

SCREENING FOR NEMATICIDAL ACTIVITIES OF *BACILLUS* SPECIES AGAINST ROOT KNOT NEMATODE (*Meloidogyne incognita*)

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ABSTRACT

Aims: The aim of this study was to isolate and select indigenous soil *Bacillus* bacteria capable in suppressing nematode populations and to understand their mechanism of action.

Study design: Randomized complete block design

Place and Duration of Study: Research and Development Division, Malaysian Pepper Board, January 2011-December 2012.

Methodology: This study was mainly focused on the isolation of rhizobacteria with nematicidal activities from root knot nematode- infected farmed. The bacterial isolated were then undergoing hydrolytic enzyme screening test as well as PCR analysis in order to determine the mechanism of action. Finally, assessment of nematode population level by bacterial isolates in pot trial experimental was conducted to determine the inhibition efficacy.

Results: Four rhizobacteria with nematicidal activity were isolated. Among these strains, nematotoxicities of *Bacillus* strains were intensively analyzed. *Bacillus* species strain MPB04 and MPB93 showed remarkable nematicidal activity by showing 76.4% and 50.6% killed tested nematodes within 2 hours and completely destroyed tested nematode within 12 hours. The results also showed that mechanism used by these bacteria were through the secretion of protease enzyme that are responsible for fungal cell wall hydrolysis. Further assays of nematicidal toxins from *Bacillus* strain MPB04 and MPB93 indicated that an extracellular cuticle-degrading protease Apr219 and Npr 219 were an important pathogenic factor against nematodes. 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 the mechanism responsible for biocontrol exhibited by *Bacillus* strain MPB04 and MPB93 is through the production of protease.

Keywords: *Bacillus*, *Meloidogyne incognita*, protease and nematicidal activity

1.0 INTRODUCTION

Root knot nematodes (*Meloidogyne incognita*) are a major constraint to successful pepper production in all pepper producing countries, causing severe damage that lead to dramatic yield losses [1]. At present, application of chemicals and the use of host resistance have been two major strategies for management of root knot nematodes. Unfortunately, based on the latest research conducted by [2], no resistant pepper cultivars to root knot nematodes are available. Currently, control of root knot nematodes has been primary accomplished through chemical nematocides [3]. However, due to the significant drawbacks of the chemical control including threats to human health and the environment [4] as well as limited availability in developed countries [5], biological control is therefore one of the attractive and alternative to synthetic fungicide due to their ability to antagonize the nematodes by different mode of action.

The mechanisms of biological control of nematodes by antagonistic bacteria and fungi have been the subjects of many studies in the past two decades [6]. Among these rhizobacteria, several strain of *Bacillus subtilis* have been reported to be a promising candidate with proven excellent characteristics like

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effective root colonization, versatile activity against multiple nematodes and promising ability to sporulate [7,8]. The most thoroughly studied *Bacillus* includes *Bacillus subtilis* and *Bacillus thuringiensis* [9]. *Bacillus thuringiensis* has drawn many research attentions due to its availability to produce one or more parasporal crystal inclusions (Cry or δ -endotoxin). Additionally, a number of studies have also reported direct antagonistic effects of other bacteria to pathogenic nematodes belonging to the genera *Heterodera* and *Meloidogyne*. These bacteria species include *B. amyloliquefaciens*, *B. cereus*, *B. megaterium* and etc [10, 11].

The mechanism involved in biological control that affecting the 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 small molecules [12]. Currently, there are several paper had reported that microbial protease have been proposed as virulence factors in their pathogenesis against nematodes. The most compelling evidences to support microbial protease as virulence factors have come from studies of protease-deficient mutant [7, 13, 14]. Besides, Siddiqui and Shaulat (2003)[15] also demonstrated that the deletion of major extracellular protease from *Pseudomonas fluorescens* CHAO reduced bacterial activity against the root knot nematodes. These entire research finding suggested that extracellular protease might play an important role in suppressing nematodes in soil.

The main focus of this work was to isolate the potential antagonistic rhizobacteria that could control nematodes and their characterization in term of antagonistic mechanism. The proteases with cuticle-degrading and nematocidal activities were found to be extensively distributed in *Bacillus spp.* Such distributions suggest that extracellular enzymes from rhizobacteria might play an important role in the bacteria-nematode-plant –environment interaction.

2.0 MATERIAL AND METHODS

2.1 Isolation and Identification 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 mol⁻¹ phosphate buffers, and appropriate dilutions were plated on NA agar and incubated at 30°C for 2 days [16]. 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 rDNA sequences method. After the genomic DNA of the *Bacillus* strains was extracted, 16S rRNA genes were amplified using P1 forward primer (5'-GGTTACCTTGTTACGACTT-3') and P6 reverse primer (5'-AGAGTTTGATCCTGGCTCAG-3') as described by Lane (1991) [17]. The sequenced of 16S rRNA genes were compared and analyzed using the clustalX 1.83 and mega version 3.1 programs [18, 19]. Candidate *Bacillus* species were stored in 30% glycerol at -20°C for further assays.

2.2 Characterization of Bacterial Isolates

Standard methods were adopted for chitinase [20], protease [21], cellulase [22] and HCN production [23].

2.3 Bioassays

All of the isolated rhizobacterial strains were inoculated into 250 ml Erlenmeyer flasks containing 50 ml YPD (yeast, peptone, glucose) medium each and grown at 30°C with rotary shaking at 150 rpm for 3 days

[24]. After centrifugation at 8500 *g* for 15 min, the culture supernatants were collected for the measurement of nematocidal activity. In bioassay, approximately 200 nematodes were added to 300 μ l culture supernatants in a 1.5 ml Eppendorf tube containing two antibiotics (50 μ g ml⁻¹ ampicillin and 30 μ g ml⁻¹ kanamycin). After incubating the tubes at 30°C for 2–10 h, the numbers of 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, YPD medium and the culture supernatant 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.

2.4 PCR Amplification of Protease Encoding Genes

Genomic DNA of *Bacillus* species were extracted, respectively, using the Wizard genomic DNA purification kit for Gram-positive bacteria (Promega, Madison, WI, USA) and stored in TE solution at -70°C for further cloning 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 genes	Primer	Primers sequence (5'-3')	Product length (bp)	References
Neutral proteases	npr1	GGGGGATTTATTGTGGGTTT	1577	[44]
Alkaline protease gene	npr2	TACAATCCGACAGCATTCCA		
	apr1	GCGCCTAGGGTGAGAGGCAAAAAGGTATG	1149	[44]
	apr2	CGCGGATCCTTACTGAGCTGCCGCCTGTAC		

PCR amplification are carried out in 50- μ L reaction mixtures containing PCR buffer (Qiagen Inc.), 1.5mM MgCl₂, 1.5U Taq DNA polymerase (Qiagen Inc.) 40 μ g of each forward and reverse primer, 200 μ M each of dATP, dGTP, dCTP, and dTTP and 2 μ L 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/>).

2.5 Biocontrol of *Meloidogyne incognita* on Pepper Vine

The experiment was performed in earthen pots of 900 g capacity with dimensions of 13×13×5 cm³. 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 treatment chart mentioned Table 2.

Table 2: Randomized design of experimental setup

Treatment set no	Treatment
T1	Bacterial strain MPB04
T2	Bacterial strain MPB04+ <i>Meloidogyne incognita</i>
T3	Bacterial strain MPB93
T4	Bacterial strain MPB93+ <i>Meloidogyne incognita</i>
T5	<i>Meloidogyne incognita</i>

Subsequently, 3-week-old pepper (*Piper nigrum* cv. Kuching) cutting were transplanted into each pot. One week after transplanting, 6 m¹ of suspension containing 2,000 freshly hatched (< 1-week-old) juveniles of *M. incognita* were 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 [25]. 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 3-5 min. Roots were washed in running tap water to remove the excess stain and macerated in a blender for 45 sec. The macerate was suspended in 100 m¹ of water and *M. incognita* females and juveniles in five samples of 5 m¹ each were counted with the aid of low power stereomicroscope (x 10).

2.6 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 $P \leq 0.05$.

3.0 RESULTS AND DISCUSSION

3.1 Isolation and Identification

A primary selection was made from the antagonism test plate where the confluent growth of bacteria from the pepper rhizosphere 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 nematocidal activity in the preliminary tested target towards *Meloidogyne incognita*. By applying additional selection criteria as described in Material and methods, the number of selected isolated was reduced to 9. Among these 9, four *Bacillus* strains (MPB04, MPB93, MPB098, and MPB115) were identified. The *Bacillus* spp. showed a range of nematocidal activity (Table 4). Most of the bodies and cuticles for the dead nematodes were degraded and destroyed by these strains.

3.2 Bacterial Isolates Characterization

Table 3 summarizes the results of the characterization on four selected strains. From the results obtained, only 2 strains (MPB04 and MPB115) were found to produce cellulase enzyme by the formation of clearing zone in the culture medium. Regarding the protease activity, out the 4 tested strains, only strain MPB04 and MPB93 produced a clear zone around the inoculated area in the skim milk agar medium. In term of chitinase activity, none of the bacteria isolates were able to grow in solid and liquid media that 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 indicated that all these strains were negative for their cyanogenic activity.

Table 3: Main Characteristics of the *Bacillus* spp.

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

nd: not detected

3.3 Bioassays for *Bacillus* Strains

All antagonist isolates were showed to be significantly different from the control ($P < 0.01$) (Table 4). The growth of root knot nematodes were significantly inhibited by antagonistic bacterial. Strain MPB04 was the antagonistic bacteria isolate that showed the most nematocidal 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 2h, 6h, and 12 hours respectively for bacterial strain MPB04, whereas for strain MPB93, the mortality of the nematode was 50.6%, 83.6% and 100% within 2h, 6h, and 12 hours respectively. After 12 hour, all dead nematodes were completely destroyed and digested. In all the controls: water, YDP medium and culture supernatant boiled for 15 minutes, the mortalities were below 25% up to 10 hours. In addition, the cuticles of the dead nematodes were intact after 10 hours.

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

<i>Bacillus</i> strains	2-h mortality of <i>M. incognita</i> % (SE)	6-h mortality of <i>M. incognita</i> % (SE)	12-h mortality of <i>M. incognita</i> % (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)

Death was determined by nudging nematodes with a stick under a light microscope, 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 $P < 0.01$

3.4 PCR Analysis and Blast Search

The specificity of the specific primers used in this study was checked with nucleotide to nucleotide Blast search to determine the presence of respective antibiotic through primer hybridization. In this experiment, 2 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. Of the 4 strains tested for the presence of the neutral protease gene, only strain MPB04 and MPB93 were produced 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](#)).

3.5 Biocontrol of *Meloidogyne incognita* on Pepper Vine

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Table 5 summarized the performance of plant growth and *Meloidogyne incognita* on pepper vine when rhizobacterial isolates were applied. Based on the results obtained, it was found out that bacterial strain MPB04 was the antagonistic bacteria isolate that showed the best inhibitory effect on the growth nematodes tested, with the reduction of nematode penetration was 60.95%, followed by strain MPB93 which showed 35.28% reduction in nematode penetration. In term of number of egg masses, treatment with MPB04 + *Meloidogyne incognita* showed 43.75% decrease in egg mass production over control while MPB93+ *Meloidogyne incognita* only showed 28.13% decrease in egg mass production over control. Thus treatment with MPB04+ *Meloidogyne incognita* was considered to be much significant in reducing number of egg masses. From the data given in the Table 2 also, 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 x 100 = -60.95	18-32/32 x 100 = -43.75	3.3
T3	0.00		
T4	68-105/105 x 100 = -35.28	23-32/32 x 100 = -28.13	3.7
T5 (Control)	105	32	4.1

4.0 DISCUSSION

At present, there are several biocontrol products from rhizobacteria have been developed [26] and many plant disease biocontrol products that contain *Bacillus* species have been used [27]. 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 [28, 29, 30, 31]. This might probably due to their ability to induce growth and defense response in host plant [32]. In addition, *B. subtilis* non-ribosomally synthesizes several kinds of small antibiotic peptides that have antifungal activities, such as iturin [33, 34], surfactin [35], fengycin [36], bacillomycin [37] and mycosubtilin [38]. *B. subtilis* also secretes proteins in abundance [39, 40]. Despite a wealth of new formulation on the genetics and physiology of *Bacillus* and related species, little is known about the 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 biocontrol agent.

In this study, main focus of this work was to isolate the potential antagonistic rhizobacteria that could control nematodes and their characterization in term of antagonistic mechanism. Four *Bacillus* species isolated from various pepper farms was identified. Among these strains, *Bacillus* species strain MPB04 and MPB93 showed remarkable nematocidal activity by showing 76.4% and 50.6% killed tested nematodes within 2 hours and completely destroyed tested nematode within 12 hours. It was well known that the cuticle of nematodes is rigid and composes of protein and chitins [41]. The results suggested that the hydrolytic enzyme might be involved in the penetration process to help bacteria kill the host [42].

An important role of hydrolytic enzymes has been well documented as a variety of microorganisms also exhibit hyperparasitic activity, attacking pathogens by excreting these enzyme [43]. From the results

obtained, cell wall degrading activities seemed to be the mechanism responsible for *Bacillus* strain MPB04 and MPB93 antagonism, since protease were detected. This mechanisms have been extensively correlated with nematocidal activity [1, 44]. This study was further elucidated by Lian *et al* (2006) [44] and Denizci (2004) [45] who reported that *Bacillus* species antagonism is related to the production of protease. Similarly, Ahman (2002) also reported that the protease was the virulence factors in the pathogenesis of nematodes and suggested that microbial protease may contributed to infection of hosts by degrading the host's protective barrier. In this project, the productions of protease was further confirmed by using PCR methods. The consistency of these nematotoxic protease from different nematocidal 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% 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 focused on the benefit of the rhizobacteria as biocontrol agent against nematodes [46, 47, 48]. Some of important genera include *Bacillus*, *Pseudomonas*, *Clostridium* and *Streptomyces*. Application of these bacteria has given very promising results [11, 15]. Similarly, Carneiro (1998) [31] also reported that the culture of *B. thuringiensis* and *B. laterosporus* caused high mortality of *Meloidogyne incognita* *in vitro* bioassay and greenhouse tests on tomato.

5.0 CONCLUSION

The coherence of results by chemical, genetic and greenhouse analysis has further strengthened the hypothesis that the mechanism responsible for biocontrol exhibited by *Bacillus* strain MPB04 and MPB93 is through the production of protease. 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 controlling nematodes. More work is required on product development on *Bacillus* strains in order to improve its biocontrol efficiency and thus provide farmers with a better and reliable products towards nematodes management.

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