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Biological Control of African Violets Root-Knot Disease: The Application Extracellular Protease *Bacillus*

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Abstract

The present study explored the efficacy of *Bacillus* spp. and protease production for biocontrol of the root-knot nematode Meloidogyne javanica in African violet media. Among 100 bacterial isolates from various soils, the highest nematode mortality was observed for treatments with isolate GM-18, which was identified as *Bacillus subtilis* based on cultural and morphological characteristics and 16S rDNA sequencing analyses. The *Bacillus* spp. showed nematicidal activity 74.01 to 28.33. Strain GM18 showed the most inhibitor zone in medium containing casein (13 mm) and nematicidal activity in our tests targeting *Meloidogyne javanica*. The effect of different variables such as time (1-24 h), pH (7- 8.5), and temperature (25- 37 °C) on the protease production was checked. The maximum enzyme production was noted when B. subtilis GM18 was grown on mineral medium and incubated at 31°C for 12 h with initial pH = 8. Three protease inhibitors that had the greatest influence on EDTA were studied. Protease produced by *B. subtilis* GM18 belonged to different groups.

Keywords: African violet media, *Bacillus*, Biological control, Root-knot disease.

INTRODUCTION

The total global agricultural damage caused by plant-parasitic nematodes is estimated to be 100 billion USD per year, among which the most important are root-knot nematodes (Oka *et al.,* 2000). Root-knot nematodes are distributed globally, infecting more than 2,000 plant species and reducing global crop yields by about 5%, mainly through root-knot gall formation and nutritional deprivation (Jiyeong *et al.,* 2014).

African violets infected with root-knot nematodes are stunted and weakened. Galls form on the roots, and the crown and leaves become thickened and distorted. Blisterlike galls also develop on the leaves and destroy all infected plants (Steinegger *et al.,* 1974). Root-knot nematodes are commonly controlled by chemicals such as soil fumigant and non-fumigant nematicides; however, they have failed to achieve full control of root-knot nematodes due to the soil-borne nature of these nematode pests. In addition, chemical methods are very toxic to humans, animals, and sometimes even plants, and can cause soil and water pollution and so, new nematicide production techniques are urgently required (Oka *et al*., 2000). Abbasi *et al.* (2014) reported that *Pleurotus* spp. killed the root-knot nematode on African violet after only a short period of exposure to their hyphae. Nematodes were immobilized once they approached the fungal colony. Limited numbers of bacterial species have been reported as biological control agents for root-knot nematode disease. Some bacterial species with nematicidal activity has been used with some success for controlling root-knot diseases including *Streptomyces* spp., *Serratia* spp., *Bacillus* spp., *Azotobacter chroococcum, Rhizobium, Corynebacterium* and *Pseudomonas* (Rahanandeh and Moshaiedy, 2014). B. megaterium was reported to reduce the penetration of both *M. chitwoodi* and *Pratylenchus penetrans* in potato by 50% (Rahanandeh *et al*., 2013).

Pasteuria spp. could parasitize most plant-parasitic nematodes, but their potential field applications are hampered because the bacteria have not been successfully cultured (Huang *et al.*, 2005). They affect nematodes by a variety of modes, for example parasitizing, producing toxins, antibiotics, or enzymes, interfering with nematode–plant-host recognition, competing for nutrients, inducing systemic resistance of plants, and promoting plant health (Siddiqui and Mahmood, 1999). Some parasporal crystal toxins from *Bacillus thuringiensis* have been manifested to infect larvae and eggs of plant-parasitic nematodes (Wei *et al*., 2003). In the molecular mechanisms of nematophagous fungi infecting their hosts, it was suggested that hydrolytic enzymes participate in several steps of host infection (Huang *et al.*, 2004). Moreover, ultrastructural and histochemical studies have revealed that the penetration of the nematode cuticle involves the activity of hydrolytic enzymes (Jansson and Nordbring-Hertz, 1988).

Microbial proteases have been proposed as virulence factors in their pathogenesis against nematodes. The most compelling evidence to support microbial proteases as virulence factors has come from the studies of protease-deficient mutants (Ahman *et al.*, 2002; Siddiqui *et al.*, 2005; Tian *et al.*, 2006). Some extracellular proteases have been detected and partly characterized from a few nematode-trapping fungi, as well as from endoparasites of cyst nematodes (Tunlid *et al*., 1994; Leger *et al.*, 1999). Results of the histopathological analysis revealed that extracellular proteases played key roles during the bacterial breaching of the proteinaceous outer cuticle and the killing of the nematode hosts (Huang *et al.*, 2005; Tian *et al*., 2007). For example, studies on the bacterial proteases of *Brevibacillus laterosporus* G4 serving as pathogenic factors in nematode infection used reverse genetics methods (Huang *et al.*, 2005; Tian *et al*., 2007). Siddiqui *et al.* (2005) also demonstrated that the deletion of a major extracellular protease from *Pseudomonas fluorescens* CHA0 reduced bacterial activity against the root-knot nematode *Meloidogyne incognita*. These studies suggest that extracellular proteases might play important pathogenic roles in suppressing nematode *M. incognita* in the soil (Siddiqui *et al.*, 2005).

The present study describes the isolation of a *Bacillus* sp. with a remarkable ability to kill nematode *Meloidogyne javanica* from the soil. The bioassay experiments of the bacteria showed that the extracellular cuticle-degrading proteases were involved in the processes to penetrate into

the cuticle and eventually digest them. These experiments also described the best conditions to produce more protease by *Bacillus subtilis*. This is the first report of application *B. subtilis* for production protease and biocontrol agent against root-knot nematode in Iran.

MATERIALS AND METHODS

Isolation and identification of rhizobacteria with nematicidal activities

Bacteria were isolated from the rhizosphere nematode-infected Guilan province in Iran. To isolate these bacteria, roots were washed in 0.1 mol 1^{-1} phosphate buffer, and appropriate dilutions were plated on NA and incubated at 28 °C for 2 days (Fang, 1998).

Bacterial suspensions were prepared by adding a mass colony of each bacterium to 100 ml of nutrient broth. The latter were allowed to grow on shaking for 48 h at 28 ºC. The cultures were then centrifuged at 5000 rpm for 15 min and the supernatants were collected and evaluated for anti-nematicidal activities against *Meloidogyne javanica.* To perform the test, a total of 30 *M. javanica* active juveniles were added into each 1 ml bacterial supernatant and incubated at 31ºC for 48 h. Sterilized distilled water was used as control. The experiment was conducted in a completely randomized design in three replicates and following formula was used to calculate the percentage of nematode juvenile mortality, normalized with controls.

Mortality (%) $=[C_1-C_2]/C_1\times 100$

where, C_1 is the number of live nematodes juveniles in control treatments and C_2 is the number of live nematodes juvenile counted in other treatments (Li Bin *et al.,* 2005).

The nematicidal bacteria were classified based on their morphological and biochemical characteristics and their phylogenetic analysis based on 16 S rDNA sequences amplified by polymerase chain reaction (PCR) using universal primers|(P1/F 5' –AGA GTT TGA TCC TGG TCA GAA CGC T–3' and P6/R 5' –TAC GGC TAC CTT GTT ACG ACT TCA CCC C –3'). Candidate bacterial were stored in 30% glycerol at -20°C for further assays.

Screening for protolithic activity

The sample culture was spread on casein agar medium containing casein 2.0%, peptone 0.5% and agar 1.5% and then incubated at 37°C for 24 h. The clear zone of casein hydrolysis was an indication of protease secretion as reported by Folasade *et al.* (2005). The isolates were selected on the basis of the larger zone on casein agar medium and further confirmed through batch wise submerged fermentation and the best one was selected for further study.

Mass production of protease through fermentation

The culture media used for mass production of protease contains dextrose 1% (w/v), peptone 0.5%, KH_2PO_4 0.2%, $MgSO_4$. $7H_2O$ 0.2%, casein 1% and pH = 8. It was maintained at 28°C for 48 h in a shaking incubator. After inoculation, fermentation was carried out at 32°C at 200 rpm for 48 h. At the end of each fermentation period, the whole culture broth was centrifuged at 10,000 rpm at 4°C for 15 min to remove the cellular, and the clear supernatant was used in enzyme preparation (Olajuyigbe and Ajele, 2005). The enzyme was purified by ammonium sulfate precipitation method.

Effect of pH and on temperature protease production

The effect of pH on protease production from B. subtilis under study was carried out using different pH's, such as 7- 8.5. The optimization media with the above pH were inoculated with the test sample and the protease assay was done after 24 h. The best pH was concluded by reading the absorbance at 680 nm.

The effect of temperature on protease production was studied by taking various temperatures

like 25°C, 31°C and 37°C. The optimization media was inoculated with the test sample at different temperatures and the protease assay was done after 24 h.

Characterization of proteases and bioassays

The effects of various inhibitors [phenylmethylsufonylfluoride (PMSF), EDTA and 1,10-phenanthroline] on the protease activity were examined by incubating the enzyme for 5 min at 37 °C and pH = 7.0 with these inhibitors. The residual proteolytic activity was measured as a percentage of that in the control without inhibitors.

In a bioassay, approximately 200 nematodes were added to protease (activity units: 930 U ml^{-1} at room temperature, pH = 7.0). After incubating the tubes for 2–10 h, the dead nematodes in each treatment were counted under a light microscope. The experiments were performed in triplicates and repeated at least three times. Controls were incubated with water, medium and protease boiled for 15 min. All the data were analyzed by the independent samples test ($P < 0.05$) using SAS software package.

RESULTS

Identification of nematicidal rhizobacteria

A total of 150 bacterial strains were isolated from the rhizosphere using the spread-plating technique on NA medium. Of these strains, nine showed nematicidal activity in the preliminary tests targeted towards *M. javanica.* According to the morphology tests, four *Bacillus* strains (GM18, GM48, GM23, GM50) were identified among these nine strains. The Bacillus spp. showed a range of nematicidal activity (Table 1). Most of the bodies and cuticles for the dead nematodes were degraded and destroyed by these strains. As shown in Table 1, strain GM18, which was identified as *Bacillus* sp., showed the most nematicidal activity in our tests targeting *M. javanica*. In order to identify GM18 strain, the internal transcribed spacer region of the 16S rDNA was amplified and sequenced.

The sequences for 16S rDNA of strain presented 82% similarity with the 16S rDNA sequences of *B. subtilis* in NCBI.

Screening for proteolytic activity

All isolates showed significant inhibitor zone (Table 2). Among them, strain GM18 exhib-

Table 1. The nematicidal activities of some antagonistic bacteria and mortality percentage of juvenile.

Bacterial strain name	Mortality (%)	Significance
GM18	74.01	A
GM48	61.95	B
GM23	32.87	C
GM50	28.33	C
Control (distilled water)	15.63	D

Data are means of three replications. Values followed by the same letters in each column are not significantly different (α= 0.05).

Table 2. Determination of zone of proteolysis by isolated strains.

Isolates	Average zone of inhibition (mm)
GM18	13
GM48	5.3
GM23	4.2
GM50	4.5

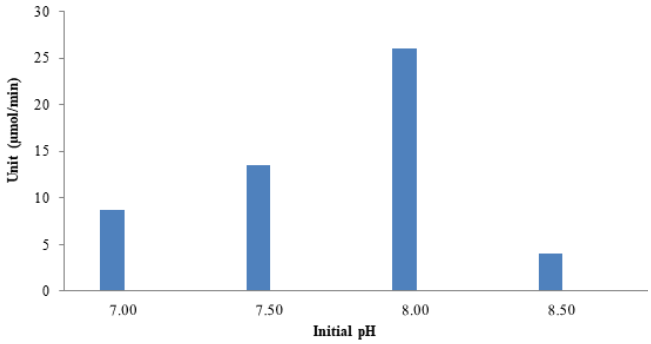

Fig.1. The effect of different pH values on protease activity.

ited maximum clear zone of proteolysis. This strain was used for further studies.

Effect of pH on protease production

Medium was adjusted to the required pH with the addition of 0.1 N HCl or 0.1 N NaOH. Protease synthesis was increased with the increase in initial pH of medium and reached maximum at pH = 8, whereas below and above this level of pH, yield was lower (Fig. 1)

Effect of temperature on protease production

Protease production was investigated in a temperature range of 26 to 37°C by *B. subtilis* when grown on mineral medium and incubated for 24 h with initial pH = 8. Maximum proteases activities were produced at 31°C (Fig. 2).

Effect of time on protease production

Maximum protease activity for protease produced by *Bacillus subtilis* strain occurred after

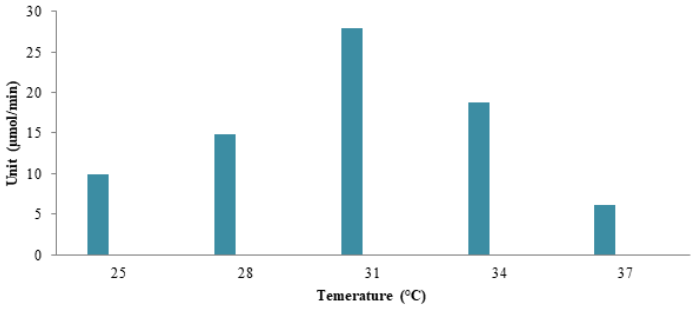

Fig.1. Fig. 2. The effect of different temperatures on protease activity.

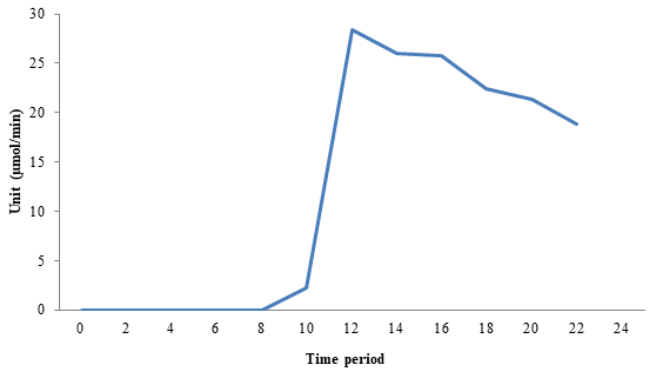

Fig. 3. Effect of incubation time on protease production.

12-h incubation period (Fig. 3).

Bioassays for protease

In the bioassay, *B. subtilis* protease killed 52.86 %, 62.23 %, 69.88 %, 72.42 %, 73.89 % and 74.01 % after 2 h, 4 h, 6 h, 8 h, 10 h, and 12 h, respectively. Under the microscope, we noticed that the cuticles of nematodes were degraded and destroyed by the proteolytic action.

Inhibitors EDTA, PMSF and 1,10-phenanthroline reduced the enzyme activity by 11%, 32% and 35% compared to the control, respectively. Protease was caused death 74.01% nematodes, on other hand, in present EDTA died only 6% nematodes. Due to the greatest effect inhibitor EDTA seems to be a combination of protease produced is more of metalloprotease.

DISCUSSION

At present, a number of commercial biocontrol products have been developed from rhizobacteria and many plant disease biocontrol products containing *Bacillus* spp. have been used (Gardener, 2004; Schisler *et al.*, 2004). Native populations of *Bacillus* spp. occur abundantly in most agricultural soils. Multiple *Bacillus* spp. can promote crop health in a variety of ways. For example, they can suppress plant pathogens and pests by producing antibiotic metabolites, or can directly stimulate plant host defenses prior to infection and promote plant growth and health. Despite a wealth of new information on the genetics and physiology of Bacillus and related species, the increased understanding of the nematotoxic mechanism of antagonist populations in the soil could potentially enhance the value of these species as effective biocontrol agents (Morton *et al.*, 2004).

The present study focused on investigating the action mode for Bacillus spp. among the nematicidal rhizobacteria. Four *Bacillus* spp. isolated from root-knot nematode-infested rhizosphere soil were identified. Folasade *et al*. (2005) identified *B. subtilis, B. licheniformis, B. cereus and B. megaterium* in soil isolations. In another study, Watanabe and Hayano (1993) identified 29 isolates as *B. megaterium* and 24 isolates as *B. subtilis* out of 306 soil samples. These agree with the results of this study that *Bacillus* genera are widespread among bacteria in soil. Among these strains, *Bacillus subtilis*. strain GM18 showed a remarkable nematicidal activity. GM18 strain killed 74.01% of the tested nematode in 12 h. Compared to the *Pasteuria* sp., an obligate bacterial parasite of plant-parasitic nematodes, *B. subtilis* strain GM18 is easy to culture *in vitro*. Laboratory test revealed that all bacteria in media containing is casein protease production. The largest clear zone was created by *Bacillus subtilis*.

It is well known that the cuticle of nematodes is rigid and is composed of proteins and chitins (Cox *et al*., 1981; Ahman, 2000). The results suggested that the hydrolytic enzymes might be involved in the penetration process to help bacteria kill the hosts (Ahman, 2000). Our further analysis of nematotoxic factors indicated that an extracellular cuticle-degrading protease was an important nematicidal factor.

Effect of time course on biosynthesis of protease by *B. subtilis* was checked and data are presented in Fig. 3. The maximum protease secretions were noted after 12 h of incubation at 31°C. Wellingta *et al.* (2004) have reported that the maximum protease production was achieved in 9 h using thermophilic *Bacillus* sp. On the other hand, Qureshi *et al.* (2011) reported *B. subtilis* most protease production after 6 hours inoculation at 37°C.

Protease synthesis was increased with the increase in the initial pH of medium and the highest level was achieved at pH = 8, whereas below and above this pH level, yield was lower. Qureshi *et al*. (2011) reported the protease production by *B. subtilis* at pH = 8.5, Prakasham *et al.* (2006) at pH = 9.0 by Bacillus sp., Ul-hang and Mukhtar (2006) at pH = 9.0 by B. subtilis, Folasade *et al.* (2005) at pH = 8.0 by *Bacillus* sp., and Moreira *et al.* (2003) at pH = 8.0.

Maximum proteases activities were produced at 31°C (Fig. 2). Many investigators have studied the correlation between protease secretions with temperature but this depends on the type of organism and culture conditions. Temperature affects all physiological activities in a living cell

and it is an important environmental factor to control the growth, microbial activities, normal functioning of enzyme and many enzymes controlling the nutritional requirement of the cell and subsequently its composition (van Demark and Batzing, 1987). Al-Shehri *et al.* (2004) reported the maximum protease secretion at 50°C with *B. licheniformis,* Chi *et al*. (2007) at 45°C with yeast A. pullulans, Camila *et al.* (2007) at 50°C with thermophilic *Bacillus* sp. and Qureshi *et al.* (2011) at 45°C with *B. subtilis.*

Data presented the effect of different inhibitors on protease activity. A remarkable decrease was observed in EDTA versus control. EDTA and 1,10-phenanthroline are metal ions chelators, which can chelate Ca ions, and are recognized as an inhibitor of metalloproteinases. The addition of EDTA to the supernatant inhibited (89%) its proteolytic activity. The effect of this inhibitor also significantly decreased the mortality rate of nematodes. Similar results have been reported by B. laterosporus, *B. amyloliquefaciens* and *B. subtilis* (Lian *et al.,* 2007).

Our findings were consistent with the findings of Haddar *et al*. (2009) who found that phenylmethylsulfonyl fluoride (PMSF) inhibited protease activity of protease produced by Bacillus stearothermophilus, Bacillus sp. NG 312 and *Bacillus* sp. I-312. This protease inhibitor is effective on serine protease.1,10-Phenanthroline was less effective than other inhibitors of the protease secreted by *B. subtilis*.

The consistency of these nematotoxic proteases from the different nematicidal bacterial strains suggested that these proteases must be highly conservative in this group of bacteria (Huang et al., 2005). The wide distribution of the serine cuticle-degrading protease in nematophagous fungi and Bacillus suggested that they must serve as an important nematicidal factor in balancing nematode populations in the soil (Huang *et al.*, 2005; Ahman 2000; Niu *et al*., 2006; Tian *et al.*, 2006).

CONCLUSION

The results obtained in this study show that *B. subtilis* is a good producer of extracellular protease. The parameters of temperature, time and pH were effective on the production of protease enzymes. This might be an indication that the Bacillus species would produce proteases which could find applications in agriculture biological control.

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