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Bio-control potential of *Bacillus* isolates against cereal cyst nematode (*Heterodera avenae*)

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Abstract

The effects of twenty *Bacillus* isolates were investigated *in vitro* on the second stage juveniles of cereal cyst nematode, *Heterodera avenae*. The isolate XZ33-3 showed significant results against juveniles (J_2s) mortality followed by the isolate of XZ 17-1, XZ 24-2-1, MH 58-60-10, MH 58-60-04 and isolate MH 01-04-01, respectively. Among them four *Bacillus* isolates were identified on molecular basis using 16S rDNA, physiologically and biochemically and grouped as plant growth promoting bacteria (PGPB). In the green house experiments identified isolates; *B.cereus* XZ 24-2-1, *B. cereus* XZ-33-3, *B. weihenstephansis* MH-58-60-01, *B. thuringiensis* MH 032-003 and a nematicide (Avermectin) were used as seed coating. All four *Bacillus* isolates significantly reduced nematode infection of wheat roots when juveniles were used as inoculum after 10 days post inoculation. Noteworthy reduction in white female cyst development was observed on roots treated with Avermectin seed coating followed by isolates *B. cereus* XZ 24-2-1, *B. cereus* XZ-33-3, *B. weihenstephansis* MH-58-60-01 and *B. thuringiensis* MH 032-003 as compared to control treatment.

Keywords: *Heterodera avenae*, *Bacillus*, biological control, green house, mortality.

The genus *Heterodera* contains cereal cyst nematode (CCN) species (*H. avenae, H. latipon* and *H. filipjevei*) that parasitize wheat (*Triticum aestivum*), oat (*Avena sativa*), barley (*Hordeum vulgare* L.) and related wild grasses and ancestors (Rivoal & Cook, 1993; Cook & Noel 2002; Nicol, 2002; Nicol & Rivoal, 2007). *H. avenae* is one of the widely distributed group ranging from Fertile Crescent land of West Asia, Australia, East Asia, North America, Southern Africa and Europe (Stone, 1979). It causes serious crop damage and annual yield losses estimated as $3.4 million in the Pacific Northwest (PNW) region of USA and $58 million in Australia (Smiley, 2009; Murray & Brennan, 2009). In China, the occurrence of *H. avenae* is distributed in 16 provinces and approximately 20 million ha (Qiao *et al*., 2016). The roots are initially subjected to soil rhizosphere during the host and soil borne pathogen interaction (Weller, 1988). In the rhizosphere certain microbes have the ability to colonize the root system, improve the plant health and reduce the soil borne nematodes (Glick, 1995; Kloepper, 2003).

Rhizobacteria like *B. subtilis*, *B. thuringiensis*, *B. amyloliquefaciens, B. cereus, B. megateriumetc* provide biocontrol such as; root colonization, sporulation and parasporal crystal formation. These bacteria release inhibitory metabolites and are antagonist to many soil

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borne plant pathogenic nematodes belonging to the genera *Heterodera* and *Meloidogyne* (Siddiqui & Mahmood, 1999; Radnedge *et al*., 2003; Krebs *et al*., 1998; Khan *et al*., 2008; Neipp & Becker, 1999). In USA the commercial products formulated from *Bacillus* isolate like *B. subtilis* strains A-13, GB03 and GB07 are being used under the trade name Quantum@, Kodiak@ and Epic @ (Broadbent *et al*., 1977).

Here we focused on *Bacillus* isolates collected from Southern China Xi Zang autonomous region and Mo He, Heilongjiang province. The objective of this study was to (i) investigate the *in vitro* inhibition activity of *Bacillus* isolates against second stage juveniles (J_2s) of *H. avenae*, (ii) biochemical and molecular identification of potential isolates and their effect on cyst in green house experiments.

Materials and Methods

Bacterial isolates: *Bacillus* isolates were collected from different wetland locations of Xi Zang autonomous region Southwestern China (29°40'N.91°8'E) and Mo He locations, Heilongjiang Province (45°42'N, 127°32'E) of China (Fig1). They were stored in the microbial culture bank Nematology Laboratory, Department of Plant Pathology, College of Plant Protection (CPP), China Agriculture University, Beijing P.R. China.

For this study the *Bacillus* isolates were (i) eleven strains from Xi Zang autonomous region, (ii) six strains from Mo He, Heilongjiang Province and (iii) three strains from College of Plant Protection (CPP) (Table 1).

All these isolates were tested *in vitro* mortality assay of second stage juveniles (J_2s) of *H. avenae*. While potential *Bacillus* isolates were identified on biochemical and molecular basis method and then those identified isolated were used in greenhouse bioassay against the *H. avenae*.

Nematode inoculum: The inoculum of *H. avenae* was collected from wheat fields of Wheat Research Station, Institute of Plant Protection, Baoding (38°50'N, 115º28'E) Hebei Province, China. Cysts were extracted by "Fenwick can" method (Fenwick, 1940) and were kept in refrigerator at 4ºC. J_2s were obtained from eggs kept in sterile distilled water at 15-18ºC for 2-10 days and were used for *in vitro* mortality test.

***In vitro* mortality assay for second stage juvenile (J_2) *H. avenae*:** Bacterial isolates were revived for getting fresh culture growth on Lysogenic Broth with addition of agar media at 25ºC for 24 hours and were further subjected to fermentation in 20ml Lysogenic Broth (L.B) medium at 27ºC under 200rpm shaking condition for 24 to 36 hours (Sambrook & Russell, 2001; Bertani, 2004).

Culture broth was centrifuged at 12000rpm for 5min (Nitao *et al*., 1999) and filtered through 0.22µm membranes filters. Second stage motile active juveniles (J_2s) of *H. avenae* were disinfected by a series of antibiotics dilutions and centrifuged at 8000rpm for 2 min. such that; (i) Streptomycin sulphate (1.0%) for 1-2 min. (ii) Ampicillin sodium salt (1.0%) for 1-2 min (iii) Amphotericin-B (1.0%) for 1-2 min. and (iv) Cetyl-trimethyle ammonium bromide (1.0%) for 1-2 min. followed by three time washing and centrifugation at 8000rpm for 2 min. with sterile distilled water (SDW).

H. avenae juveniles (J_2s) concentration was adjusted as 50 juveniles per 50µl with the ratio of 850µl water (SDW) and 100µl of culture filtrates (CF) was pipetted into each well of cell culture plates.

Sterile culture medium (L.B) containing 100µl sample treated as control for the *H. avenae* mortality assay. Cell culture plates were kept for incubation at 25ºC for 12 h and 24 h observation of dead juveniles (J_2s). Each treatment of bacterial strains was replicated four times and the experiment was repeated three times. Results were means of three separate experiments. *H. avenae* cadaver mortality was mathematically calculated according to the equation;

Mortality of J_2 (%) = (Dead cadaver / Total number of Juvenile) X 100

Molecular Identification of *Bacillus* isolates: Molecular identification of four *Bacilli* isolates *viz*., of XZ33-3, XZ24-2-1, MH 032-003 and MH 58-60-01 was conducted. Bacteria Genome DNA was extracted by using DNA Extraction Kit (Sangon Biotech Co., Shanghai, China). The 16S rDNA gene was amplified by primers set, 27F(C) AGAGTTTGATCCTGGCTCAG and 1492R(C) TACGGCTACCTTACGACTT (Marchesi *et al*., 1998).

The reaction mixture was comprised as: 1µl of template DNA (60ng), 1.5µl of each forward and reverse primers (10µM/L) 15µl of 2 x Taq PCR master mix (Gen Star Company, Beijing, China) and 11µl of double-distilled water. PCR cycle program was established as: 5min. denaturation at 94°C, 30cycles for 30 seconds at 94°C, then 30 seconds at 52°C, 1.30 min. at 72°C, followed by chain elongation for 10 min. at 72°C, then the mixture was cooled to 10°C. PCR digested product was separated by electrophoresis on standard (1.0%) (wt/vol) agarose gels with ethidium bromide staining.

PCR products were then cloned and sequenced (TsingKe Biological Technology, Beijing, China). The sequence results were compared to the GeneBank database in the http://www.ncbi.nlm.nih.gov using the BLAST program. Phylogenic tree was constructed with neighbor joining algorithms (MEGA 4: Molecular Evolutionary Genetics Analysis (http://www.megasoftware.net).

Physiological and biochemical test of *Bacillus* isolates: *Bacillus* isolates were characterized for their morphological, physiological and biochemical characters. Morphology of colonies were described on LB plates at 30ºC for 24 hours, however, other distinguished tests, including Voges-Proskauer reaction, gelatin hydrolysis, growth at pH_5 and growth at 8 and 38ºCwere carried out as described by Staley, 1989. The API50CHB strips were used to determine the carbon source utilization from substrates and other physiological and biochemical characteristics, according to the bio-Merieux instructions manual (Logan & Berkely, 1984).

Effect of *Bacillus* isolates on *H. avenae* in green house: The cereal cyst nematode susceptible wheat cultivar cv. Aikang 58, provided by Institute of Plant Protection, Baoding, Hebei Province of China and used in greenhouse bioassay. Wheat seeds were surface sterilized by immersion for 3min. in a solution of 2.5% sodium hypochlorite (NaOCl) rinsed in sterile distilled water and dried overnight under sterile air stream. *Bacillus* isolates were grown on Lysogenic Broth (LB) suspended in a solution of 1.0% methyl cellulose and mixed with wheat seeds (Weller & Cook 1983).

Avermectin 1.8% (Syngenta Investment Co., Ltd, Beijing, China) was dressed on wheat seeds as nematicide control while seeds without any dressing (only surface sterilized) were used as untreated control (CK). Cyst inoculum of *H. avenae* was previously collected and cysts were extracted from the soil by wet sieving and sucrose flotation centrifugation methods (Riggs *et al*., 2000).

J_2s were collected as described earlier. Individual wheat seeds were sown in thirty PVC tubes. 200 *H. avenae* juveniles J_2s were inoculated in each treatment soon after seedling germination.

After 10 days post inoculation, wheat plants from 15 tubes per treatment were observed. Roots were stained with acid fuchsin and observed for nematode infection (Byrd *et al*., 1983). Wheat plants and soil of remaining 15 tubes of each treatment were washed in a 300µm sieve and observed after 60 days post inoculation (DPI) for *H. avenae* white female development. The greenhouse environment was maintained at 16-20°C with photoperiod of 15:9 h Light: Dark and 80% relative humidity (RH).

Quantitative data of white female attached on roots and detached from roots in soil were determined under the stereoscope.

The reduction rates of cysts of white female were calculated as;
RRWF (%) = (CWF-TWF)/CWF x 100

Where RRWF imply for reduction rate of white female, CWF denotes the number of white female per plant in the control and TWF signify the number of white females per plant/treatment. Each experiment was done three times in a complete randomized design (CRD).

Statistical Analysis: One-way analysis of variance was performed on data from *in vitro* and greenhouse experiments. Comparisons among means were made via Least Significance Difference (LSD) test using SPSS package (SPSS V16.0. SPSS Ltd., Chicago, IL) at the 0.05% probability level.

Results

***In vitro* mortality assay for second stage juvenile of *H. avenae*:** Inhibition of *H. avenae* juveniles increased by the tested *Bacillus* isolates culture filtrates (CF) as compared to control (sterile LB) broth culture medium after 12 and 24 hr exposure time. Isolate XZ33-3 showed 70.0% juveniles inhibition mortality after 12 hrs followed by isolate XZ17-1(66.0%), isolate XZ24-2-1(65.0%), isolate XZ12-2 (62.0%) and isolate XZ27-7(58.0%). However, isolates XZ21-1 and XZ53-1 exhibited 54.0% mortality while XZ5-1 and XZ21-4 showed (53%) similar effects on juvenile's mortality. Other isolates XZ21-6 and XZ28-2 revealed the mortalities as 50.0% and 49.0%, respectively after 12 hrs exposure time (Fig 2). The corresponding data of exposure time duration of 24 hours revealed no significant increase in juveniles mortality ($P \geq 0.05$) except increased up to 10% to 15% (Fig 2).

Bacillus isolate MH-01-04-10 showed juveniles (J_2s) mortality as (64.0%) followed by isolate MH-58-60-04 (63.5%) isolate MH-58-60-01 (63.0%), isolate MH032-003 (55.0%), isolate MH-61-63-03 (52.0%) and isolate MH-05-10-03 (48.0%) with exposure time duration of 12 hrs to cell free culture filtrates. After 24 hrs it was observed that the isolates had no effect on juvenile mortality except mortality percent increase up to 10.0% to 15.0% (Fig 2).

Bacillus isolate QZ-7 showed juveniles (J_2s) mortality as (57.0%) followed by isolate XMH-14 (55.0%) and isolate XMH-1 (52.0%) with exposure time of 12 hours to cell free culture filtrates. After 24 hour these isolates had no noteworthy effect on juvenile mortality except mortality percent increase up to 10.0% (Fig 2).

Molecular Identification of *Bacillus* isolates used in greenhouse bioassay: *Bacillus* isolates for greenhouse assay were belongs to *B. cereus* group. The bacterial colonies were large round or sub orbicular and opaque on L.B agar plates. Gram staining and observation under light microscope showed that all four isolates were gram positive and rod shaped. Based on the 16S rDNA sequencing, isolate MH-58-60-01 and isolate MH032-003 had 98.0% similarity with *B. cereus* (GeneBank accession KF831382.1) and *B. cereus* (GeneBank accession KC293998.1).

The isolate XZ33-3 had 99.0% similarity with *B. weihenstephansis* (GeneBank accession KU902434.1) and isolate XZ24-2-1 had 99.0% similarity with *B. thuringiensis* (GeneBank accession KJ767350.1). All four isolates were clustered with *B. cereus* group based on phylogenic analysis of the 16S rDNA sequences (Fig.3).

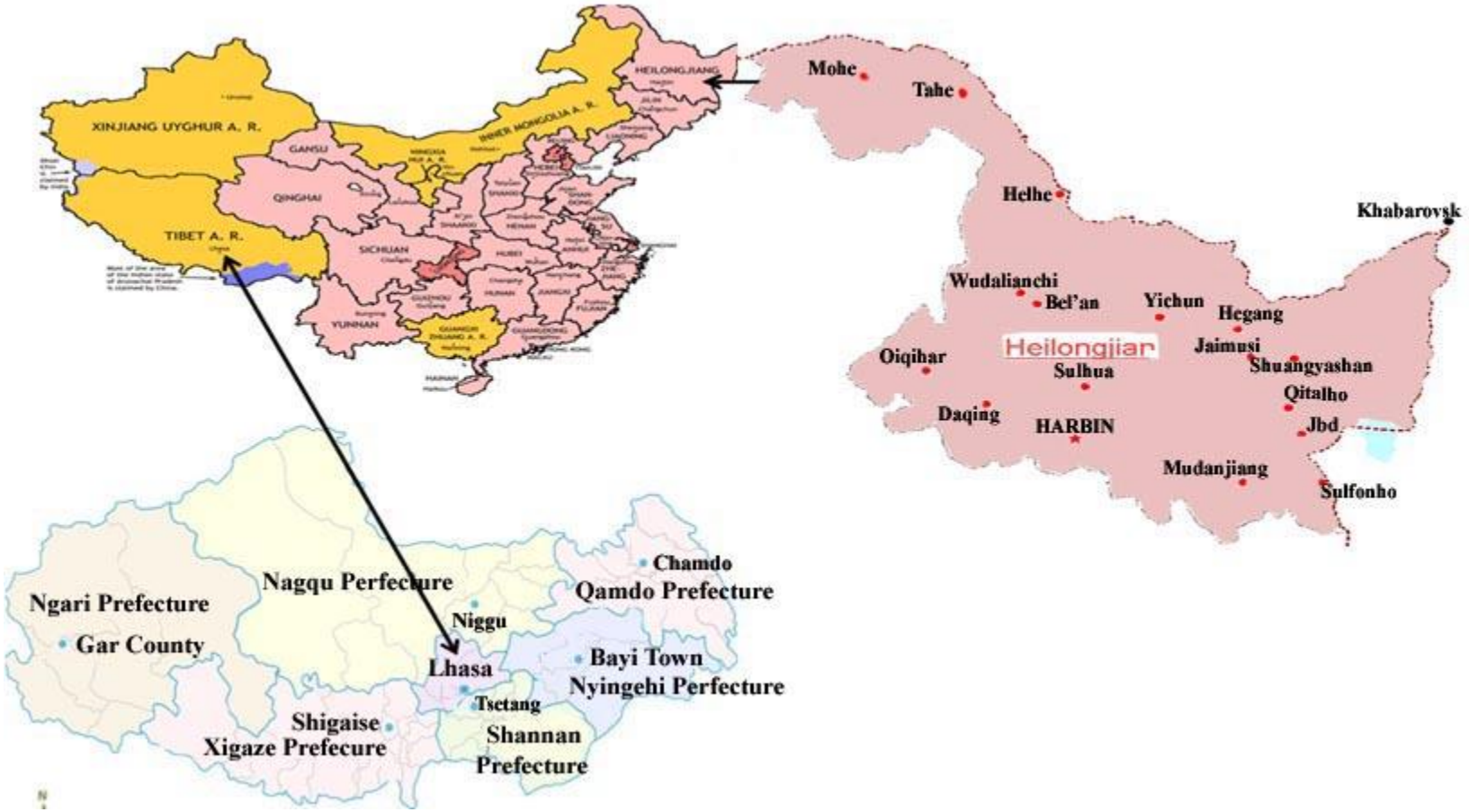

Fig.1. *Bacillus* isolates from Xi Zang autonomous region of Southwestern China and MoHe locations of Heilongjiang Province of China.

Table 1. *Bacillus* isolates used in this study.

Bacterial isolates	Province	Location	City
XZ 27-7	Tibet	Xi Zang	Zhu Feng Da Ben Ying
XZ 24-2-1	Tibet	Xi Zang	Zhu Feng Da Ben Ying
XZ 12-2	Tibet	Xi Zang	Lin Zhi
XZ 5-1	Tibet	Xi Zang	Lin Zhi
XZ 21-1	Tibet	Xi Zang	Lin Zhi
XZ 21-4	Tibet	Xi Zang	Lin Zhi
XZ 17-1	Tibet	Xi Zang	Lin Zhi
XZ 21-6	Tibet	Xi Zang	Lin Zhi
XZ 53-1	Tibet	Xi Zang	Lang KaZi
XZ 33-3	Tibet	Xi Zang	Gong Bu Jiang Da
XZ 28-2	Tibet	Xi Zang	Gong Bu Jiang Da
MH -58-60-01	Heilongjiang	Mo He	
MH -58-60-04	Heilongjiang	Mo He	
MH -61-63-03	Heilongjiang	Mo He	
MH-01-04-10	Heilongjiang	Mo He	
MH-05-10-03	Heilongjiang	Mo He	
MH-032-003	Heilongjiang	Mo He	
XMH-1	Beijing	CPP, CAU	
XMH-14	Beijing	CPP, CAU	
QZ-7	Beijing	CPP, CAU	

CPP= College of Plant Protection; CAU= China Agriculture University.

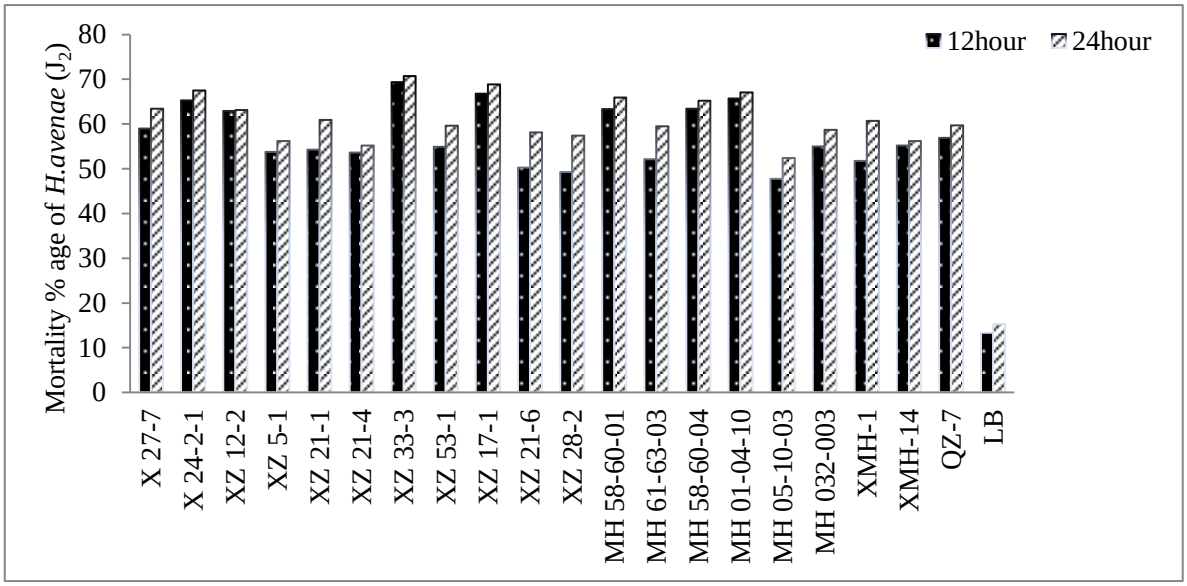

Fig. 2. *In-vitro* inhibition rates of motility *by Bacillus* isolates culture filtrates relative to the negative control against second stage juveniles (J_2s) *H. avenae* on 12 and 24 hour duration exposure time.

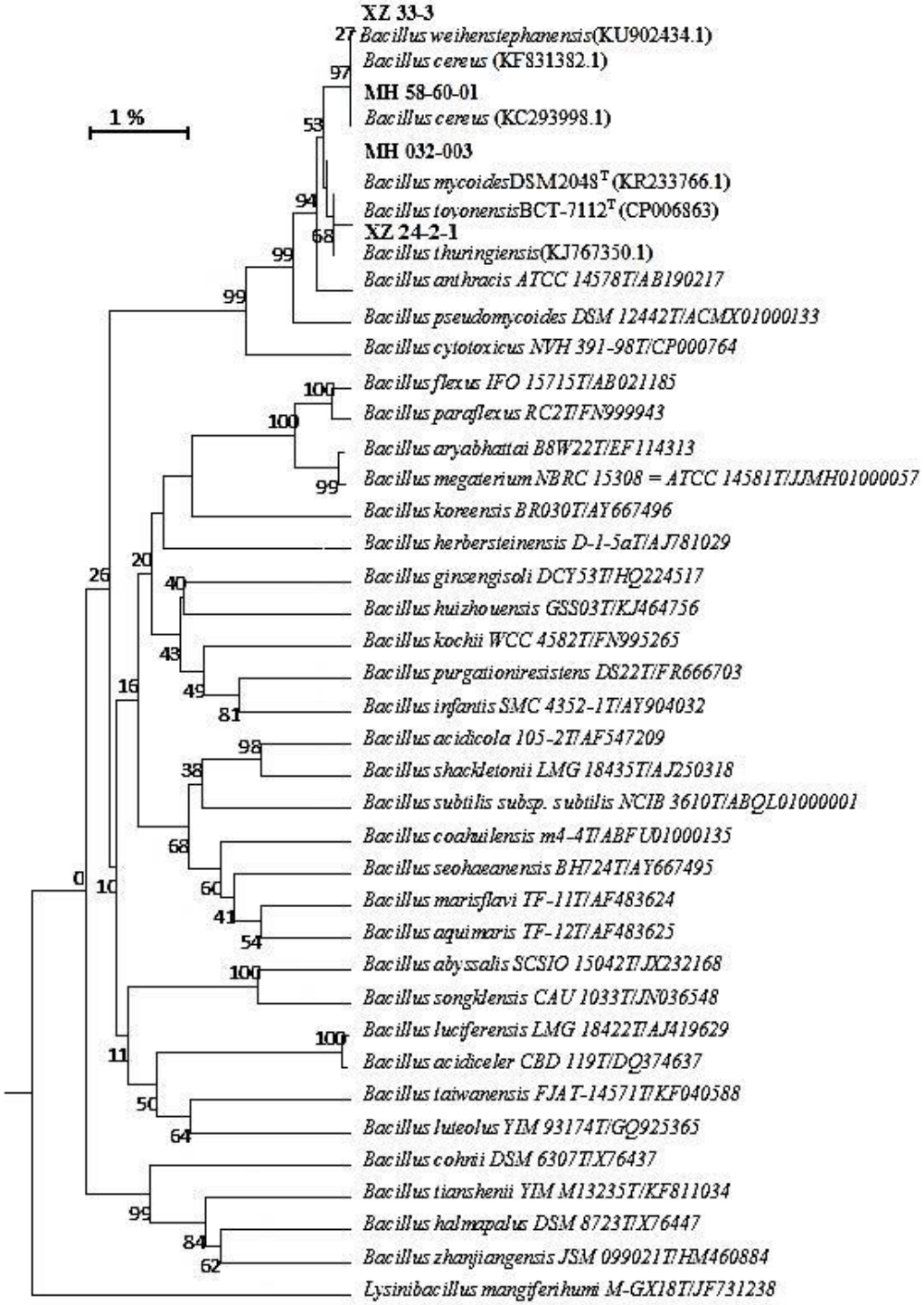

Fig. 3. Neighboring-joining dendrogram showing the relationship of tested strains XZ 33-3, XZ 24-2-1, MH 58-60-01 & MH 032-003 and other known *Bacillus* spp. based on 16S rDNA sequence comparison, using *Lysinibacillus mangiferihumi* M-GX18T/JF731238 as an out group.

Table 2. Physiological and biochemical characterization of four *Bacillus* isolates.

Biochemical test	XZ 33-3	XZ 24-2-1	MH 58-60-01	MH 032-003
Motility	+	+	+	+
Parasporal crystal	-	+	-	-
Catalase	+	+	+	+
Anaerobic growth	+	+	+	+
Voges-Proskauer	+	+	+	+
Hydrolysis of				
Starch	+	+	+	+
Casein	+	+	+	+
Gelatin	+	+	+	+
Utilization of citrate	+	+	+	+
Nitrate reduction	+	+	d	d
Growth at;				
5°C	-	-	-	-
10°C	+	+	d	d
40°C	+	+	+	+
Acid from:				
Glycerol	+	+	+	+
Glycogen	+	+	+	+
API 50CHB tests[z]				
Glycerol	+	+	+	+
Ribose	+	+	+	+
Galactose	+	-	+	+
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+
D-Mannose	-	+	-	-
Inositol	+	-	+	+
α-Methyle-D-Glucoside	+	+	+	+
N-Acetyl glucose amine	+	+	+	+
Amygdalin	+	+	+	+
Arbutin	+	+	+	+
Aesculin	+	+	+	+
Salicin	+	+	+	+
Cellobiose	+	+	+	+
Maltose	+	+	+	+
Lactose	+	-	+	+
Sucrose	+	+	+	+
Trehalose	+	+	+	+
Starch	+	+	+	+
Glycogen	+	+	+	+

[y] *Bacillus* spp. according to Shivaji *et al*., 2006; + and – represents positive and negative reactions, respectively.
[z] Data were obtained by use of API50CHB test strips.

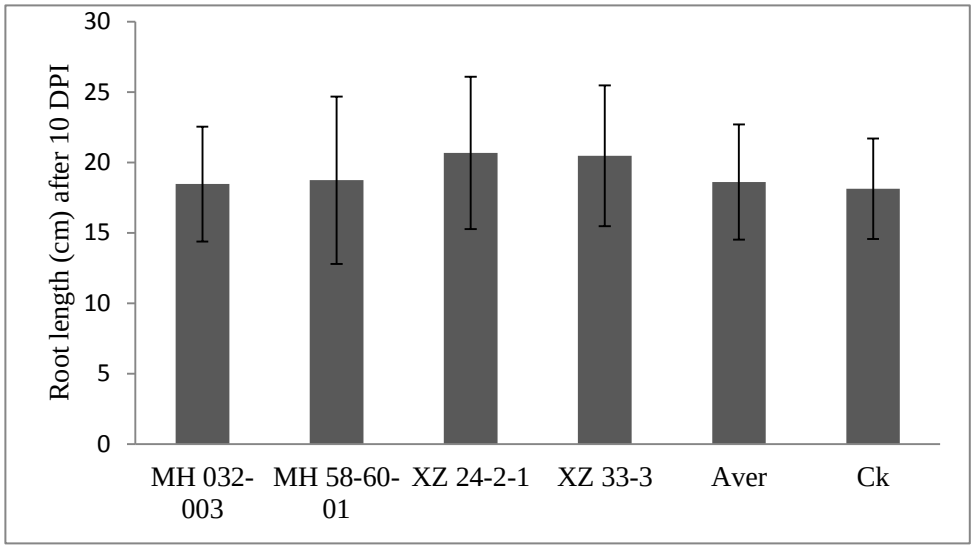

Fig.4. Effect of selected *Bacillus* isolates and Avermectin on root length (cm).

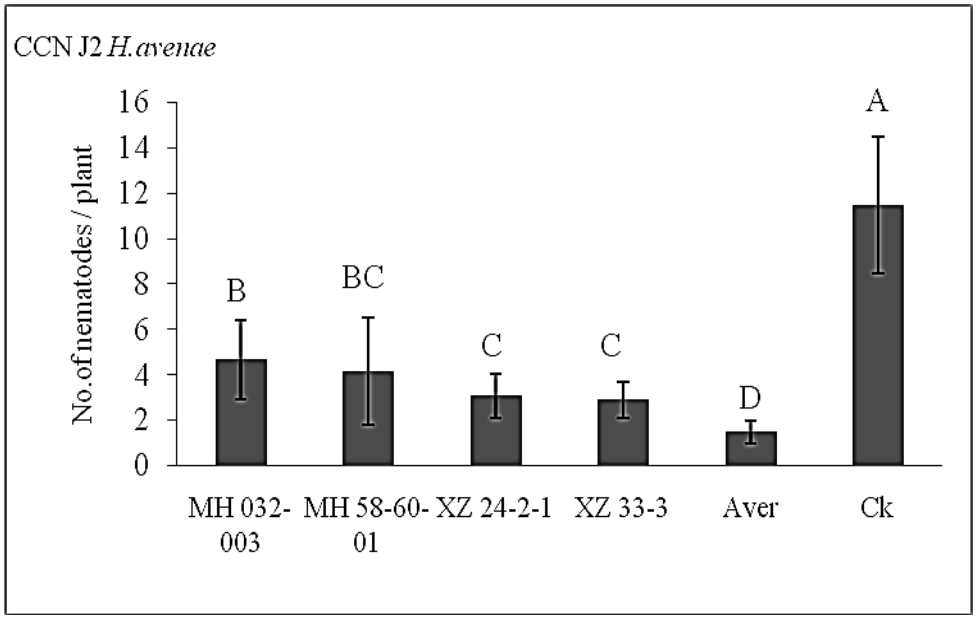

Fig.5. Reduction of *H. avenae* second stage juveniles (J_2s) infection of wheat roots after treatment by *Bacillus* isolates. Nematodes were counted in stained roots 10 days after inoculation.

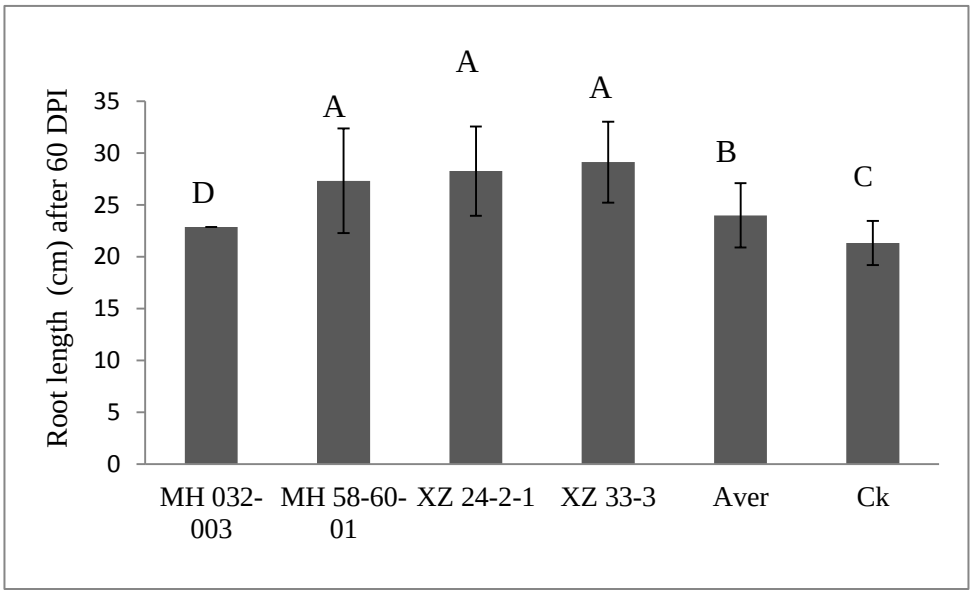

Fig.6. Effect of selected *Bacillus* isolates and Avermectin on root length (cm) after 60 DPI.

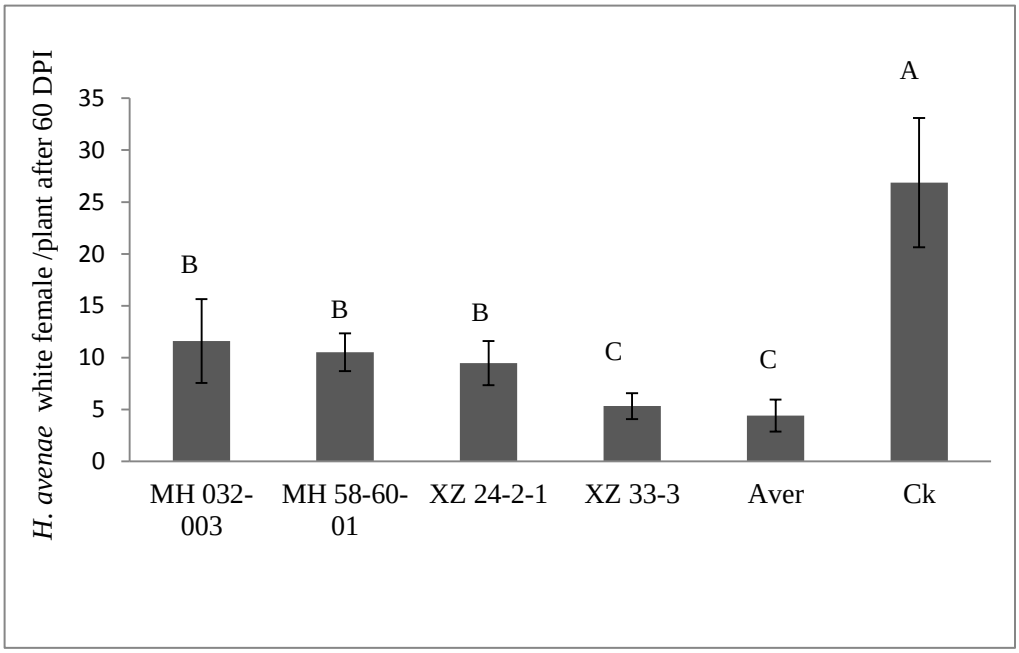

Fig. 7. Number of *H. avenae* white females found in roots after 60 DPI.

Physiological and biochemical test of *Bacillus* isolates: Physiological and biochemical characterization of these four *Bacillus* isolates indicates a great similarity to *B. cereus* group (Table 2). The API 50CHB strips also exhibited 99.9% similarity with *B. cereus* group (Table 2). Based on the physiological and biochemical characteristics, the carbohydrates utilization biochemical profile from API 50CHB strips and phylogenic taxonomy isolate XZ33-3, isolate XZ24-2-1, isolate MH-58-60-01 and isolate MH 032-003 was identified as *B. weihenstephansis*, *B. thuringiensis* and *B. cereus* and grouped in *B. cereus* group (Table 2).

Effect of *Bacillus* isolates on *H. avenae* in green house: *Bacillus* isolates including *B. weihenstephansis* MH-58-60-01, *B. thuringiensis* MH 032-003, *B. cereus* XZ24-2-1, *B. cereus* XZ33-3 and Avermectin coated wheat seeds significantly ($P \leq 0.05$) reduced the second stage juveniles (J_2s) infection in wheat roots, when wheat seedlings were inoculated with 200 J_2s compared to control treatment after 10 days post inoculation DPI (Fig 5). Reduced mean number of *H. avenae* juveniles J_2 on roots was observed in treatments Avermectin (1.47 ± 0.50) followed by *B. cereus* XZ33-3 (2.87 ± 0.81) and *B. cereus* XZ24-2-1 (3.07 ± 1.00), *B. thuringiensis* MH 032-003 (4.67 ± 1.74) and *B.weihenstephansis*MH-58-60-01(4.13 ± 2.36), as compare to control treatment (CK) (11.47 ± 3.01) (Fig 5).There were no any significant difference in root length was observed in among the different treatments and control ($P \geq 0.05$) (Fig. 4).

Noteworthy, numbers of *H. avenae* white female cyst was observed in roots treated with Avermectin (4.40 ± 1.54) followed by *B. cereus* XZ33-3 (5.33 ± 1.25) and *B. cereus* XZ24-2-1 (9.47±2.12), *B. weihenstephansis* MH-58-60-01(10.53 ± 1.82), *B. thuringiensis* MH 032-003(11.60 ± 4.03) ($P \leq 0.05$). However, increased number of white female cyst development was observed in control treatment (CK) (26.87 ± 6.22) after 60 days post inoculation DPI (Fig 7). There were no significant difference observed among the wheat roots in seed coating treatments with *Bacillus* isolates ($P \geq 0.05$) while there were significant differences within seed coating teatments of Avermectin and control ($P \leq 0.05$) (Fig 6).

Discussion

Biocontrol products containing *Bacillus* species are being developed from rhizobacteria for the management of different soil borne diseases of field crops (Gardner, 2004). Among *Bacillus* spp., *B. subtilis, B. licheniformis, B. amyloliquefaciens* and *B. cereus* have been reported effective against soil borne pathogens (Hassan *et al*., 2010; Carneiro *et al*., 1998).

We tested twenty *Bacillus* isolates for inhibition activity of second stage juveniles *in vitro*. Noteworthy inhibitory effects were found among all the isolates tested against *H. avenae* juveniles (J_2s) for the period of 12 hours exposure, which was about 50.0% and later observations after 24 hours exposure time with increasing trend about 70.0%. Although the *H. avenae* juveniles inhibition mortality rate from isolates; XZ33-3, XZ17-1, XZ24-2-1, XZ12-2, MH 58-60-01 and MH032-002, after 24 hours exposure time to culture filtrates was not 100%, but was substantial. Nematode cuticle is rigid and composed of proteins and chitins hence the possible activity of hydrolytic enzyme of isolates might be involved in the penetration process to help bacteria kill the juveniles (Cox *et al*., 1981; Ahman *et al*., 2002). Rhizospheric microorganism exhibits hyperplastic activity and by excreting hydrolytic enzyme can easily attacks the pathogen.

Bacillus isolate *Streptomyces* spp. isolated from marine environment was used against Pine wood nematode (*Bursaphelenchus xylophilus*) showed 79.0% inhibition effect (Lei *et al*., 2007). Rhizospheric microorganism associated with cyst and wheat roots comprised on spore forming *Bacillus* and *Proteobacteria* (*Stentrophomonos* spp.) showed 70.0-90.0% *in vitro* inhibition of juveniles of *H. avenae*

(Unpublished data). Rhizoshperic *Bacillus* from sugar beet reduced hatch of the cyst nematode *Heterodera schachtii* and potato cyst nematode, *Globodera rostochinensis* and *G. pallida* (Ryan & Jones, 2004).

Greenhouse experiments revealed that wheat seed coated with *Bacillus* isolates; *B. weihenstephansis* MH-58-60-01, *B. thuringiensis* MH 032-003, *B. cereus* XZ24-2-1, *B. cereus* XZ33-3 significantly reduced the J_2s infection on wheat roots. This indicated that these bacterial isolates could reduce the ability of *H. avenae* infection on roots. Rhizospheric microorganism important genera include *Bacillus, Pseudomonas, Clostridium* and *Streptomyces* are being used as biocontrol agents against soil born nematodes (Khan *et al*., 2008; Priest, 1993).

The coherence of *Bacillus* isolates results *in vitro* mortality assay, molecular and biochemical analysis and green house has further strengthened the hypothesis that biocontrol exhibited in isolates is thought to be production of hydrolytic enzymes. On the basis of results, it is concluded that bacterial strains are the valuable candidates for the production of biopesticides for soil borne nematodes. More work is required on product development of *Bacillus* isolates to provide the reliable products for soil borne nematode management.

Phytonematodes are obligate parasite on plants and spend different amounts of time in the rhizosphere, depending upon their mode of infection, longevity, multiplication and their life cyle completion. In development of rational biocontrol strategies for these nematodes, studies on their interaction between nematode and natural enemies in the rhizosphere should be documented. Moreover, molecular biological and immunological techniques that confirm and quantify individual isolates of microbial agents are needed to help understand the host parasite dynamics in the soil. Furthermore in combination with biological methods they would provide powerful tools for studies of the multitrophic interactions that occur in the rhizoshere especially the factors that affect the switch from the saprophytic to the parasitic state bacteria or fungi. Bioactive compounds are also being produced from the natural enemies that could be developed as new pesticides or nematicides which can be incorporated in a transgenic method or strategy for management of phytonematodes.

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