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Screening for Nematicidal Activities of *Bacillus* Species Against Root Knot Nematode (*Meloidogyne incognita*)

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Author's contribution

The only author performed the whole research work, made the statistical analysis, wrote the first draft of the paper, read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: The aim of this study is to screen for nematicidal activities of *Bacillus* species against root knot nematode (M*eloidogyne incognita*).
Study Design: Randomized complete block design was used for this study
Place and Duration of Study: This work was done in Research and Development Division, Malaysian Pepper Board in January 2011 to December 2012.
Methodology: In this study, bacteria were isolated from pepper farm, the use of these strains was evaluated to control root knot nematode *in vitro* and the effectiveness of the antagonistic strains in greenhouse condition was investigated.
Results: Four rhizobacteria with nematicidal activity were isolated. Among these strains, nematotoxicities of *Bacillus* strains were intensively analyzed. *Bacillus* spp strains MPB04 and MPB93 showed remarkable nematicidal activity of 76.4 and 50.6%, killed tested nematodes within 2 h and completely destroyed tested nematode within 12 h. The results also showed that nematicidal activity displayed by *Bacillus* strains is related with their proteolytic activity. The pot trial also revealed that the application of *Bacillus* strain MPB04 and MPB93 reduced the root population of *M. incognita* by 60.95 and 35.28%, respectively over control. This indicated that these bacterial isolates could reduce the ability of *M. incognita* to reproduce in soil.
Conclusion: The coherence of results by chemical, genetic and greenhouse analysis has further strengthened the hypothesis that nematicidal activity displayed by *Bacillus* strains is

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related with their proteolytic activity responsible for nematode penetration by bacteria strains.

Keywords: Bacillus; Meloidogyne incognita; protease; nematicidal activity.

1. INTRODUCTION

Root knot nematode (*Meloidogyne incognita*) is a major constraint to successful pepper production in all pepper producing countries; it causes severe damage that leads to dramatic yield losses [1]. At present, application of chemicals and the use of host resistance are two major strategies for management of root knot nematodes. Unfortunately, based on recent research conducted [2], no resistant pepper cultivars to root knot nematodes are available. Currently, control of root knot nematodes has been primary accomplished through chemical nematicides [3]. However, due to the significant drawbacks of the chemical control including threats to human health and the environment, as well as limited availability in developed countries, biological control is therefore one of the attractive and alternative to synthetic fungicide due to its ability to antagonize the nematodes by different mode of action.

The mechanisms of biological control of nematodes by antagonistic bacteria and fungi have been used as the subjects of many studies in the past two decades [4]. Among these rhizobacteria, several strains of *Bacillus subtilis* have been reported to be promising candidates with proven excellent characteristics. Examples are effective root colonization, versatile activity against multiple nematodes and promising ability to sporulate [5,6]. The most thoroughly studied *Bacillus* includes *Bacillus subtilis* and *Bacillus thuringiensis* [7]. *B. thuringiensis* has drawn many researchers' attention due to its availability to produce one or more parasporal crystal inclusions (Cry or δ-enotoxin). Additionally, a number of studies have also been reported that direct antagonistic effects of other bacteria to pathogenic nematodes belonged to the genera *Heterodera* and *Meloidogyne*. These bacteria species include *Bacillus amyloliquefaciens, Bacillus cereus, Bacillus megaterium* and etc [8,9].

The mechanisms involved in biological control affecting root gall development, egg hatching or nematode survival were either directly through the production of toxic metabolites or indirectly by induction of systematic resistant in plant. In most cases, the biological mechanism consists of production of metabolites such as protease, chitinase, glucanase, antibiotics or antimicrobial peptides of low molecular weight [10]. Currently, several papers have reported that microbial proteases have been proposed as virulence factors in their pathogenesis against nematodes. The most compelling evidences to support microbial proteases as virulence factors come from those studies of protease-deficient mutant [11,12]. Besides, Siddiqui and Shaulat [13] also demonstrated that the deletion of major extracellular protease from *Pseudomonas fluorescens* CHAO reduced bacterial activity against the root knot nematodes. These entire research findings suggested that extracellular protease might play an important role in suppressing nematodes in soil.

However, no attempt has been made to control *M. incognita* infection in pepper vine using *Bacillus* strains as biological agents. In this paper, we address the isolation of bacteria from pepper farm, evaluate the use of these strains to control root knot nematode and investigate the effectiveness of the antagonistic MPB04 strain in greenhouse condition. The objectives of this study include the selection of potential bacteria that control the root knot nematodes and to determine their potential mechanism of action.

2 MATERIALS AND METHODS

2.1 Isolation of Rhizobacteria with Nematicidal Activities

Bacteria were isolated from the rhizosphere of pepper in five root knot nematode-infested farms in Malaysia (Serian, Betong, Julau, Johor and Melaka). To isolate these bacteria, roots were washed in 0.1 M phosphate buffers, and appropriate dilution was placed on nutrient agar and incubated at 30ºC for 2 days. Nematicidal activities of isolated strains were tested according to the methods described below using the free-living nematode *M. incognita* as the target nematode. The nematicidal bacteria were identified by using 16S rRNA sequences method. Candidate *Bacillus* species were stored in 30% glycerol at −20ºC for further assay.

2.2 Genomic DNA Extraction

A total DNA miniprep procedure was used to isolate cellular DNA from the bacterial isolates. 1.5 ml of a late log-phase culture was pelleted. The cell pellet was resuspended in 567 µl of TE buffer and 30 µl of 10% sodium dodecyle sulphate (SDS). 3 µl of proteinase K (20 mg/ml) was added to each cell suspension and the mixture was incubated for one hour at 37ºC. Following incubation, 100 µl of 5 M NaCl and 80 µl of cetyltrimethylammonium bromide (CTAB)/NaCl were added. The mixture was then re-incubated at 65ºC for 10 min. An equal volume of chloroform: isoamyl: alcohol (24:1) was added, and the entire solution was centrifuged for 5 min. The supernatant was transferred to a fresh tube, and an equal volume of phenol:chloroform: Isoamyl alcohol (25:24:1) was added. Following centrifugation for 5 min, 0.6 volume of isopropanol was added, and the solution was mixed until the DNA precipitated. The supernatant was discarded, and the pellet was washed with 70% ethanol. The suspension was then centrifuged, the supernatant discarded, and the pellet was allowed to air-dry before being resuspended in 100 µl of TE buffer. These genomic DNA preps were stored at –20ºC until used. The DNA concentrations and purity were then measured using a spectrophotometer based on absorbance at wavelengths of 260 and 280 nm (A_{260} and A_{280}). The purity of the DNA was estimated based on the ratio of 260 nm//280 nm. A ratio of ≥ 1.8 indicated that the DNA is free from contaminating protein.

2.3 16S rRNA Analysis

All isolates of *Bacillus* species were taken up for 16S rRNA-polymerase chain reaction (PCR) amplification. Genomic DNA was amplified by mixing the template DNA with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase chain reaction was performed in a total volume of 100 µl, containing 78 ul deionized water, 10 µl 10× Taq pol buffer, 1 µl of Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1 µl of template DNA, 1.5 µl of 100 mM P1 forward primer (5′-GGTTACCTTGTTACGACTT-3) and P6 reverse primer (5′-AGAGTTTGATCCTGGCTCAG-3′). PCR was programmed with an initial denaturing at 94ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 59ºC for 1 min and extension at 70ºC for 2 min and the final extension 70ºC for 10 min in thermocycler (Eppendorf). Amplified products were finally separated by electrophoresis on 1.2% agarose gel stained with ethidium bromide and visualized under ultraviolet light. Desired products were eluted from gels using the gel extraction kit (Qiagen Inc.) and finally sent for sequencing at FIRST BASE Laboratories Sdn Bhd. Nucleotide sequences were identified using the basic local alignment search tool (BLAST) and GenBank nucleotide data bank from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/).

2.4 Nematidical Activity of *Bacillus* Strains

All of the isolated rhizobacterial strains were inoculated into 250 ml Erlenmeyer flasks containing 50 ml YPD (1% yeast extract, 2% peptone, 2% glucose, pH 7.0) medium each and grown at 30ºC, with rotary shaking at 150 rpm for three days. After centrifugation at 8,500 *g* for 15 min, the culture supernatants were collected for the measurement of nematicidal activity. In bioassay, approximately 200 nematodes were added to 300 µl culture supernatants in a 1.5 ml Eppendorf tube containing two antibiotics (50 µg ml^{-1} ampicillin and 30 µg ml^{-1} kanamycin). After incubating the tubes at 30ºC for 2 to 10 h, the numbers of dead nematodes (immobile nematode) 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, YPD medium and the culture supernatant was boiled for 15 min. All the data were analyzed by the independent samples test ($P = 0.05$ or $P = 0.01$), using procedures of the Statistical Package for Social Sciences (SPSS, version 10·0 for Windows, SPSS Inc., Chicago, IL, USA). Standard error (SE) was recorded.

2.5 Characterization of Bacterial Isolates

2.5.1 Detection for HCN production

Production of hydrogen cyanide (HCN) was observed according to the method of Castric and Castric(1980) [14]. Freshly grown cells were spread on Lysogeny broth (LB) medium containing glycine (4.5 g/L). A sterilized filter paper saturated with 2% solution of sodium carbonate and 1% solution of picric acid was inserted in the upper lid of a Petri plate. The Petri plate was sealed with parafilm and incubated at 30ºC for four days. A change in colour of the filter paper from yellow to reddish brown was used as an index of cynogenic activity.

2.5.2 Detection for chitinase production

Chitinases were detected on chitin agar medium as described by [15]. Bacterial strains were streaked on this medium**.** Plates were incubated at 37ºC. After five days of incubation, plates were checked. Formation of a hollow zone around the spot indicates chitinase production.

2.5.3 Detection of protease production

Protease production was tested as described by [16] on Skim milk agar medium that contained 0.1% glucose, 0.2% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.5% skim-milk (skim-milk was sterilized separately). The pH of the medium was adjusted to 7 by addition of 10% Na_2CO_3 solution after sterilization. Inoculated plates were incubated for 72 h at 30ºC. Formation of a hollow zone around the spot indicates protease production.'

2.5.4 Detection for Cellulase production

Cellulase production was tested as described by [17] on carboxymethylcellulose (CMC) plates. Bacterial strains were streaked on this medium. Plates were incubated at 37ºC. After three days of incubation, the CMC plates were flooded with Gram's iodine. The formation of sharp and distinct zone around the cellulase-producing microbial colonies within 3 to 5 min indicates cellulase production.

2.6 Nucelotide Sequences Analysis and Protease Gene Identification

Genomic DNA of *Bacillus* species were extracted by using total DNA miniprep procedure and stored in TE solution at −70ºC for further identification of protease genes. Primers used for the detection of protease encoding genes are presented in Table 1.

Table 1. Specific primer sequence used in this study

Protease encoding gene	Primer	Primers sequence (5'-3')	Product length (bp)	Reference
Neutral proteases	npr1 npr2	GGGGGATTTATTGTGGGTTT TACAATCCGACAGCATTCCA	1577	[18]
Alkaline protease gene	apr1 apr2	GCGCCTAGGGTGAGAGGCAAAAAAGGTATG CGCGGATCCTTACTGAGCTGCCGCCTGTAC	1149	[18]

PCR amplification was carried out in 50 µl reaction mixtures containing PCR buffer (Qiagen Inc.), 1.5 mM $MgCl_2$, 1.5 U Taq DNA polymerase (Qiagen Inc.) 40 µg of each forward and reverse primer, 200 µM each of deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), deoxythymidine triphosphate (dTTP) and 2 µl of template DNA (approximately 100 ng of bacterial genomic DNA). The amplifications were performed using a thermocycler (Eppendorf). Amplified products were finally separated by electrophoresis on 1.2% agarose gel stained with ethidium bromide and visualized under ultraviolet light. Desired products were eluted from gels using the gel extraction kit (Qiagen Inc.) and finally sent for sequencing. Nucleotide sequences were identified using the basic local alignment search tool (BLAST) and GenBank nucleotide data bank from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/).

2.7 Biocontrol of *Meloidogyne incognita* on Pepper Vine

The experiment was performed in earthen pots of 900 g capacity with dimension of 13 × 13 × 5 cm^3. Five (5) sets of treatments were prepared in triplicates. Loam soil obtained from agricultural field was autoclaved for 15 min twice on alternate days. Appropriate quantity of soil as per capacity of pots was mixed with test agents according to the treatment chart in Table 2.

Table 2. Randomized design of experimental setup

Treatment set no	Treatment
T1	Bacterial strain MPB04
T2	Bacterial strain MPB04 + *Meloidogyne incognita*
T3	Bacterial strain MPB93
T4	Bacterial strain MPB93 + *Meloidogyne incognita*
T5	*Meloidogyne incognita*

Death was determined by nudging nematodes with a stick under a light microcope, the immobilized nematodes were counted as dead. Mortality of nematode = number of dead

nematodes/all tested nematodes x 100%. The mortality with the letter b indicated statistically significant difference against its corresponding water control within a column. The mortality with the letter B indicated statistically significant difference against its corresponding YPD control medium control within a column. SE: standard error of mortality. For all these treatment, an independent samples test showed P< 0.01

Subsequently, 3-weeks-old pepper *(Piper nigrum* cv. Kuching) cuttings were transplanted into each pot. One week after transplanting, 6 ml of suspension containing 2,000 freshly hatched (< 1-week-old) juveniles of *M. incognita* was introduced into the soil via three holes made in the soil around the cuttings. The experiment was terminated 60 days after the addition of the nematodes, and fresh root weight and numbers of galls induced by *M. incognita* were recorded. The root systems were thoroughly washed with running tap water, cut into small segments and divided into two equal portions. The root-knot nematodes in bacteria treated and untreated pots were extracted using a modified Baermann funnel technique. To determine nematode penetration, one of the root portions was cut into smaller segments, wrapped in a muslin cloth and dipped in boiling 0.25% acid fuchsin in lactic acid for 3 to 5 min. Roots were washed in running tap water to remove the excess stain and macerated in a blender for 45 s. The macerate was suspended in 100 ml of water and *M. incognita* females and juveniles in five samples of 5 ml each were counted with the aid of low power stereomicroscope (×10).

2.8 Data Analysis

Data were statistically analyzed using procedures of the Statistical Package for Social Sciences (SPSS, version 10·0 for Windows, SPSS Inc., Chicago, IL, USA). All data were analyzed by analysis of variance (ANOVA) and the treatment means were separated by using Duncan's multiple range test at P ≤ 0.05.

3 RESULTS

3.1 Isolation of Nematicidal Bacteria

A primary selection was made from the antagonism test plate where the confluent growth of bacteria from the pepper rhizophere inhibited the growth of nematodes. Pure bacterial cultures isolated from these plates were tested for nematode antagonism. This procedure resulted in isolation of 150 potential bacterial strains with nematicidal activity in the preliminary tested target towards *M. incognita.* By applying additional selection criteria as described in Material and Methods, the number of selected isolated was reduced to nine. Among these nine, four *Bacillus* strains (MPB04, MPB93, MPB098, and MPB115) were identified. The *Bacillus* spp. showed a range of nematicidal activity (Table 4). The initial screenings were performed by determining the reduction of immobile nematodes after 3 h of incubation with overnight bacterial cultures. The bacterial strains that showed more than 50% nematode reduction were selected for further analysis. Most of the bodies and cuticles for the dead nematodes were destroyed by these strains.

3.2 Bacterial Isolates Identification

The four bacterial strains with high nematicidal activity were analyzed by PCR method to determine their taxonomic status. PCR products of approximately 1.5 kb representing 16S ribosomal RNA sequences were obtained following PCR experiment using genomic DNA as

template. When the 16S rRNA sequences were aligned against the ribosomal sequence database, these four (4) bacterial isolates were identified as *Bacillus* group. The 16S rRNA sequence analyses showed that the strain MPB098 and MPB115 had identical pattern to those of the reference strains of *Bacillus* species, genebank accession no: KC687087.1 and HF934967.1 . The strain MPB04 was classified as belonging to *B. firmus* by showing 96% similarity to *B. firmus* genebank accession no: (AY833571.2, HQ116811.1, HQ622343.1 and KC734527.1). In the case of strain MPB93, 16S rRNA gene sequence similarity result showed that this strain was classified as *B. subtilis* with 98% sequence similarity with *B. subtilis* genebank accession no: FJ973542.1).

3.3 Bacterial Isolates' Characterization

Table 3 summarizes the results of the characterization on four selected strains. The results obtained showed that two strains (MPB04 and MPB115) produced cellulase. The strain MPB04 and MPB93 produce protease by forming a clear zone around the inoculated area in the skim milk agar medium (Table 3). In terms of chitinase activity, none of the bacteria isolates were able to grow in solid and liquid media containing chitin as the sole carbon source. Thus, all tested strains were considered as negative for chitinase production. Similar result was also reported for the cyanogenic activity where no remarkable change in colour from yellowish to reddish brown was detected. This indicated that all these strains were negative for their cyanogenic activity.

Table 3. Main characteristics of the *Bacillus spp*

Bacterial isolate	Chitinolytic	Cellulolytic	Proteolytic	HCN production
MPB04	nd	+	+	nd
MPB93	nd	nd	+	nd
MPB098	nd	nd	nd	nd
MPB115	nd	+	nd	nd

nd: not detected

3.4 Nematicidal Activity of *Bacillus* Strains

All antagonist isolates were shown to be significantly different from the control ($P < 0.01$) (Table 4). The growth of root knot nematodes was significantly inhibited by antagonistic bacterial. Strain MPB04 was the antagonistic bacteria isolate that showed the most nematicidal activity against *M. Incognita*, although all bacteria also showed inhibitory effect on nematodes tested. Based on the bioassay results, the mortality of the nematode was 76.4, 98.2 and 100% within 2, 6, and 12 h, respectively for bacterial strain MPB04, whereas for strain MPB93, the mortality of the nematode was 50.6, 83.6 and 100% within 2, 6, and 12 h, respectively. After 12 h, all dead nematodes were completely destroyed and digested. In all the controls, water, YDP medium and culture supernatant were boiled for 15 min; the mortalities were below 25% up to 10 h. In addition, the cuticles of the dead nematodes were intact after 10 h.

Table 4. Mortality of *Meloidogyne incognita* in culture supernatants from *Bacillus* spp. The percentage of dead nematodes was determined after 2, 6, and 12 h exposures to the supernatant

Bacillus strain	2-h mortality of *M. incognita* % (SE)	6-h mortality of *M. incognita* % (SE)	12-h mortality of *M. incognita* % (SE)
MPB04	76.4 (1.26)^{b,B}	98.2 (2.5)^{b,B}	100(0)^{b,B}
MPB93	50.6 (2.03)^{b,B}	83.6 (0.6)^{b,B}	100(0)^{b,B}
MPB098	33.6 (3.25)^{b,B}	66.5 (0.85)^{b,B}	85.6 (0.6)^{b,B}
MPB115	23.6 (1.03)^{b,B}	59.5 (3.65)^{b,B}	83.5 (1.26)^{b,B}
Water	5 (0)	6 (0)	12 (0.52)
YPD medium	7 (0)	8 (0)	10 (0.25)

3.5 Nucelotide Sequences Analysis and Protease Gene Identification

Proteases have been proposed as virulence factors in the pathogenesis of nematodes by microorganisms. Previous studies have suggested that microbial proteases may contribute to infection of hosts by degrading the host's protective barriers [19,20]. To further comfim this hypothesis, this study employed specific PCR primer for the detection of biosysnthesis gene of protease enzyme. In this experiment, two primer pairs were used for the amplification of genes involved in the protease biosynthesis from the *Bacillus* strains. Protease Npr219 gene was amplified by PCR with previously designed primers. Among the four strains tested for the presence of the neutral protease gene, only strains MPB04 and MPB93 were produced by the product of 1577 bp. The PCR products of *npr1/npr2* were sequenced and analyzed using the National Center for Biotechnology Information nBlast database. The PCR products of all strains showed a very high similarity to the sequence of the protease gene from *Bacillus* species (GenBank accession no. DQ983789). Primer *apr1/apr2*, specific for alkaline protease gene, amplified the expected 1149 bp PCR product from all the strains tested. The sequenced product of strains MPB04, MPB03 and MPB115 showed a high similarity to cuticle degrading proteases from *Brevibacillus laterosporus* (GenBank accession no. AAU81559).

3.6 Biocontrol of *Meloidogyne incognita* on Pepper Vine

Table 5 summarized the performance of plant growth and *M. incognita* on pepper vine when rhizobacterial isolates were applied. The results obtained show that bacterial strain MPB04 was the antagonistic bacteria that showed the best inhibitory effect on the growth of nematodes tested, reducing nematode penetration by 60.95%, followed by strain MPB93 (35.28%). In terms of number of egg, the bacteria strain MPB04 managed to decrease the mass egg production by 43.75% over control while strain MPB93 only decrease mass egg production by 28.13%. Thus, treatment with MPB04 was considered to be much significant in reducing number of egg. From the results obtained, it was evident that the plants treated with the MPB04 had lowest gall index over control, with the index value of 3.3 followed by MPB93 with the index value of 3.7.

Table 5. Performance of plant growth and *Meloidogyne incognita* on pepper vine when rhizobacteria isolates were applied in greenhouse

Treatment	No. of *juveniles*/g of fresh roots (% increase/decrease over control)	No. of egg masses/g of fresh roots (% increase/decrease over control)	Galling Index (% increase/decrease over control)
T1	0.00	0.00	0.00
T2	41-105/105 × 100 = -60.95	18-32/32 × 100 = -43.75	3.3
T3	0.00	0.00	0.00
T4	68-105/105 × 100 = -35.28	23-32/32 × 100 = -28.13	3.7
T5 (Control)	105	32	4.1

4. DISCUSSION

At present, there are several bio-control products from rhizobacteria which have been developed and many plants' disease bio-control products that contain *Bacillus* species have been used [21]. Among these agents, there is an increasing interest in the introduction of *Bacillus* spp for managing fungal and nematode infection. *Bacillus* spp such as *B. subtilis, B. licheniformis, B. amyloliquefaciens and B. cereus* have been reported effective against foliar and soil-borne fungal pathogens and nematodes [22,23]. This might probably be due to their ability to induce growth and the defense response in host plant. In addition, *B*. *subtilis* non-ribosomally synthesizes several kinds of small antibiotic peptides that have antifungal activities, such as iturin [24], surfactin [25] and fengycin [26]. *B*. *subtilis* also secretes protease enzymes in abundance [27]. Despite a wealth of new formulation on the genetics and physiology of *Bacillus* and related species, little is known about the mechanism of action against nematode population. Increased understanding of the nematotoxic mechanism of antagonist populations in the soil could potentially enhance the value of these species as effective bio-control agent.

In this study, main focus of this work was to isolate the potential antagonistic rhizobacteria that could control nematodes and their characterization in terms of antagonistic mechanism. Four *Bacillus* species isolated from various pepper farms were identified. Among these strains, *Bacillus* spp strains MPB04 and MPB93 showed remarkable nematicidal activity of 76.4 and 50.6%, killed tested nematodes within 2 h and completely destroyed tested nematode within 12 h. It is well known that the cuticle of nematodes is rigid and composes of protein and chitins [28]. The results suggested that the nematicidal activity displayed by *Bacillus* strains is related with their proteolytic activity responsible for nematode penetration by bacteria strains.

An important role of hydrolytic enzymes has been well documented as a variety of microorganism also exhibit hyperparasitic activity, attacking pathogens by excreting these enzyme [29]. From the results obtained, cell wall degrading activities seemed to be the mechanism responsible for *Bacillus* strain MPB04 and MPB93 antagonism, since proteases were detected. This mechanisms have been extensively correlated with nematicidal activity. This study was further elucidated by [18] and [16] who reported that *Bacillus* species antagonism is related to the production of protease. Similarly, [19] also reported was

protease is the virulence factor in the pathogensis of nematodes and suggested that microbial protease may contribute to infection of hosts by degrading the host's protective barrier. In this project, the production of protease was further confirmed by using PCR methods. The consistency of these nematotoxic protease from different nematicidal bacteria strains suggested that these protease must be highly conservative in this group of bacteria.

The present pot experiment revealed that the application of *Bacillus* strain MPB04 and MPB93 reduced the root population of *M. incognita* by 60.95 and 35.28%, respectively over control. This indicated that these bacterial isolates could reduce the ability of *M. incognita* to reproduce in soil. Currently, there are several reports that focus on the benefit of the rhizobacteria as bio-control agent against nematodes [20, 30]. Some of the important genera include *Bacillus, Pseudomonas*, *Clostridium* and *Streptomyces*. Application of these bacteria has given very promising results [9, 13]. Similarly, [23] also reported is that the culture of *B. thuringiensis* and *B. laterosporus* caused high mortality of *M. incognita in vitro* bioassay and greenhouse tests on tomato.

5. CONCLUSION

The coherence of results by chemical, genetic and greenhouse analysis has further strengthened the hypothesis that nematicidal activity displayed by *Bacillus* strains is related with their proteolytic activity responsible for nematode penetration by bacteria strains. On the basis of the results, it is concluded that bacterial strains MPB04 and MPB93 are valuable candidates for the development of broad spectrum biopesticides for controling nematodes. More work is required on product development of *Bacillus* strains in order to improve their bio-control efficiency and thus provide farmers with a better and reliable product towards nematodes management.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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