J. Agric. Food. Tech., 3(12)31-40, 2013

ISSN 2090 – 424X
Journal of Agriculture and Food Technology
www.textroad.com

Efficiency of the Novel Strain *Bacillus alvei* NRC-14 for Biocontrol of Parasitic Nematode

Shadia M. Abdel-Aziz[1]*, Wafaa A. El-Nagdi[2] and Maysa E. Moharam[1]

[1]Microbial Chemistry Department and [2]Nematology Department
National Research Center, Dokki, Cairo, Egypt

ABSTRACT

In the present study, the strain *Bacillus alvei* NRC-14 showed potentiality against nematode's eggs and larvae. The strain used hydrolytic enzymes to directly hydrolyze nematode's eggs and larvae. Action of the strain or its metabolites against eggs and larvae was associated with release of high levels of reducing sugars. *B. alvei* NRC-14 was evaluated for its efficiency (*In vitro*) against *Meloidogyne javanica* eggs and larvae to reduce the incidence and pathogenicity of the root-knot nematode. Experiments were applied by culture supernatant of *B. alvei* NRC-14 at different concentrations or by the whole cells. *In vitro* studies revealed that *M. javanica* eggs and larvae were inhibited by the crude supernatant and this inhibition was positively correlated with increase in the concentration of crude supernatant. The strain produced lytic enzymes *viz.* chitinase, chitosanase, proteases, as well as other potential bioactive metabolite(s). The lytic enzymes in combination with the metabolite(s) showed a strong synergistic inhibitory effect on *M. javanica* eggs and larvae in comparison with antibiotics and chemical nematicides. Reduction percentage in number of females, galls, and egg masses were 77, 45, and 62 %, respectively, by application of whole cells, whereas it reached 70, 52.4, and 58.6 % when the produced metabolites were applied. In comparison, reduction percentages were 46.7, 88.0, 48.3% and 53.3, 64.3, 55.2 % when antibiotics and chemical nematicides were applied, respectively. Application of strain NRC-14 or its metabolites may have potential for development as a single microbial control agent. To our knowledge, biocontrol of phytoparasitic nematode by the antagonist, *Bacillus alvei* NRC-14 is reported for the first time.

KEY WORDS: lytic enzymes, *Bacillus alvei*, parasitic nematodes, synergistic action, root-knot nematode.

1. INTRODUCTION

Plant-parasitic nematodes cause serious crop losses worldwide and are among the most important agricultural pests. The management of nematodes is more difficult than that of other pests because nematodes mostly inhabit the soil and usually attack the underground parts of the plants (Pakeerathan *et al*. 2009). Chemical nematicides are effective, easy to apply, and show rapid effects. However, they are hazardous concerning public health and environmental safety (Tian *et al*. 2007). The search for novel, alternatives that are environmental-friendly to manage plant-parasitic nematode populations has therefore become increasingly important. Biological control of soil-borne plant pathogens and nematodes by antagonistic microorganisms is a potential non-chemical means of plant disease control (Stirling 1991). A wide range of bacterial and fungal agents have been used to reduce a range of plant parasitic nematodes (Hallmann *et al*. 2001, Bird *et al*. 2003). The egg shell of nematodes contains chitin, and it is presumed that exposing eggs to chitinase can disrupt egg hatch (Bird and Bird 1991). Both chitinase and chitinase-producing bacteria have been considered for use in reducing numbers of plant-parasitic nematodes in soil (Spiegel *et al*. 1991). Bacteria and fungi are extensively tested to control plant-parasitic nematodes (Mankau, 1980). Bacteria are numerically the most abundant organisms in soil, and some of them, for example members of the genera *Pasteuria*, *Pseudomonas*, and *Bacillus*, have shown great potential for the biological control of nematodes (Emmert and Handelsman, 1999, Tian *et al*. 2007). Extensive investigations have been conducted over the last twenty years to assess their potential to control plant-parasitic nematodes. These research efforts have found that nematophagous bacteria are distributed broadly, possess diverse modes of action, and have broad host ranges.

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). *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). Induction of induced-systematic resistance (ISR) in plant has been reviewed by *Bacillus* spp., for protection of plants such as

***Corresponding Author:** Shadia M. Abdel-Aziz, Microbial Chemistry Department, National Research Center, Dokki, Cairo, Egypt. abdelaziz.sm@gmail.com

tomato and soybean. 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 previous work, strain *Bacillus alvei* NRC-14 has proved to be an efficient agent for protection of tomato plant (Abdel-Aziz 2012) in biocontrol against *Fusarium exosporum* (Fig. 1).

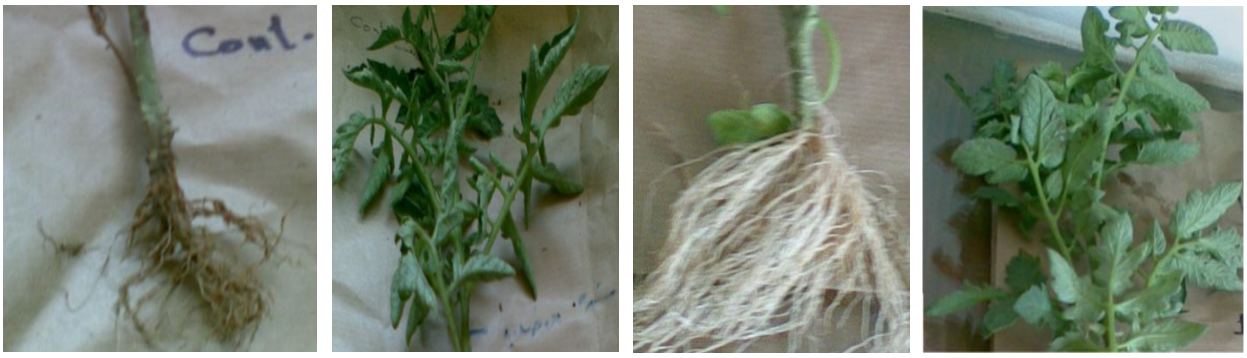

Fig. 1. *In vivo* potential effect of extracellular metabolites from strain *Bacillus alvei* NRC-14 in biocontrol for protection of tomato plant against *Fusarium exosporum.* Images show: leaves/roots of fungal-infested tomato plant (*left 2-images*), and leaves/roots (*right 2-images*) of a healthy plant treated with the crude extracellular metabolites produced by strain *B. alvei* NRC-14, and harvested after, approximately, 3-months growth.

On other hand, as a group of important natural enemies of nematode pests, *Bacillus* spp. exhibit diverse modes of action: these include parasitizing; producing toxins, antibiotics, or enzymes; competing for nutrients; inducing systemic resistance of plants; and promoting plant health (Tian *et al*. 2007). They act synergistically on nematodes through the direct suppression of nematodes, promoting plant growth, and facilitating the rhizosphere colonization and activity of microbial antagonists (Tian *et al.* 2007). *Bacillus alvei* NRC-14 was found to be an effective agent in biological control of several fungal pathogens (Abdel-Aziz 2013). Suppression of fungal pathogens by strain *B. alvei* NRC-14 is attributed in part to chitinase, chitosanase, β-glucanase production. In the present study, *B. alvei* NRC-14 is introduced for the first time as a potent agent in biological control against parasite nematode. A previous work described the effectiveness of *B. alvei* NRC-14 on nematode eggs because the bacteria produce chitinolytic enzymes and other potentially antagonistic enzymes and compounds (Abdel-Aziz 2013). This strain has never been reported previously in literatures despite of its efficiency for production of carbohydrate-active enzymes, capability for production of bioactive compounds and secretion of a variety of extracellular metabolites and lytic enzymes. The present study describes: 1) effects of extracellular metabolites produced by the strain against economically an important plant-parasitic nematode and examined the influence of strain NRC-14 on the survival of egg and larvae; 2) studying some properties of these metabolites; and 3) to evaluate the synergistic effect and activity of these metabolites against parasitic nematode under *in vitro* conditions.

2. MATERIALS AND METHODS

2.1 Bacterial strain and secondary metabolite secretion

Bacillus alvei NRC-14, a potent chitosanase producer, was isolated from soil and identified as described previously (Abdel-Aziz 1999), maintained on nutrient agar slants, and kept at 4^{o}C. This strain was found to produce a variety of carbohydrate-active enzymes and bioactive compounds. The strain has proved to be an efficient antagonist in biocontrol against plant-fungal pathogens (Abdel-Aziz 2013). For production of extracellular metabolites, cells of the fungus *Mucor rouxii* were used as carbon source. A pre-culture (24h old) of strain NRC-14 was grown with fungal biomass and incubated at 30^{o}C (130 rpm) for 48h, after which cell free culture supernatant was obtained by centrifugation of culture broth at 5000 x g for15 min (Abdel-Aziz 2013). Activity of enzymes in the culture 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.

2.2 Enzymatic activities determination

Activities of chitinase and chitosanase were determined as described previously (Abdel-Aziz *et al*. 2012*a*). Activity of ß-1,3 glucanase was estimated by the method of Wichetra *et al.*, (2006) using laminarin as substrate. The enzyme activity was defined as micromoles equivalents of glucose released/ml of culture/min. Activity of cellulase

and ß-1,4 glucanase was estimated using carboxymethyl cellulose as the substrate (Diby *et al.* 2005). Protease activity was estimated as reported previously (Abdel-Aziz *et al*. 2004*b*).

2.3 Purification of enzyme

All purification steps were carried out at 4^{o}C unless otherwise noted. Culture broth (500 ml) was centrifuged at 7000 x g for 15 min to remove culture debris. The supernatant was subjected to precipitation by ammonium sulphate (30-80%), and allowed to be kept at 4^{o}C overnight (Frank *et al.*, 2005**)**. The solution was centrifuged at 7000 x g for 15 min, and the resultant precipitate was dissolved in a minimal volume of acetate buffer (pH 5.6) and dialyzed against the same buffer. The dialyzed sample was loaded onto sephadex G-100 column (50 x 2.5 cm) equiliberated with the same buffer. The enzyme was eluted at a rate of 1ml/2 min. The active fractions (3 ml each) were collected and checked for chitinolytic and chitosanolytic activities with colloidal chitin and soluble chitosan as the substrates. Activities of the purified enzyme against chromogenic substrates, i.e. *pNP-N*-acetyl-*B-D*-glucosaminide, *pNP-B*-D-galactosaminide, and *pNP*-glucopyranoside were also assayed.

2.4 Kinetic Parameters

Initial reaction rates using soluble chitosan as substrate were determined at substrate concentrations of 0.16–2.0 mg/mL in 50mM acetate buffer (pH 5.6) at 40^{o}C. The kinetic constants, K_m and V_{max}, were estimated using the linear regression method of Lineweaver and Burk (Lineweaver and Burk 1934).

2.5 Nematode parameters

Nematode parameters of *Meloidogyne javanica*, i.e., numbers of females, galls, egg masses, and % reduction were recorded. *In vitro* experiments were achieved as indicated by Abd-El-Fattah *et al.* (2013). Females of *M. javanica* were isolated as described previously (Abd-El-Fattah *et al.* 2013).

2.6 Influence of *B. alvei* NRC-14 on *M. javanica*

2.6.1 Effect of whole cells against eggs and larvae

Eggs and larvae of *M. javanica*, obtained from the Nematology Dept., were washed with sterile distilled water thrice and transferred to a pree 24-h old culture of *B. alvei* NRC-14, incubated at 28^{o}C for 120h, and tested for formation of reducing sugars (Naserinasab *et al.* 2011). Efficacy of whole cells of strain NRC-14 against egg mass and larvae of *M. javanica* was detected by growing the strain on nutrient broth in which the carbon source was replaced with nematode eggs or larvae (Abdel-Aziz 2013).

2.6.2 Effect of bioactive metabolites on eggs and larvae

In vitro synergistic activity of the crude supernatant (comprises a mixture of enzymes and metabolite bioactive compound), produced by *B. alvei* NRC-14 against nematode's egg and larvae, was determined. The concentrations of the initial enzymes and metabolite solutions used for this test were serially dilutions of 1:1, 1:3, 1:5, 1:7 with 50mM acetate buffer (pH 5.6). A mixture of equal volumes of acetate buffer and 80% ethanol was used as control (Wichitra *et al.* 2006).

3. RESULTS AND DISCUSSION

3.1 Preparation of extracellular metabolites

In the present study, biomass of the fungus *Mucor rouxii* was used as a low-cost carbon source for preparation of extracellular metabolites by strain NRC-14 (cell wall of this fungus contains mainly chitosan). In our previous study (Abdel-Aziz *et al.* 2012*b*), 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. In the present study, surprisingly, when the strain was grown at 40^{o}C using fungal biomass as carbon source, parallel formation of hydrolytic enzymes and bioactive compound (as indicated by zone of inhibition) were detected in the culture broth. Effect of carbon source revealed that, maximum secretion of reducing sugars was obtained with fungal biomass as a carbon source followed by flaked chitosan, soluble chitosan, and glucose (Fig. 2).

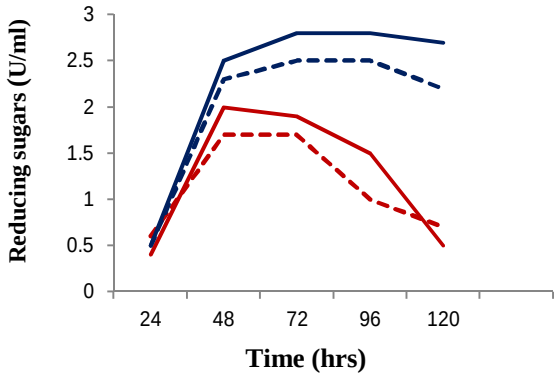

Fig. 2. Effect of carbon source: soluble chitosan (*Red solid line*) flaked chitosan (*Blue dashed line*); fungal biomass (*Blue solid line*); or glucose (*Red dashed line*), on production of reducing sugars by strain NRC-14 when grown at 40°C.

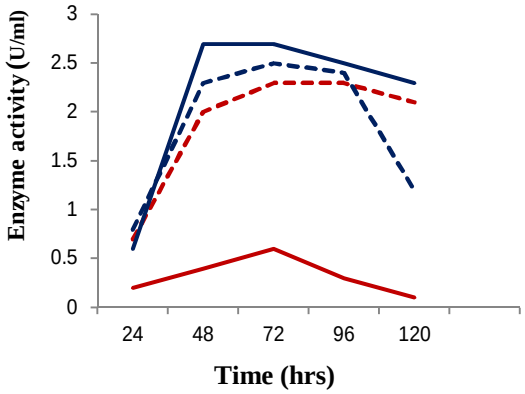

Fig. 3. Lytic enzymes: chitinase (*Red solid line*), chitosanase (*Blue dashed line*), β-glucanase (*Blue solid line*), and protease (*Red dashed line*), produced by strain NRC-14 when grown with fungal biomass as a carbon source at 40°C.

On other hand, time course for production of enzymes with fungal biomass was estimated. As shown in Fig. 3, lytic enzymes such as chitinase, chitosanase, β-glucanase, and protease were detected in the culture broth of strain NRC-14. Thus, in this study, fungal biomass is found to be the best carbon source for production of enzymes and secondary metabolite(s). 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. Glycerol was also found to be a very important medium component for the production of antifungal antibiotics from microorganisms (Fukuda *et al.* 2005, Siti *et al.* 2011). It could be concluded that, formation of enzymes and secondary metabolite(s) by strain NRC-14 was often stimulated by slowly assimilation of complex carbohydrates (flaked chitosan and fungal biomass) from the productive media, whereas with glucose as a carbon source, more easily constitutive utilization of this monosaccharides resulted in formation of chitosanase (Abdel-Aziz 1999). On other hand, although accessibility of soluble chitosan as an easier substrate for enzymatic degradation, it was noticed that, strain NRC-14 degraded flaked chitosan and fungal biomass more effectively (Fig. 2). However, when grown with glucose as a carbon source, the strain produced high levels of chitosanase (Fig. 2). These results indicated that, strain NRC-14 could, constitutively, produces a wide range of carbohydrate-active enzymes and that it has a great affinity for the natural biopolymers such as flaked chitosan and fungal biomass.

Many factors may represent an important role in the production process of antimicrobial and anti-nematode compounds, and consequently affect the antagonistic activity against the microbial species (Siti *et al.* 2011). Carbon is an important ingredient in the medium to determine the parameter of a product. Furthermore, 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 nematicidal substances. When strain NRC-14 was grown with different carbon sources, maximum activities of lytic enzymes and bioactive compounds (inhibition zone) were detected before 48h of growth. Strain *B. alvei* NRC-14, as a soil isolate, was found to produce lytic enzymes (Abdel-Aziz *et al.* 2012*b*), polysaccharide bioflocculant (Abdel-Aziz *et al.* 2011*d*), and bioactive compound(s) effective for the biological control of fungal pathogens (Abdel-Aziz 2012). Metabolites produced by strain NRC-14 exhibited potential efficacy in

biocontrol either in liquid culture or on agar plates. Synergistic activity of lytic enzymes and bioactive compounds might serve as a tool to reduce the use of hazardous chemical fungi- and nematcides, and to reduce the impact of some chemical pesticides on animals. 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 (Haran *et al.*1996). On other hand, it was reported that, the temperature has a profound effect on activities of enzymes and antibiotics produced by the biocontrol species. Metabolism occurs by strain NRC-14 at 30°C differed at 40°C (Abdel-Aziz *et al*. 2012*b*).

3.2 Purification and specificity of enzymes

After the supernatant was applied to sephadex G-100 column, the purification procedure yielded three protein peaks (Fig. 4) corresponding to three enzymes: Enz-1, Enz-2, and Enz-3. The enzyme, CHS-2, showed the highest activity (Fig. 4). Enz-2was found to be specific toward chitosan, Avicel, and carboxymethyl-cellulose (CM-C), whereas Enz-1 and Enz-3 exhibited great activity toward Avicel and CM-C. The other two enzymes showed lower activity, therefore, Table 1, represented only the purification scheme of Enz-2. As shown, the enzyme activity was purified about 10-fold with an overall yield of 23%. The final specific activity was approximately 31 U/mg.

Table 1. Purification steps of Enz-2 produced by *Bacillus alvei* NRC-14

Step	Total activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Purification fold	Yield (%)
Culture broth	1220	340	3.6	1	100
0-70% $(NH_4)_2SO_4$	980	33	29.7	8.3	80
Sephadex G-100	460	3.5	131.4	36.5	38

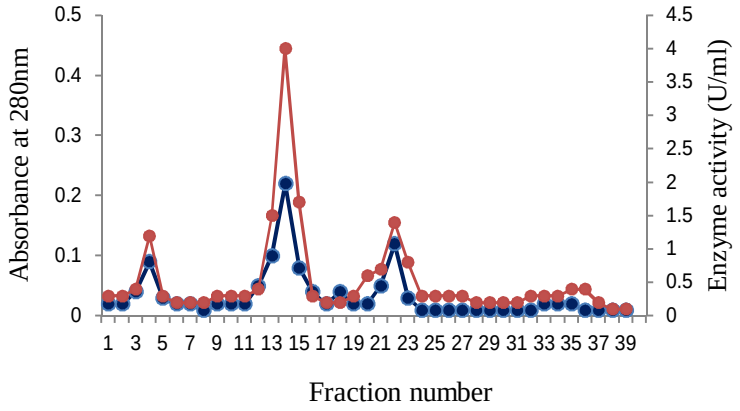

Fig. 4. Sephadex G-100 chromatography of the partially purified enzymes produced by strain NRC-14. Absorbance at 280 nm (●); enzymes activity (■).

3.3 Kinetic pararameters

The kinetic parameters K_m and V_{max} values were determined from Lineweaver-Burke plot of Enz-2activity at 40°C using various concentrations of soluble chitosan as substrate. The K_m of Enz-2 was 1.218 mg/ml, and the V_{max} was 0.5 µmol/min/mg (Fig. 5). Pelletier and Sygusch (1990) reported that, the K_m and V_{max} values of a chitosanase from *Bacillus megaterium* P1 were 0.8 mg/ml and 280 U/mg, respectively. Jo *et al*. (2003) reported the values of 0.52 mg/ml and 7.71×10^{-6} , respectively, for K_m and V_{max} of a chitosanase from *Bacillus* sp. P16, whereas, the K_m and V_{max} values of a chitosanase from *Achatina fulica* were 0.154 µM and 0.005 µM/min (Sun *et al*. 2012). Kinetic studies were only undertaken for Enz-1 because it showed the higher activity and exhibited specificity towards chitosan. The reciprocal plot of the initial velocity data (Fig. 5) deviates from linearity at high substrate concentration, which is characteristic of a substrate-inhibited enzyme (Pelletier and Sygusch 1990).

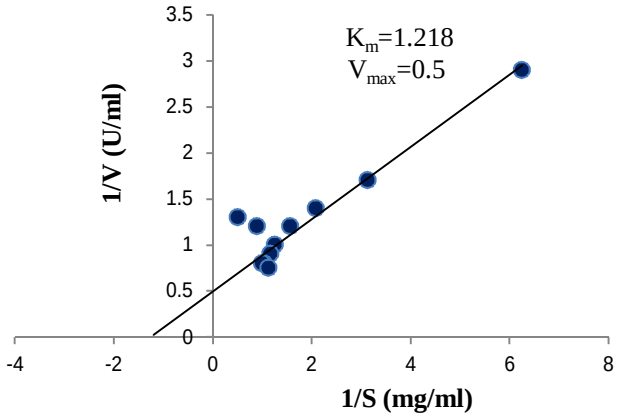

Fig. 5. Double reciprocal plot for determining the K_m and V_{max} values of Enz-2 against soluble chitosan at 40ºC.

3.4 Specificity of the purified enzymes

Some properties of Enz-1, Enz-2, and Enz-3 were studied (Table 2). Enz-1 was active towards chitosan, CM-cellulose, Avicel, and, whereas Enz-2 and Enz-3 exhibit great activities towards CM-cellulose, Avicel, and cellobiose. It is suggested that, Enz-2 may, probably be a chitosanase. On other hand, activity of the three enzymes on chromogenic substrates were estimated and the results revealed that Enz-1was found to hydrolyze *pNP-N*-acetylglucosaminide, *pNP-N*-acetylgalactosaminide, and *pNP*-glucopyranoside, whereas Enz-2 and Enz-3 displayed high activities only toward *pNP*-glucopyranoside (data not shown). These results indicated that, co-occurrence of enzymes such as chitinase, chitosanase, chitobiase, and cellobiase in the crude preparation of strain NRC-14 may confirm the important role of the strain in natural soils. Hydrolysis of *pNP-N*-acetylglucosaminide by Enz-1 could suggest an involvement of a random, endo-acting hydrolysis of glycosidic bonds, followed by an exo-acting type of enzyme, chitobiosidase (*N*-acetylglucosaminidase). In nature, such consecutive mechanisms would favor a rapid and complete degradation of chitin which is the main component in fungal cell wall and eggs of nematode. On other hand, complete degradation of chitin microfibrils by such enzymes producing monomers for nutrition of beneficial microflora in soil, and induction of further enzymes synthesis (Orikoshi *et al.*, 2005).

Table 2. Some properties of the partially purified enzymes produced by *Bacillus alvei* NRC-14

Characteristic	Enz-1	Enz-2	Enz-3
Optimum pH	5.0	6.0	5.0
Optimum temperature (ºC)	45	40	50
pH stability	5.5-7.0	5.0-6.3	7.0-9.5
Hydrolysis of:			
***pNP-N*-acetylglucosaminide**	-	+	-
***pNP-N*-acetylgalactosaminide**	-	+	-
***pNP*-glucopyranoside**	+	+	+
Substrate specificity:			
Chitin	-	++	-
Chitosan	-	++++	-/+
Avicell	+	+++	++++
CM-cellulose	+	+++	++++

3.5 Susceptibility of *M. javanica* eggs and larvae to different treatments

Efficiency of culture supernatant and whole cells of strain NRC-14 on inhibition of nematode eggs and larvae was investigated. *In vitro* tests showed that, both whole cells and culture metabolites of the strain exhibited potential effects in comparison with other treatments (Table. 3). As shown, reduction percentage in number of females, galls, and egg masses were 77, 45, and 62 %, respectively, by application of whole cells, whereas it reached 70, 52.4, and 58.6 % when the produced metabolites were applied. In comparison, reduction percentages were 46.7, 88.0, 48.3% and 53.3, 64.3, 55.2 % when antibiotics and chemical nematicides were applied, respectively. These results suggested that, enzymes or other bioactive compounds in the bacterial culture supernatant exhibit activity against specific stages in the nematode life cycle. It was concluded that natural substances produced by *B. alvei* NRC-14 are important factors involved in suppression of nematode damage.

Table 3. *In vitro* relative susceptibility of *M. javanica* eggs and larvae with different treatments.

Treatment	No. of females	Reduction* %	No. of galls	Reduction* %	No. of egg masses	Reduction* %
Untreated	30	-	42	-	29	-
Whole cells[a]	7	77.0	23	45.0	11	62.0
Metabolites[b]	9	70.0	20	52.4	12	58.6
Antibiotic	16	46.7	5	88.0	15	48.3
Chemical nematicides	14	53.3	15	64.3	13	55.2

*Reduction % = Untreated sample-treated sample / Untreated sample x 100. Whole cells[a], were used at A_{580}=0.25-0.3, equivalent to about $1x10^8$ cells/ml. Metabolites[b], concentration of reducing sugars in the crude metabolites is 3.4U/ml.

3.6 Influence of *B. alvei* NRC-14 on *M. javanica*

3.6.1 Effect of whole cells against eggs and larvae

When cells of the strain were grown with eggs and larvae high levels of reducing sugars were detected. Surprisingly, a dramatic influence was observed as indicated by the release of reducing sugars due to the degradation of nematode's eggs and larvae (Fig. 6A and B). Potential effect of secondary metabolites produced by the strain (at 40^{o}C) against eggs and larvae may, probably, be due to secretion of lytic enzymes and inhibitory substances (Abdel-Aziz *et al*. 2013*c*). Several studies have demonstrated that extracellular lytic enzymes and metabolites from *Bacillus* spp., showed a potential effect against parasitic nematode, acting alone or synergistically through degradation of the cell walls (Wichitra *et al*. 2006). Because nematodes often occur in high numbers in soil, it is not surprising that a wide variety of soil microorganisms exploit nematodes as food, i.e., as a source of carbon, nitrogen, and energy. Organisms that seek out and consume nematodes are called predators such as protozoa (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 (Ferris 1993). Interaction between plant-parasitic nematodes and other competitors may cause increased damage to a particular food source. In the present study, mechanisms of action include potential lytic enzymes and bioactive compound(s). Results against nematode by strain NRC-14 or its metabolites suggest that the enzymes and other active compounds in the culture supernatant 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 supernatant of the strain against nematode. However, effect of whole cells may be attributed to the ingestion by bacteria as well as to the dissolved metabolites (Jousset *et al*. 2006). Noteworthy is that, secretion of lytic enzymes and antimicrobial compound(s) by the strain when grown at 40^{o}C (Abdel-Aziz *et al*. 2013*c*) may indicate its capability to overcome the fluctuation conditions in soil during different seasons.

3.6.2 Effect of cultural metabolites on egg masses and larvae

Surprisingly, eggs were effectively affected by bacterial supernatants and the amount of reducing sugars increased by increasing the concentration, probably due to the potential effect of lytic enzymes in these metabolites in degradation of egg walls which contain mainly chitin (Fig. 7A). However, after denaturation of enzymes in the culture supernatant to examine the effect of other metabolite(s), an obvious positive effect against eggs was also observed (data not shown). Thus, it was not possible to distinguish between the contributions of the secondary metabolites produces by strain NRC-14. On other hand, different dilutions of culture supernatants were more effective against larvae of nematode and revealed a great role in the inhibition of larvae (Fig. 7B). 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 also be predictive under *in vivo* system to prevent nematode damage on plants (Abdel-Aziz 2012). *In vitro* tests by the strain or its metabolites, the variety of lytic enzymes, bioactive compounds, as well as its potentiality against plant pathogens and event effects under *in vivo* experiments (Abdel-Aziz 2012), undoubtedly, confirm that this strain play an important role as a biocontrol agent. Further tests regarding the strain against nematodes in soil is, however, essential in future. Successful management of parasitic nematode eggs and larvae depends on enzymes such as chitinases and chitosanase which are responsible for suppression of the parasite nematode life cycle. Possible mechanisms involved in biological control by biocontrol species have led to several alternative explanations, i.e., these enzymes function by breaking down the polysaccharide, chitin, which is a main component in eggs and responsible for the rigidity of cell wall, thereby destroying cell wall integrity.

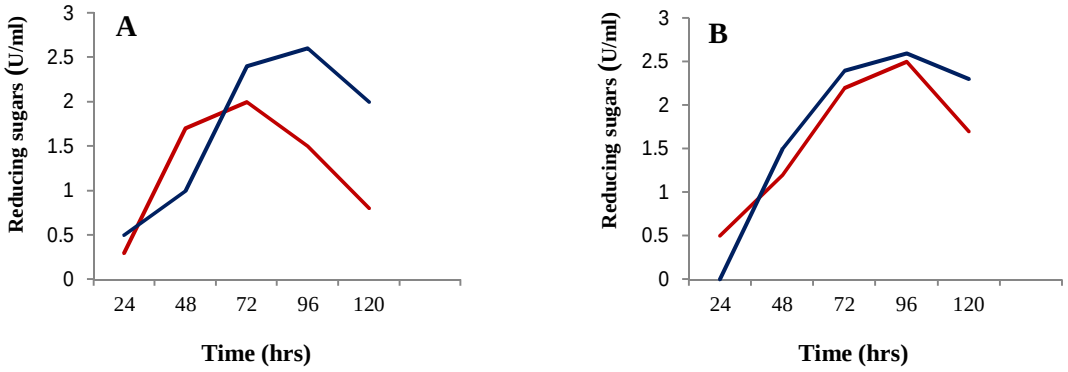

Fig. 6. Reducing sugars released by the action of strain NRC-14 against: eggs (*blue line*) and larvae (*red line*) when grown at 30°C (A) or 40°C (B).

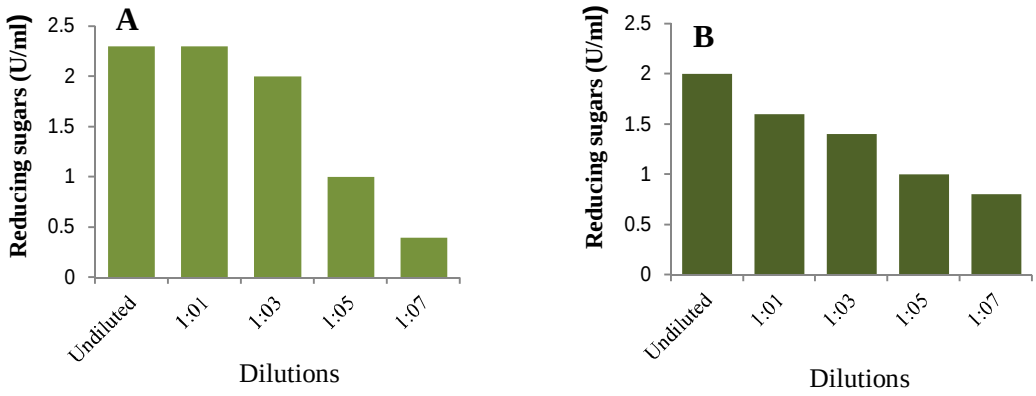

Fig. 7. Efficacy of metabolites (culture supernatant) produced by strain NRC-14 against eggs (A, ■) or larvae (B, ■) of *M. javanica* using different concentrations.

Thus, chitinase and chitosanase act as key enzymes in the lyses of nematode and enzymes from strain NRC-14 is of special importance in the defense mechanisms. Worthy mention is that, individual use of the three purified enzymes, i.e., chitinase, chitosanase, β-glucanase from strain NRC-14 showed lower effect in *in vitro* tests, in comparison with the crude metabolites, whereas a combination of the three purified enzymes, relatively, have a minor effect against eggs and larvae in comparison with the crude metabolites, suggesting potentiality of other substance(s) involved in these metabolites, which considering, however, more economically.

3.7 Multiple plant-growth promoting properties

In the previous work, when strain NRC-14 was used as a biocontrol agent against fungal-plant pathogens, an enhancement in shoot- and root-length, plant height, and fortifying plant stand and healthy were observed, in comparison with controls (Abdel-Aziz 2012). Such improvements may probably be an indicator for the plant-growth promoting properties of strain NRC-14 which resulted in enhancing plant systemic resistance (Shahi *et al*. 2011). 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 (Fig. 1). In the present study, the strain or its culture metabolites showed a potential influence against nematode eggs and larvae which may indicate an obvious role of the strain for protection of plant against phytopathogens and parasitic nematode. In fact, during the long-term of work with the strain, it was found to adapt with abiotic stress conditions and this may suggest a direct antagonism with the pathogens for essential nutrients and space. Rhizosphere competence is an important feature because a biocontrol agent can't compete for space and nutrients if it is unable to grow in the rhizosphere area. On other hand, strain NRC-14 was also found to be a good producer for siderophore and indole acetic acid (Abdel-Aziz 2012). Secretion of indole acetic acid promotes roots to grow directly and stimulating plant cell elongation, plant fortification, and healthy (Fig. 1). In the previous work for protection of tomato plant, a reduction in incidence of root wilt (94%) was observed, and the growth-promoting efficiency and biocontrol efficiency reached 180 and 151%, respectively (Abdel-Aziz 2012).

The antagonistic action of *Bacillus* spp. against phytopathogenic fungi and nematode, *in vitro*, has been reported in many studies. *In vivo* experiments by utilizing *Bacillus* spp. as antagonists are, however, inconclusive with respect to the efficacy of these microorganisms under field conditions (Choudhary and Johri 2008).

Conclusion

In soil ecosystems, bacteria must overcome any abiotic stress conditions. Strategies for development of lytic enzymes and antimicrobial substances may help bacteria maintain higher populations and persist longer in the soil. To be faced with nematode, bacteria can secrete many extracellular hydrolytic enzymes such as proteases and chitinases to digest and penetrate nematode/egg-cuticles and larvae. 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, it could be concluded that the strain:

1) revealed multiple plant growth-promoting properties and could be applied as an excellent plant-growth promoting agent;

2) provided evidence for antibiosis as a mechanism of antagonism because it causes significant antimicrobial effects;

3) produces an inhibitory substance, when exposed to a heat-shock, which with the lytic enzymes exhibit a synergistic effect against nematode' egg and larvae.

In nature, probably, these lytic enzymes and bioactive compounds produced by such strains could function as key fungicides and nematicides for biological control of plant diseases.

REFERENCES

Abdel-Aziz, SM. 1999. Production and some properties of two chitosanases from *Bacillus alvei.* J. Basic Microbiol., 39, 79-87.

Abdel-Aziz, SM. 2013. Extracellular Metabolites Produced by a Novel Strain, *Bacillus alvei* NRC-14: 5. Multiple Plant-Growth Promoting Properties. J. Basic Appl. Sci. Res., 3, 670-682.

Abdel-Aziz, SM., Gad-Allah, M. and Mouafi, F. 2004*a*. Recovery of chitin from crustacean wastes by proteolytic enzymes. New Egyptian J. Microbiol., 9:137-151.

Abdel-Aziz, SM., Moharam, M., Hamed, H., and Mouafi, F. 2012*b*. 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. 2013*c*. Amazing Abiotic Stress Response of a Novel Strain *Bacillus alvei* NRC-14 Isolated from Egyptian Soil. J. Basic Appl. Sci. Res., accepted for publication.

Abdel-Aziz, SM., Hamed, H., Mouafi, F., and Abdelwahed, N. 2011*d*. 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.

Abd-El-Fattah, A., Shalaby, N. and El-Nagdi, W. 2013. Impact of two bio-products on *Meloidogyne javanica* and their influence on yield components of some sugar beet varieties. J. Appl. Sci. Res., 9, 4627-4637.

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.

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.

Bird, A. and Bird, J. 1991. The structure of nematodes (S. Diego, CA, Academic Press).

Bird, D., Opperman, C. and Davies, K. 2003. Interaction between bacteria and plant-parasitic nematodes: now and then. Int. J. Parasitol., 33, 1269–1276.

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.

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.

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

Emmert, E. and Handelsman, J. 1999. Biocontrol of plant disease: a (Gram+) positive perspective. FEMS Micriobiol. Lett., 171, 1–9.

Frank, H., Jessica, E. and Rolf, D. 2005. Enrichment of chitinolytic microorganisms: isolation and characterization of a chitinase exhibiting antifungal activity against phytopathogenic fungi from a novel *Streptomyces* strain. Appl. Microbiol. Biotechnol., 66, 434-442.

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.

Hallmann, J., Quadt-Hallmann, A., Miller, W., Sikora, R. and Lindow, S. 2001. Endophytic colonization of plants by the biocontrol agent *Rhizobium etli* G12 in relation to *Meloidogyne incognita* infection. Phytopathol., 91, 415–422.

Jo, Y., Jo, K., Jin, Y., *et al*. 2003. Characterization and kinetics of a 45kDa chitosanase from *Bacillus* sp. P16. Biosci. Biotechnol. Biochem., 67, 1875-1882.

Jousset, A., Lara, E., Wall, L. and Valverde, C. 2006. Secondary Metabolites Help Biocontrol Strain *Pseudomonas fluorescens* CHA0 To Escape Protozoan Grazing. Appl. Env. Microbiol., 72, 7083–7090.

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

Lineweaver, L. and Burk, D. 1934. The determination of enzyme dissociation constants. J.Amr. Chemical Society, 56, 658–666.

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

Mankau, R. 1980. Biological control of nematodes pests by natural enemies. Ann. Rev. Phytopathol., 18, 415–440.

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

Naserinasab, F., Sahebani, N. and Etebarian, H. 2011. Biological control of *Meloidogyne javanica* by *Trichoderma harzianum* BI and salicylic acid on Tomato. Afric. J. Food Sci., 5, 276-280.

Orikoshi, H., Nakayama, S., Miyamoto, K., *et al*. 2005. Roles of four chitinases in the chitin degradation system of marine bacterium *Alteromonas* sp. Strain O-7. Appl. Env. Microbiol., 71, 1811-1815.

Pakeerathan, K., Mikunthan, G. and Tharshani, N. 2009. Effect of different animal manures on *Meloidogyne incognita* (Kofoid and white) on Tomato. World J. Agr. Sci., 5, 432-435.

Pelletier, A. and Sygusch, J. 1990. Purification and Characterization of Three Chitosanase Activities from *Bacillus megaterium* P1. Appl. Env. Microbiol., 56, 844-848.

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.

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.

Spiegel, Y., Cohn, E., Galper, S., Sharon, E. and Chet, I. 1991. Evaluation of a newly isolated bacterium, *Pseudomonas chitinolytica* sp. nov., for controlling the root-knot nematode *Meloidogyne javanica.* Biocontrol Sci. Technol., 1, 115–125.

Stirling, G. 1991. Biological Control of Plant Parasitic Nematode: Progress, Problems and Prospects. CAB International, Wallington, UK.

Sun, M., Zhu, Y., Guo, C., Su, L. and Xin, Y. 2012. A chitosanase purified from the snail of Achatina fulica. Afric. J. Biotechnol., 11. 10242-10247.

Tian, B., Yang, J. and Zhang, K. 2007. Bacteria used in the biological control of plant-parasitic nematodes: populations, mechanisms ofaction, and future prospects. FEMS Microbiol. Ecol., 61, 197-213.

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.

Wichitra, L., Pranom ,S. and Phongpaichit, S. 2006. Purification, characterization and synergistic activity of β-1,3 -glucanase and antibiotic extract from an antagonistic *Bacillus subtilis* NSRS 89-24 against rice blast and sheath blight. Enz. Microbial Technol., 38, 990–997.