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Research Article

EFFICACY TESTING OF *PAENIBACILLUS* SPP. AGAINST PENETRATION, HATCHING AND SURVIVAL OF THE ROOT-KNOT NEMATODE (*MELOIDOGYNE CHITWOODI)* AND THE ROOT LESION NEMATODE (*PRATYLENCHUS PENETRANS*).

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ABSTRACT

Root-knot and root lesion nematodes are economically very important nematodes. They cause severe damage every year to a wide variety of crops. The use of chemical nematicides is one of the most widely used control measures for nematode management but their negative effect on health and environment and ineffectiveness after prolonged use have led to a need of safe and more effective control options. Biological control could be the best alternative option to chemicals. Experiment was conducted in the year 2016under *in vitro* conditions at laboratory of ILVO, Belgium. Application of *Paenibacillus* spp. with two different suspensions (10% and 100%) was found effective and caused significant reduction in root penetration, hatching from eggs and substantial mortality of juveniles in both *Meloidogyne chitwoodi* and *Pratylenchus penetrans.* Increased concentration with increased exposure duration increased the mortality of both species. Great reduction in hatching of both *M. chitwoodi* and *P. penetrans* was observed in 100% bacterial suspension and to a lesser extent in 10% bacterial suspension. For *M. chitwoodi* only 4.56% hatching was observed when egg masses were exposed to 100% bacterial suspension and 15.5% hatching was reported for *P. penetrans* in 100% bacterial suspension. Ninety two percentage of *M. chitwoodi* juveniles found dead after 6 hours and 99.2% after 24 hours in 100% bacterial suspension. For *P. penetrans* this was 78.2% after 6 hours and 100% after 24 hours.

Key words: Invitro, nematodes, Paenibacillus spp., bio-control, concentration

INTRODUCTION

Root-knot nematodes are an economically important obligate plant parasites and are worldwide distributed. They exist in soil in areas with hot climates or short winters. About 2000 plants worldwide are susceptible to infection by root-knot nematodes and they cause approximately 5% of global crop loss (Sasser *et al*., 1985). Root-knot nematode larvae infect plant roots, causing the development of root-knot galls.The most important species of

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Meloidogyne sometimes also referred to as the four major species are the tropical-*M. arenaria, M.incognita* and *M. javanica* and the temperate one-*M. hapla* (Jones *et al.*, 2013). *Meloidogyne chitwoodi* commonly known as columbia root-knot nematode. It has a wide host range among several plant families (O'Bannon *et al*., 1982), including crop plants and common weed species. Potatoes (*Solanum tuberosum*) and tomatoes (*Lycopersicon esculentum*) are good hosts, while barley (*Hordeum vulgare*), maize (*Zea mays*), oats (*Avena sativa*), sugarbeet (*Beta vulgaris* var. *saccharifera*), wheat (*Triticum aestivum*) and various Poaceae (grasses and weeds) will maintain the nematode.

The root lesion nematode *Pratylenchus penetrans*, is also an economically important polyphagous, migratory, endoparasitic pest of different crops of temperate areas and it also favours disease complexes with other pathogens (Hackenberg *et al.*, 2000). It ranks third position in term of impact on crops after root-knot and cyst nematodes. Sixty species are identified which are worldwide in distribution (Jones *et al*., 2013). The demand of effective and eco-friendly bio-control measures is increasing day by day. Bio-control agents received a lot of attention in the recent times and are being incorporated in integrated pest management programmes (Zuckerman and Esnard, 1994).

Different types of bacteria and fungi have been isolated from nematode populations under laboratory conditions to reduce nematodes populations (Galper *et al.*, 1995; Liu and Chen, 2000). Though, detailed study on biological control of nematodes is not adequate but some general principles have emerged, well documented and effective biological control has been established (Sayre and Walter, 1991). Biological control measure is more robust than disease management strategies with synthetic chemicals (Emmert and Handelsman, 1999). Involvement of numerous disease suppression mechanisms, complexity of organismal interactions and adeptness to the environment has made them more durable than synthetic chemicals.They absorb nutrients from the invaded host body or indirectly by the action of toxic, diffusible metabolites that are produced by one or more microorganisms in various developmental stages of nematodes (Zuckerman and Esnard, 1994). These toxins often make nematodes, especially the eggs and juveniles, more vulnerable to infection or target to the activities of organisms nonvirulent, slightly pathogenic or basically saprophytic in nature (Karakas, 2007).

Different kinds of studies are going on to investigate the use of microorganisms as bio-control agents against nematode pest populations. This study was carried out to assess the efficacy and potential of *Paenibacillus*spp. (ILVO strain) as biological control agent to control root-knot (*M. chitwoodi*) and lesion (*P. penetrans*) nematodes.

MATERIALS AND METHODS

Paenibacillus spp. culture

Paenibacillus strain (similar to *P. polymyxa*) was obtained from Institute for Agricultural and Fisheries Research (ILVO), Belgium where it was isolated and maintained for different

experiments. *Paenibacillus* spp. was cultured in DifcoTM Pseudomonas nutrient agar (37 g/l) with different dilution series (-6, -7, -8, -9, -10) to determine the bacterial CFU/ml. Fifty µl bacterial suspension from each dilution series was transferred to the nutrient agar plates and spread well with a sterile glass rod until the media surface felt dry. This was repeated for all dilution series and then agar plates were incubated at 28˚C for 24 hours. The bacterium cells from the culture were checked with a Spectrophotometer and found to be10^8CFU/ml. This density was taken as standard. Bacterium was cultured and maintained in brain-heart infusion (BHI) media (CM1135, Oxoid LTD, England) with 10% Glycerol broth. Periodic transfer of bacterium and maintenance of culture was carried out to use in different experiments. Paenibacillus pre-culture was prepared by taking a single isolated colony from the culture that was grown in Petri dish at 28°C for 24 hour using BHI agar media (37 g/l BHI + 15 g/l Agar + 975ml distilled water and 25ml 10% sterilized glycerol after autoclaving). One isolated bacterial colony was transferred to the glass tube with lid containing 3ml BHI liquid media (without agar) and incubated at 28°C for 24 hour with shaking at 200 rpm. A culture of 1l BHI liquid media was prepared using 3 ml pre-culture and incubated for 24 hours as mentioned above. The bacterial suspension was then poured into centrifuge tubes and centrifuged with rotor 5.3, speed 4000 rpm, temperature 4°C for 15 minutes. The bacterium pallet retained at the bottom of the centrifuge tubes. Media from the centrifuge tubes was discarded and the bacterial pallets were diluted using sodium potassium phosphate buffer. Buffer was added to the tubes, shaken well and transferred to a sterile bottle to obtain the final volume 1l. That stock solution was considered as 100% and required dilutions were made by adding buffer to use in different experiments.

Nematode culture

M. chitwoodi

Root-knot nematode (*M. chitwoodi*) and root lesion nematode (*P. penetrans*) were obtained from the pure cultures that were maintained on tomato plants in green house and on carrot disc in incubator at 21°C, respectively. Potato tubers were surface sterilized with 5% NaOCl for 4 minutes and washed thoroughly. Then the potatoes were kept in light condition to allow sprouting for 3 weeks. Sprouted potato tubers were put in plastic closed container with 200 g sterilized sand to allow root growth. Second stage juveniles of *M. chitwoodi* extracted from pure culture were inoculated in potato roots at the rate of 2000 nematodes per plant and the containers were kept in a dark room at 20°C to grow. Eight weeks after inoculation nematodes were extracted from the closed container cultures and used in greenhouse pot experiment as well as in in vitro experiments.

P. penetrants

Big size fresh carrots with leaves attached were brought from vegetable market. Leaves were removed and the carrots were washed with tap water and then sterile water. The top and tip of carrots were removed and the carrots were surface sterilized by dipping in alcohol for 2 minutes under laminar flow and passing through the flame holding by forceps. Carrots

were peeled from top to tip flaming the knife between peeling. Carrot discs were prepared by cutting the carrot into 3mm thickness with flaming the knife between successive discs. Each disc was transferred immediately to 2.5 cm diameter Petri dish with sterilized tweezers and sealed with parafilm. All the Petri dishes with carrot discs were kept in a plastic bag arranging in rows and incubated 3 weeks at 21°C in dark. Contamination free carrot discs were then inoculated with sterilized nematodes at the rate of 30 nematodes/disc and incubated for two and half months at 21°C. Nematodes were sterilized by 4000 ppm streptomycin sulphate. Equal amount of nematode suspension and streptomycin sulphate solution were taken which reduces the streptomycin suspension to 2000ppm. The suspension was shaken well and stored overnight at 4°C. Supernatant above the settled nematodes was removed with a pipette. Afterwards, sterile water was added up to half of the tube shake well and allowed for nematodes to settle after which supernatant was removed. Washing of nematode was done 3 times.

Nematode extraction

Baermann funnel

Nematodes were extracted using baermann funnel technique for *M. chitwoodi* and baermann funnel with mistifier for *P. penetrans* based on the motility of nematodes. The funnel stem was connected with a rubber tube that was closed with a clip. Roots or carrot discs were cut into small pieces (1cm size using scissor to extract *M. chitwoodi* and carrot disc were cut using scalpel to extract *P. penetrans*) and put on a filter paper (EderolPund filter, 40g/m^2, Munketell filter AB, Sweden) lined in a 2 mm mesh sieve. The sieve was then put on top of a glass funnel filled with tap water up to a level that just covered the bottom of sieve and plant tissue. The hatched juveniles pass through the filter paper and sieve to funnel and accumulated in the bottom of the rubber tube. The nematodes were collected in a beaker by loosening the clip for few seconds as per need. The water level of the funnel was adjusted by adding water to compensate evaporation loss.

Effect of different bacterial suspensions on penetration

Two bacterial suspensions, 100% and 10% were prepared. Tomato seedlings grown in Petri dishes were taken and root systems dipped in bacterial suspensions and buffer and distilled water as control for 30 minutes. Then the seedlings were transferred to 3.9 cm diameter agar plates (prepared by autoclaving 1% bacterial agar and pouring to Petri dishes under laminar flow) at one seedling per plate for each treatment. Each treatment had five replicates. Second-stage juveniles (J2) were counted under microscope and 100 J2 were transferred to each seedling about 1 cm far from root of seedlings and kept in dark room at 20°C. Nematodes touching and 0.5 cm around the roots were counted after 24 hour, 48 hour and 5 days. After final observation, roots were stained (as described by Byrd *et al.*, 1983) to assess the nematode penetration in roots. Roots were cut into 1cm pieces in glass beakers (200 ml) and 1.5% NaOCl. Roots were kept in this solution for 4 minutes and stirred frequently. Then

the NaOCl solution was discarded and the roots were rinsed in abundant water on a sieve. 30 ml water plus 1 ml staining solution (3.5g Fuchsin acid, 250 ml acetic acid, and 750 ml distilled water) was added into a beaker with the roots and heated until boiling point for 30 seconds. After cooling to room temperature, the roots were rinsed with abundant water on a sieve. Roots were then collected in a beakers and 20 ml glycerol was added and heated till boiling point and poured into Petri dish. The nematodes inside roots were counted under microscope. Penetration experiment was carried out for both *M. chitwoodi* and *P. penetrans*.

Effect of different bacterial suspensions on hatching

M. chitwoodi

Two bacterial suspensions 10% and 100% and two controls (buffer and distilled water) were used to see the effect of *Paenibacillus*spp. on nematode egg hatching. Egg masses of *M. chitwoodi* from potato roots were separated with root pieces and transferred to small plastic tubes (1 cm diameter, 2.5 cm height) with sieve (48 µm mesh) at the bottom so that hatched juveniles can pass easily through the sieve but egg masses, root pieces and dirt remain on the sieve surface. The tubes were placed inside bigger tubes (2 cm diameter, 4.5 cm height) and 5 ml of each test solution was added. Tubes with the submerged egg masses were kept in a dark room at 20°C to allow hatching. Each treatment had four replicates. Every sixth day the hatched juveniles were counted and the test solutions were replaced by fresh test solutions. This was done during one month. After one month, egg masses were treated with 1% NaOCl solution to liberate the eggs from the gelatinous matrix using forceps and needle. Unhatched eggs were counted and cumulative hatching percentage was calculated.

P. penetrans

Eggs of *P. penetrans* were separated from the carrot discs culture. The carrots discs were chopped and macerated in a blender for 1 minute at high speed using commercial electrical blender. Eggs and vermiform stages were separated by sieving the nematode suspension through 50 µm sieve. Then the filtrate was filtered through 20 µm sieve. The eggs retained on the sieve were collected by gently rinsing with water and filter several times through 50 µm to remove vermiform nematodes. Hatching test was done in small containers (2 cm diameter, 4.5 cm height) equipped with 10 µm sieve (plastic tube having 1 cm diameter and 2.5 cm height). 500µl suspension containing 100 eggs was transferred onto the sieve of small container kept inside the big container and 5ml test solutions were added into the big containers. The container was closed with parafilm with small holes to allow air circulation. The containers were kept in incubator at 21°C. Every 6 days the hatched juveniles were counted and the test solutions were replaced by fresh test solutions. This was done during one month and cumulative hatching percentage was calculated.

Effect of different bacterial suspension on juvenile survival

Bacterial suspensions of 10% and 100% were prepared. Freshly hatched J2 of *M. chitwoodi* and mixed stages of *P. penetrans* extracted by baermann funnel methods were transferred to

the 5ml test solutions in plastic container. Buffer and distilled water were taken as control. Immobilized nematodes in test solutions were observed after 6 hour and 24 hour incubation at 20°C. Nematodes which did not move when probed with fishing needle were considered as dead. Immobile nematodes were transferred to distilled water after 6 hours of exposure to bacterial suspensions to check the recovery of nematodes.

Statistical Analysis

Computer package R studio was used to analyse data. The normal distribution and homogeneity of data were checked and log transformation done for the data which were not normally distributed. Treatment effects of the experiments were determined byanalysis of variance (One-way ANOVA) considering *P*- value.

RESULTS

Effect of bacterial suspensions on in vitro hatching of *M. chitwoodi* and *P. penetrans*

The effect of bacterial suspensions on in vitro hatching of *M. chitwoodi* from egg masses and *P. penetrans* from eggs are shown in figure1&2. Significant differences were observed in maximum hatching percentage of both *M. chitwoodi*(*P=0.01*) and *P. penetrans* (*P=0.02*). For *M. chitwoodi*almost no hatching was observed when egg masses were exposed to 100% bacterial suspensions after two weeks of exposure. The highest cumulative hatching percentage 56.85 was found in distilled water followed by 52.13 in buffer. The lowest cumulative hatching (4.56%) was found in 100% bacterial suspension followed by 42.67 in 10% bacterial suspension *in M. chitwoodi*. Likewise, the highest cumulative hatching for *P. penetrans* 45.5% was found in distilled water followed by 37.25% in buffer. The lowest cumulative hatching 15.5% was found in 100% bacterial suspension followed by 25.75% in 10% bacterial suspension in *P. penetrans*. No significant differences were found in hatching rate (b) and the time where 50% of the total hatching was reached (m) for both *M. chitwoodi* and *P. penetrans*. The highest time for 50% hatching (m) for *M. chitwoodi*was 2.08 weeks in 10% bacterial suspension followed by 1.93 weeks in distilled water and 1.78 weeks in buffer. Similarly, the highest time for 50% hatching (m) for *P. penetrans*was 1.23 weeks in buffer, 0.85 weeks in distilled water and 0.67 weeks in 100% bacterial suspension and 0.75 weeks in 10% bacterial suspension. The highest hatching rate (1.58) for *M. chitwoodi* was found in buffer, 1.53 in distilled water and 1.03 in 10% bacterial suspension. In the same way, the highest hatching rate (b) for *P. penetrans* was found highest in distilled water (1.65) followed by 1.53 in 100% bacterial suspension. The lowest hatching rate (1.18) was found in 10% bacterial suspension followed by 1.38 in buffer.

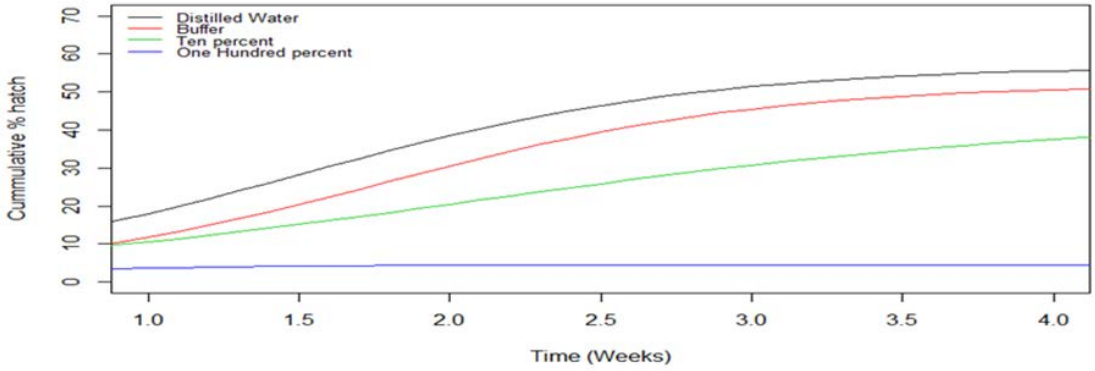

Fig. 1 : Hatching curves showing the cumulative percentage hatch of *M. chitwoodi* exposed to different bacterial suspensions (buffer and distilled water as control) with time in weeks (top line represent the distilled water, second last line for ten percent suspension and the bottom line represent the hatching at hundred percent suspension).

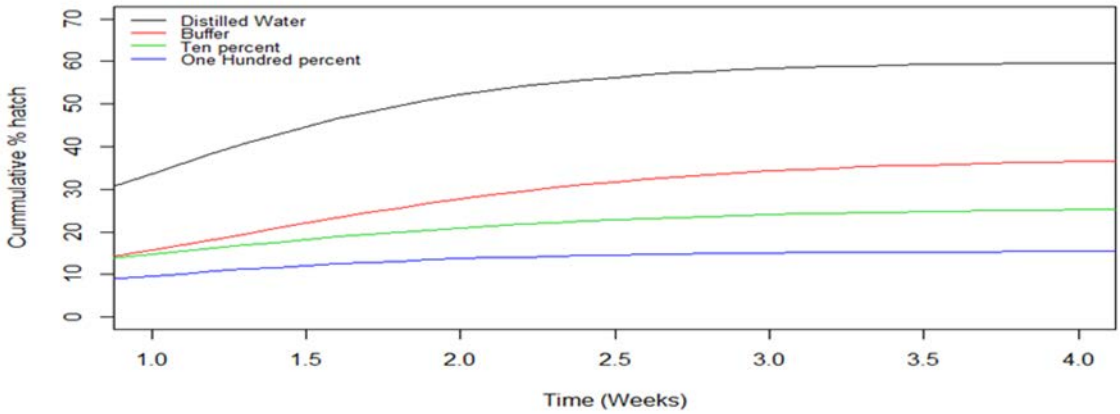

Fig. 2 : Hatching curves showing the cumulative percentage hatch of *P. penetrans* exposed to different bacterial suspensions (buffer and distilled water used as control) with time in weeks (top line represent the distilled water, second last line for ten percent suspension and the bottom line represent the hatching at hundred percent suspension).

Mortality of *M. chitwoodi* and *P. penetrans* in in vitro assay

Mortality of both *M. chitwoodi* and *P. penetrans* was found higher after exposure to bacterial suspensions. No nematodes were found dead in distilled water and negligible in buffer. Significant increase in J2 mortality of *M. chitwoodi* from 3.4% after 6 (*P=0.0003*) hours to 68.4% after 24 hours (*P=0.0008*) was observed after exposure to 10% bacterial suspension. In 100% bacterial suspension 92% and 99.2% was observed dead after 6 (*P=0.0003*) and 24 hours (*P=0.0008*) exposure, respectively (Figure 3). For *P. penetrans* a

mortality of 2.4% and 39% was observed after 6 hours and 24 hours exposure to 10% bacterial suspension. 78.2% and 100% nematodes were found dead after 6 hours (*P=0.0007*) and 24 hours (*P=0.0005*) exposure to 100% bacterial suspension, respectively (Figure 4).

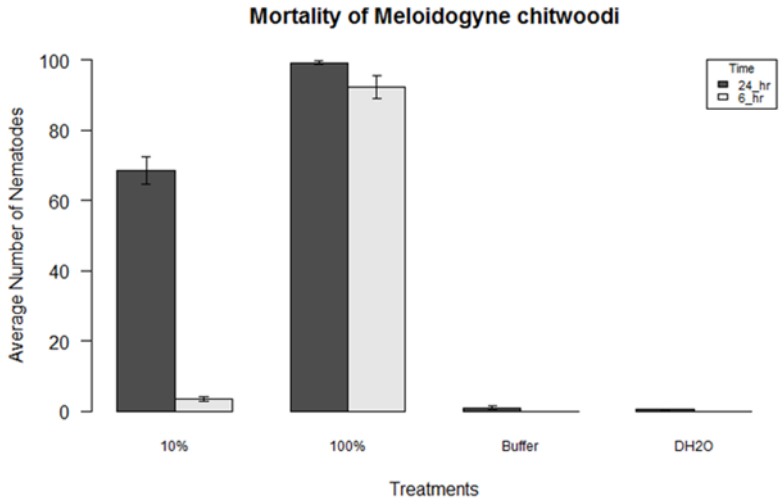

Fig. 3 : Mortality of *M. chitwoodi* juveniles after exposure during 6 hours and 24 hours to different bacterial suspensions (buffer and distilled water as control).
Number of nematodes inoculated was 100.

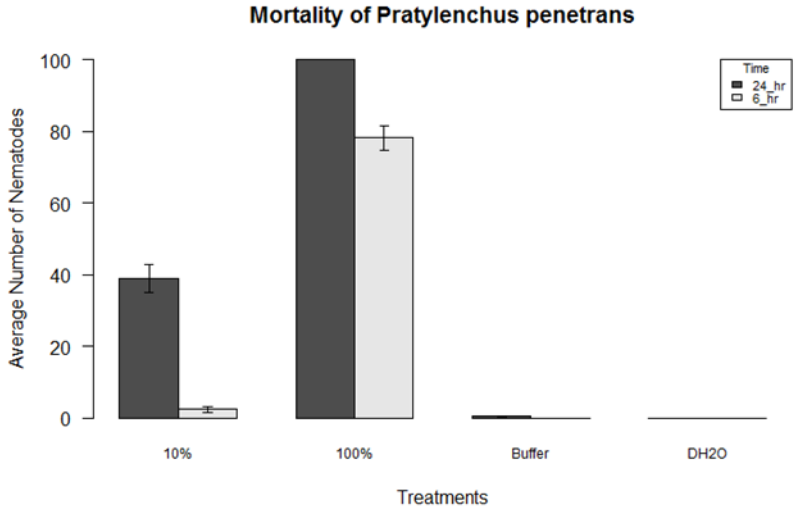

Fig. 4 : Mortality of *P. penetrans* after exposure during 6 hours and 24 hours to different suspensions (buffer and distilled water as control).
Number of nematodes inoculated was 100.

Effect of bacterial suspensions on root penetration

Effect of bacterial suspension on root penetration of *M. chitwoodi* and *P. penetrans* was observed after 24 hours, 48 hours and 120 hours. No significant difference ($P \geq 0.05$) was found between the treatments in the in vitro experiment of nematodes attraction to the root zone. Highest number of nematodes found around root zone of tomato seedlings was in buffer. On average 7.7 *P. penetrans* were found concentrated around the root zone of seedlings after 24 hours, 7.8 after 48 hours and 7.2 after 120 hours in buffer. The lowest numbers of nematodes (5.2 after 24 hours, 2.4 after 48 hours and 3.8 after 120 hours) were found in 10% bacterial suspension. The average numbers of nematodes found in 100% bacterial suspension were 4.4 after 24 hours, 6.2 after 48 hours and 5.8 after 120 hours (Figure 5). For the *M. chitwoodi*, the lowest numbers of juveniles found around root zones was in 10% bacterial suspension which was 7.4 after 24 hours, 8.2 after 48 hours and 9.2 after 120 hours. The average numbers of *M. chitwoodi* found in 100% bacterial suspension treatment were 14.4 after 24 hours, 30.6 after 48 hours and 17.4 after 120 hours. The average number found in buffer was 24.6 after 24 hours, 17.6 after 48 hours and 12 after 120 hours. The average nematode number found in distilled water treatments was 25.6 after 24 hours, 19.8 after 48 hours and 11.4 after 120 hours (Figure 7). Significant reduction in root penetration after exposure to bacterial suspensions was observed in both *M. chitwoodi* (*P=0.0007*) and *P. penetrans* (*P=0.01*). The lowest penetration was found in 100% bacterial suspension and highest in distilled water treatment for both nematode species. The average numbers of nematodes penetrated inside root 120 hours after inoculations was 4 in 10% bacterial suspension, 1.2 in 100% bacterial suspension, 8.6 in buffer and 8.8 in distilled water treatment for *M. chitwoodi* and 0.8 in 10% bacterial suspension, 0.4 in 100% bacterial suspension, 2.2 in buffer and 2.4 in distilled water treatment for *P. penetrans* (Figure 6 and 8).

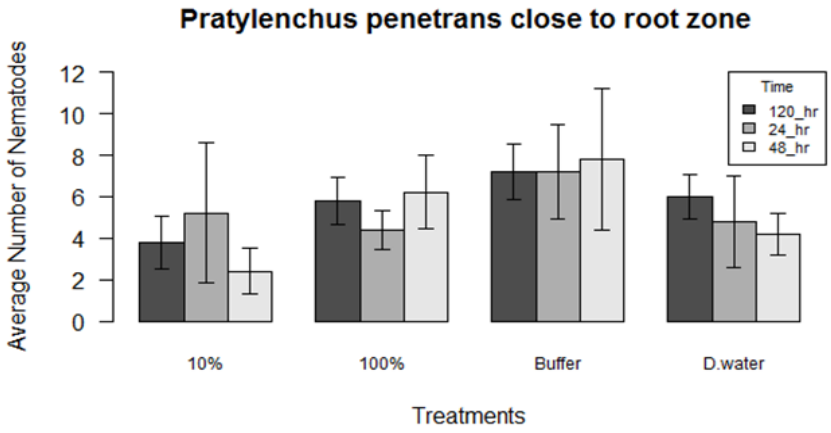

Fig. 5 : Numbers of *P. penetrans* observed at or around (0.5 cm) roots after 24, 48 and 120 hours in 10% and 100% bacterial suspensions treatment, buffer and distilled water. The number of nematode inoculated was 100.

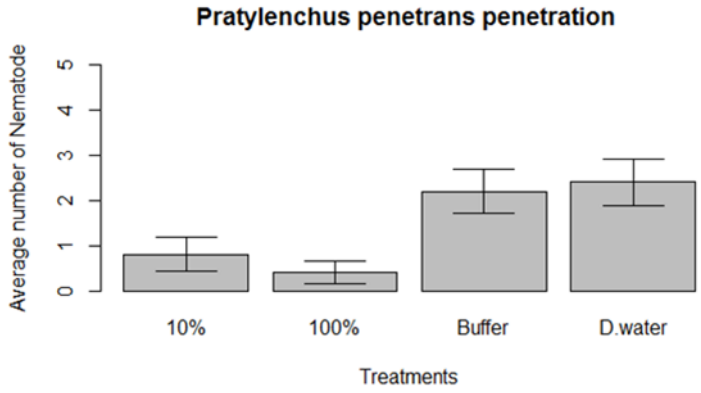

Fig. 6 : The average number of *P. penetrans* observed inside roots 120 hours after inoculation with 100 nematodes (mix of adults and juveniles) and treatment with 10% and 100% Paenibacillus suspensions, buffer and distilled water.

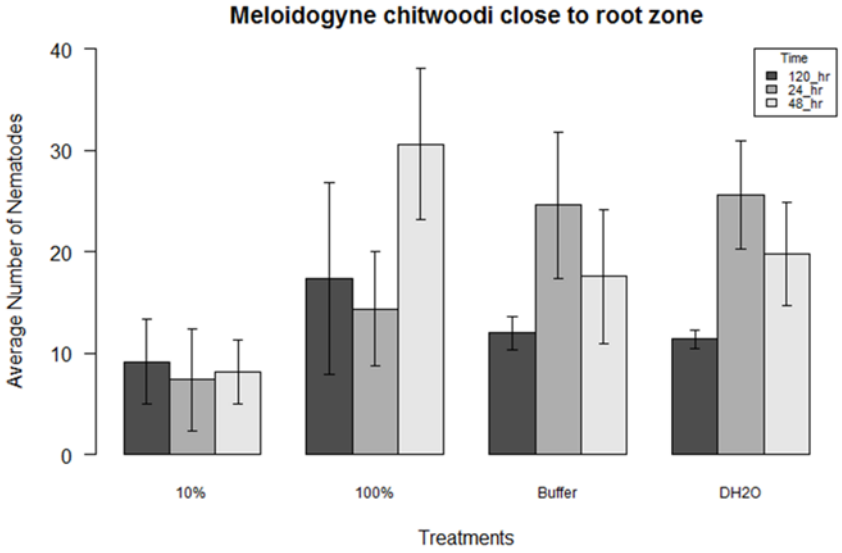

Fig. 7 : Number of *M. chitwoodi* observed at or around (0.5 cm) roots after 24, 48 and 120 hours in 10% and 100% bacterial suspension, buffer and distilled water treatment. The number of nematodes inoculated was 100.

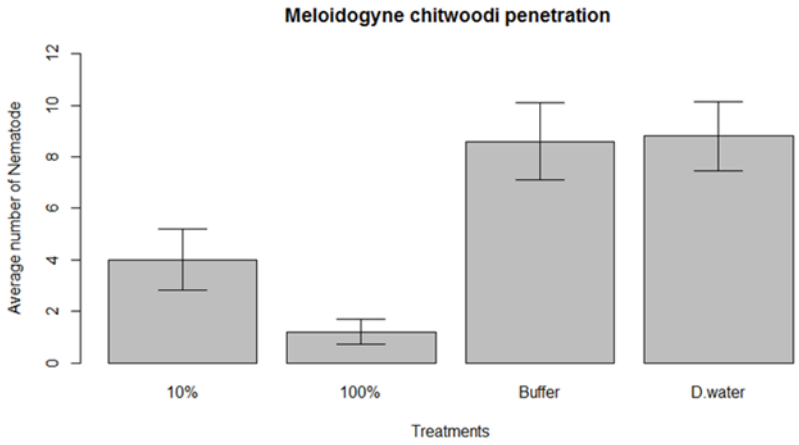

Fig. 8 : Average number of *M. chitwoodi* observed inside roots 120 days after inoculation with 100 juveniles and treatment with 10% and 100% Paenibacillus suspensions, buffer and distilled water.

DISCUSSION

Paenibacillus spp. used for these experiment was previously known as *P. polymyxa* but in molecular study some differences were noticed and suggested to write *P. spp.* until the species name confirmed. Significant reduction in hatching and increased mortality of *M. chitwoodi* and *P. penetrans* was found in bacterial suspension (*Paenibacillus* spp.) which is similar to the findings of Khan *et al.* (2008) who worked with *P. polymyxa* and *M. incognita* in tomato. The highest hatching was found during first two weeks and lowered down thereafter (Pudasaini *et al.*, 2008) for *P. penetrans* in normal condition. No hatching of *M. chitwoodi* and very few hatching of *P. penetrans* was occurred after 2 weeks in 100% bacterial suspension. The production of chitinase by *Paenibacillus illinoisensis* KJA-424 in vitro caused the lysis of egg shell in *M. incognita* resulting in the inhibition of egg hatching (Jung *et al.*, 2002). The average mortality of 68.4% in 10% bacterial suspension and 99.2% was observed in *M. chitwoodi* after 24 hours of exposure. 92% of *P. penetrans* found dead in 10% bacterial suspension and all most all nematodes found dead after 24 hours of exposure. This confirmed the findings that the bacterial suspension (CFU) and exposure duration had direct effect on nematode mortality (Khan *et al.*, 2008). The presence of toxic compounds in metabolites, lysis of egg shell and bacterial antibiotics are probably the cause of reduced hatching and increased mortality.

Significant reduction in root penetration was observed in both *M. chitwoodi* and *P. penetrans* in120 hours after inoculation. Four *M. chitwoodi* juveniles in average found penetrated inside root in 10% bacterial suspension and 1.2 in 100% bacterial suspension. Very few *P. penetrans* nematodes were observed around the root zone. They were migrated far from roots and towards shoot regions. The average of 0.8 and 0.4 nematodes penetrated inside root in 10% and 100% bacterial suspension, respectively. The less penetration of

nematodes in bacterial suspension than control is probably due to the production of Biofilms by *Paenibacillus spp*. Timmusk *et al.* (2005) have explained about formation of biofilm after invasion of *P. polymyxa*. The application of bacterial suspension (*P. polymyxa*) induced systemic resistance so that when applied to one half of the split root system caused reduced penetration of J2 in the untreated other half of the split roots and inhibited giant cell formation (Khan *et al.*, 2012).

CONCLUSION

Paenibacillus spp. was found effective against both nematode species *M. chitwoodi* and *P. penetrans* due to its toxic effectand control on eggs hatching, penetration and survival. Therefore, it can be helpful to substitute the use of synthetic chemicals.

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