Potential of *Paenibacillus* spp. as bio control agent for root-knot nematodes (*Meloidogyne* spp.)

Jackline A. Bakengesa

**Promoter & Supervisor:** Prof. Dr. Ir. Wim Wesemael

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Potential of *Paenibacillus* spp. as bio control agent for root-knot nematodes (*Meloidogyne* spp.)

Jackline A. BAKENGESA¹, Wim M. L. WESEMAEL¹,²,³

¹Department of Biology, Faculty of Sciences, Ghent University, K.L. Ledeganckstraat 35, B-9000, Ghent, Belgium
²Institute for Agricultural and Fisheries Research (ILVO), Burgemeester Van Gansberghelaan 96, 9820 Merelbeke, Belgium
³Laboratory for Agrozoology, Faculty of Bioscience engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium
Abstract; Root-knot nematodes are the most damaging plant-parasitic nematodes. Potential of *Paenibacillus* spp. as a bio-control agent for root-knot nematodes (*M. fallax, M. chitwoodi, M. javanica, M. enterolobii, M. incognita* and *M. hapla*) was examined *in vitro* and *in vivo*. This includes its effect on nematode attraction, penetration, mortality, hatching and multiplication. Plant growth and phytotoxic effect were also assessed. All *in vitro* experiments were done at room temperature. Plants in pot experiment were grown in the growth chamber (16 hours light, 8 hours dark with temperature between 22°C and 24°C). 10% and 100% bacterial suspension (BS) were used as treatments with buffer and distilled water as control. 100% *Paenibacillus* spp. BS strongly caused J2 mortality and reduced hatching (p<0.05). Hatching was inhibited by 97%-99% for all tested species. More than 90% J2 mortality occurred after 24 hours of exposure in 100% BS. Some J2 recovered when exposed in distilled water with increasing time. 10% BS also reduced final population of all tested species of root-knot nematodes in a pot experiment. Root gall index was the same for all treatments. Phytotoxic effect on plants was observed at 100% BS but seems to be influenced by climatic conditions particularly temperature. Increase of plant growth due to *Paenibacillus* spp. was also observed. *Paenibacillus* spp. was found to have nematostatic and nematicidal effects on root-knot nematodes.

**Key words;** Biological control, mechanism, *Paenibacillus* spp., root-knot nematodes
Plant-parasitic nematodes cause a loss of about $125 billion worldwide due to annual crop losses (Chitwood, 2003). Among all, root-knot nematodes have increased in importance in different parts of the world including Europe and Africa (Wesemael et al., 2011; Onkendi et al., 2014) and cause substantial damage. Different species of root-knot nematodes have been found from warmer to cooler areas. For example the more aggressive species *M. enterolobii* has been found in Europe and different parts of Africa such as DRC, Burkina Faso, Malawi, South Africa Mozambique, Togo, and Senegal.

Root-knot nematodes, (genus *Meloidogyne* Goldi, 1887) penetrate within plant roots, feed, reproduce and induce small to large galls triggering a plant disease known as root-knot. They are of great economic importance as they disrupt plant physiology and may reduce crop yield and quality due to their endoparasitic form of living and feeding (Karssen et al., 2013).

Chemical control has been worldwide used as a primary means of control, not only for root-knot nematodes but also for other plant pathogens as well. These chemicals have several negative impacts to the environment, biodiversity and humans at large which led to a total prohibition or restricted use of some of the nematicides (Nyczepir & Thomas, 2009; Haydock et al., 2013). Hence causes the impetus for new, urgent, safe and more effective approaches. This is necessary to ensure higher agriculture production to fit the growing world population while keeping the environment safe for present and future generations.

To fulfill this goal organic inputs and microbial inoculants have become main interests (Chauhan et al., 2015). This is due to the fact that these could be the best alternative for the chemical products for sustainable agriculture (Ashraf et al., 2013) such as the use of biocontrol agents. Promising results have been obtained and repeatedly tested on control of plant-parasitic nematodes by using antagonistic bacteria (Giannakou et al., 2004) such as *Pseudomonas aeruginosa* (Siddiqui et al., 2000) on control of *Meloidogyne* spp. Rhizobacteria assists on triggering plants endogenous defense mechanisms i.e. *Pseudomonas fluorescens* (M'piga et al., 1997). Root occupation by rhizosphere bacteria also lessen nematode invasion (Siddiqui & Shaukat, 2004). Xiong et al. (2015) showed the efficacy of bacterium *Bacillus firmus* YBf-10 as a biocontrol agent. It exhibits nematicidal activity against root-knot nematodes including motility, inhibition of hatching and above all lethal activity. Nematicidal effect is due to activity
of bioactive secondary metabolites. Its efficacy is comparable with that of fenamiphos, the broadly used chemical nematicide.

Biological control basically involves the usage of microbial agents (living organisms) for management of plant diseases and pests (Karssen et al., 2013). Bio-control can be geared by some organisms including fungi, mites and bacteria. The study by Hashem and Abo-Elyousr (2011) reported significant results due to application of different biocontrol agents such as; mortality of nematodes, induction of systemic resistance in plants and significant growth due to availability of enough nutrients. Among others, one of the extensive studied biological control agents are plant growth promoting rhizobacteria (PGPR) (Khan et al., 2008). PGPR have beneficial effects on seed germination, emergence and colonization of roots, mineral nutrition and water utilization, suppression of diseases and hence overall plant growth (Siddiqui et al., 2007).

PGPR operation mechanisms is either directly, indirectly or combination of both (Martínez-Viveros et al., 2010; Chauhan et al., 2015). Direct mechanisms include assisting on uptake of essential nutrients and secretion of plant growth promoting metabolites like cytokinins, indole acetic acid (IAA), gibberellins, etc., Indirect mechanism is through production of antibiotics to reduce or prevent pathogenic effects, such compounds are siderophores, hydrogen cyanide (HCN), etc. Within PGPRs some have reached the stage of commercial success such as Azospirillium and Bacillus while others not yet as Paenibacillus (Ashraf et al., 2013).
Regardless reasonable public and legislative pressure to lessen the use of nematicides due to possible health and environmental risks, only a minority of biocontrol agents have been established and none is widespread in use (Viaene et al., 2013). But researches are done worldwide on different organisms for their possibility to be used on control, a rhizobacteria *Paenibacillus* being one.

*Paenibacillus* spp. is among PGPR that forms a biofilm around roots (root tips). In this respect it can hamper penetration of harmful organisms to plants particularly root-knot nematodes hence the potential to be used as biocontrol agent. Timmusk et al. (2005) recommended *P. polymyxa* as potential biocontrol agent for commercial purpose due to its ability to form endospores, production of several kinds of antibiotics and possibility to colonize several host plants. Khan et al. (2008) found exposure of root-knot nematode *M. incognita* to various concentrations of culture filtrate of *P. polymyxa* GBR-1 under *in vitro* conditions significantly reduced hatching from eggs and caused substantial mortality of its juveniles.
Studies on *P. polymyxa* as a potential tool for bio control mostly has been based on antibiotics production (Timmusk *et al.*, 2005). Its mechanism of action on nematode reduction was suggested area of study by Khan *et al.* (2008). In this study, the potential of *Paenibacillus* spp. and its mechanism of action as a biocontrol agent for *Meloidogyne* spp. were assessed on concentration basis. The experiments were conducted under *in vitro* and greenhouse conditions. The effects of *Paenibacillus* spp. on nematode attraction, penetration, mortality, hatching and multiplication were examined and reported.

**MATERIALS AND METHODS**

**Nematode culture**

Species of root-knot nematodes (*M. fallax*, *M. chitwoodi*, *M. javanica*, *M. enterolobii*, *M. incognita* and *M. hapla*) were obtained from pure cultures maintained at ILVO, Merelbeke, Belgium. Cultures were multiplied on tomato and/or in transparent closed containers on potato tubers.

Multiplication on tomato plants, cultivar Marmande was used. Seedlings were allowed to develop a dense root system in organic soil (PeltrAcom N.V, NPK fertilizer added (14-16-18) 1.4kg/m³, PH 5.8). After 4 weeks they were transplanted in 2l pots filled with heat sterilized soil (100°C, 16h). Then 2000 of pure culture root-knot nematode juveniles (*J2*) were inoculated. Each day the plants were watered to field capacity. Nematodes were left to multiply for three months and then harvested.

In closed containers, potato tubers (cultivar Bintje) were used. Tubers were cleaned with tap water and then submerged in a 5% sodium hypochlorite (NaOCl) solution and left for four minutes to disinfect the tubers. The potatoes were then rinsed with tap water to remove residual NaOCl. Potato tubers were dried and left to sprout for three weeks.

Transparent closed containers (11.5cm diameter and 8.5cm length) were filled with 200g of sterilized white sand soil and watered with 30ml of sterile tap water. Per container one sprouted potato tuber was placed with the point of sprout touching the sand. These closed containers with potato tubers were then stored in the dark room at 20°C until formation of roots.
Then 2000 freshly hatched J2 of root-knot nematodes were inoculated into each container and left to reproduce. After two months the nematodes were ready to be harvested.

Nematode inoculums were prepared from maintained pure cultures. Infected tomato and potato plants were uprooted and the entire root system gently cleaned with water to remove the soil. The roots were then cut into small pieces of about 2mm and placed on Baermann funnel (Baermann, 1917). Freshly hatched J2 were collected after every 24 hours. After each collection the water in the Baermann funnel was refreshed. The collected nematode suspension was then thoroughly homogenized and a subsample of 5ml was poured on a counting dish. With the aid of a microscope and a counter, the nematodes were counted three times in 5ml aliquots to calculate the nematode density. The inoculum was used for both in vitro and pot experiments.

**Bacteria culture**

*Paenibacillus* spp. was grown in Brain Heart Infusion (BHI) (OXOID LTD, Basingstoke, and Hampshire, England) agar plates and liquid medium.

For BHI agar plates, 18.5g of BHI was mixed with 7.5g of bacterial agar and dissolved in 475ml of distilled water. The mixture was shaken to dissolve and autoclaved at 121°C for 15 minutes. 25ml of sterilized 10% glycerol was added, shaken thoroughly, then poured on agar plates and left to cool and solidify under laminar flow. The plates were stored in the refrigerator at 4°C.

Liquid medium was prepared by dissolving 18.5g of BHI medium in 475ml of distilled water. The mixture was autoclaved at 121°C for 15 minutes. 25ml of sterilized 10% glycerol was added and shaken thoroughly. The medium was then stored at room temperature.

*Paenibacillus* spp. culture was obtained from bacteriology department –ILVO. A single bacterial colony was isolated and transferred to BHI agar plates and incubated overnight at 28°C. This was repeated twice in order to obtain pure fresh cultures. Then bacteria agar plates were maintained at 4°C in the refrigerator. Bacteria were continuously cultured whenever required in order to obtain fresh bacteria.

To obtain inoculum, the *Paenibacillus* spp. single colony was transferred into 3ml BHI liquid medium as pre-culture and incubated overnight in an incubator shaker at 28°C and continuously shaken at 200rpm. 1.5ml of pre culture bacteria was poured into 500ml liquid medium as the main culture. This was also incubated overnight at 28°C and 200rpm.
The *Paenibacillus* spp. suspension from the main culture was transferred to the sterilized centrifuge bottles 175ml each. The suspension was centrifuged at 4°C for 15 minutes at 4000rpm to form pellet. The supernatant was discarded and 500ml buffer (sodium phosphate) solution added. Buffer was prepared by dissolving 4.26g of sodium phosphate dibasic with 2.72g of potassium phosphate monobasic (Sigma-Aldrich Co, USA) on a magnetic hot plate to make one liter of 0.05M buffer. This was filtered into sterilized bottles through a plastic sterilized disposable filter (250ml volume) under vacuum. The filter has a nozzle where the tubing from the vacuum outlet is connected to. The vacuum was turned on and the buffer poured slowly through the filter.

The *Paenibacillus* spp. pellets and buffer solution were gently mixed and this was taken as stock solution. Dilutions were made with buffer to obtain different concentrations (10% and 100%). The use of buffer was important to remove nutrients agar and medium which could have influence on bacteria and plant during experiments. All the activities were done under laminar flow.

*Paenibacillus* spp. density from the stock solution was determined by optical density and serial dilution. Optical density was done with a spectrophotometer. 1ml of the solutions, BHI liquid medium and buffer as blank and *Paenibacillus* spp. stock solution in different transparent tubes (cuvette) were placed in the machine. Beam light was allowed to pass to determine the density of each solution.

Colony forming units after different serial dilutions of *Paenibacillus* spp. stock solution (10^-1 to 10^-10) were also observed. 50µl of bacterial solution after each dilution was plated on pseudomonas agar plate. The bacteria were allowed to grow overnight in the incubator at 28°C and the number of colonies were counted.

(Pseudomonas agar plates were prepared by dissolving 18.8g of difco pseudomonas powder (Becton, Dickinson and Company, Sparks, USA) in half liter of distilled water; mixed thoroughly and autoclaved at 121°C for 15 minutes. Then poured on agar plates and left to solidify under laminar flow. Agar plates were stored in the refrigerator at 4°C until use.)

Actual *Paenibacillus* spp. cells were 10^8 Colony Forming Unit /ml, this was considered as stock bacterial suspension (BS).
**In vitro experiment**

Tomato seeds (cultivar Marmande) were grown in petri dishes (14cm diameter) with a 4 layer tissue paper moistened with distilled water. The plates were covered with parafilm and stored in the dark room at 20°C. Seeds were left to germinate for two weeks.

**Paenibacillus spp. phytotoxicity test on tomato seedlings**

20 tomato seedlings were dipped into BS of concentrations 10% and 100% each for 2 hours. Distilled water and buffer were taken as control. The status of the seedlings was observed in two experimental setups, one during a sunny day and the second during a cloudy and cool day. The experiments were done at room temperature with average temperature 25-26°C on a sunny day and 20-21°C on a cloudy, cool day.

**Effect of Paenibacillus spp. on J2 mortality**

Into tubes (10ml total volume) 5ml of different treatments (10% BS, 100% BS, buffer and distilled water) were placed. 100 freshly hatched J2 were added and stored into dark at 20°C. Observation of mortality was done after 3 hours and 24 hours. During observation the immobile J2 were probed with picking needle, if not moving they were considered dead. After observation the nematodes were transferred in distilled water to check for recovery for 3 hours and 24 hours.

**Effect of Paenibacillus spp. on hatching**

Infected potato and tomato roots from the stock cultures were gently cleaned. Under microscope egg masses were picked from the root system. Three egg masses were placed on a sieve (length 2.5cm, diameter 1cm and 48µm mesh) and placed in tubes of 10ml volume. 5ml of distilled water and buffer as control, and *Paenibacillus* spp. suspension (10% and 100%) was poured into each tube. Then tubes were covered with a perforated lid to allow oxygen exchange but limit evaporation and stored in the dark room at 20°C. The numbers of hatched J2 were counted weekly during four weeks. After each count, the solution was replaced according to each treatment.

After four weeks all the solutions were replaced with distilled water. Observation on hatching recovery was done for two weeks on egg masses previously treated with 100% BS. The numbers of non-hatched eggs were then counted for all egg masses. Therefore egg masses were submerged with 1% NaOCl solution and eggs separated from the gelatinous matrix with bluntly forceps. With aid of microscope all non-hatched eggs were counted.
**Paenibacillus spp. effect on nematode attraction and penetration**

Tomato seedlings were dipped into BS of different concentrations (10%, 100%), distilled water and buffer as control for 2 hours. Seedlings were then transferred on agar plate (1% of basic technical agar (OXOID LTD, Basingstoke, Hampshire, England) and 100 freshly hatched J2 of RKN inoculated 1cm from the rootlet. Observation was done after 24 hours, 48 hours and 5 days. Roots of the seedling were then stained (Byrd Jr et al., 1983) to count the number of penetrated nematodes. Roots of each plant were cut into small pieces of about 1cm and placed into 25ml of tap water. 10ml of NaOCl solution was added into each and left for 4 minutes. This was important to soften the roots tissues for good staining. The roots were then rinsed well with flowing tap water. 30ml of tap water with 1ml of staining solution (3.5g acid fuschin dissolved in 250ml acetic acid and 750ml distilled water) was added into each and heated on a hot plate until boiling point for 30 seconds. After cooling the roots were well rinsed, covered with glycerol, heated until boiling point and left to cool. Then numbers of penetrated J2 were counted.

Liquid pluronic gel was also used. It was prepared by dissolving 287.5g of powder pluronic gel into 800ml of distilled water in order to make one liter (Wang et al., 2009). The mixture was then stirred with a magnetic stirrer for 24 hours in cold room (4°C) to dissolve. The gel was then stored in the refrigerator at 4°C until use. During experiment the gel was poured in plates to solidify at room temperature. Tomato seedlings dipped into different treatments (bacterial suspension, buffer and distilled water) were placed on it individually. 100 freshly hatched J2 were inoculated 1cm from the rootlet. Numbers of non-penetrated nematodes were counted. After staining (see above section) penetrated nematodes were also counted.

**Greenhouse experiments**

Tomato seeds were planted in plastic pots (8.8cm diameter and length) with organic soil and left to germinate and grow. At the fourth leaves stage the plants were shifted to the growth chamber (16 hours light, 8 hours dark with temperature between 22°C and 24°C) and inoculated with 10ml of 10% and 100% BS, distilled water and buffer as control. Inoculation was repeated after two days.

After one week the plants were trans-planted in 1l plastic pots filled with heat sterilized soil (100°C, 16h) and inoculated with 500 freshly hatched J2 of root-knot nematodes. Plants were left to grow for eight weeks; watered at field capacity on daily basis.
8 weeks after nematode inoculation, different data were first collected, this includes; plant height and fruits weight. Plants were then carefully uprooted from pots and the soil attached to the roots gently removed, root weight, gall index, fresh and dry mass of shoots were also recorded.

Then by Automatic Zonal Centrifugation (AZC) method (Hendrickx, 1995) nematodes were extracted from each plant whole root system as organic sample and from 200cc of homogenized soil.

**Experimental design and data analysis**

All experiments were set up in a completely randomized design (factorial). Five replications for each treatment and experiment were done for six *Meloidogyne* spp. (*M. fallax*, *M. chitwoodi*, *M. javanica*, *M. enterolobii*, *M. incognita* and *M. hapla*).

Package Rstudio (R 3.2.3) was used for all statistical analyses. The assumptions for ANOVA were tested using Shapiro test for normality and Levene’s test for homogeneity of variances. Duncan multiple range test was performed as post-hoc test. When the assumptions were not met, data were transformed using log(x+1) or log x. And a non-parametric Kruskal-Wallis test was carried out.

**RESULTS**

**In vitro experiment**

*Paenibacillus* spp. phytotoxicity test on tomato seedlings

Observations on phytotoxic effect of *Paenibacillus* spp. on tomato seedlings (Table 1) showed contradicting results on different days. On a sunny day (25-26°C), all seedlings dipped in 100% BS became weak after 2 hours of exposure while on a cooler day (20-21°C) the seedlings in all treatments were healthy.
Table 1: *Paenibacillus* spp. phytotoxic effect on tomato seedlings at room temperature

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Setup 1, on sunny day (25-26°C)</th>
<th>Setup 2, on a cloudy day (20-21°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH2O</td>
<td>20 healthy</td>
<td>20 healthy</td>
</tr>
<tr>
<td>Buffer</td>
<td>20 healthy</td>
<td>20 healthy</td>
</tr>
<tr>
<td>10%</td>
<td>20 healthy</td>
<td>20 healthy</td>
</tr>
<tr>
<td>100%</td>
<td>20 weak and wilt</td>
<td>20 healthy</td>
</tr>
</tbody>
</table>

**Effect of *Paenibacillus* spp. on J2 mortality**

*Paenibacillus* spp. BS caused significant mortality of J2 of tested species of root-knot nematodes (*P* < 0.0001) compared to distilled water and buffer. This is shown in figures 2 and 3. 100% of BS caused more than 40% mortality within three hours of exposure; *M. hapla* and *M. chitwoodi* were less killed compared to other tested species. J2 mortality caused by 10% of BS was more pronounced after 24 hours than 3 hours. The effect of BS on J2 mortality of root-knot nematodes increased with time of exposure. *M. hapla* and *M. incognita* seem more resistant as after 24 hours of exposure in 10% BS, J2 mortality was less than 10% compared to *M. enterolobii, M. javanica M. fallax* and *M. chitwoodi* with up to 60% of mortality. For both species after 24 hours of exposure in 100% *Paenibacillus* spp. BS, there was more than 90% of mortality.

*Paenibacillus* spp. significant effect (p-values) on J2 mortality compared to control for all the species are: *M. enterolobii* p-value = 1.868e-05, *M. javanica* p-value = 6.986e-06, *M. incognita* p-value = 1.476e-05, *M. hapla* p-value = 1.66e-05, *M. fallax* p-value = 5.79e-06, *M. chitwoodi* p-value = 7.628e-06.
Figure 2: Mortality (%) of J2 of six species of root-knot nematodes exposed to 10% and 100% of *Paenibacillus* spp. BS, and distilled water and buffer as control for 3 hours. Significant differences (p < 0.05) between treatments and species are marked with a different letter.
Figure 3: Mortality (%) of J2 of six species of root-knot nematodes exposed to 10% and 100% of *Paenibacillus* spp. BS, and distilled water and buffer as control for 24 hours. Significant differences (p < 0.05) between treatments and species are marked with a different letter.

Observations on recovery of J2 in distilled water after treatment with 10% and 100% *Paenibacillus* spp. dosage during different times are shown in Table 2. The percentage recovery increased with time the J2 were exposed in distilled water. Total recovery (100%) was observed for J2 treated with 10% BS during 3 hours and then exposed in distilled water for 3 hours. J2 treated with 100% BS during 3 or 24 hours showed no recovery when exposed for 3 hours in distilled water. Exposure of J2 in distilled water for 24 hours after treatment with 10%
Paenibacillus spp. BS resulted to more than 90% J2 recovery and less than 50% J2 recovery after treatment with 100% BS.

Table 2: Average recovery (%) of J2 of all tested species of root-knot nematodes in distilled water after being exposed to different concentrations of Paenibacillus spp. BS during different times

<table>
<thead>
<tr>
<th>Species</th>
<th>Time for recovery</th>
<th>10%BS</th>
<th>100%BS</th>
<th>10%BS</th>
<th>100%BS</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. enterolobii</td>
<td>3 hours</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>100%</td>
<td>65%</td>
<td>90%</td>
<td>40%</td>
</tr>
<tr>
<td>M. javanica</td>
<td>3 hours</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>100%</td>
<td>60%</td>
<td>81%</td>
<td>35%</td>
</tr>
<tr>
<td>M. incognita</td>
<td>3 hours</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>100%</td>
<td>72%</td>
<td>99%</td>
<td>42%</td>
</tr>
<tr>
<td>M. hapla</td>
<td>3 hours</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>100%</td>
<td>59%</td>
<td>98%</td>
<td>48%</td>
</tr>
<tr>
<td>M. fallax</td>
<td>3 hours</td>
<td>99%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>99%</td>
<td>61%</td>
<td>85%</td>
<td>40%</td>
</tr>
<tr>
<td>M. chitwoodi</td>
<td>3 hours</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>100%</td>
<td>60%</td>
<td>90%</td>
<td>31%</td>
</tr>
</tbody>
</table>

Effect of Paenibacillus spp. on hatching of root-knot nematodes

Paenibacillus spp. BS showed strong effect (p < 0.01) on inhibition of hatching of J2 for all tested species of root-knot nematodes (Figure 4). Treatment with 100% of BS resulted in less than 4% J2 hatched for the consecutive four weeks. Hatching was reduced by 97% to 99%. This was the case for all tested species of root-knot nematodes. Hatching parameters m (the time at which 50% of the total hatching is reached), b (the hatching rate) and c (the maximum hatching percentage) were calculated except for 100% BS where m and b values could not be calculated due to strong inhibition of hatching. For 10% BS, buffer and distilled water the numbers of
hatched J2 were not significantly different. No recovery on hatching was observed in distilled water for egg masses treated previously with 100% BS.

Figure 4: Fitted curves showing the expected cumulative (%) hatch of six species of root-knot nematodes exposed to 10% and 100% of *Paenibacillus* spp. BS, and distilled water and buffer as control for four consecutive weeks.

Due to inhibition of hatching by 100% *Paenibacillus* BS no hatching curves could be calculated for this concentration and analysis was only done for the final (low) hatching percentage (Table 3).
Table 3: Parameters of the logistic curve $y = c/(1 + \exp(-b\times(time-m)))$ describing J2 hatching of root-knot nematodes at different concentration of *Paenibacillus* spp. treatment and control. The results are the means of five replicates of the time at which 50% of the total hatching is reached (m), the hatching rate (b) and the maximum hatching percentage (c). Significant differences between treatments for each species are marked with a different letter.

<table>
<thead>
<tr>
<th>Species</th>
<th>B</th>
<th>m</th>
<th>c</th>
<th>B</th>
<th>m</th>
<th>c</th>
<th>B</th>
<th>m</th>
<th>c</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>dH2O</td>
<td>Buffer</td>
<td>10%</td>
<td>dH2O</td>
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<td>10%</td>
<td>dH2O</td>
<td>Buffer</td>
<td>10%</td>
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<tr>
<td><em>M. enterolobii</em></td>
<td>1.80a</td>
<td>1.66a</td>
<td>1.22a</td>
<td>0.28a</td>
<td>0.54a</td>
<td>0.25a</td>
<td>33.49b</td>
<td>66.59a</td>
<td>45ab</td>
</tr>
<tr>
<td><em>M. javanica</em></td>
<td>1.96a</td>
<td>2.04a</td>
<td>2.58a</td>
<td>1.74a</td>
<td>2.60a</td>
<td>1.86a</td>
<td>58.87a</td>
<td>41.89a</td>
<td>51.73a</td>
</tr>
<tr>
<td><em>M. incognita</em></td>
<td>1.66a</td>
<td>2.28a</td>
<td>2.28a</td>
<td>2.42a</td>
<td>2.36a</td>
<td>2.60a</td>
<td>66.65a</td>
<td>62.9a</td>
<td>61.35a</td>
</tr>
<tr>
<td><em>M. hapla</em></td>
<td>1.78a</td>
<td>2.36a</td>
<td>1.78a</td>
<td>2.12a</td>
<td>2.64a</td>
<td>2.54a</td>
<td>74.01a</td>
<td>43.51b</td>
<td>35.8</td>
</tr>
<tr>
<td><em>M. fallax</em></td>
<td>1.50a</td>
<td>1.44a</td>
<td>2.18a</td>
<td>1.1a</td>
<td>1.4a</td>
<td>1.3a</td>
<td>45.76a</td>
<td>64.63a</td>
<td>50.26a</td>
</tr>
<tr>
<td><em>M. chitwoodi</em></td>
<td>1.54a</td>
<td>1.70a</td>
<td>1.38a</td>
<td>1.60a</td>
<td>2.00a</td>
<td>1.96a</td>
<td>63.43a</td>
<td>52.95a</td>
<td>38.24b</td>
</tr>
</tbody>
</table>

*Paenibacillus* spp. effect on nematode attraction and penetration

After 48 hours the number of J2 of *M. enterolobii* around the tomato root treated with buffer was significantly higher compared to the one treated with *Paenibacillus* spp. but not with distilled water ($p = 0.04319$). J2 of *M. fallax* around tomato root treated with distilled water was significantly higher than the other treatments ($p = 0.003766$). For *M. chitwoodi*, *M. hapla*, *M. incognita* and *M. javanica* no effect on attraction due to *Paenibacillus* spp. was observed after 24 hours and five days ($p$-value > 0.05) (results not shown).

Similarly, *Paenibacillus* BS had no significant effect on penetration (Figure 5) of J2 of root-knot nematodes compared to the control ($p > 0.05$). On average penetration was less than 30%. For all the species tested, penetration was lower when the seedlings were treated with 100% BS but the difference was not significant (*M. enterolobii* $p$-value = 0.7895, *M. javanica* $p$-value = 0.2605, *M. incognita* $p$-value = 0.6874, *M. hapla* $p$-value = 0.8982, *M. fallax* $p$-value =
0.07056 and *M. chitwoodi* p-value = 0.7139). The average percentage penetration for each species and treatments is shown in figure 5. Highest penetration was observed for *M. hapla*.

![Box plots showing penetration of J2 of six species of root-knot nematodes exposed to 10% and 100% of Paenibacillus spp. BS, and distilled water and buffer as control after five days. Significant differences (p < 0.05) between treatments and species are marked with a different letter.](image)

**Figure 5**: Penetration (%) of J2 of six species of root-knot nematodes exposed to 10% and 100% of *Paenibacillus* spp. BS, and distilled water and buffer as control after five days. Significant differences (p < 0.05) between treatments and species are marked with a different letter.

**Greenhouse experiments**

*Paenibacillus* spp. BS reduced nematode final population density (Pf) compared to buffer and distilled water (Table 4). *Paenibacillus* spp. BS significantly reduced the Pf of *M. fallax* (P = 0.0445) compared to control. For the other tested root-knot nematodes species BS had no significant Pf reduction (*M. enterolobii* p-value = 0.7417, *M. javanica* p-value = 0.1396, *M.*
incognita p-value = 0.2865, M. hapla p-value = 0.6388, M. chitwoodi p-value = 0.3409). 10% BS rendered a lower Pf compared with the 100% BS (p-value > 0.05).

Table 4: Average Pf eight weeks after inoculation of root-knot nematodes on tomato plants treated with 10% and 100% Paenibacillus spp. BS, buffer and distilled water as control. Significant differences between treatments and species are marked with a different letter.

<table>
<thead>
<tr>
<th>Species</th>
<th>Distilled water</th>
<th>Buffer</th>
<th>10% BS</th>
<th>100% BS</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. enterolobii</td>
<td>4919d</td>
<td>3928d</td>
<td>3047d</td>
<td>4334d</td>
</tr>
<tr>
<td>M. javanica</td>
<td>10910e</td>
<td>9393e</td>
<td>6326e</td>
<td>9567e</td>
</tr>
<tr>
<td>M. incognita</td>
<td>36485g</td>
<td>28847g</td>
<td>22700g</td>
<td>25220g</td>
</tr>
<tr>
<td>M. hapla</td>
<td>31749f</td>
<td>32951f</td>
<td>26042f</td>
<td>28083f</td>
</tr>
<tr>
<td>M. fallax</td>
<td>2592b</td>
<td>3054b</td>
<td>1560a</td>
<td>1747a</td>
</tr>
<tr>
<td>M. chitwoodi</td>
<td>4900c</td>
<td>4792c</td>
<td>3293c</td>
<td>3728c</td>
</tr>
</tbody>
</table>

A significant effect of Paenibacillus spp. BS on increase of plant growth was found on plants inoculated with M. javanica where the length (p = 0.03555), shoot fresh (p = 0.000508) and dry weight (p = 0.004822) was significantly higher (Figure 6, 7 and 8). Root weight of tomato plants treated with bacteria and inoculated with M. incognita was also significantly higher (p = 0.03889) compared to control (Figure 9). For plants inoculated with M. enterolobii, the mean length, shoot fresh and dry weight was higher when treated with 100% BS (p > 0.05).

For the plants inoculated with M. chitwoodi, M. hapla and M. fallax, Paenibacillus spp. didn’t increase plant growth parameters compared to buffer and distilled water. Plant growth trend was also not consistent due to different treatments. Some plants treated with buffer and distilled water showed increased growth, for example plants inoculated with M. chitwoodi, means values for different growth parameters were higher compared to the one treated with BS (p > 0.05).

Galling index was similar for all the plants with less than 10% galling. Tomato plants inoculated with M. incognita, M. enterolobii and M. javanica showed more large galls and very obvious egg masses. In all the plants flowers were present.
Figure 6: Average tomato plant length eight weeks after inoculation with six species of root-knot nematodes and treated with 10% and 100% of *Paenibacillus* spp. BS, and distilled water and buffer as control. Significant differences (p < 0.05) between treatments and species are marked with a different letter.
Figure 7: Average tomato plant fresh shoot weight eight weeks after inoculation with six species of root-knot nematodes and treated with 10% and 100% of *Paenibacillus* spp. BS, and distilled water and buffer as control. Significant differences (p < 0.05) between treatments and species are marked with a different letter.
Figure 8: Average tomato plant dry shoot weight eight weeks after inoculation with six species of root-knot nematodes and treated with 10% and 100% of *Paenibacillus* spp. BS, and distilled water and buffer as control. Significant differences (p < 0.05) between treatments and species are marked with a different letter.
Figure 9: Average tomato plant root weight eight weeks after inoculation with six species of root-knot nematodes and treated with 10% and 100% of Paenibacillus spp. BS, and distilled water and buffer as control. Significant differences (p < 0.05) between treatments and species are marked with a different letter.
DISCUSSION

Root-knot nematodes cause qualitative and quantitative damage in the production of different crops. The use of chemicals as control measure is declining day to day due to negative impact on the environment. Among alternatives different antagonistic microorganisms have been tested to be used as bio-control agents for root-knot nematodes. Microorganisms that showed potential are Bacillus spp. (Park et al., 2014) for M. hapla, Purpureocillium lilacinum (Kiewnick & Sikora, 2006) and P. polymyxa (Khan et al., 2008) for M. incognita.

In this study potential of Paenibacillus spp. was studied. Preliminary observations of phytotoxic effect of the bacteria on tomato seedlings showed that the effect can occur due to 100% BS at room temperature between 25-26°C. Higher temperature might result on increasing phytotoxicity. Phytotoxic effect by biological control agent was also reported by Terefe et al. (2009) on tomato seedlings where application of B. firmus at rate of 16 g/pot resulted in seedling mortality. In this study phytotoxic effect was observed on plants on in vitro assays. Plants were exposed direct into higher BS which is not the case for plants in the pots and field condition. Phytotoxic effect of PGPR is related to production of IAA which is toxic at high concentration (Lebuhn et al., 1997). Paenibacillus spp. can cause phytotoxicity on plants but seems to depend on climatic conditions (temperature) and BS concentration. Before its application preliminary testing can be suggested e.g. dipping of seedlings in BS.

Treatment with BS of Paenibacillus spp. in vitro showed significant effect on J2 mortality and hatching for all tested species of root-knot nematodes. Reduction of hatching and J2 mortality occurred to a varying degree depending on the concentration of BS as compared to control (buffer and distilled water). Mortality and immobility was observed on J2 of M. enterolobii, M. javanica, M. incognita, M. hapla, M. fallax and M. chitwoodi treated with BS after 3 hours and 24 hours. The effect was more pronounced with 100% BS whereby just after three hours of exposure more than 50% of immobility occurred. Less than 10% hatching was observed for all the species treated with 100% BS for four consecutive weeks. The reason might be secondary metabolites and antibiotics produced by Paenibacillus spp. (Timmsk et al., 2005). Inhibition of hatching and J2 mortality were also reported by Khan et al. (2008). P. polymyxa GBR-1 was found to significantly inhibit hatching and caused mortality of M. incognita J2.
Observations on J2 recovery suggest that the mechanism of action of *Paenibacillus* spp. can be paralysis. This implies that antibiotics and secondary metabolites found in *Paenibacillus* spp. may act as nematistat and nematicidal. The study by Park *et al.* (2014) also reported strong nematicidal effect of *B. cereus* on *M. hapla*. Recovery increased with time that J2 were exposed in distilled water after the treatment. On the other hand number of recovery decreased with increasing time of exposure into BS. The study by Jung *et al.* (2002) reported the antagonistic effects to significantly increase with duration of the treatments.

Chitin degradation of nematode egg shells caused by chitinase may be a reason for inhibition of hatching. Treatment with 100% BS completely reduced hatching and no recovery was observed after exposure in distilled water for consecutive two weeks. This suggests that chitinase effect on inhibition of hatching can persist for a particular amount of time. Chitinase lysis of the nematode egg shell by *Paenibacillus* spp. was reported by Jung *et al.* (2002) where *P. illinoisensis* KJA-424 caused degradation of *M. incognita* eggshell and resulted in the inhibition of hatching *in vitro*.

Hatching of *M. incognita* was also completely inhibited by *P. polymyxa* GBR-462, GBR-508 and *P. lentimorbus* GBR-158 cultures (Son *et al.*, 2009). The strains were also responsible for increased plant growth. *M. incognita* hatch inhibition and J2 mortality rate was also found to be enhanced with the increase in the concentration of BS and the crude enzymes (gelatinase and chitinase) of *P. elgii* HOA73 (Jung *et al.*, 2002).

A reduction in penetration into the seedlings treated with 100% *Paenibacillus* spp. (p > 0.05) may be attributed to both direct and indirect mechanism of this PGPR such as production of secondary metabolites and root colonization. The study by Timmusk *et al.* (2005) showed the possibility of *P. polymyxa* to form biofilms in plant roots after predominant colonization which was not examined in this study. Biofilms can suggest inhibition of J2 penetration as they were found to protect pathogen infection sites (Timmusk *et al.*, 2005). Investigation on biofilm formation is recommended for future studies to prove its possibility on protecting infection sites that may assure failure of nematode establishment and reproduction.

Root colonization by rhizosphere bacteria was also reported by Siddiqui and Shaukat (2004) to reduce nematode invasion. *M. incognita* penetration of roots, reproduction, and root-knot disease were decreased more by dual inoculations with AM fungi and PGPR (Li *et al.*, 2004).
In pot experiment, some data indicated significant increase of plant growth parameters while others did not. The increase of plant growth parameters were also reported in other studies like Jung et al. (2002) and Khan et al. (2008). The reason for the increase may be attributed to synthesis of plant hormones such as cytokinin and auxin (Schroth & Loper, 1986), facilitation of nutrient availability through nitrogen and phosphate metabolism (Eastman et al., 2014). PGPR also acts as biofertilizer (Chauhan et al., 2015). Study of PGPR P. putida, P. alcaligenes, P. polymyxa and B. pumilus found these species to substantially increase growth of inoculated plants (Siddiqui et al., 2007). Son et al. (2009) reported that the tested strains of Paenibacillus species (P. polymyxa and P. lentimorbus) also promoted plant growth. Application of different biocontrol agents such as P. fluorescens, P. lilacinum and P. guilliermondii has been also found to strengthen the growth of plants via production of natural growth hormones and supplying many nutritional elements, induction of systemic resistance in plants and lethal effect on nematodes (Hashem & Abo-Elyousr, 2011).

Plant growth promotion by Paenibacillus spp. and other PGPR is not always the case due factors like cultivar specificity hence failure of productive association, optimum inoculation density and excess nutrient at the site (Lebuhn et al., 1997). This may result in inconsistency in results between experiments. The study by Timmusk et al. (2003) reported 30% reduction of plant growth and stunted root system on A. thaliana inoculated with P. polymyxa in the absence of biotic or abiotic stress. Under these conditions, Paenibacillus spp. can be considered as a deleterious rhizobacterium. But in abiotic and biotic stress P. polymyxa can induce drought tolerance and antagonizes pathogens (Timmusk & Wagner, 1999).

The conditions under which Paenibacillus spp. application is done should be taken into consideration to ensure that no deleterious effects occur. The induction of drought tolerance can be interesting especially in field conditions in places where water is the limiting factor. PGPR being isolated from natural environments (Lal & Tabacchioni, 2009) can be an advantage for easier plant colonization and persistence in the field.
Galling Index was similar for all plants in this study. But higher reduction of galling and nematode multiplication due to PGPR has been reported in several studies (Khan et al., 2008; Park et al., 2014). This may be due to factors like production of antibiotic and toxic metabolites which enhance bio-controlling effects on phytopathogenic microorganisms (Laslo et al., 2012).

The PGPR can also be responsible for induction of plants endogenous defense mechanisms (M’piga et al., 1997). The activities of enzymes of phenylpropanoid metabolism and antioxidant have been found to be induced by strain CF05 of *P. polymyxa* (Mei et al., 2014). This shows the potential of PGPR on nematode multiplication reduction hence lessen final population density.

10% BS resulted a higher Pf reduction (p-value>0.05) of *M. enterolobii*, *M. javanica*, *M. incognita*, *M. hapla*, *M. fallax* and *M. chitwoodi* than 100% BS treatment and control. The study by Khan et al. (2008) also concluded that “potted soil treated with 10% concentrations of BS at the rate of 10 ml/plant at 2 days before nematode inoculation is the most appropriate because it provided a reasonable level of protection against *M. incognita* without any phytotoxic effect”. A higher Pf on tomato plants treated with 100% than 10% BS may be related to the improved plant growth, hence nematodes had a better environment. Significant Pf reduction seems to depend on concentration and inoculum amount and this should be further examined. Optimum dosage and time for colonization are crucial elements for the success of any bio-control agent.

The interpretation of the results can predict the potential of *Paenibacillus* spp. for its use especially in greenhouse production and organic farming where nutrients and specific plant pathogens are the limiting factor. Small scale production also facilitates application methods. This can be later extended to field conditions with several biotic and abiotic stresses.

Most studies have been reported on species like *M. incognita*, *M. javanica*, and *M. hapla*. This study shows the potential of *Paenibacillus* spp. on other root-knot species like *M. fallax*, *M. chitwoodi* and *M. enterolobii*. Antagonism of *Paenibacillus* spp. is not limited to root-knot nematodes only but also to other plant pathogens like fungi *F. oxyporum* (Son et al., 2009; Mei et al., 2014).
CONCLUSION AND RECOMMENDATIONS

Tested *Paenibacillus* spp. showed nematostatic and nematicidal effects. Some root-knot nematode J2s were able to recover in distilled water after exposure into *Paenibacillus* BS, while hatching from eggs was completely inhibited with 100% BS. This suggests that its mode of action for the biological control of root-knot nematodes may be paralysis and antibiosis which caused mortality, immobility, prevention of invasion and inhibition of hatching. Improvement of plant growth is also an interesting feature for sustainable production although this may favor plant pathogens as well. *Paenibacillus* spp. have potential to reduce deleterious effects of root-knot nematodes hence seem promising as a biological control agent for root-knot nematodes.

For its effective use as a successfully bio-control agent further studies are required on optimal dosage and inoculum amount. Application timing in order to ensure colonization, biofilms formation and persistence for effective control should also be figured out. This can differ between plant species. Increase in number of replicates may also result into statistical significant results.

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At last, I would like to dedicate my thesis to my parents, Jasson Bakengesa and Sixta Imelda Nagabona for their never-ending support and love.
REFERENCES


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