sterile potato-dextrose broth in the control tubes. Samples were filtrated and dried biomass was obtained by drying at 100^{o}C upon constant weight (Carissimi *et al*. 2009).

Production of siderophore

Production of siderophore by strain NRC-14 was detected (Brian *et al.* 2011). Many bacteria utilize siderophores to help the process of ferric iron uptake in the environment. This process is necessary for many microorganisms to obtain the environmental iron needed for essential processes. Various assays have been developed to detect different phenotypes of siderophores (Brian *et al.* 2011). In presence of ferric iron, a blue color is formed and when a strong iron chelator such as a siderophore removes iron from the dye complex, the color changes from blue to orange (Brian *et al*. 2011).

Production of IAA

The Salkowski reagent (150 ml H_2SO_4, 250 ml distilled water, 7.5 ml (0.5 M) $FeCl_3.6H_2O$) was used to colorimetrically assay the production of IAA (Gordon and Weber 1951). The strain was tested for their quantified production of indole compounds by growing in flasks containing 10 ml of yeast extract mineral broth supplemented with 5 mM of L-tryptophan with agitation (150 rpm) at 28 ºC for 4 days. The cultures were centrifuged at 7000 x g, after which, 2 ml of the supernatant was mixed with 2 ml of the Salkowski reagent and incubated at room temperature for 30 min (Aris*et al.* 2011). Development of pink color indicates the presence of IAA and the concentration of IAA was measured spectroscopically at 520 nm (Gordon and Weber 1951). Non inoculated and untreated controls were kept for comparison. The experiment was independently performed twice with two replicates of the bacterial strain each time.

***In vivo* efficacy of extracellular metabolites**

Efficacy of whole cells as well as the culture metabolites in biocontrol was investigated. Preparation of tomato seedlings were carried out as described in the previous work (Hamed *et al.* 2011). In the present study, no treatment for tomato seeds was achieved. Five strains of fungal pathogens were used as test strains: *Fusarium oxysporum, Fusarium solani, Rhizoctonia solani, Schloretium rolfsii, and Pythium ultimum*. Preparation of pathogen cultures was performed as described previously (Hamed *et al*. 2011). In pot trials were performed where soil was treated using two sets of preparations: culture extract or a bacterial cell suspension. Cell suspensions for strain NRC-14 were adjusted to OD_{620} = 0.1, corresponding with a cell density of approximately 10^8 cells per ml (Algam *et al*. 2010). After removing of tomato seedings (45 days-old) to the pots, soil was infected with the fungal spores and mixed in the soil with the bacterial cell suspension or culture extract and allowed to be grown for another 45 days with water drench at intervals. All experiments were performed at least twice, and the best was chosen.

Plant growth promotion

Plant growth was measured in terms of shoot-height and –length after planting both in the absence and presence of strain NRC-14 and *F. oxysporum.* Reduction in wilt incidence was calculated as described by Song *et al*. (2004). The relative growth promotion efficacy (GPE) and biocontrol efficacy (BE) by the antagonistic bacterial strain were calculated (Algam *et al*. 2010) as follows:

GPE (%) = {(Plant parameter by antagonist- Plant parameter of control) / Plant □parameter of control} X100.

BE (%) = {(Disease incidence of control -Disease incidence of antagonist-treatment)□/ Disease incidence of control} X100.

RESULTS AND DISCUSSION

Effect of carbon source

Carbon is an important ingredient in the medium to determine the parameter of a product. Regarding the effect of different carbon sources on production of lytic enzymes and bioactive compound(s) by strainNRC-14, flaked chitosan and fungal cell wall were found to be the best carbon

sources for production of lytic enzymes and bioactive compound(s) that suppressing the growth of *F.oxysporum.* Enzyme activities reached a maximum with chitosan and fungal cell wall (FCW-1) as sole carbon sources (Table 1). Interestingly, other reports revealed that glucose was the best carbon source for *M. spinosa* to produce effective metabolites, whereas, fructose was found to be the best carbon sources for strain UPMKB4, and glycerol for *S. maltophilia* to suppress the growth of *F. oxysporum* and *C. gloeosporioides* (Siti *et al.*2011). Qureshi *et al.* (2001) and Fukuda *et al.* (2005) have reported that, glycerol was also found to be very important medium component for the production of antifungal antibiotics from microorganisms. On other hand, in the previous study (Abdel-Aziz *et al.* 2012 *a*), it was reported that a bioactive compound was synthesized by strain NRC-14 at 40^{o}C with chitin as a carbon source, while the culture broth was found to be completely free of enzymes. Surprisingly, in the present study, when the strain was grown at 40^{o}C using chitosan or fungal cell wall as carbon source, parallel formation of hydrolytic enzymes and bioactive compound(s) were detected in the culture broth. The time course for production of enzymes and bioactive compound(s) at 40^{o}C was estimated. As shown in Fig. 1(A and B) chitosan and fungal cell wall were the best carbon sources for production of both lytic enzymes and bioactive compound, whereas glucose was a poor carbon source for the production of bioactive compound (Fig. 1,B). It could be concluded that secondary metabolite production from strain NRC-14 was often stimulated by slowly assimilation of complex carbohydrates from the productive media and is decreased when more rapidly utilized monosaccharides such as glucose are present as sole carbon source (Bertasso *et al*., 2001). Worthy mention is that, high levels of cellulase were detected when FCW-2 (*P. ultimum*) was used as a carbon source (Table 1); these species contains, mainly, cellulose as a major component in their cell walls. The specific activity of chitinase, chitosanase, and ß-1,3glucanase reached 0.97, 5.3, and 2.8 U/mg protein, respectively (data not shown). Many factors may represent an important role in the process of antifungal compound production and consequently affect the antagonistic activity of the bacterial species. Carbon compounds constitute the major requirement for growth as they enter in different metabolic process resulting in the production of primary and secondary metabolites including antifungal substances (Siti *et al.* 2011). When strain NRC-14 was grown with different carbon sources, maximum activities of lytic enzymes and bioactive compounds were detected before 48 hrs of growth. The strain produces a variety of fungal cell wall-degrading enzymes, such as chitinases, chitosanases, ß-1,3glucanases and protease when grown on fungal mycelium as a carbon source. Strain *Bacillus alvei* NRC-14, as a soil isolate, was found to produce lytic enzymes (Abdel-Aziz *et al.* 2012*a*), polysaccharide bioflocculant (Abdel-Aziz *et al.* 2011), and bioactive compound(s) efficient for the biological control of fungi (Abdel-Aziz *et al.* 2012 *a*). Metabolites produced by strain NRC-14 exhibited potential efficacy in biocontrol either in liquid culture or on agar plates (Abdel-Aziz *et al.* 2012 *a,b*). Synergistic activity of lytic enzymes might serve as a tool to reduce the use of hazardous chemical fungicides and to reduce the impact of some chemical pesticides on animals (Haran *et al.* 1996). The level of synergism appeared to be higher when enzymes act with the bioactive compound(s) having primary sites of action associated with membrane structure, as compared to pesticides action.

Table 1. Effect of carbon source on production of lytic enzymes by strain *B. alvei* NRC-14.

Carbon source	Chitinase	Chitosanase	ß-1,3 glucanase	Protease	Cellulase
Glucose	–	++++	+	–	–
Flaked chitin	+	+++	+	+++	–
Flaked chitosan	++	+++++	+++	+++	+++
FCW-1*	++++	+++++	+++++	+++++	–
FCW-2*	–	++++	++	+++	+++++
FCW-3*	++	+++++	+	++	–

***FCW-1, *A. niger*; **FCW-2, *P. ultimum*; FCW-3, *Rhizopus sp.*

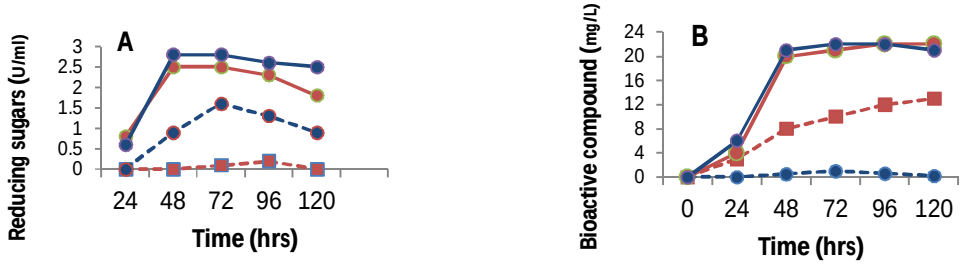

Fig. 1. Time course for production of lytic enzymes (A) and bioactive compounds (B) by strain NRC-14 when grown at 40^{o}C with: glucose (--●--); chitin (--■--); chitosan (●); or FCW-1 (●) as carbon sources.

Synergism of lytic enzymes and bioactive compound

In vitro tests showed that, culture suspensions of the strain exhibited potential against all the tested fungi as well as its culture supernatant and dilutions up to 3-fold (Fig. 2), whereas at 4-fold fungal strains sharply exhibited obvious resistance. Only *P. ultimum*, unlike other fungi, exhibited pronounced resistance with the diluted culture supernatant (2-4 fold dilutions).

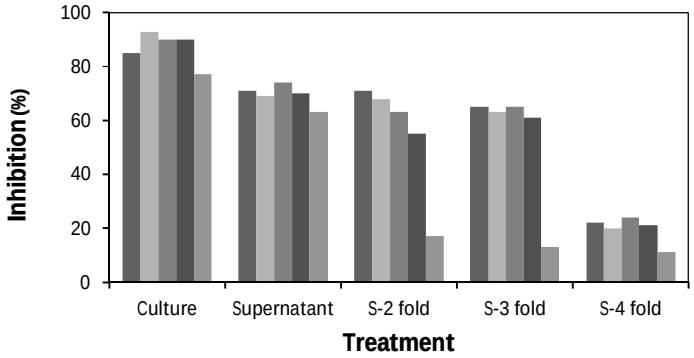

Fig. 2.Synergistic effect of lytic enzymes and bioactive compound (s) produced by strain NRC-14 upon fungal growth. Culture suspension and culture supernatant with different dilutions (S-2 fold, S-3 fold, and S-4 fold) were used against: *F. oxysporum* (■), *F. solani* (■), *R. solani* (■), *S. rolfsii* (■), and *P. ultimum* (■).

Effect of whole cells and culture filtrate of the strain against nematode

Efficacy of both culture supernatant and whole cells of strain NRC-14 on inhibition of nematode (*Meloidogyne incognita*) eggs and larvae was investigated. Whole cells of the strain proved to be relatively more effective than the culture supernatant (Fig. 3). Pure protease and chitinase from the strain showed less efficacy in biocontrol (data not shown) although chitinase and protease have often been associated with biocontrol of the root-knot nematode. When soil was treated with the strain as a biocontrol agent and grown in nematode infested soil, it exhibited a drastic reduction in root galling when compared with the control (yet unpublished data). Because nematodes often occur in high numbers in soil, it is not surprising that a wide variety of soil organisms exploit nematodes as food, i.e., as source of carbon, nitrogen, and energy. Those organisms that seek out and consume nematodes are called predators such as mites and protozoa; these organisms are called parasites. Parasites of plant-parasitic nematodes include fungi, bacteria, and mycoplasma-like organisms (Ferris 1993). Other organisms may have a detrimental effect on nematodes without utilizing them as a substrate, by competing for food, space, and necessary resources. Interaction between plant-parasitic nematodes and other competitors may cause increased damage to a particular food source. Some organisms may antagonize nematodes by producing nematicidal or nemastatic compounds such as ammonia and certain fatty acids (Ferris 1993). This mode of actions is referred to as antibiosis, and involves bacteria and fungi. In the present study, mechanisms of action include potential lytic enzymes, bioactive compound(s) as well as, probably, competition for nutrients.

The results of strain NRC-14 against nematode suggested that the enzymes and other active compounds in the culture broth of the strain exhibited potential activity against nematode‘s egg and larvae by which it may disrupt its life cycle. Moreover, no significant differences were observed regarding the effects of the whole cells and culture broth of the strain against nematode. It is concluded that the natural substances produced by the strain are important factors for suppression of the nematode under *in vitro* experiments and which may be also predictive under *in vivo* system to prevent nematode damage on plants. However, further tests regarding the strain against nematode in soil is essential in future. There is no evidence to believe that the observed traits of strain NRC-14 is specific for this strain or for other biocontrol strains in the soil. However, *in vitro* tests by the strain and the variety of lytic enzymes, bioactive compounds, bioflocculat it produces as well as potentiality against nematode and event effects under *in vivo* experiments (Fig. 6) undoubtedly confirm that this strain play an important role as a biocontrol agent.

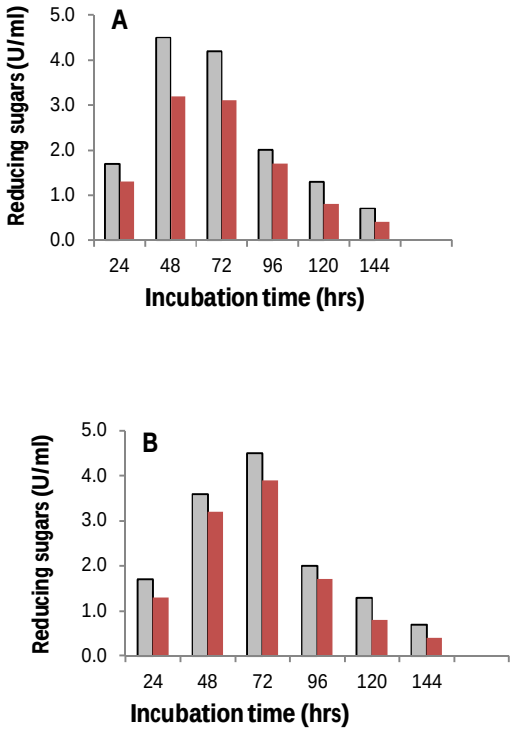

Fig. 3. Efficacy of strain NRC-14 (■) and its culture metabolites (■) against: (A) eggs and (B) larvae of nematode.

Thermal stability of bioactive metabolite(s)

The bioactive metabolite(s) was submitted to a thermal treatment in order to evaluate the stability of the antifungal effect under different temperatures. Results showed that, the antifungal activity kept constant when the supernatant was submitted to temperatures from 50-80ºC, showing an inhibition activity similar or near to that of control (Fig. 4). There was a significant loss of activity at temperature of 90ºC when compared to the control and other treatments. Even though, at this temperature, more than 60% of activity was observed, whereas the antifungal activity for metabolites treated at 100°C was less than 50% (Fig. 4). This result is in accordance with that reported by Carissimi *et al.* (2009) but differs from the one reported by Shirokov *et al.* (2002) were 60% of the activity of the filtrate was lost when it was submitted to 70ºC.

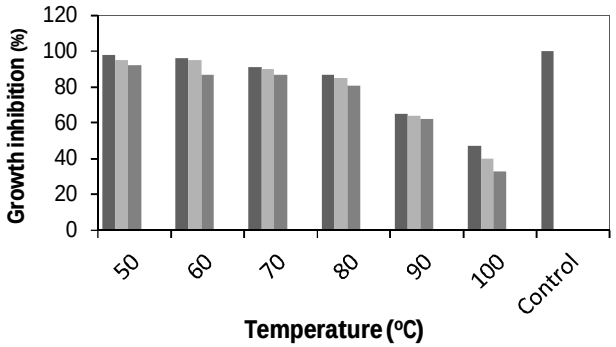

Fig. 4. Stability and activity of the bioactive metabolite(s) of strain NRC-14 after thermal treatments, against *F. oxysporum*. Time of treatments was: (■) 30, (■) 50, and (■) 70 minutes in different temperatures in comparison with control.

The pH stability of bioactive metabolite(s)

The stability of bioactive metabolite(s) was tested at different pH treatments varying from pH 4-10. From the mycelium dry weights it is observed that the inhibited effect upon fungal growth was stable over a wide range of pH values (5-9), when the supernatant was submitted to pH treatment, whereas a decrease in the inhibition effect was observed with the culture supernatant as the pH increased (Fig. 5). However, there was no significant loss of antifungal activity at pH 5-9; inhibition activity was between 75-90 % for the culture supernatant and culture broth (Fig. 5).

On other hand, bioactive metabolite(s) produced by the strain was found to be resistant to hydrolysis by proteases (data not shown). It was suggested previously that biocontrol of phytopathogens might be due, in part, to the actions of produced proteases that inactivate the hydrolytic enzymes produced by a pathogen (Elad and Kapat 1999). So, the stability of the bioactive metabolite(s) to temperature, pH, and proteases as well as *in vitro* antagonism against a wide range of phytopathogenic fungi might be responsible for the biocontrol efficiency by strain NRC-14.

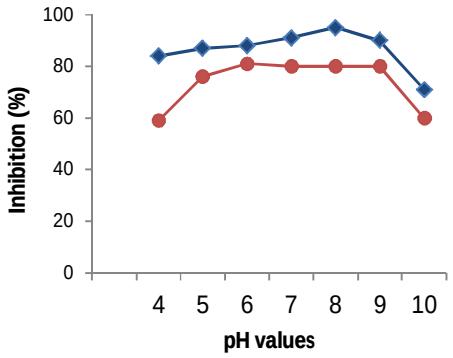

Fig. 5. Stability of the bioactive metabolite(s) produced by strain NRC-14 after treatment at different pH values. Inhibition effect of the bioactive metabolite(s) was against *F. oxysporum*, grown on potato dextrose broth with the culture broth (◆), or the culture supernatant (●), for 72hrs.

Possible mechanisms involved in biological control by biocontrol species has led to several alternative explanations for successful biocontrol. One idea that has been advanced is that; enzymes such as chitinases, chitosanase, and/or ß-glucanases produced by the biocontrol agents are responsible for suppression of the plant pathogen. These enzymes function by breaking down the polysaccharides, chitin, and ß-glucans that are responsible for the rigidity of fungal cell walls, thereby destroying cell wall integrity. Besides chitin and ß-glucan, filamentous fungal cell walls contain lipids and proteins. Proteases may therefore play a significant role in the cell-wall lyses that occurs during pathogen-host interactions. Therefore, another interesting concept related to enzymes as a mechanism in the biocontrol process is production of proteases by an antagonist. Proteases break down the hydrolytic enzymes from a pathogen into peptide chains and/or their constituent amino acids and thereby destroy their effect to act against plant cells. Protease solutions when produced by a biocontrol agent, partially deactivated hydrolytic enzymes from a pathogen and reduced disease severity by 56 to 100% when the solutions were used to treat leaves infected with the pathogen (Elad and Kapat 1999). Another mechanism proposed to explain activity by biocontrol species is that the induction of resistance in the host plant by treatment with the biocontrol agent; pretreating hyphal walls of *F.oxysornm* with proteolytic enzymes increases their susceptibility to lyses by chitinase and ß-1,3 glucanase (Sivan and Chet 1989); it could be suggested that hydrolyses of the protein or protein-like constituent(s) by proteases may increase susceptibility of the fungus to lytic enzymes and enhance accessability for chitin and laminarin degradation, in fungal cell wall, thereafter. These observations, together with the fact that chitin and ß-1,3-glucan are the main skeletal polysaccharides of fungal cell walls (except for oomycetes such as *Pythium* spp. which contain mainly cellulose), suggest that chitinase and ß-1,3glucanases act as key enzymes in the lyses of phytopathogenic fungi during the antagonistic action. Hence, fungal cell wall- degrading enzymes of strain NRC-14 is of special importance in plant defense mechanisms. Growth of some strains under conditions in the laboratory may probably be not predictive in the field. Temperature has a profound effect on the production and activities of enzymes and antibiotics produced by the biocontrol species. Metabolism occurs at 28°C in a petri dish may not occur at all in the soil around a germinating seed at 40°C. The presence of other members of the soil microflora may also influence biocontrol activity by inhibiting the growth and development of the

biocontrol agent or alter the metabolism of its enzymatic and/or antibiotic products. However, these factors did not affect the biocontrol activities of strain NRC-14 or limit their efficacy in terms of time-length (3-months period in pots) that they stay effective and exhibit biocontrol influence.

***In vivo* efficacy of extracellular metabolites**

The *in vivo* experiments against *F. oxysporum* revealed efficacy of the strain as a PGPR in most replicates comparing to the control samples (Fig. 6). Inoculation with strain NRC-14 resulted in a large increase in root-weight and length and enhanced the shoot-weight and plant height which resulted in an overall plant healthy. Root length in some replicates (Rep-3 and 4, Fig. 6) was found to be shorter. In addition, reduction of root numbers and length in some replicates resulting in a large decrease in the shoot/root ratio which has been suggested to be an indicator of plant stress (Naseby *et al.* 2000). Causes of plant stress include nutrient limitations (including oxygen), and therefore a decrease in shoot/root ratio, may indicate such stress. It should be recognized, however, that such stressed plants may be more effective in acquiring water and nutrients as a result of the expanded root system. Thus this is a positive adaptive response to such stresses and this could be a useful trait in low-nutrient or dry soils (Naseby *et al*. 2000). The antagonistic phenomenon between microorganisms occurs naturally in soil, around the root surface (rhizosphere) and is easily observed in pathogens cultures.

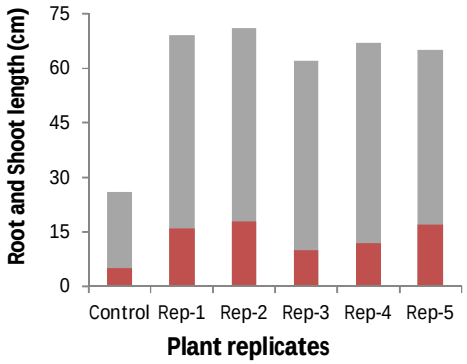

Fig. 6. Synergistic effect of lytic enzymes and bioactive compound(s) produced by strain NRC-14 against *F. oxysporum* for protection of tomato plant: root (■) and shoot (■) length, in comparison with control.

Multiple Plant-growth promoting properties

Enhancement in shoot- and root-length, plant height, and fortifying plant stand and healthy were observed, in comparison with controls. Such improvements may probably be indicators for the plant-growth promoting properties of strain NRC-14 which may result in enhancing plant systemic resistance (Table 2). The inhibitory effect of the strain metabolites upon fungal growth was observed in early stages of plant growth. As shown in Fig. 7, wilt, browning color in roots and wrapping of blade in leaves are occurred (Fig. 7, above images; control). Treatment of soil with strain NRC-14, therefore, has the potential to fortify the plant and improve overall plant healthy, and might be particularly important in suboptimal enrichment of soil conditions due to the sustainability of the strain under a wide range of temperatures. It is suggested that, improvement of plant nutrition was directly related to the general beneficial growth effect on the root system of inoculation with the strain. During the long-term of work, it was found that the strain showed resistance to biotic and abiotic stress factors and this may increase the nutritional status of the plant when the strain is applied as a biocontrol agent and this suggesting a direct antagonism with the pathogens for essential nutrients. Rhizosphere competence is important because a biocontrol agent can't compete for space and nutrients if it is unable to grow in the rhizosphere. Strain NRC-14 was found to be a good producer for siderophore and IAA. Secretion of IAA promotes root growth directly by stimulating plant cell elongation and cell division (Shahi *et al.* 2011). A reduction in incidence of root wilt (94%) was observed. Moreover, the growth-promoting efficiency (GPE) and biocontrol efficiency (BE) reached 180 and 151%, respectively (Table 2). Despite of antagonistic action of *Bacillus* spp. against phytopathogenic fungi has been observed by many workers in *in vitro*, however, *in vivo* experiments by utilizing *Bacillus* spp. as antagonists are inconclusive with respect to the efficacy of these microorganisms under field conditions.

Table 2. Multiple plant-growth promoting activities of strain *B. alvei* NRC-14 against *F. oxysporum*, (some of which cited from Loon 2007).

The nature of systemically induced resistance in plants	Activities of strain NRC-14
(A) Characteristics of systemically induced resistance	
Enhancement of defense capacity of the plant (enhanced defense capacity is expressed systemically through the plant).	+
Induced systemic resistance is maintained for prolonged periods	+
Thickness of plant stem and roots are vigorous	+
Reduction in disease symptoms	+
(B) Mechanisms of induced systemic resistance	
Induction of phytoalexins and gibberellins	ND**
Induction of cell wall reinforcement	+
Induction of pathogenesis-related proteins	ND**
(C) Strain activities and properties	
Antimicrobial compound	+
Prodution of IAA	+
Production of siderophore	+
Enzymatic activities	+
*Growth promotion efficiency (GPE %)	180%
*Biocontrol efficiency (BE %)	151%

ND**; not detected, *represented the highest values.

Fig. 7. Protection of tomato plants by strain NRC-14 against *Fusarium oxysporum*. Control samples for a plant infested with the fungus without bacteria or culture broth (above images); and the inhibitory effect after treatment of soil with: 1) culture broth of the strain (middle images); or 2) after inoculation of soil with the strain as a PGPR (bottom images) before plant growth. Plants were harvested after approximately, 3 months.

Spontaneous mutation of the strain

Worthy mention is that, during more than twenty years of experiments using the novel strain *Bacillus alvei* NRC-14, a spontaneous mutation has been occurred for this strain after about ten generations of isolation from soil. Spontaneous mutation is a mutation that arises naturally and not as a result of exposure to mutagens. Spontaneous mutations arise from a variety of sources, including errors in DNA replication, spontaneous lesions, and transposable genetic elements. Strain NRC-14 was found to adapt abiotic stress and hard environmental conditions. The stress response is a mechanism used by microorganisms to adapt to and overcome a stress stimulus in order to survive. Whilst facilitating survival, the stress response may generate a weakly mutant. However, the mutant of strain NRC-14 was

found to be strong and different from the wild-type. This strain was, firstly, reported to produce two chitosanases with low activity towards other substrates such as chitin, CM-cellulose, and starch. However, after long term of work, the strain was found to be an excellent producer of carbohydrate-active enzymes, bioflocculant, aminosugars, as well as bioactive compound(s). Under well aerated conditions, the cells were observed to form three types of colonies on nutrient agar or chitosan-based medium: 1) circular, flat, glistening, and smooth; 2) circular, very small and convex; and 3) circular and edges irregular, wrinkled and glistening. The first and second types were mainly dominant after isolation of the strain from soil, while the third type was developed after a long period. Of interest is that, exactly similar results are reported by Chung *et al.* (2000). Oxidase, catalase, and indole activities were positive by the strain (Abdel-Aziz *et al.* 2012 *a*). The long-term storage viability of the strain either being on agar slants or in liquid culture is high; this may be due to the formation of a biopolymer flocculant.

A systematic illustration of important mechanisms known for plant growth promotion by PGPR is represented in Fig. (8). Enhanced lateral root formation increases the capacity to take up nutrients. Moreover, the larger leaf area plays an important role in better photosynthesis and respiration (Yang *et al*. 2009). PGPR may facilitate plant growth either indirectly or directly. The ability of PGPR to act as biocontrol agents against phytopathogens and thus indirectly stimulate plant growth may result from one or more of a variety of mechanisms including antibiotic production, depletion of iron from the rhizosphere, induced systemic resistance, production of fungal cell wall lysing enzymes, and competition for binding sites on the root (Anderson *et al.* 1993). PGPR can directly facilitate plant growth as biofertilizers by performing nitrogen fixation, synthesize siderophores which can sequester iron from the soil to support plant cells and to synthesize phytohormones such as auxins, cytokinins and gibberelins (Anderson *et al.* 1993), solubilize minerals such as phosphorus to be more available for plant growth and healthy (Fig. 8).

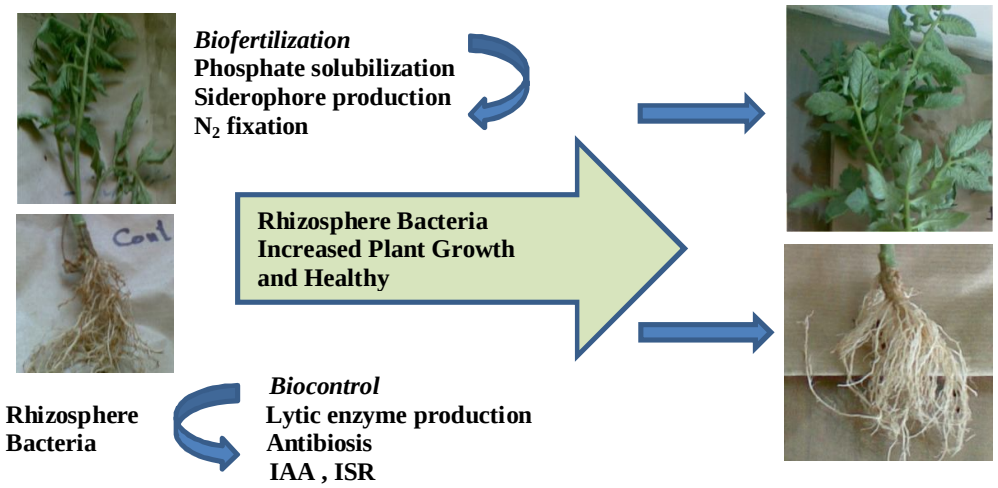

Fig. 8. Schematic illustration for important mechanisms known of plant growth promotion by PGPR. Mechanisms of pathogen inhibition can be performed by: 1) Biofertilization (Phosphate solubilization, Siderophore production**,** N2 Fixation); and (2) Biocontrol of pathogens (Lytic enzyme production, Antibiosis, andInduction of Systemic Resistance (ISR) of host plant by PGBR, leading to increased plant growth.

Biological control by *Bacillus* strains and other biocontrol agents is important corresponds to disease-suppressive soils, in which disease-susceptible plants can grow without being extensively damaged by virulent root pathogens (Couillerot *et al.* 2009). In contrast, non-suppressive soils (i.e. conducive soils) allow plant infection and spread of the disease (Couillerot *et al.* 2009). Suppressive soils are extensively documented in the case of fungal soil-borne pathogens, noticeably *Gaeumannomyces graminis* var. *tritici* (take-all of wheat), *Fusarium oxysporum* (wilt diseases of several crop plants), *Rhizoctonia solani* (seedling damping-off of various crops), *Pythium ultimum* (damping-off of cucumber), and *Thielaviopsis basicola* (black root rot of tobacco and other species), and to a lesser extent phytoparasiticoomycetes e.g. *Phytophthoracinnamoni* (root rot of eucalyptus), nematodes e.g. *Meloidogyne incognita* (root-knot galls on several tropical and subtropical crops) and bacteria e.g. *Streptomyces scabies* (potato scab) and *Ralstonia solanacearum* (bacterial wilt of several crops).

The advantages of crop treatments with fungicides are reliable, simple handling, and of low costs. However, under long periods of low soil temperatures and high humidity, microorganisms may offer results against fungi, which outdo those of fungicides. This behavior is probably due to the growth of those microorganisms within the seeds that, when the plant shoots be forth, eventually reach the root surface and protect the tissues (Couillerot *et al.* 2009). Therefore, research is still needed to test the use of several microorganisms sequentially or as a mixture and to ensure safety for human and animals.

Conclusion

Strain *Bacillus alvei* NRC-14, was applied in agriculture as a biocontrol agent. The strain not only has biocontrol properties but also can adapt and survive under various abiotic stress conditions. Lytic enzymes and bioactive compounds were produced using fungal spores as low-cost natural carbon source. Thus, in response to treatment of soil with the strain, it could be concluded that the strain: 1) revealed multiple plant growth-promoting properties and could be applied as an excellent PGPB; 2) provided evidence for antibiosis as a mechanism of antagonism because it causes significant antimicrobial effect; and 3) could providing long-term induced resistant for tomato plants against various soil-borne pathogens.

Acknowledgement

The author is indebted to Prof. Dr. Hoda Hamed and Dr. Yomna Moustafa, Microbial Chemistry Dept., for supporting the filed experiments, performed in pot trials in: National Research Center. I deeply thank Prof. Dr. W. Elnagdy, Nematology Lab., for supplementation with nematode's egg and larvae.

REFERENCES

Abdel-Aziz, SM., Hamed, H., Mouafi, F., and Abdelwahed, N. 2011. Extracellular metabolites produced by a novel strain, *Bacillus alvei* NRC-14: 3. Synthesis of a bioflocculant that has chitosan Like structure. Life Sci. J., 8: 883-890.

Abdel-Aziz, SM., Moharam, M., Hamed, H., and Mouafi, F. 2012 *a.* Extracellular metabolites produced by a novel strain, *Bacillus alvei* NRC-14: 1. Some properties of the chitinolytic system. New York Sci. J., 5: 53-62.

Abdel-Aziz, SM., Mouafi, F. and Keera, A. 2012 *b.* Extracellular metabolites produced by a novel strain, *Bacillus alvei* NRC-14: 2. Molasses-based growth for high production of *N*-acetylglucosamine. Aust. J. Basic Appl. Sci., 6: 251-261.

Aktuganov, G., Galimzyanova, N., Melent'ev, A. and Yu, L. 2007. Extracellular hydrolases of strain *Bacillus* sp. 739 and their involvement in the lysis of micromycete cell walls. Microbiology, 76: 413-420.

Algam, S., Xie1, G., Li1, B., *et al.* 2010. Effects of *Paenibacillus* strains and chitosan on plant growth promotion and control of Rarstonia wilt in tomato. J. Plant Pathol.,92: 593-600.

Anderson, R. D. *et al.*1993. In: *Cellular and Molecular Aspects of the Plant Hormone Ethylene* (eds. Pech, J. C., Latché, A. &Balague, C.) 197–204. (Kluwer, Dordrecht, 1993).

Aris, T., Rina, P., Asri, W., Anja, M. and Asih, N. 2011.Characterization of *Bacillus* sp. strains isolated from rhizosphere of soybean plants for their use as potential plant growth for promoting Rhizobacteria. J. Microbiol. Indonesia, http://repository.ipb.ac.id/handle/123456789/54530.

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. J. Appl. Microbiol., 108: 386–395.

Bertasso, M., Hulzenkampfer, A., Zeexk, F., Antonia, D. and Fiedler, H. 2001. Bahremycin A and B, novel antibiotics from *Streptomycete*sp. TU. 4128. J. Antibiotic, 54: 730–736.

Brian, C., Daniel, H. and Aeron, M. 2011. Use of blue agar CAS assay for siderophore detection. J. Microbiol. Biol. Education, 12, DOI: 10.1128/jmbe.v12i1.249.

Carissimi, M., Giraudo, M., Germani, G. and Sand, S. 2009. Antifungal activity of *Bacillus* sp. E164gainst *Bipolaris.*Biociencias, Porto Alegre, 17: 48-58.

Chen XH, Koumaoutsi A, Scholz R. and Borriss, R. 2009. More than anticipated-production of antibiotics and other secondary metabolites by *Bacillus amyloliquefaciens* FZB42. J. Mol. Microbiol. Biotechnol., 16:14–24.

Chernin, L. and Chet, I. 2002. *Enzymes in the environment: activity, ecology, and applications.* Burns, R. G. and Dick, R..P. (eds.). pp. 171-226.

Choudhary, D., and Johri B. 2008. Interactions of *Bacillus* spp. and plants with special reference to induced systemic resistance (ISR). Microbiol. Res., 164: 493–513.

Gordon, S. and Weber, R. 1951. Colorimetric estimation of indole acetic acid. Plant Physiol.,26: 192-195.

Couillerot, O., Prigent, C., Caballero, J. and Moenne, Y. 2009. *Pseudomonas fluorescens* and closely-related fluorescent *pseudomonads* as biocontrol agents of soil-borne phytopathogens. Lett. Appl. Microbiol., 48: 505-512.

Diby, P., Saju, K., Jisha, P., *et al*. 2005. Mycolytic enzymes produced by Pseudomonas fluorescens and Trichoderma spp. against Phytophthoracapsici, the foot rot pathogen of black pepper (Pipernegrum L). Ann. Microbiol., 55: 129-133.

Elad, Y. and Kapat, A. 1999. The role of *Trichoderma harzianum* protease in the biocontrol of *Botrytis cinerea*. Eur. J. Plant Pathol., 105:177-189.

Ferris, H. 1993. New frontiers in nematode ecology. J. Nematology, 25, 374-382.

Fukuda, T., Matsumoto, Y. Takahashi, H. and Omura, S. 2005. Phenatic acids A and B, new Potentiators of antifungal miconazole activity produced by *Streptomyces* sp. K03-0132. J. Antibiotic, 58: 252–259.

Haran,S, Schickler, H. and Chet I. 1996. Molecular mechanisms of lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum*. Microbiology, 142: 2321-2331.

Hurek, T. and Reinhold-Hurek, B. 2003. *Azoarcus* spp. strain BH72 as a model for nitrogen fixing grass endophytes. J. Biotechnol., 106:169.

Jayalakshmi, S., Raju, S., Usha, R., Benagi, V. and Sreeramulu, K. 2009. *Trichoderma harzianum*-L1as a potential source for lytic enzymes and elicitor of defense responses in chickpea (*Cicerarietinum*L.) against wilt disease caused by *Fusarium oxysporum*f. sp. *ciceri*. Aust. J. Crop Sci., 3: 44-52.

Khan, A., Hamayun, M., Javid, H., Kang, S. and Lee, I. 2012. The Newly Isolated Endophytic Fungus *Paraconiothyrium* sp. - LK1 Produces Ascotoxin. Molecules, 17, 1103-1112.

Kloepper, J., Ryu, C. and Zhang, S. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathol., 94:1259–126.

Loon, L. C. 2007. Plant responses to plant growth-promoting rhizobacteria. Eur. J. Plant Pathol., 119: 243-254.

Lowry O, Rosebrough N., Farr, A. and Randall, R. 1951. Protein measurement with the folinphenol reagent.J. Biol. Chem., 193: 265-275.

Miller, GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem., 31:426-428.

Naseby, D., Pascual, J. and Lynch, J. 2000. Effect of biocontrol strains of *Trichoderma* on plant growth, *Pythium ultimum* populations, soil microbial communities and soil enzyme activities. J. Appl. Microbiol., 88:161–169.

Pal, K. and Brian, G. 2006.Biological Control of Plant Pathogens. DOI: 10.1094/PHI-A-2006-1117-02.

Picard C., Baruffa E. and Bosco M. 2008. Enrichment and diversity of plant-probiotic microorganisms in the rhizosphere of hybrid maize during four growth cycles. Soil Biology and Biochem., 40:106-115.

Picard C., Bosco M. (2008): Genotypic and phenotypic diversity in populations of plant-probiotic *Pseudomonas* spp. colonizing roots. Naturwissenschaften, 95:1-16.

Ramyasmruthi S., Pallavi O, Pallavi S, Tilak K and Srividya S. 2012. Chitinolytic and secondary metabolite producing *Pseudomonas fluorescens* isolated from Solanaceae rhizosphere effective against broad spectrum fungal phytopathogens. Asian J. Plant Sci. Res., 2: 16-24.

Sahebani, N. and Hadavi, N. 2008. Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum.* Soil Biol. Biochem., 40: 2016-2020.

Shahi, K., Rai, A., Tyagi, M., *et al*. 2011. Rhizosphere of rice plants harbor bacteria with multiple plant growth promoting features. Afr. J. Biotechnol., 10, 8296-8305.

Shircov, A., Loginov, O., Melent'ev, A. and Aktuganov, G. 2002. Protein and peptide factors from *Bacillus* sp. 739 inhibit the growth of phytopathogenic fungi. Appl.Biochem. Microbiol., 38: 139-143.

Siti, M., Biviand, M. and Kherulmazmi, A. 2011. Effect of carbon sources on bacterial production of metabolites against *Fusarium oxysporum* and *Colletotrichum gloeosporioides.* Intr. J. Agr. Biol., 13: 1-8.

Song, W., Zhou L., Yang C., Cao X., Zhang L., and Liu X. 2004. Tomato *Fusarium* wilt and its chemical control strategies in a hydroponic system. Crop Prot., 23: 243-247.

Validov, S., Kamilova, F., Qi1, S. 2007. Selection of bacteria able to control *Fusarium oxysporum* f. sp. radicis-lycopersici in stonewool substrate. J. Appl. Microbiol., 102: 461–471.

Wang, H., Rui, F.and Wei, Q. 2012. Antifungal Activity of *Bacillus coagulans* TQ33, Isolated from Skimmed Milk Powder, Against *Botrytis cinerea.* Tianjin University of Science and Technology, Tianjin, PR China.

Yang Z., Liu, X., Zhong, L., *et al.* 2009. Effect of *Bacillus Subtilis* SY1 on antifungal activity and plant growth. Int. J. Agric. Biol. Eng., 2: 55-61.

Yedidia, I., Benhamou, N., and Chet, I. 1999. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum.* Appl. Env. Microbiol., 65:1061-1070.

Zhan, Y., Xu, Q., Liu, H., Wang, Y. and Guo, J. 2011. Effect of different concentrations of bacterial suspensions on biocontrol efficacy and storage stability of *Bacillus cereus* AR156, *Pantoea ananatis* YT11 and *Pseudomonas fluorescens* ABc9. Afr. J. Microbiol. Res., 5, 4019-4022.