Saudi Journal of Biological Sciences 28 (2021) 1318–1323

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Functional evaluation of culture filtrates of *Bacillus subtilis* and *Pseudomonas fluorescens* on the mortality and hatching of *Meloidogyne javanica*

Sukalpa Das [a], Md. Abdul Wadud [b], Md. Atiqur Rahman Khokon [c,*]

[a] Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh, Bangladesh & Department of Agricultural Extension, Bangladesh
[b] Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh & Bangladesh Agriculture Research Institute, Bangladesh
[c] Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh, Bangladesh

ARTICLE INFO

Article history:
Received 29 September 2020
Revised 15 November 2020
Accepted 15 November 2020
Available online 24 November 2020

Keywords:
Meloidogyne javanica
Bacillus subtilis
Pseudomonas fluorescens
Mortality
Hatching

ABSTRACT

Rhizospheric bacteria *Bacillus subtilis* and *Pseudomonas fluorescens* are two widely tested biological control agents against root-knot nematodes (RKN) of different crops. However, their performance as biocontrol agents varies with their place of origin. Culture filtrates of rhizospheric bacteria contain some intermediary metabolites that have nematicidal activity. An *in vitro* experiment was undertaken to evaluate the functionality of culture filtrates of *B. subtilis* (MN252542.1) and *P. fluorescens* (MN256394.1) at different concentrations (1.0%, 2.5%, 5.0%, 7.0%, 10.0% and 25.0%) on the hatching and mortality of *Meloidogyne javanica* at different time span. Bacterial strains were isolated from rhizospheric soils of Bangladesh. At three days after incubation (DAI), 25.0% concentration of culture filtrates of both *B. subtilis* and *P. fluorescens* showed 100.0% mortality of second stage juveniles (J_2) of *M. javanica*. Additionally, 25.0% concentration of culture filtrates of both bacteria showed 100.0% inhibition of hatching at one week after incubation (WAI). A decreasing trend in hatching of *M. javanica* was observed with the increment of the concentration of culture filtrates and progression of incubation time. The findings of this experiment reveal that culture filtrates of these accessions of *B. subtilis* and *P. fluorescens* are effective for controlling *M. javanica* and would be potential candidates for developing bio-nematicides.

1. Introduction

It has been estimated that 12.6% of global crop loss, equivalent to 215.77 billion dollar, is incurred due to the infestation of plant pathogenic nematodes (PPN) (Abu-Elgawad and Askary, 2015). PPN are pseudocoelomate, unsegmented worm-like animals, mostly subterranean and comprise about 15% of all forms of nematodes that exist in different habitats having various feeding behaviors (Decraemer and Hunt, 2006). PPN are considered as the hidden enemy of farmers because the symptoms expressed on plants by their infestation are very similar to that with fungal attack, water stress or other physiological disorders. Among PPN, root-knot nematodes (RKN), belonging to the genus *Meloidogyne*, are distributed worldwide and comprise relatively small but important polyphagous group of highly adapted obligate plant pathogens that can parasitize more than 3000 species of plant, causing an estimated crop loss of worth 100 billion dollar annually (Hunt and Hando, 2009; Dejene, 2014). Under the genus *Meloidogyne*, 106 species have been described so far (Karssen and Moens, 2006). Among them, only four species, *Meloidogyne incognita*, *M. arenaria*, *M. javanica* and *M. hapla* are responsible for 95% of global infestations (Sasser et al., 1983).

Traditionally, farmers opt synthetic chemical pesticide applications to control of PPN infestations. The widely used chemical nematicides against PPN include soil fumigants, organophosphate and carbonate group of pesticides (Dejene, 2014). However, besides increasing the production cost to a greater extent, these broad spectrum non-selective pesticides are detrimental for many non-target organisms, as they are highly toxic to environment (Kepenekci et al., 2017). Moreover, long term use of these chemicals have resulted in the prohibition or restrictions on various

* Corresponding author.
E-mail address: atiq.ppath@bau.edu.bd (Md. Atiqur Rahman Khokon).
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2020.11.055
1319-562X/

molecules employed worldwide due to the emergence of resistance-breaking nematode pathotypes on many important crops (Abu-Elgawad and Askary, 2015; Silva et al., 2017). Therefore, scientists are looking for non-chemical and efficient alternative methods for the management of RKN (Huang et al., 2016). Biological control could be one of the sustainable control methods against RKN as this a low risk, economically viable and can be used for a long period due to its ecological acceptability (Sheppard et al., 2005; Sehebani and Hadavi, 2008; Moosavi et al., 2010).

Biological control is an eco-friendly pest management strategy that utilizes deliberate introduction of living animals to lower the population level of a target pest (Delfosse, 2005; Brand et al., 2010). RKN control by biological means is gaining popularity mostly by utilizing the soil dwelling microorganism like fungi and bacteria (Crawford and Clardy, 2011). Some bacterial species of the genus *Bacillus* and *Pseudomonas* are widely tested and commercially formulated biological control agents against RKN of different crops (Abu-Elgawad and Askary, 2015). They produce metabolites such as enzymes and toxins which inhibit nematode reproduction, egg hatch and juvenile survival (Siddiqi and Mahmood, 1999). Although, considering the importance of biological control of pests, now-a-days multinational firms with microbial product portfolios are investing in biotechnology researches, still there are not much registered biological nematicides available in market (Wilson and Jackson, 2013). As bio-nematicides represent living systems, a number of difficulties like their culture and formulation, variable gap between laboratory and field performance, potential negative effects on non-target and beneficial organisms exist in developing commercial product (Abu-Elgawad and Askary, 2015). Moreover, imported biological control agents may less adapt to local climatic condition resulting in less success (Stirling, 1991). Therefore, RKN species- and climate-specific further researches are necessary. In Bangladesh, there is no report available about the potentiality of the locally isolated *Bacillus* spp. and *Pseudomonas* spp. in controlling RKN. It can be hypothesized that the presence of secondary metabolites in the culture filtrates of bacterial solutions would be helpful in controlling the infestation of RKN by limiting their hatchability and causing mortality within the shortest time in field. It is also important to investigate *in vitro* the potentiality of bacterial inoculant at minimal concentration to predict their antagonism against RKN in natural condition. Thus, the aim of the present study was to evaluate the efficacy of different concentration of culture filtrates of local strains of *Bacillus subtilis* (MN252542.1) and *Pseudomonas fluorescens* (MN256394.1) in causing mortality and inhibiting the hatching of RKN (*M. javanica*).

2. Materials and methods

2.1. Preparation of culture filtrates of bacterial strains

Two bacterial strains *viz B. subtilis* (MN252542.1) and *P. flurorescens* (MN256394.1), used in this experiment were previously isolated from the rhizospheric soil of Meherpur (24°N, 89°E) and Mymensingh (25°N, 90°E), respectively. The bacterial strains were identified at molecular level and obtained National Centre for Biotechnology Information (NCBI) accession. The bacterial strains were stored at −80 °C at the Department of Plant Pathology, Bangladesh Agricultural University (BAU) maintaining proper condition (Schaad, 1980; Kreig and Holt, 1984). For *B. subtilis*, 2.8 g of nutrient agar medium, and for *P. fluorescens*, 4.2 g of King's B medium was dissolved in 100 ml of distilled water and allowed to get solidified in 9 cm petri dish. Bacterial inoculum was streaked on the solidified medium in the petri dish. The media was then incubated at 28 °C at for 24 h (Mahesha et al., 2017). A loop full of bacterial colony was taken out of the petri dish and aseptically transferred to the nutrient broth in a 250 ml conical flask. Conical flask was then incubated in an electric shaker for 48 h at 200 rpm at 32 °C (Sela et al., 1998). After incubation, culture filtrates were harvested by centrifugation of the nutrient broth at 6000 rpm for 10 min (Mahesha et al., 2017). Afterwards, the supernatant was passed through membrane filters having the pore size of 0.45 and 0.20 µm (CHROMAFIL® Xtra) subsequently and stored as stock solution in the refrigerator. Different concentrations (1.0%, 2.5%, 5.0%, 7.0%, 10.0% and 25.0%) of culture filtrates were prepared by diluting the stock solution with required amount of double distilled water (DDW) and considered as treatments.

2.2. Nematode inoculum

Egg masses and J_2 used in this experiment were randomly collected from previously characterized pure culture of *M. javanica*, maintained and raised in brinjal (*Solanum melongena* L.) plants at the net house of the Seed Pathology Centre (SPC) of Bangladesh Agricultural University (BAU).

2.3. Mortality study

The brinjal plants inoculated with the egg masses of *M. javanica* were uprooted from soil and the root system was washed gently with running tap water to remove adhering soil. Egg masses of *M. javanica* were gently picked using a forceps. Eggs were incubated for 48 h using Baermann funnel method (Baermann, 1917) to obtain J_2. Population density of J_2 was calculated from 5 replications of one ml aliquots of an inoculum suspension. Freshly hatched one hundred (48 h old) J_2 were put in 2.5 cm diameter petri plate containing 5 ml solution of each treatment. J_2 kept in tap water was treated as control. Plates were covered with lid and incubated at room temperature (25 ± 2 °C) during the experiment period. Each treatment was replicated 3 times. Data on mortality was recorded at every 3 days after incubation (DAI) and continued up to 9 DAI. Mortality of the J_2 was assessed by observing the mobility of the J_2 under stereo microscope (Zeiss, Carl Zeiss Microscopy GmbH, Germany) at 60× magnification and expressed as the percentage of the total population. The moribund and non-mobile J_2 were prodded using a 'fishing' needle to check for mobile responses (Das et al. 2011).

2.4. Assessment of hatching inhibition

Five egg masses of *M. javanica* were kept on a 48-µm-sieve fixed at the perforated cap of an inverted eppendorf tube and immersed in 5 ml solution of each treatment in a small plastic bottle (Khokon et al., 2009). Egg masses kept in tap water was treated as control. Each treatment was replicated 3 times. The bottles were kept at room temperature (25 ± 2 °C). Number of hatched J_2 was counted in a counting dish under stereo microscope (Zeiss, Carl Zeiss Microscopy GmbH, Germany) and the solution of each treatment was replaced after every counting. Data was recorded at every 1 week interval and continued until 6th week. Percent egg hatch inhibition over control was calculated using the formula (Mahesha et al., 2017):

$$\text{Percent egg hatch inhibition} = (C - T)/C \times 100$$

where C = Number of hatched J_2 in control and T = Number of hatched J_2 in treatment

2.5. Statistical analysis

Statistical analyses were done by Statistix 10 (© 1985–2013 Analytical Software, Miller Landing Rd, Tallahassee, FL 32312)

and MS Excel. Two-way ANOVA was performed to determine the significance of the interaction effect of different concentrations of culture filtrates of the bacterial strains and time on the mortality and hatching inhibition. Tukey's HSD test was performed at 5% level of probability to find the significant difference among means.

3. Results

The influence of different concentrations of culture filtrates of two rhizospheric bacteria *viz. B. subtilis* and *P. flurorescens* on the hatching and mortality of J_2 of *M. javanica* was evaluated considering different incubation time. For both hatching and mortality experiments, six different concentrations (1.0%, 2.5%, 5.0%, 7.0%, 10.0%, and 25.0%) of culture filtrates of both bacteria were used as treatments with tap water as control.

In this experiment, mortality of J_2 of *M. javanica* was found to be significantly different ($p < 0.0001$) among the interaction effect of treatments and incubation time for both bacteria (Tables 1 and 2). For *B. subtilis*, 100.0% mortality was found at three days after incubation (DAI) in 25.0% concentration of the culture filtrate, suggesting its superiority in affecting the survival of J_2 over other treatments (Table 1). For rest of the treatments, percentage of mortality increased over prolonged exposure time. Mortality of J_2 reached up to more than 50.0% on 9 DAI for 10.0%, 7.0% and 5.0% of the culture filtrates, whereas, the mortality was only 7.0% for the control at that time (Table 1). Almost similar pattern of mortality was observed for the set-ups treated with *P. fluorescens* (Table 2). Concerning *B. subtilis*, on 3 DAI, 100.0% mortality of J_2 was observed in 25.0% concentration of the culture filtrate of *P. fluorescens*. However, on 9 DAI more than 50.0% of the mortality was recorded only in 10.0% concentration of this bacterium.

Throughout the experiment period, number of hatched J_2 was notably lower in all treatments of both bacteria in comparison to untreated control (Figs. 1 and 2). In control, number of hatched J_2 was the highest at the second week after incubation (WAI) and then it declined gradually in the following weeks of experiment. In the experiment, hatching was found to be significantly inhibited ($p < 0.0001$), considering culture filtrate concentration and incubation time for both *B. subtilis* and *P. fluorescens*, and differed significantly ($p < 0.0001$) among the treatments of both bacteria at different time intervals (Tables 3 and 4). The hatching experiment was continued up to 6 WAI and at the 1 WAI 100.0% of the hatching was found to be inhibited in 25.0% concentration of culture filtrates of both bacteria. It was also noteworthy that in 10.0% concentration of two bacteria, more than 90.0% of the hatching was inhibited from 2 WAI. During the experiment period, in all treatments of two bacteria, inhibition of hatching increased with the progress of time and 90.0% hatching inhibition was found at 5 WAI (Tables 3 and 4).

We further examined the effect of higher concentrations (50.0%, 75.0% and 100.0%) of culture filtrates on the mortality and hatching of *M. javanica*. All these higher concentrations of culture filtrates responded similarly as 25.0% concentration (data not shown). It was observed from both mortality and hatching experiment that 25.0% concentration of culture filtrates of both bacteria was the most effective in causing 100.0% mortality and inhibition of hatching by the earliest time.

Table 1
Interaction effect of different concentrations of culture filtrate of *B. subtilis* (treatment) and incubation time on the mortality of J_2 of *M. javanica*.

Time	Treatment (%)	Mortality (%)
3 DAI*	1.0	4.67 ± 0.88 fg
	2.5	4.00 ± 0.57 fg
	5.0	6.00 ± 1.00 (e–g)
	7.0	12.67 ± 2.02 e
	10.0	25.66 ± 0.33 d
	25.0	100.00 ± 0.00 a
	Water	2.33 ± 0.88 g
6 DAI	1.0	11.00 ± 1.73 ef
	2.5	13.33 ± 1.85 e
	5.0	25.33 ± 2.02 d
	7.0	24.00 ± 2.64 d
	10.0	44.67 ± 1.76c
	25.0	100.00 ± 0.00 a
	Water	4.66 ± 1.20 fg
9 DAI	1.0	40.67 ± 1.73c
	2.5	46.00 ± 2.51c
	5.0	55.00 ± 2.31b
	7.0	56.33 ± 1.85b
	10.0	54.67 ± 2.60b
	25.0	100.00 ± 0.00 a
	Water	7.33 ± 1.67 (e–g)
Level of significance	**	
CV (%)	5.08	

Values are the mean ± Standard Error of three replicates. Treatment means were compared by two-way ANOVA. Same letter in a column do not differ significantly according to Tukey's test at 5% probability.
* DAI = Days after incubation ** 1% level of probability.

Table 2
Interaction effect of different concentrations of culture filtrates of *P. fluorescens* (treatment) and incubation time on the mortality of J_2 of *M. javanica*.

Time	Treatment (%)	Mortality (%)
3 DAI*	1.0	14.00 ± 0.57 gh
	2.5	12.00 ± 0.57 gh
	5.0	12.66 ± 0.88 gh
	7.0	16.33 ± 1.45 g
	10.0	23.33 ± 1.45f
	25.0	100.00 ± 0.00 a
	Water	2.33 ± 0.88 i
6 DAI	1.0	25.00 ± 2.08f
	2.5	26.33 ± 1.76f
	5.0	24.00 ± 1.00f
	7.0	29.00 ± 1.52f
	10.0	58.00 ± 2.51c
	25.0	100.00 ± 0.00 a
	Water	4.66 ± 1.20 i
9 DAI	1.0	36.66 ± 1.85 e
	2.5	40.66 ± 1.45 de
	5.0	44.66 ± 1.45 d
	7.0	45.00 ± 1.52 d
	10.0	67.33 ± 2.18b
	25.0	100.00 ± 0.00 a
	Water	7.33 ± 1.67 hi
Level of significance		**
CV (%)		4.23

Values are the mean ± Standard Error of three replicates. Treatment means were compared by two-way ANOVA. Same letter in a column do not differ significantly according to Tukey's test at 5% probability.
* DAI = Days after incubation ** 1% level of probability.

4. Discussions

This experiment was undertaken with an aim to generate important information for effective formulation of bio-pesticides of *B. subtilis* and *P. fluorescens*, isolated from the soil of Bangladesh, against *M. javanica*. For RKN, J_2 is the infective stage that hatches out and look for the suitable host to penetrate (Karssen and Moens, 2006). Therefore, effective management of RKN is possible by impacting the hatching and survival of J_2. Here, we tested the influence of different concentrations of culture filtrates of these two bacteria on hatching and mortality of J_2 of *M. javanica*. The experiment was conducted in a controlled condition. In our experiment, number of hatched J_2 was significantly higher in untreated control than the treatments and that was maximum at the 2nd week after incubation followed by a gradual decline. In a favourable situation, each female of RKN may lay several hundred eggs with an average of 30–80 per day, however, hatching of those

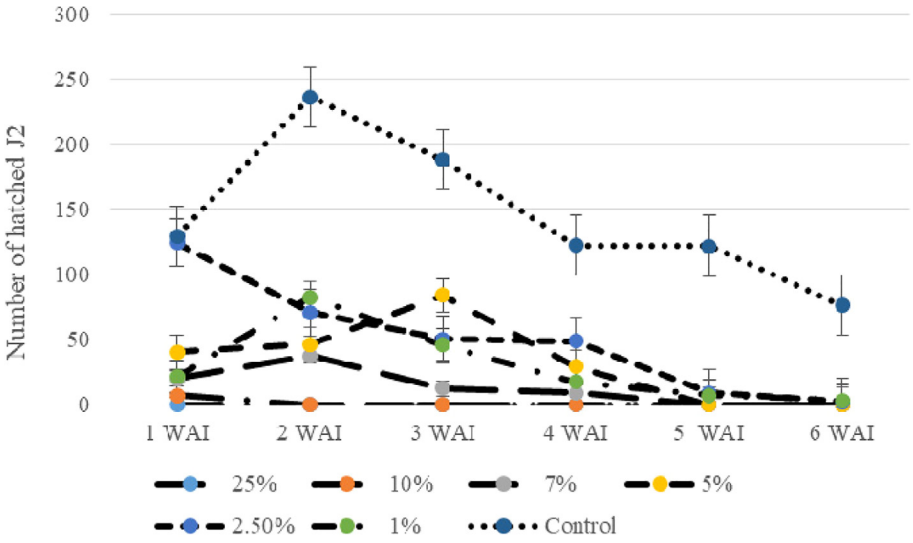

Fig. 1. Number of J_2 of *M. javanica* hatched at different weeks after incubation (WAI) in different concentrations of culture filtrates of *B. subtilis*. J_2 hatched in water was treated as control.

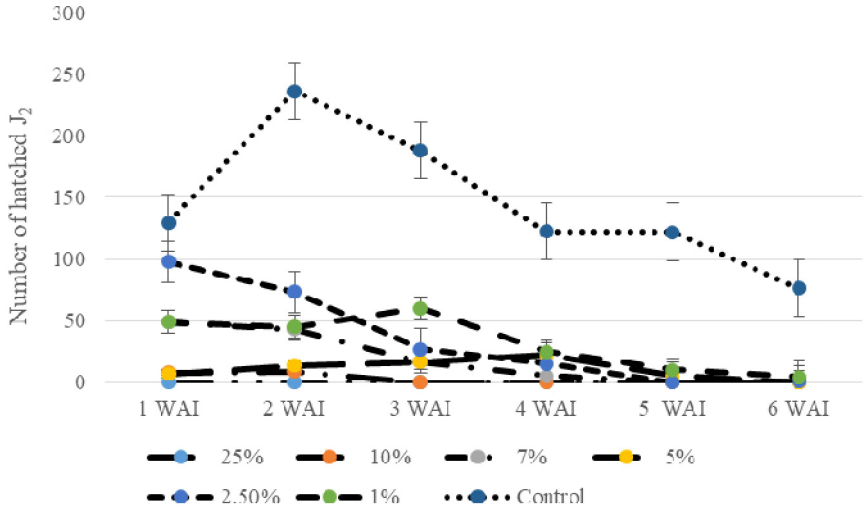

Fig. 2. Number of J_2 of *M. javanica* hatched at different weeks after incubation (WAI) in different concentrations of culture filtrates of *P. fluorescens*. J_2 hatched in water was treated as control.

are dependent partly on external sources of heat and water (Bird and Wallace, 1965; Karssen and Moens, 2006).

In this experiment, hatching was found to be 100.0% inhibited by 25.0% concentration of culture filtrates of both *B. subtilis* and *P. fluorescens* at 1 WAI. It was also noted that 90.0% of hatching got inhibited at 2 WAI by 10.0% concentration of both bacteria. In a similar experiment, Mahesha et al. (2017) tested the bio-efficacy of different strains of *Bacillus* spp., including *B. subtilis*, against *M. incognita* and observed significantly different degrees of hatching inhibition after 24 and 120 h of incubation. They observed 90.0% of hatching inhibition by 100.0% concentration of culture filtrates of *B. subtilis* after 24 h of incubation and 70.0% of inhibition by 25.0% concentration after 120 h. Jamily et al. (2018) also found around 80.0% of hatching inhibition by exposing the egg mass of RKN for 4 days to 100.0% concentration of culture filtrates of different strains of *Bacillus* spp. However, we worked with lower concentration (1.0%, 2.5%, 5.0%, 7.0%, 10.0% and 25.0%) of culture filtrates and had our first reading at 1 WAI.

In the mortality study, we have recorded 100.0% mortality of J_2 of *M. javanica* in 25.0% concentration of culture filtrates of both *B. subtilis* and *P. fluorescens* at 3DAI. Jamily et al. (2018) observed more than 80.0% mortality of J_2 of RKN by exposing them for 4 days to 100.0% concentration of culture filtrates of different strains of *Bacillus* spp. But they did not evaluate the efficacy of lower concentrations (1.0%, 2.5%, 5.0%, 7.0%, 10.0% and 25.0%). Xia et al. (2011) evaluated the effect of different dilutions and diluents of culture filtrates of five *B. subtilis* strains on the mortality of J_2 of *M. javanica* and observed 89.0–100.0% mortality. From the supernatant, they identified the *pur*L gene that regulates the synthesis of intermediary metabolites of purine and assumed that it might have nematicidal activity. Conducting a mortality experiment, Abo-Elyousr et al. (2010) found 50.0–60.0% of immobilized J_2 of *M. incognita* in 10^8 CFU/ml suspension of *P. fluorescens* for 24 and 48 h. It was seen from these experiments that culture filtrates of bacteria might have better efficacy in causing mortality of J_2 of RKN than its suspension. Mohammad et al. (2008) worked with several strains of *B. thringiensis* and observed 100.0% mortality of J_2 of *M. incognita* applying supernatant of bacterial solution, whereas applying purified Cry protein 90.0% mortality was seen. They opined that supernatant of bacterial solution contained some vegetative protein

Table 3
Interaction effect of different concentrations of culture filtrates of *B. subtilis* (treatment) and incubation time on the hatching of egg mass of *M. javanica*.

Time	Treatment (%)	Inhibition of hatching (%)
1 WAI*	1.0	83.46 ± 3.41 (e–g)
	2.5	3.61 ± 0.93 m
	5.0	68.73 ± 3.17 (i–k)
	7.0	84.49 ± 2.23 (c–g)
	10.0	100.00 ± 0.00 a
	25.0	100.00 ± 0.00 a
2 WAI	1.0	64.97 ± 1.66 jk
	2.5	69.91 ± 2.48 ij
	5.0	80.36 ± 1.62 (f–h)
	7.0	84.03 ± 1.97 (d–g)
	10.0	94.57 ± 1.61 ab
	25.0	100.00 ± 0.00 a
3 WAI	1.0	75.53 ± 2.45 (g–i)
	2.5	73.22 ± 1.87 (h–j)
	5.0	55.31 ± 2.01 l
	7.0	93.26 ± 0.77 (a–d)
	10.0	100.00 ± 0.00 a
	25.0	100.00 ± 0.00 a
4 WAI	1.0	85.79 ± 2.38 (b–f)
	2.5	59.83 ± 4.73 kl
	5.0	76.22 ± 1.89 (f–i)
	7.0	92.34 ± 2.42 (a–e)
	10.0	100.00 ± 0.00 a
	25.0	100.00 ± 0.00 a
5 WAI	1.0	93.98 ± 0.98 (a–c)
	2.5	92.62 ± 1.63 (a–e)
	5.0	100.00 ± 0.00 a
	7.0	100.00 ± 0.00 a
	10.0	100.00 ± 0.00 a
	25.0	100.00 ± 0.00 a
6 WAI	1.0	95.61 ± 1.16 a
	2.5	96.92 ± 0.87 a
	5.0	100.00 ± 0.00 a
	7.0	100.00 ± 0.00 a
	10.0	100.00 ± 0.00 a
	25.0	100.00 ± 0.00 a
Level of significance		**
CV (%)		3.38

Values are the mean ± Standard Error of three replicates. Treatment means were compared by two-way ANOVA. Same letter in a column do not differ significantly according to Tukey's test at 5% probability. * WAI = Week after incubation ** 1% level of probability.

Table 4
Interaction effect of different concentrations of culture filtrates of *P. fluorescens* (treatment) and incubation time on the hatching of egg mass of *M. javanica*.

Time	Treatment (%)	Inhibition of hatching (%)
1 WAI*	1.0	61.75 ± 2.46f
	2.5	24.03 ± 1.61 g
	5.0	94.83 ± 1.29 (a–c)
	7.0	62.01 ± 1.79f
	10.0	94.05 ± 1.12 (a–c)
	25.0	100.00 ± 0.00 a
2 WAI	1.0	84.03 ± 1.97 de
	2.5	68.92 ± 1.80f
	5.0	94.20 ± 0.98 (a–c)
	7.0	81.77 ± 1.29 e
	10.0	96.32 ± 0.74 ab
	25.0	100.00 ± 0.00 a
3 WAI	1.0	67.02 ± 1.33f
	2.5	85.63 ± 1.91 de
	5.0	91.31 ± 2.04 (b–d)
	7.0	91.31 ± 1.77 (b–d)
	10.0	100.00 ± 0.00 a
	25.0	100.00 ± 0.00 a
4 WAI	1.0	85.24 ± 3.31 de
	2.5	87.70 ± 1.89 (c–e)
	5.0	82.24 ± 1.97 e
	7.0	95.90 ± 2.36 ab
	10.0	100.00 ± 0.00 a
	25.0	100.00 ± 0.00 a
5 WAI	1.0	93.98 ± 1.19 (a–c)
	2.5	100.00 ± 0.00 a
	5.0	95.90 ± 0.94 ab
	7.0	100.00 ± 0.00 a
	10.0	100.00 ± 0.00 a
	25.0	100.00 ± 0.00 a
6 WAI	1.0	98.24 ± 1.75 ab
	2.5	98.68 ± 1.31 ab
	5.0	100.00 ± 0.00 a
	7.0	100.00 ± 0.00 a
	10.0	100.00 ± 0.00 a
	25.0	100.00 ± 0.00 a
Level of significance		**
CV (%)		2.67

Values are the mean ± Standard Error of three replicates. Treatment means were compared by two-way ANOVA. Same letter in a column do not differ significantly according to Tukey's test at 5% probability. * WAI = Week after incubation ** 1% level of probability.

which might be responsible for the higher mortality rate of J_2 of RKN. Antibiosis is one of the mechanisms, by which *B. subtilis* work against RKN (Engelbrecht et al., 2018). Antibiosis is the production of volatile organic compounds, toxins and diffusible antibiotics (Rahman et al., 2018). Jamily et al. (2018) reported that culture filtrates of the several strains of *Bacillus* spp. significantly increased J_2 mortality and decreased the egg hatch of RKN through antibiosis.

It was seen in our mortality experiment that at 9 DAI more than 50.0% of J_2 were dead in 10.0%, 7.0% and 5.0% concentrations of the culture filtrates of *B. subtilis*, whereas, at the same time, similar percentage of mortality was observed only for 10.0% concentration of *P. fluorescens*. It suggests that between the two rhizospheric bacteria, *B. subtilis* might have better capability in affecting the survival of J_2 of RKN than *P. fluorescens*. *B. subtilis* produces antibiotics zwittermicin A, kanosamine, lipopeptides, bacisubin, endotoxins, a variety of antibiotics of bacilomycin group, iturin, fungistatin, mycobacilin and mycosubtilin and hydrolytic enzymes such as proteases, chitinase, lipases, b-glucanases, cllulase which exhibit a broader spectrum of activity in nematode supression (Leifert et al., 1995; Pal-Bais et al., 2004; Chaurasia et al., 2005; Kudryashova et al., 2005; Liu et al., 2007). Proteases and chitinase extracted from the culture filtrates of *Bacillus* spp. can hydrolyze peptide bonds and N-acetyl-D-glucosamine polysaccharide chains, respectively, found in the chitin/protein complex of eggs of RKN (Castaneda-Alvarez and Aballay, 2016). On the other hand, Siddiqui and Shaukat (2003) reported that *P. fluorescens* produce 2, 4-diacetylphloroglucinol and hydrogen cyanide that inhibit egg hatch and induce juvenile mortality of *M. javanica*. *Bacillus* spp. are considered excellent candidate for the formulation of a stable bio-nematicide as they can form highly resistant endospore under aerobic condition and can show tolerance against extreme environmental condition in dormant state (Padgham and Sikora, 2007; Cawoy et al., 2011).

There are a number of *B. subtilis* and *P. fluorescens* based bio-nematicides available in market *viz* Rhizo Plus, SERENADE, Bio-Cure-B, Biocomp-X, SHEATHGUARD, BioStart, Stanes Sting, Quratzo, Pathway Consortia etc. (Berlitz et al., 2014; Abu-Elgawad and Askary, 2018; Engelbrecht et al., 2018). Formulation of biological control agents is not a straightforward task, because their performance largely depends on their ability to establish in and disperse through new environment (Bordeur, 2012; Engelbrecht et al., 2018). Thus, it is very important to isolate effective biological control agents from local environmental condition (Ramezani et al., 2013). In this experiment, we have worked with two locally isolated stains of rhizospheric bacteria and found that 25.0% concentration of their culture filtrate could cause 100.0% mortality and inhibition of hatching of *M. javanica*. Our findings will help in the formulation of *B. subtilis* and *P. fluorescens* based bio-nematicides while determining LC_{50} against RKN. Although this experiment was conducted in a controlled condition and the

efficacy of any biological control agent depends on many factors in natural soil, our research findings provide strong support for undertaking similar research in field involving other RKN.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was a part of PhD research of the first author financially supported by the World Bank funded National Agricultural Technology Program, Phase – II (NATP - 2) Project (Project ID: P149553) in Bangladesh.

References

Abo-Elyousr, K.A., Khan, Z., Award, M.E., Abedel-Moneim, M.F., 2010. Evaluation of plant extracts and *Pseudomonas* spp. for control of root-knot nematode, *Meloidogyne incognita* on tomato. Nematropica. 40, 289–299.

Abu-Elgawad, M.M.M., Askary, T.H., 2015. Impact of phytonematodes on agriculture economy. In: Askary, T.H., Martinelli, P.R.P. (Eds.), Biological control Agents of Phytonematodes. CABI, Wallingford, pp. 3–49.

Abu-Elgawad, M.M.M., Askary, T.H., 2018. Fungal and bacterial nematicides in integrated nematode management strategies. Egypt. J. Biol. Pest Control. 28, 74–98.

Baermann, G., 1917. Eine eifache Methode Zur Auffindung von Anklyostomum (Nematoden) larven in Erdproben. Geneesk. Tijdschr. Ned. -Indie. 57, 131–137.

Berlitz, D.L., Knaak, N., Cassal, M.C., Fiuza, L.M., 2014. *Bacillus* and Biopesticides in control of phytonematodes. In: Sahayaraj, K. (Ed.), Basic and Applied Aspect of Bio-nematicides. Springer, India, pp. 3–16.

Bird, A.F., Wallace, H.R., 1965. The influence of tempertaure on *Meloidogyne hapla* and *Meloidogyne javanica*. Nematologica. 11, 581–589.

Bordeur, J., 2012. Host specificity in biological control: insights from opportunistic pathogens. Evol. Appl. 5, 470–480.

Brand, D., Soccol, C.R., Sabu, A., Roussos, S., 2010. Production of fungal biological control agents through solid state farmentation: a case study on *Paecilomyces lilacinus* agent root-knot nematodes. Microbiol. Aplicada Int. 22 (1), 31–48.

Castaneda-Alvarez, C., Aballay, E., 2016. Rhizobacteria with nematicide aptitude: enzymes and compounds associated. World J. Microbiol. Biotechnol. 32, 137–140.

Cawoy, H., Wagner, B., Fickers, P., Ongena, M., 2011. Bacillus-based biological control of plant diseases. In: Stoytcheva, M. (Ed.), Pesticides in the modern world – pesticides use and management. InTech, Rijeka, pp. 272–302.

Chaurasia, B., Pandeya, A., Palnib, L.M.S., Trivedia, P., Kumara, B., Colvine, N., 2005. Diffusible and volatile compounds produced by an antagonistic *Bacillus subtilis* strain cause structural deformations in pathogenic fungi in vitro. Microbiol. Res. 160, 75–81.

Crawford, J.M., Clardy, J., 2011. Bacterial symbionts and natural products. Chem. Commun. 47, 7559–7566.

Das, S., Wesemael, W.M.L., Perry, R.N., 2011. Effect of temperature and time on the survival and energy reserves of juveniles of *Meloidogyne* spp. Agric. Sci. Res. J. 1, 102–112.

Decraemer, W., Hunt, D.J., 2006. Structure and classification. In: Perry, R.N., Moens, M. (Eds.), Plant nematology. CABI, Wallingford, pp. 4–32.

Dejene, T.A., 2014. Opportunities for biological control of root-knot nematodes in organic farming systems: a review. Int. J. Org. Agric. Res. Develop. 9, 87–107.

Delfosse, E.S., 2005. Risk and ethics in biological control. Biol. Control 35, 319–329.

Engelbrecht, G., Horak, I., Rensburg, P.J.J.V., 2018. Bacillus-based bio-nematicides: development, modes of action and commercialisation. Biol. Control Sci. Technol. 28, 629–653.

Hunt, D.J., Handoo, Z.A., 2009. Taxonomy, identification and principal species. In: Perry, R.N., Moens, M., Star, J.L. (Eds.), Root-knot nematodes. CABI, Wallingford, pp. 55–88.

Huang, W., Cui, J.K., Liu, S., Peng, D., 2016. Testing various biological control agents against the root-knot nematode (*Meloidogyne incognita*) in cucumber plants identifies a combination of *Syncephalastrum racemosum* and *Paecilomyces lilacinus* as being most effective. Biol. Control 92, 31–37.

Jamily, A.S., Toyota, K., Koyama, Y., 2018. Isolation of *Bacillus* spp. from afganistan soils and their potential in suppressing the root-knot nematodes on tomato. Soil Microorgan. 72, 39–49.

Karssen, G., Moens, M., 2006. Root-knot nematodes. In: Perry, R.N., Moens, M. (Eds.), Plant nematology. CABI, Wallingford, pp. 59–88.

Kepenekci, L., Dura, O., Dura, S., 2017. Determination of nematicidal effects of some bio-pesticides against root-knot nematode (*Meloidogyne incognita*) on kiwifruit. J. Agric. Sci. Technol. 7, 546–551.

Khokon, M.A.M., Okuma, E., Rahman, T., Wesemael, W.M.L., Murata, Y., Moens, M., 2009. Quantitative analysis of the effects of diffusates from plant roots on the hatching of *Meloidogyne chitwoodi* from young and senescing host plants. Biosci. Biotechnol. Biochem. 73, 2345–2347.

Kudryashova, E.V., Vinokurova, N.G., Ariskina, E.V., 2005. *Bacillus subtilis* and phenotypically similar strains producing hexane antibiotics. Appl. Biochem. Microbiol. 41, 486–489.

Krieg, N.R., Holt, J.G., 1984. Bergey's Manual of Systematic Bacteriology. Williams and Wilking Company, Baltimore, MD, USA.

Leifart, C., Li, H., Chidburee, S., Hampson, S., Workman, S., Sigee, D., Epton, H.A.S., Harbour, A., 1995. Antibiotic production and biological control activity by *Bacillus subtilis* CL27 and *B. pumilus* CL45. J. Appl. Biotechnol. 78, 97–108.

Liu, Y.F., Chen, F., Ng, T.B., Zhang, J., Zhou, M.G., Song, F.P., Lu, F., Liu, Y.Z., 2007. Bacisubin, an antifungal protein with ribonuclease and hemagglutinating activities from *Bacillus subtilis* strain B-916. Peptides 28, 553–559.

Mahesha, H.S., Ravichandra, N.G., Rao, M.S., Narasegowda, N.C., 2017. Bio-efficacy of different strains of *Bacillus* spp. against *Meloidogyne incognita* under in vitro. Int. J. Curr. Microbiol. Appl. Sci. 6 (11), 2511–2517.

Moosavi, M.R., Zare, R., Zamanizadeh, H.R., Fatemy, S., 2010. Pathogenecity of *Pochona* species on egg of *Meloidogyne javanica*. J. Invertebr. Pathol. 104, 125–133.

Mohammad, S.H., Anwer, M., Saedy, E., Mohamed, R., Enam, N., Ibrahim, E., Ghareeb, A., Salah, A.M., 2008. Biological control efficiency of *Bacillus thuringiensis* toxins against root-knot nematode, *Meloidogyne incognita*. J. Cell Mol. Biol. 7, 57–66.

Padgham, J.L., Sikora, R.A., 2007. Biological control potential and modes of action of *Bacillus megaterium* against *Meloidogyne graminicola* on rice. Crop Prot. 26, 971–977.

Pal-Bais, H., Fall, R., Vivanco, J.M., 2004. Biological control of *Bacillus subtilis* against infection of arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiol. 134, 307–319.

Rahman, S.F.S.A., Singh, E., Pieterse, C.M.J., Schenk, P.M., 2018. Emerging microbial biological control strategies for plant pathogens. Plant Sci. 267, 102–111.

Ramezani, M.M., Mahdikhani, M.E., Baghaee, R.S., Rouhani, H., 2013. The nematicidal potential of local *Bacillus* species against the root-knot nematode infecting greenhouse tomatoes. Biocontrol Sci. Technol. 24, 279–290.

Sasser, J.N., Eisenback, J.D., Carter, C.C., Triantaphyllou, A.C., 1983. The International Meloidogyne project - its goals and accomplishments. Annu. Rev. Phytopathol. 21, 271–288.

Schaad, N.W., 1980. Laboratory guide for identification of plant pathogenic bacteria. Bacteriology Committee of American Phytopathological Society, Minnesota, USA.

Sehebani, N., Hadavi, N., 2008. Biological control of the Root-Knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. Soil Biol. Biochem. 40, 2016–2020.

Sela, S., Schickler, H., Chet, I., Spiegel, Y., 1998. Purification and characterization of *Bacillus cereus* collagenolytic/proteolytic enzyme and its effect on *Meloidogyne javanica* cuticular proteins. Eur. J. Plant Pathol. 104, 59–67.

Sheppard, A.W., Shaw, R.H., Sfroza, R., 2005. Environmental weeds for classical Biological Control in Europe: a review of opportunities, regulations and other barriers to adoption. Weed Res. 46, 93–117.

Siddiqui, Z.A., Mahmood, I., 1999. Role of bacteria in the management of plant parasitic nematodes; a review. Bioresour. Technol. 69, 167–179.

Siddiqui, I.A., Shaukat, S., 2003. Suppression of root-knot disease by *Pseudomonas fluorescens* CHA0 intomato: importance of bacterial secondary metabolites 2,4-diacetylphloroglucinol. Soil Biol. Biochem. 35, 1615–1623.

Silva, J.D.O., Santana, M.V., Freire, L.L., Ferreira, B.D.S., Rocha, M.R.D., 2017. Biological control agents in the management of *Meloidogyne incognita* in tomato. Ciência Rural Santa Maria. 47, (10) e20161053.

Stiriling, G.R., 1991. Biological control of plant parasitic nematodes. Progress, problems and prospects. CAB International, UK, p. 282.

Wilson, M.J., Jackson, T.A., 2013. Progress in the commercialisation of bio-nematicides. Biol. Control 58, 715–722.

Xia, Y., Xie, S., Ma, X., Wu, H., Wang, X., Gao, X., 2011. The *purl* gene of Bacillus subtilis is associated with neamticidal activity. FEMS Microbial. Lett. 322, 99–107.