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RESEARCH ARTICLE

Nematicidal potential of rhizobacteria against *Meloidogyne graminicola* in the rice-wheat cropping system

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Abstract

The rice-wheat cropping system (RWCS) is one of the most important cropping sequences for food self-security. In this RWCS, rice root-knot nematode, *Meloidogyne graminicola* is an emerging problem. To manage this nematode, the rhizospheric bacteria were isolated from three treatments using three media, NA, Kings' B and TSA (Rice - Wheat - *Crotalaria*; Rice - Wheat - Mungbean; Rice - Wheat - Fallow) followed by RWCS. Out of six isolated bacteria, four bacteria showed the antagonist potential against *M. graminicola. In vitro* conditions, the bacteria isolated from fallow showed up to 31 % J2s mortality, 55-61 % in mungbean and 54-96 % in *Crotalaria*. The isolate, SRB7 showed 100 % juvenile mortality and 98.6 % hatching inhibition; finally, the isolates followed a pattern of SRB7 > SRB13 > SRB9 > SRB6 > SRB2 > SRB9. The bacterial isolates, SRB7 and SRB13, performed better than others in the attraction and penetration test. Through molecular characterization by 16s rRNA sequencing, the isolates SRB7, SRB13, SRB9 and SRBS6 were identified as *B. subtilis* (OL716087), *B. cereus* (OL716088), *B. megaterium* (OM816754) and *Pseudomonas stutzeri* (OL716089) in the soil collected from long-term RWCS fields. In the case of the pot study, these isolates decrease the nematode infestation by 83 % and increase plant growth by 82 % over the control. In the case of the pot study, these isolates decrease the nematode infestation by around 50 to 80 % over the control. The plant defence enzymes, PO, PPO and PAL activity reached a maximum on the sixth day and started to decrease. The long-term incorporation of green manure crop *Crotalaria* and mung bean in RWCS increases the beneficial soil microbes, suppressing the *M. graminicola* population and increasing the yield in the rice-wheat cropping system.

Keywords: antagonistic potential; *Bacillus* spp; *M. graminicola;* plant defense enzyme; *Pseudomonas* spp.; rice-wheat cropping system

Introduction

The rice (*Oryza sativa*)-wheat (*Triticum aestivum*) cropping system (RWCS) plays a vital role in providing staple food for the global population (1). In Asia, the RWCS is practised on 13.5 million hectares (mha), with 57 % of this area in South Asia (2). Among the biotic factors affecting crop yields, nematode infestations are particularly significant, causing up to 72 % yield loss in rice production (3). The major plant-parasitic nematodes in rice include *Meloidogyne graminicola*, *Aphelenchoides besseyi*, *Ditylenchus angustus*, *Hirschmanniella oryzae* and *Hoplolaimus* spp., all of which damage upland, lowland and deepwater rice (4). *M. graminicola*, the rice root-knot nematode, has emerged as a particularly severe threat to paddy production (3). This obligate sedentary endoparasite is highly detrimental to rice crops in various growing conditions (4). Infection by *M. graminicola* causes symptoms such as chlorosis, poor tillering, stunted growth and unfilled spikelets. The nematode induces the formation of characteristic root galls that resemble hooks or horseshoes, disrupting the roots' ability to absorb water and nutrients. This result in stunted plant growth, poor tillering and yellowing leaves (5). The economic impact of *M. graminicola* is significant, with rice yield losses amounting to Rs. 23 million (6). Reported that a pre-plant population of 1500 juveniles of *M. graminicola* per kg of soil could reduce rice yield by 27-35 % (7). In contrast, other studies suggest that the nematode causes around 10% yield loss, with a minor reduction in crop value (4).

To mitigate these agricultural losses, effective nematode management strategies are essential. Traditional methods often rely on resistant rootstocks, chemical nematicides, cultural practices and biological control agents. However, synthetic chemicals have been phased out in many regions due to their harmful environmental and human health impacts (8). Cultural practices such as soil solarization, fallowing, crop diversification and rotation have also shown some efficacy. Among these, the use of nematode-suppressive crops, like *Crotalaria juncea* (sunn hemp), in the rice-wheat cropping system offers several benefits. Sunn hemp, a member of the Fabaceae family, improves soil structure, reduces erosion and enhances soil

nutrient levels and organic matter content. Additionally, it suppresses plant-parasitic nematodes and boosts beneficial microorganisms, including bacteria, fungi and invertebrates in the soil food web (9). Long-term use of green manure crops such as *Crotalaria* has been observed to alter nematode populations, potentially providing an eco-friendly alternative for managing rice root-knot nematodes.

Biological control agents have emerged as a promising alternative strategy for nematode management. Farmer communities are increasingly adopting environmentally friendly alternatives to traditional practices, driven by the need for sustainable solutions. Bacterial and fungal antagonists are among the widely recognized biocontrol methods. In this regard, genera like *Bacillus, Clostridium* and *Pasteuria* are known to produce endospores. These endospores have a prolonged shelf life and are also extremely resistant to desiccation and chemical degradation. Among the various biocontrol methods like organic amendments and nematode-resistant cultivars, plant growth -promoting rhizobacteria (PGPR) have demonstrated high efficacy against sedentary endoparasites that complete their life cycle within the host hydrolytic enzyme production, nutritional competition, antibiotic compound production and systemic resistance in host plants (10). The use of natural beneficial antagonists is not only effective but also cost-efficient for managing rice root-knot nematodes. These biocontrol agents can target multiple stages of the nematode life cycle by inhibiting nematode penetration, reducing reproductive capacity, preventing egg hatching and restricting nematode movement (11). Several antagonistic bacteria have been isolated from various crops and tested against a range of pathogens, including nematodes. In particular, rhizospheric bacteria isolated from *Crotalaria* treatments have shown antimicrobial activity (8) and have been identified through 16S rRNA gene sequencing (12). However, few studies have focused on isolating and formulating such antagonistic bacteria for the management of *Meloidogyne graminicola* in rice within the rice-wheat cropping system (RWCS) (7). To explore the feasibility of using bioagents for managing *M. graminicola* in rice, this study was carried out.

Materials and methods

Experimental field

The samples were collected from the field (latitude 77°9'48.66" E and longitude 28°38'24.81" N) with rice-wheat cropping sequences maintained since 2015. Cropping sequences were followed, as under Rice - Wheat - Fallow; Rice - Wheat - *Crotalaria*; Rice - Wheat - Mung bean, for the last 5 years.

Isolation of rhizospheric bacteria in the rice-wheat cropping system

Rhizospheric soil (1 g) was weighed, added to 50 mL of distilled water and mixed thoroughly. It was later kept in a shaker for 15-20 min for complete dispersion. Only 1 mL of this suspension was added to 9 mL of water and subjected to serial dilutions. Approximately 100 µL of appropriate dilutions to the Petri plates having nutrient agar medium, Kings' B medium and TSA medium. Incubated for 48 hr and two replications were maintained. Observations were taken on each organisms' colour, consistency and colony character. The colonies per plate were counted and the populations were calculated. Each colony is referred to as a colony-forming unit (cfu) for bacteria. After purification, the isolates were maintained in glycerol stock (50 % concentration) at -80°C for further analysis.

Morphological characters

The isolated bacterial strain was evaluated for its difference in colony morphology, colour, shape and size by visual and microscopic observations (13).

Biochemical characterization of bacterial strains

Starch hydrolysis

During a 72 hr incubation period, bacterial isolates were streaked over a Petri plate containing nutritional agar medium mixed with 400 mg of soluble starch. After incubation, 10 mL of iodine solution was flooded over the plates, allowed for 30 seconds and drained. The zone surrounding the bacterial growth in the mediums' blue background suggested that the isolate had positive amylase activity, as described in the method (14).

KOH test

A drop of KOH (3 %) was applied to a transparent glass slide. It was covered with a loopful of bacterial culture, which was thoroughly mixed with the help of a needle. Developing a fine thread-like structure demonstrated the pathogenicity of the isolated bacteria as the given method (15).

Gelatin hydrolysis

Bacterial isolates were streaked over a Petri plate containing soluble gelatin (200g) and nutrient agar medium and the mixture was cultured for 72 hr. After incubation, the plates were soaked with a 0.2 % mercuric chloride (Hg_2Cl_2) solution for 30 sec before being drained. The absence of colour around the bacterial growth in contrast to the mediums' blue backdrop revealed the isolates' positive gelatin activity as given method (16).

Chitinase activity

The isolated bacteria culture was incubated overnight to achieve their growth and 10 µL of the culture broth was spot inoculated on minimal media (M9 media) agar plates on sterilized glass fibre strips placed and supplemented with colloidal chitin 1 % as substrate and incubated at 30 °C for 48 hr. Those plates were stained with congo red 0.1 % and destained with 1M NaCl to observe the colony with a clear zone as described in the procedure (17).

Protease activity

This protease assay was like a chitinase assay with minimal media supplemented with casein 1% as substrate. Protease production was observed by a clear zone around the colonies as given method (18).

Molecular characterization of bacterial strains

24h old bacterial culture in NB broth was used for bacterial genome isolation. The final DNA quality and quantity were examined under Nanodrop (260/280 value) and verified using 0.8 % gel electrophoresis. PCR amplification

reactions were carried out using the standard protocol of 16S RNA universal primers were 27F (5'-AGAGTTTGAT CAT GGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3'). The resultant PCR product was confirmed with 1.2 % agarose gel and the image was documented with Alpha imager TM1200 documentation and analysis system. The sample was sent for sequencing to AgriGenome Labs Private Limited. Blast analysis matched the sequenced data to previously existing data in the NCBI data bank by BLAST analysis. Data were arranged with MEGA7 software. Identified isolates were analysed at the genus and species levels and the dendrogram was drawn (19).

In vitro screening of isolated bacterial strain

Effect on juvenile mortality and hatching inhibition studies

The isolated bacteria were inoculated in NB broth for 48h and centrifuged at 10000 rpm for 15 min at 25 °C. The supernatant was passed through a bacterial filter (0.22 µm) and used for *in vitro* screening to obtain cell-free culture filtrate. Effect of different concentrations (25, 50, 75 and 100 %) of cultural filtrates one mL was transferred to a sterilized multi-vial glass plate to tested on egg hatching and juvenile mortality at regular intervals after inoculation (20, 21). 100 J_2S and 100 eggs of *M. graminicola* were incubated at 28 °C with sterile distilled water and autoclaved media as control. For each isolate, five replications were maintained in a completely randomized design.

Effect of attraction and penetration studies

The four-day-old PB1121 rice seedlings were dipped in a bacterial suspension containing 10^8 cfu/mL for 30 min and were placed in Pluronic F-127 medium poured in a 90mm petri dish (22). Approximately 100 J_2s per plate of *M. graminicola* was inoculated in the petri dish and incubated at BOD with a temperature of 27 ± 2ºC. The nematodes attracted towards the rice root were counted at 6, 12, 18 and 24 h after inoculation in the plates under the stereo-zoom microscope (23). For penetration, the roots were stained with acid fuchsin-lactophenol and plain lactophenol was used for destaining and the roots were observed under a microscope for nematode penetration after 12, 24, 36 and 48 h after inoculation in the plates.

Biocontrol potential of isolated bacterial strain against *M. graminicola* in rice roots under pot culture studies

The experiment was conducted in 10 cm pots with sterilized pot mixtures of transplanted ten-day-old rice (var. PB1121) seedlings. A bacterial culture, 3 % (10^8 CFU/mL), was poured after a week of establishment. Freshly hatched *M. graminicola* $2J_2$/g of soil was inoculated. The experiment terminated after 45 days of inoculation and observations were recorded for the final nematode population (250cc) No. of galls/5g root, No. of females/galls, No. of eggs/egg mass, plant height, shoot weight and root weight. The soil samples collected were washed using Cobbs' decanting and sieving method, followed by a modified Baermann funnel technique (24, 25). The root samples were stained with acid fuchsin lactophenol and plain lactophenol. Treatments were: 1. *B. subtilis* 2. *B. cereus* 3. *B. megaterium* 4. *P. stutzeri* 5. Positive control - *P. fluorescence* and *B. amyloliquefaciens* 6. Chemical control - Velum prime 7. Control - water. Three replications were maintained.

Biochemical changes induced by antagonist bacterial strains

The plants maintained an inoculation of bacterial culture the same as the previous experiment after the inoculation of $2J_2$/g of soil. One gram of root samples was collected on 2, 4, 6 and 8 days of inoculation. The treatments were: 1. *B. subtilis* 2. *B. cereus* 3. *B. megaterium* 4. *P. stutzeri* 5. Positive control - *P. fluorescence* 6. Negative Control 7. Healthy plants only. Three replications were maintained.

Estimation of peroxidase (PO)

Spectrometric analysis was used to assess the peroxidase activity (26). One-gram plant root sample was homogenized in 2 mL of 0.1M sodium phosphate buffer (pH 6.5) before being centrifuged at 16000 rpm for 15 min. An enzyme source was the supernatant. At room temperature (28 ± 2ºC), the reaction mixture of 1.5 mL of 0.05M pyrogallol, 0.5 mL of enzyme extract and 0.5 mL of hydrogen peroxide (1 %) was incubated. At intervals of 30 sec throughout 3 min, the spectrophotometer recorded the changes in absorbance at 420 nm. The enzyme activity was measured as a change in the absorbance of the reaction mixture on a $min^{-1}g^{-1}$ fresh weight basis (27).

Estimation of polyphenol oxidase (PPO)

The method determined the polyphenol oxidase (PPO) activity (28). A root sample weighing one gram was homogenized in two ml of 0.1M sodium phosphate buffer (pH 6.5) before being centrifuged at 16000 rpm for fifteen min. The reaction mixture combined approximately 200µL of enzyme extract with 1.5 mL of sodium phosphate buffer, 0.1 M. 200µL of 0.01M catechol was injected to start the reaction. During the next three minutes, the change in the absorbance value at 495 nm was measured every 30 seconds. The variations in absorbance $min^{-1}g^{-1}$ fresh weight of tissue were used to represent the enzyme activity

Estimation of phenylalanine ammonia-lyase (PAL)

The method used to analyze phenylalanine ammonia-lyase (PAL) activity was used (29). A plant root sample weighing one gram was macerated in 5 mL of cold, 25 mM borate HCl buffer with a pH of 8.8 and 5 mM mercaptoethanol. The sample was centrifuged for 20 min at 12000 rpm and the supernatant was employed as an enzyme source. The reaction mixture comprises 0.5 mL of borate buffer (0.2M), 0.2 mL of enzyme extract and 1.3 mL of sterile distilled water. One mL of 0.1M L-phenylalanine was added to the reaction, then proceeded at room temperature for 30 to 60 min. Trans-cinnamic acid synthesis, a precursor for specific phenolic compound formation, was used to measure PAL activity. Adding 0.5 mL of 1M trichloroacetic acid halted the process and the change in absorbance was recorded at 290 nm using a spectrophotometer. Micromole trans-cinnamic acid min^{-1} g^{-1} of the sample was used to express the reaction rate.

Statistical analysis

STAR, Statistical Tool for Agricultural Research, was used to determine the significant difference through one-way ANOVA (P<0.05). Turkeys' honestly significant difference test (Turkeys' HSD) was used for multiple mean comparisons.

Results

Characterization of bacterial isolates

Six bacteria were isolated from the rice-wheat cropping system fields and used against *M. graminicola.* The isolated bacteria, such as SRBS6 and SRBS9, showed J_2s mortality ranging from 61.4 and 55.2 %. Followed by the bacterial isolates, SRB2, SRB7, SRB9 and SRB13 showed J_2s mortality as 54.2, 96.8, 75 and 95.4 %, respectively. The six antagonist bacterial isolates used for further experiments were SRB2, SRB7, SRB9, SRB13, SRBS6 and SRBS9.

Morphological, biochemical and molecular characterization of bacterial strains

Preliminary characterization showed that four isolates belonged to gram-positive and two isolates belonged to gram-negative bacteria. The isolates, *viz*., SRBS6 produced yellow and SRBS9 produced orange pigments. In the KOH test, isolates of all the bacterial strains showed negative results, which proved that our isolates were non-pathogenic. The isolated bacterial strains were subjected to physiological and biochemical characterization (Table 1). The molecular identification (Fig. 1) revealed that the atagonistic bacterial strains belonged to *B. subtilis* (OL716087), *B. cereus* (OL716088), *B. megaterium* (OM816754) and *Pseudomonas stutzeri* (OL716089) were identified based on 16s rRNA present in the soil collected from long-term RWCS fields and Phylogenetic analysis was done using MEGA 7 Software (Fig. 2, Table 2).

In-vitro screening of culture filtrates on *M. graminicola*

In vitro assay was done with six bacterial isolates. The mortality percentage of J_2s differed significantly ($P<0.05$) among the bacterial strains. All bacterial strains showed a mortality rate from 60% to 100% at 48h. It followed a pattern of SRB7>SRB13>SRB9> SRBS6>SRB2>SRBS9. The results indicated that an increase in concentration and period of exposure led to a progressive increase in juvenile mortality. In hatching inhibition, the results showed that SRB7 and SRB13 effectively inhibited the hatching 98.6 % and 97.8 %, respectively, at 100 % concentration on the third day of exposure and became constant. However, SRBS6, SRB2 and SRBS9 significantly reduced the hatching rate of eggs to 94 %, 75.4 % and 72.2 % respectively (Fig. 3a-d & 4a-d).

Effect on attraction and penetration of *M. graminicola* J_2s towards the rice roots

From the result of *in vitro* screening, four potential

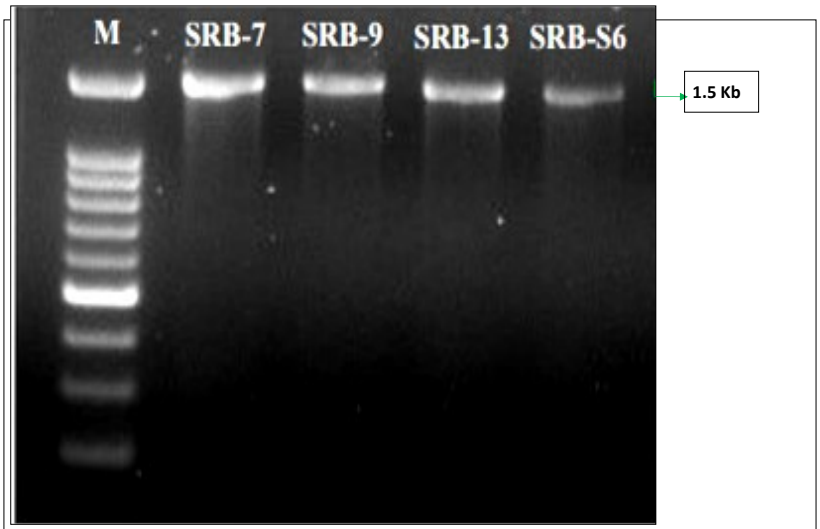

Fig. 1. Molecular characterization of promising bacterial isolates (Agarose gel electrophoresis of PCR-amplified with 16s RNA, 100bp ladder; Lane M-Marker, Lane 1-SRB7, Lane 2 - SRB9, Lane 3- SRB13 and Lane 4 - SRBS6). SRB7 - *Bacillus subtilis,* SRB9 - *Bacillus cereus,* SRB13 - *Bacillus megaterium,* SRBS6 - *Pseudomonas stutzeri*).

Table 1. Biochemical characterization of bacterial isolates

S. No	Biochemical test	Rhizospheric bacterial isolates					
		SRB2	SRB7	SRB9	SRB13	SRBS6	SRBS9
1	Gram staining	+ve	+ve	+ve	+ve	- ve	- ve
2	Cell morphology	rod	rod	rod	rod	rod	Rod
3	Starch hydrolysis	+	+	+	+	+	+
4	KOH test	+	-	+	+	-	+
5	Gelatin hydrolysis	-	+	+	+	+	+
6	Protease	+	+	+	+	+	+
7	Chitinase	+	+	+	+	+	+
8	Pigment production	-	-	-	-	Yellow	Orange

The rhizospheric bacteria, such as SRB2, SRB7, SRB9, SRBS13, SRB2 and SRBS9 were isolated from the rice-wheat cropping system.

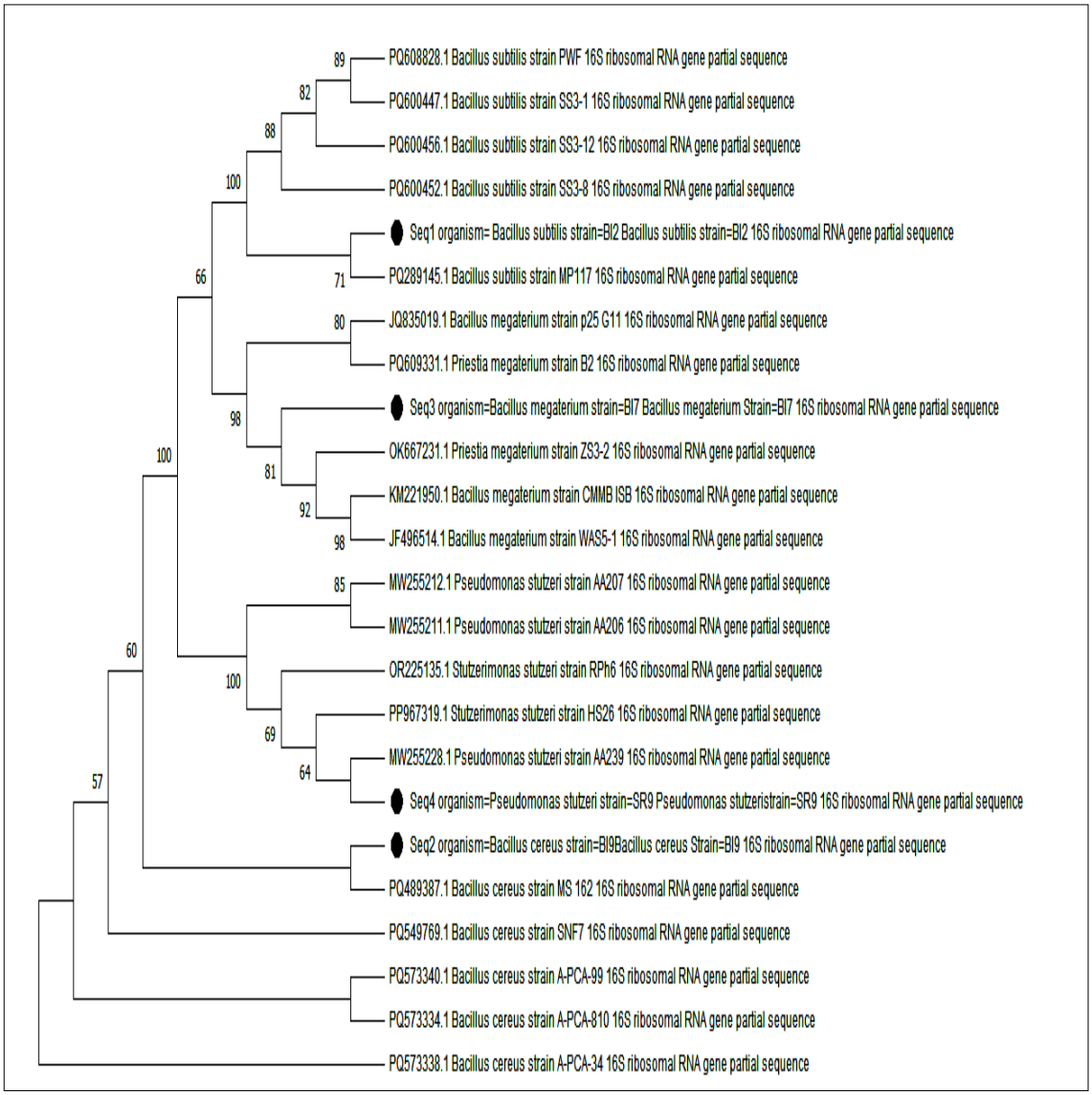

Fig. 2. Phylogenetic analysis of identified bacterial isolates in rice-wheat cropping system with selected reference strains.

Table 2. Species identification of bacterial isolates by 16S rRNA and ITS gene sequence homology

Bacterial isolates	Gene sequence homology			
	Species identified	NCBI accession number	No. of bases sequenced	Homology %
SRB7	*Bacillus subtilis*	OL716087	1145	99.76
SRB9	*Bacillus cereus*	OL716088	1044	98.22
SRB13	*Bacillus megaterium*	OM816754	1106	99.63
SRBS6	*Pseudomonas stutzeri*	OL716089	1303	100

Species identified based on 16S rRNA gene similarity of rhizospheric bacteria isolated from the rice-wheat cropping system.

antagonist bacterial isolates, SRB7, SRB13, SRBS9 and SRBS6, were used for further study. To determine the attraction effect of *M. graminicola* J_2s, attracted towards the root was counted at a regular interval of 12 h. In SRB7 and SRB13 at 12h post-inoculation, there was a significant reduction of 18.43 ± 0.47 and 20.26 ± 0.47 in the number of J_2s attraction after 36h no attraction (Fig. 5a). But in the remaining treatments, it gradually increased with an increase in the period of exposure. The result in (Fig. 5b) showed that the penetration of J_2s was significantly reduced in SRB7, followed by SRB13, SRB9 and SRBS6 (67.76, 63.83, 55.30, 48.35 % respectively) after a period of 12h exposure. Moreover, the maximum reduction was observed with SRB7 compared to other isolates after 48h of exposure.

Effect of bacterial agent on plant growth factors

The bacterial isolate treatment increases plant height, shoot and root weight compared to the control. The percent increase in plant height varies from 30 to 55 %, shoot weight from 35 to 70 % and root weight ranges from 40 to 80 %in different treatments with the nematode inoculation. There was no significant difference between the *B. amyloliquefacians* and *P. fluorescence* in fresh root weight (Table 3). The application of these bacteria significantly showed a positive result. The chemical treatment velum prime records maximum shoot and root length compared to other treatments.

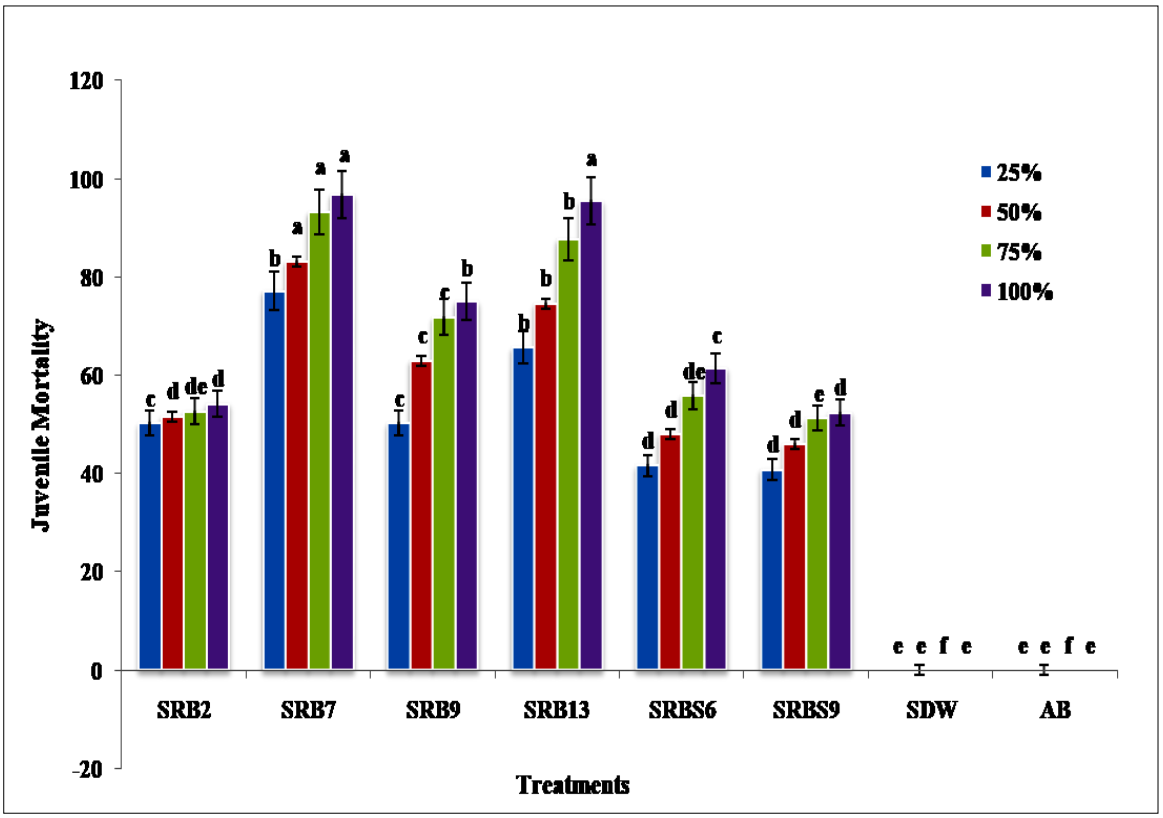

Fig. 3a. Juvenile mortality percentage (charts represent means ± SE; n = 3 recorded at 12h) of *M. graminicola* juveniles by different bacterial culture filtrates (25, 50, 75 and 100 % concentrations) at 12 h intervals. Abbreviations: SDW - sterile distilled water; AB - Autoclaved broth; h-hour. Controls are sterile distilled water and autoclaved broth. One-way ANOVA followed by Tukeys' honestly significant difference (HSD) post hoc test was performed among the control and each treatment at each time interval. In each time interval, values marked with the same letters are not significantly different from each other (P <0.05: Tukeys' HSD post hoc test).

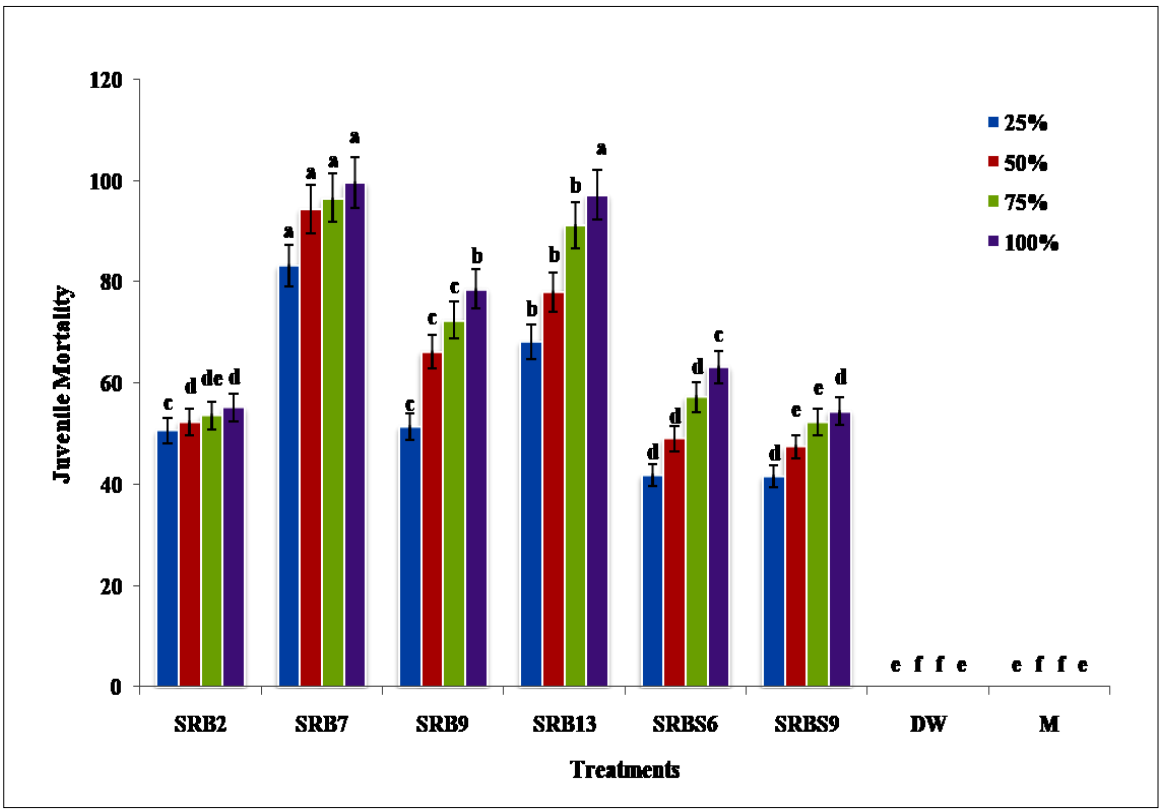

Fig. 3b. Juvenile mortality percentage (charts represent means ± SE; n = 3 recorded at 24h) of *M. graminicola* juveniles by different bacterial culture filtrates (25, 50, 75 and 100% concentrations) at 24 h intervals. Abbreviations: SDW - sterile distilled water; AB - Autoclaved broth; h-hour. Controls are sterile distilled water and autoclaved broth. One-way ANOVA followed by Tukeys' honestly significant difference (HSD) post hoc test was performed among the control and each treatment at each time interval. In each time interval, values marked with the same letters are not significantly different from each other (P <0.05: Tukeys' HSD post hoc test).

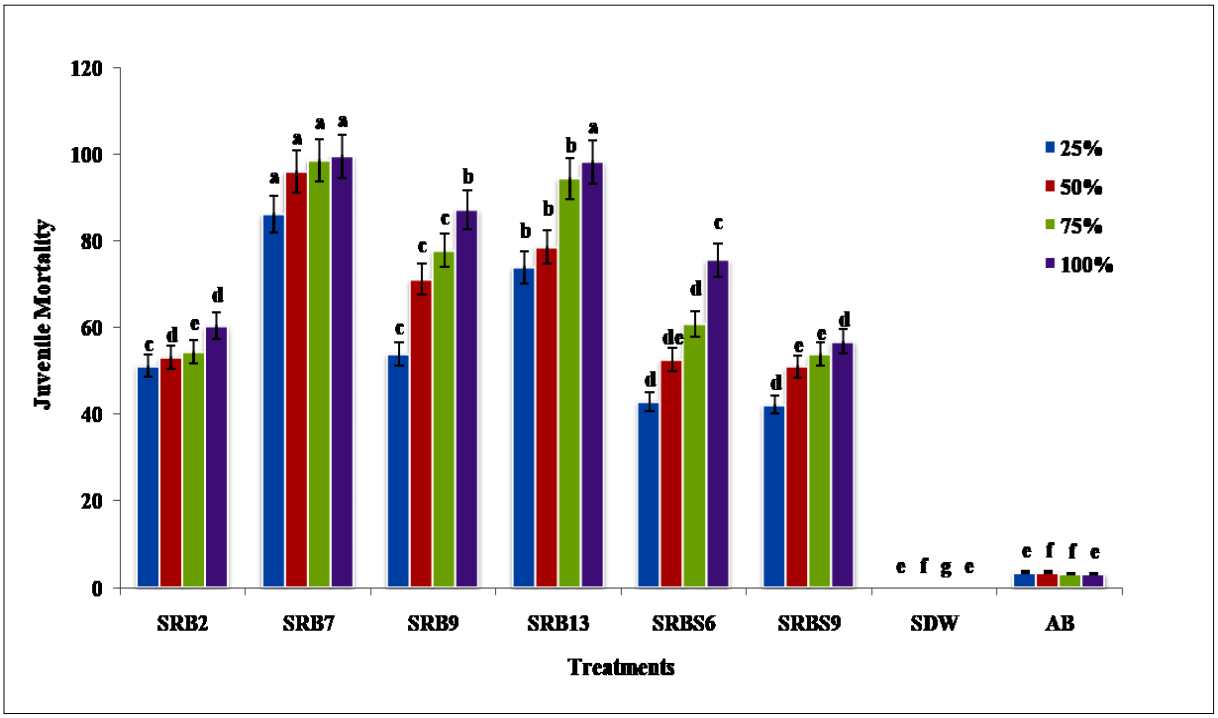

Fig. 3c. Juvenile Mortality Percentage (charts represent means ± SE; n = 3 recorded at 36 h) of *M. graminicola* juveniles by different bacterial culture filtrates (25, 50, 75 and 100% concentrations) at 36 h intervals. Abbreviations: SDW - sterile distilled water; AB - Autoclaved broth; h-hour. Controls are sterile distilled water and autoclaved broth. One-way ANOVA followed by Tukeys' honestly significant difference (HSD) post hoc test was performed among the control and each treatment at each time interval. In each time interval, values marked with the same letters are not significantly different from each other (P <0.05: Tukeys' HSD post hoc test).

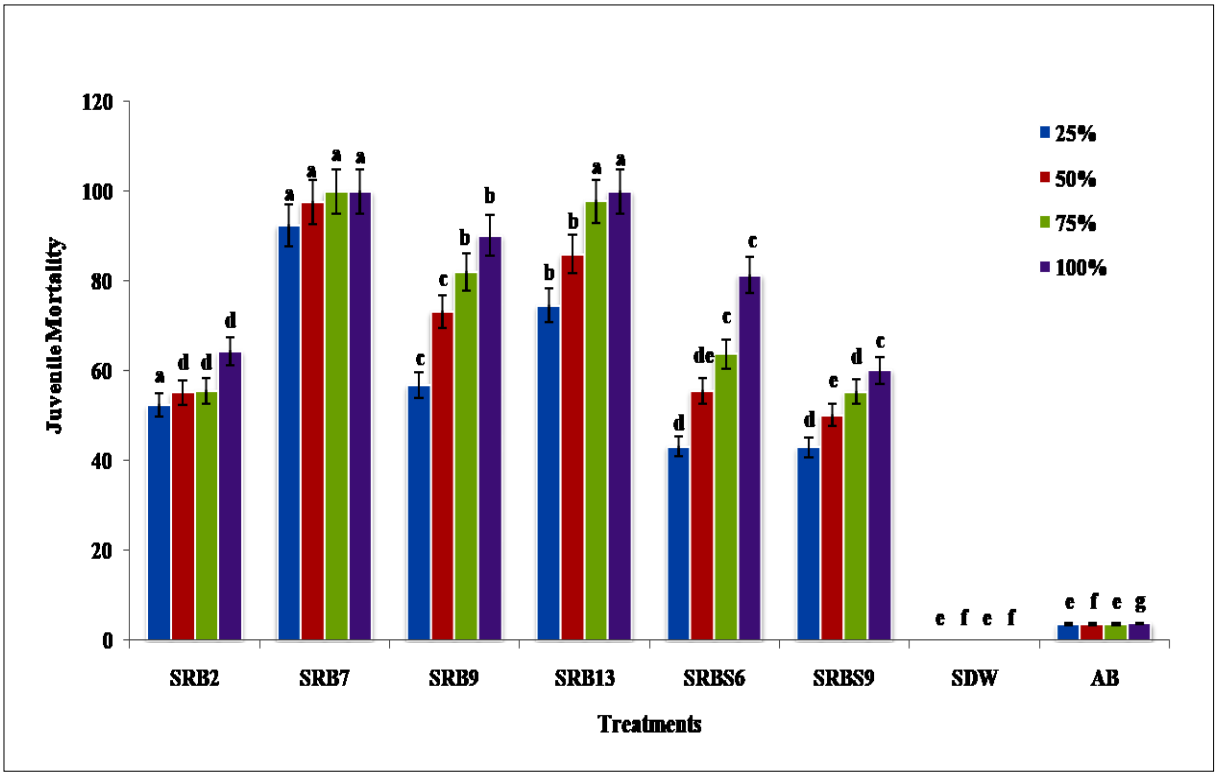

Fig. 3d. Juvenile mortality percentage (charts represent means ± SE; n = 3 recorded at 48 h) of *M. graminicola* juveniles by different bacterial culture filtrates (25, 50, 75 and 100 % concentrations) at 48 h intervals. Abbreviations: SDW - sterile distilled water; AB – Autoclaved broth; h-hour. Controls are sterile distilled water and autoclaved broth. One-way ANOVA followed by Tukeys' honestly significant difference (HSD) post hoc test was performed among the control and each treatment at each time interval. In each time interval, values marked with the same letters are not significantly different from each other (P <0.05: Tukeys' HSD post hoc test).

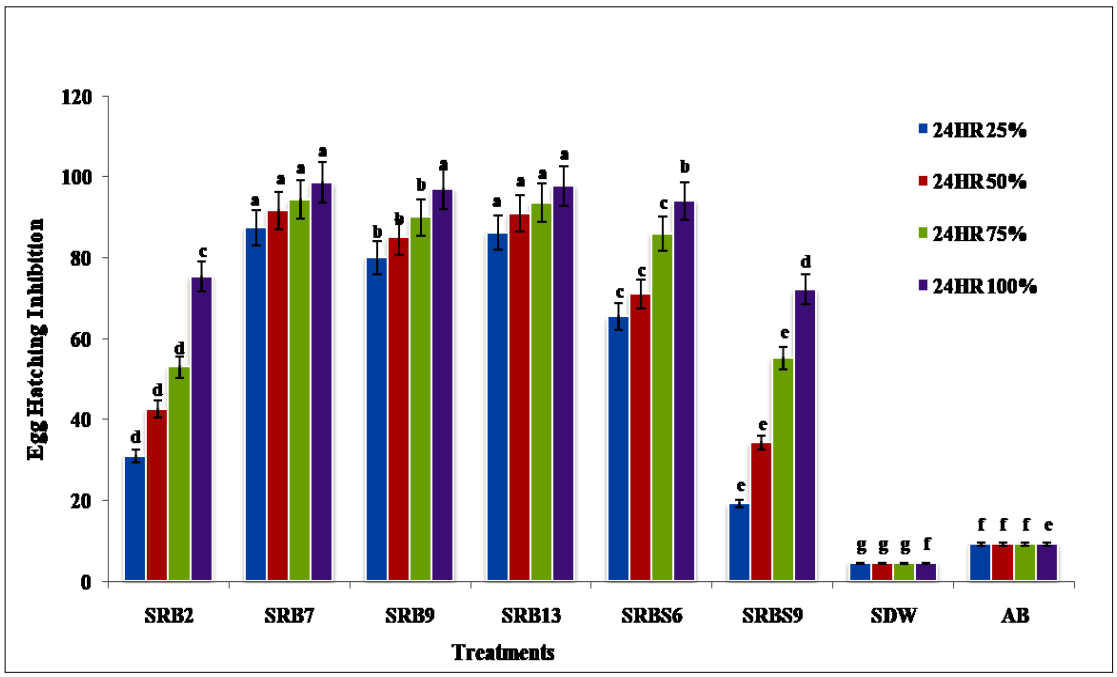

Fig. 4a. Hatching inhibition percentage (charts represent means ± SE; n = 3 recorded at 24 h) of *M. graminicola* juveniles by different bacterial culture filtrates (25, 50, 75 and 100 % concentrations) at 24 h intervals. Abbreviations: SDW - sterile distilled water; AB - Autoclaved broth; h-hour. Controls are sterile distilled water and autoclaved broth. One-way ANOVA followed by Tukeys' honestly significant difference (HSD) post hoc test was performed among the control and each treatment at each time interval. In each time interval, values marked with the same letters are not significantly different from each other (P <0.05: Tukeys' HSD post hoc test).

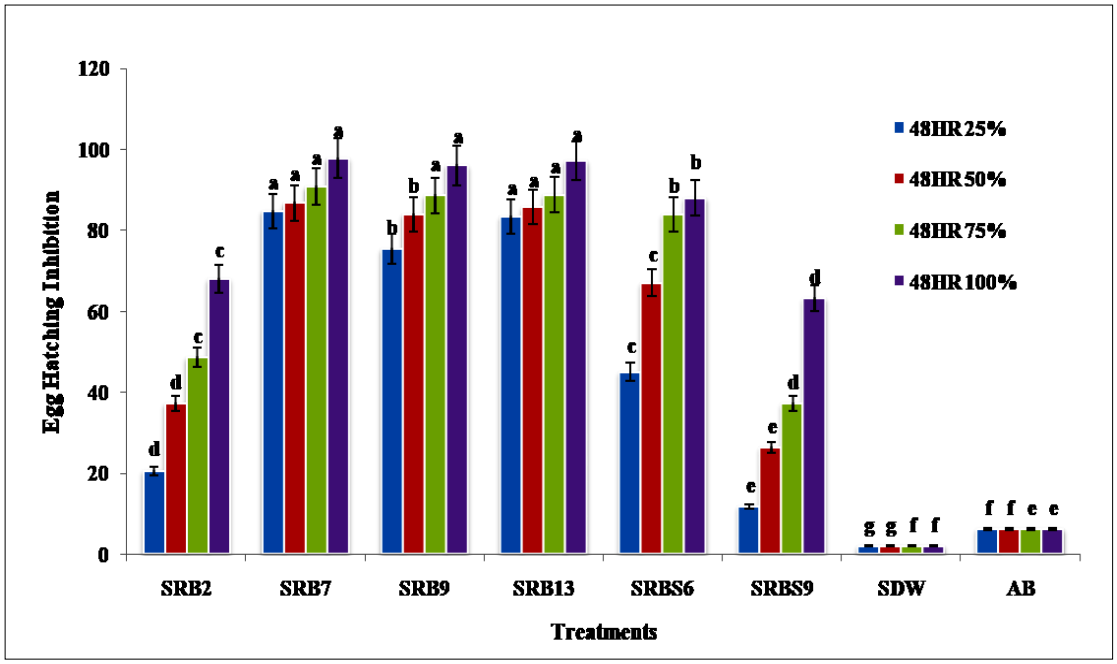

Fig. 4b. Hatching inhibition percentage (charts represent means ± SE; n = 3 recorded at 48 h) of *M. graminicola* juveniles by different bacterial culture filtrates (25, 50, 75 and 100% concentrations) at 48 h intervals. Abbreviations: SDW - sterile distilled water; AB - Autoclaved broth; h-hour. Controls are sterile distilled water and autoclaved broth. One-way ANOVA followed by Tukeys' honestly significant difference (HSD) post hoc test was performed among the control and each treatment at each time interval. In each time interval, values marked with the same letters are not significantly different from each other (P <0.05: Tukeys' HSD post hoc test).

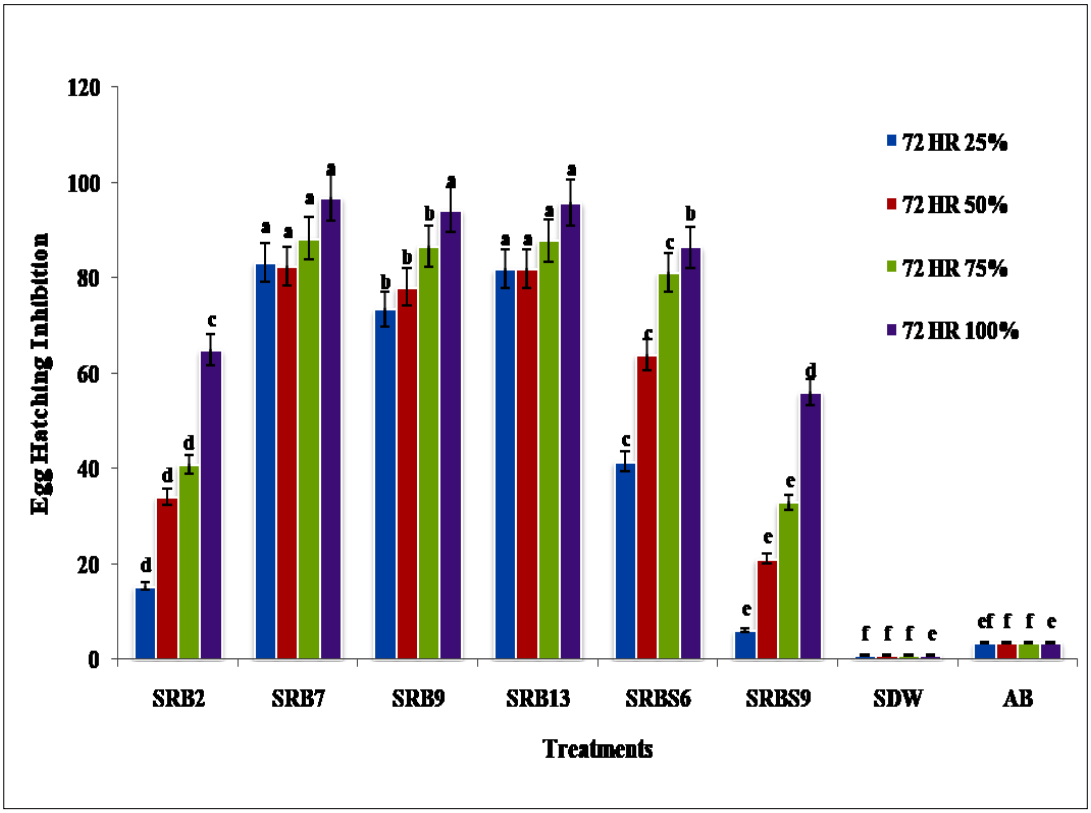

Fig. 4c. Hatching Inhibition Percentage (charts represent means ± SE; n = 3 recorded at 72 h) of *M. graminicola* juveniles by different bacterial culture filtrates (25, 50, 75 and 100% concentrations) at 72 h intervals. Abbreviations: SDW - sterile distilled water; AB – Autoclaved broth; h- hour. Controls are sterile distilled water and autoclaved broth. One-way ANOVA followed by Tukeys' honestly significant difference (HSD) post hoc test was performed among the control and each treatment at each time interval. In each time interval, values marked with the same letters are not significantly different from each other (P <0.05: Tukeys' HSD post hoc test).

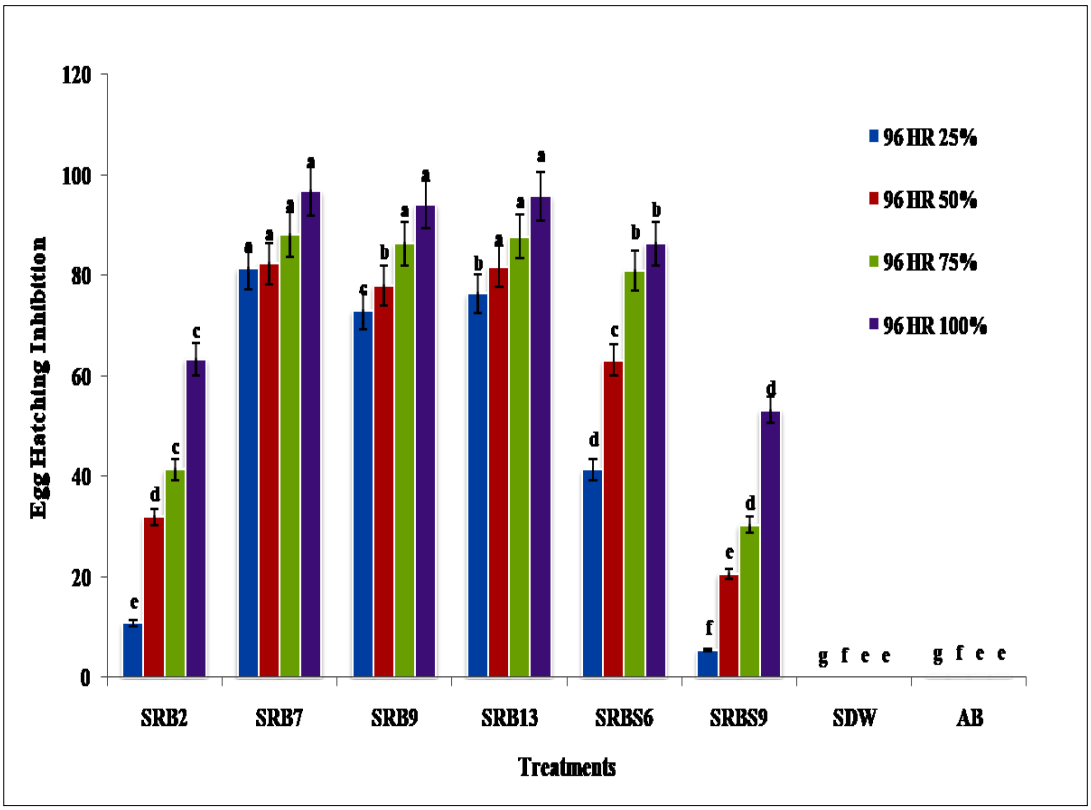

Fig. 4d. Hatching inhibition percentage (charts represent means ± SE; n = 3 recorded at 96 h) of *M. graminicola* juveniles by different bacterial culture filtrates (25, 50, 75, and 100 % concentrations) at 96 h time intervals. Abbreviations: SDW - sterile distilled water; AB – Autoclaved broth; h- hour. Controls are sterile distilled water and autoclaved broth. One-way ANOVA followed by Tukeys' honestly significant difference (HSD) post hoc test was performed among the control and each treatment at each time interval. In each time interval, values marked with the same letters are not significantly different from each other (P <0.05: Tukeys' HSD post hoc test).

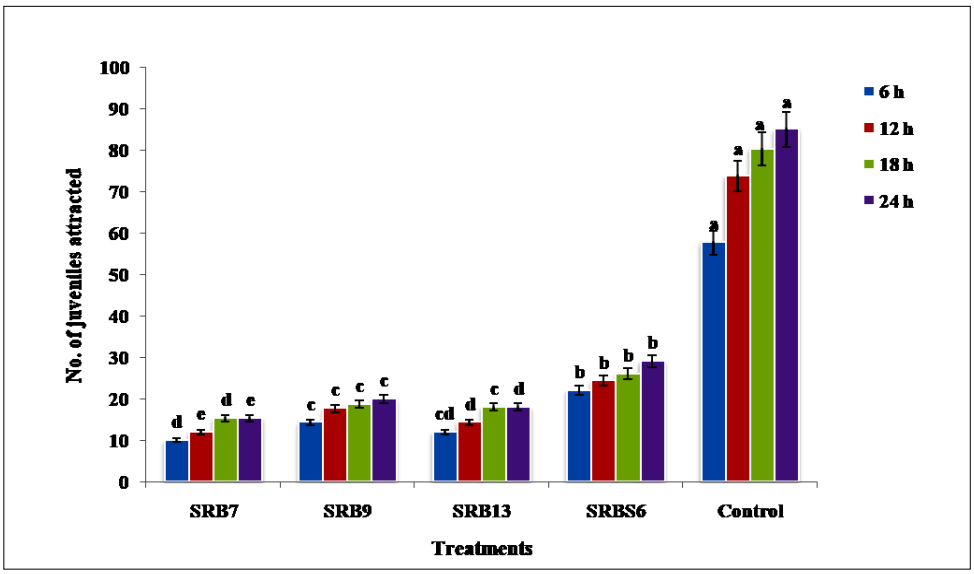

Fig. 5a. Effect of different bacterial culture filtrates on attraction of *M. graminicola* juveniles at 6-hour time intervals (6h, 12h, 18h and 24h). This charts represent means ± SE; n = 3. Note: (SRB7 - *Bacillus subtilis,* SRB9- *Bacillus cereus,* SRB13- *Bacillus megaterium,* SRBS6- *Pseudomonas stutzeri,* h- hour). One-way ANOVA followed by Tukeys' honestly significant difference (HSD) post hoc test was performed among the control and each treatment at each time interval. In each time interval, values marked with the same letters are not significantly different from each other (*P* <0.05: Tukeys' HSD post hoc test).

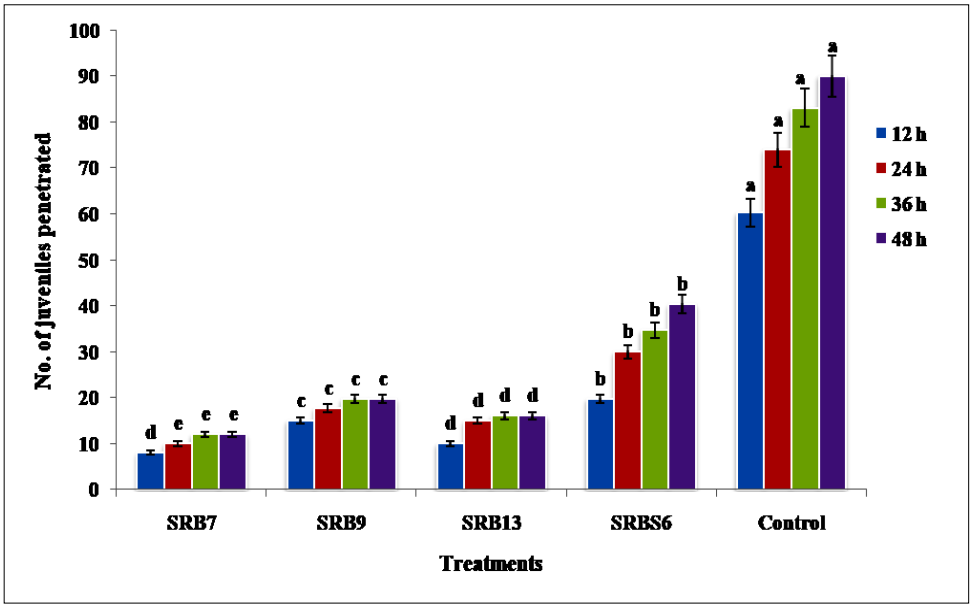

Fig. 5b. Effect of different bacterial culture filtrates on penetration of *M. graminicola* juveniles at 12-hour time intervals (12h, 24h, 36h and 48h). This charts represent means ± SE; n = 3. Note: (SRB7 - *Bacillus subtilis,* SRB9- *Bacillus cereus,* SRB13- *Bacillus megaterium,* SRBS6- *Pseudomonas stutzeri,* h- hour). One-way ANOVA followed by Tukeys' honestly significant difference (HSD) post hoc test was performed among the control and each treatment at each time interval. In each time interval, values marked with the same letters are not significantly different from each other (*P* <0.05: Tukeys' HSD post hoc test).

Table 3. Effect of bacterial isolates on plant growth parameters under pot culture studies

Treatments	Mean ± SE					
	Plant height (cm)	% increase over control	Fresh Shoot weight (g)	% increase over control	Fresh root weight (g)	% increase over control
SRB7	84.5 ± 0.41^{a}	51	2.95 ± 0.10^{a}	68	0.81 ± 0.01^{ab}	82
SRB9	77.0 ± 0.41^{c}	47	1.92 ± 0.02^{c}	52	0.54 ± 0.04^{c}	74
SRB13	80.5 ± 0.65^{b}	49	2.51 ± 0.07^{b}	63	0.73 ± 0.02^{b}	80
SRBS6	65.5 ± 0.65^{d}	37	1.39 ± 0.06^{d}	33	0.22 ± 0.02^{e}	36
BA	61.0 ± 0.91^{e}	33	1.52 ± 0.10^{d}	39	0.41 ± 0.03^{d}	65
Pf	67.0 ± 2.20^{d}	39	1.82 ± 0.03^{c}	49	0.41 ± 0.01^{d}	65
VP	87 ± 0.91^{a}	53	3.23 ± 0.17^{a}	71	0.85 ± 0.02^{a}	83
Control	40.75 ± 0.48^{f}		0.92 ± 0.06^{e}		0.14 ± 0.01^{f}	
CD(0.05)	1.91		0.53		0.316	
F(cal)	411.12		127.41		134.47	

(SRB7 - *Bacillus subtilis,* SRB9- *Bacillus cereus,* SRB13- *Bacillus megaterium,* SRBS6- *Pseudomonas stutzeri*, BA - *Bacillus amyloliquefaciens,* PF - *Pseudomonas fluorescence* and VP - Velum prime). Data shown correspond to the mean of four replicates ± standard error. According to Turkeys' honestly significant difference (HSD) test between control and each treatment, the same letter indicates data in each column is not significantly different from each other (*P*<0.05).

Effect of potential bacterial isolates (a pot study)

The four bacterial isolates SRB7, SRB9, SRB13 and SRBS6 showed significant reduction (P<0.05) in nematode infestation. The percentage decrease in the number of galls varies from 50 to 80; the number of adult females per galls varies from 60 to 80 %; and the percentage of eggs per egg mass decreases from 70 to 80. The reproduction factor for *B. subtilis* is 1.5 and it varies from 1.5 to 2.9. The velum prime showed a maximum decrease in nematode infestation with a 0.8 reproduction factor. These bacteria proved their potential against nematodes by decreasing the nematode infestation and acting as a plant growth-promoting factor (Table 4).

Changes in plant defence enzymes induced by antagonist bacterial isolates

The antagonist bacterial strain showed improved activity of defence enzymes such as peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) by inducing systemic resistance in nematode-infested plants. The *B.subtilis* and *B.megaterium* showed increased PO (1.84 and 1.76); PPO (1.54 and 1.5); PAL (4.2 and 4.0) activity when compared to control (Fig. 6a-c). All defence enzyme activity in bacteria-treated plants with nematode inoculation showed a 50 percent boost over the inoculated control plant roots. The maximum level of defence enzyme activity was observed on the sixth day of inoculation and then it slowly decreased.

Discussions

The soil biota contains diverse microbial communities that significantly influence soil health and plant productivity in rice-wheat cropping systems. A notable decrease in the population of plant parasitic nematodes, especially *M. graminicola*, led to the objective of understanding the reasons behind this reduction. It is hypothesized that beneficial bacterial communities may contribute to this decline. The *Bacillus* was the dominant genus isolated from the rice root rhizosphere (30).

In a study, six bacterial strains were isolated from the rice-wheat cropping system (31). This is consistent with those who reported that rhizobacterial strains isolated from *Crotalaria juncea* fields exhibited significant antimicrobial activity (12). Among the bacterial endophytes they isolated, 80 % were Gram-positive and 20 % were Gram-negative, a distribution similar to the current studys' findings (32).

In line with the morphological and biochemical characterization of six bacterial isolates (SRB7, SRB13, SRB9, SRBS6, SRB2, SRBS9) was conducted (33). The endophytic bacterium *Bacillus subtilis* demonstrated positive amylolytic, catalase and urease activity, whereas *Stenotrophomonas maltophilia* strain psk2 showed negative results for amylolytic and urease activity reported (34).

The molecular characterization of four potential biocontrol isolates was performed (35). The present study tested four bacterial isolates - *B. subtilis*, *B.* cereus, *B. megaterium* and *P. stutzeri*- in pot culture experiments to evaluate their antagonistic activity against *M. graminicola.* According to this report, bacterial isolates such as SRB7, SRB9, SRB13 and SRBS6 demonstrated biocontrol potential (36). The culture filtrates of these isolates - SRB7, SRB9, SRB13 and SRBS6- were highly effective in inducing juvenile mortality and inhibiting hatching (37). Also reported that the bacterial strain SRB7 caused more than 95% egg-hatching inhibition and juvenile mortality compared to the control (38).

Compared to untreated ones, the reduced movement of M. graminicola towards treated roots is likely due to the impaired host-finding ability caused by bacterial colonization around the roots. This movement and nematode penetration reduction has been reported in crops treated with *Bacillus cereus* and *Bacillus megaterium* against *M. graminicola*, *M. incognita* and *Pratylenchus penetrans* (37, 39). The bacterial isolates significantly reduced *M. graminicola* infestation by 50 to 80 (40, 41). This reduction is likely due to the broad-spectrum efficacy of the bacterial isolates, which enhanced their effectiveness in

Table 4. Effect of potential bacterial isolates against *Meloidogyne graminicola* under pot culture studies

Treatments	Mean ± SE						
	No of galls/5 g root	% decrease in control	No. of females/ galls	% decrease over control	No. of eggs/ egg mass	% decrease over control	Reproduction factor
SRB7	22.5 ± 0.65^{ef}	83.33	9.75 ± 0.71^{d}	82.43	28.50 ± 0.65^{f}	83.04	1.5
SRB9	45.0 ± 0.65^{c}	66.62	17.00 ± 0.48^{b}	69.36	52.50 ± 0.87^{c}	68.75	2.4
SRB13	26.25 ± 0.41^{de}	80.55	10.75 ± 0.41^{cd}	80.62	31.50 ± 0.65^{e}	81.25	1.7
SRBS6	65.00 ± 0.63^{b}	51.86	19.00 ± 0.48^{b}	65.76	59.50 ± 0.65^{b}	64.58	2.9
BA	33.75 ± 0.29^{d}	75.00	11.50 ± 0.63^{c}	78.30	44.25 ± 0.41^{d}	73.66	2.1
PF	32.5 ± 0.48^{d}	75.92	12.00 ± 0.65^{c}	79.20	43.50 ± 0.85^{d}	74.12	2.0
VP	17.5 ± 0.41^{f}	87.03	5.25 ± 0.96^{e}	90.54	10.00 ± 0.41^{g}	94.05	0.8
Control	135 ± 0.91^{a}		55.50 ± 0.41^{a}		168.00 ± 0.65^{a}		6.1
CD(0.05)	1.89		1.55		1.92		
F(CAL)	104.86		400.24		5361.60		

(SRB7 - *Bacillus subtilis,* SRB9- *Bacillus cereus,* SRB13- *Bacillus megaterium,* SRBS6- *Pseudomonas stutzeri*, BA - *Bacillus amyloliquefaciens,* PF - *Pseudomonas fluorescence* and VP - Velum prime). Data shown correspond to the mean of four replicates ± standard error. According to Turkeys' honestly significant difference (HSD) test between control and each treatment, the same letter indicates data in each column is not significantly different from each other (P <0.05).

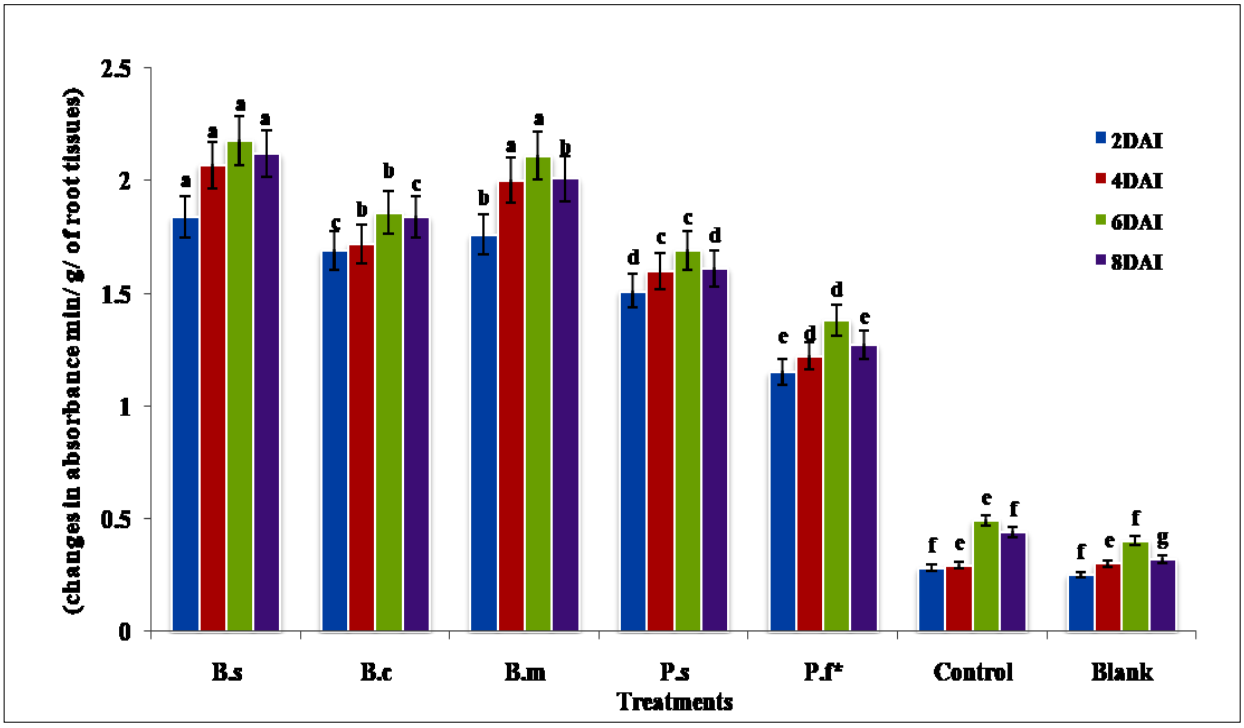

Fig. 6a. Changes in Peroxides (PO) activity in rice root treated with bacterial isolates against *M. graminicola* juveniles under pot condition at 2-day intervals (2, 4, 6 and 8 DAI). This charts represent means ± SE; n = 3. (Note: DAI – Day of Inoculation, B.s – *Bacillus subtilis*, B.c- *Bacillus cereus*, B.m – *Bacillus megaterium*, P.s - *Pseudomonas stutzeri* and *P.f*- Pseudomonas fluorescence*). In each time interval, values marked with the same letters are not significantly different from each other (*P* <0.05: Tukeys' HSD post hoc test).

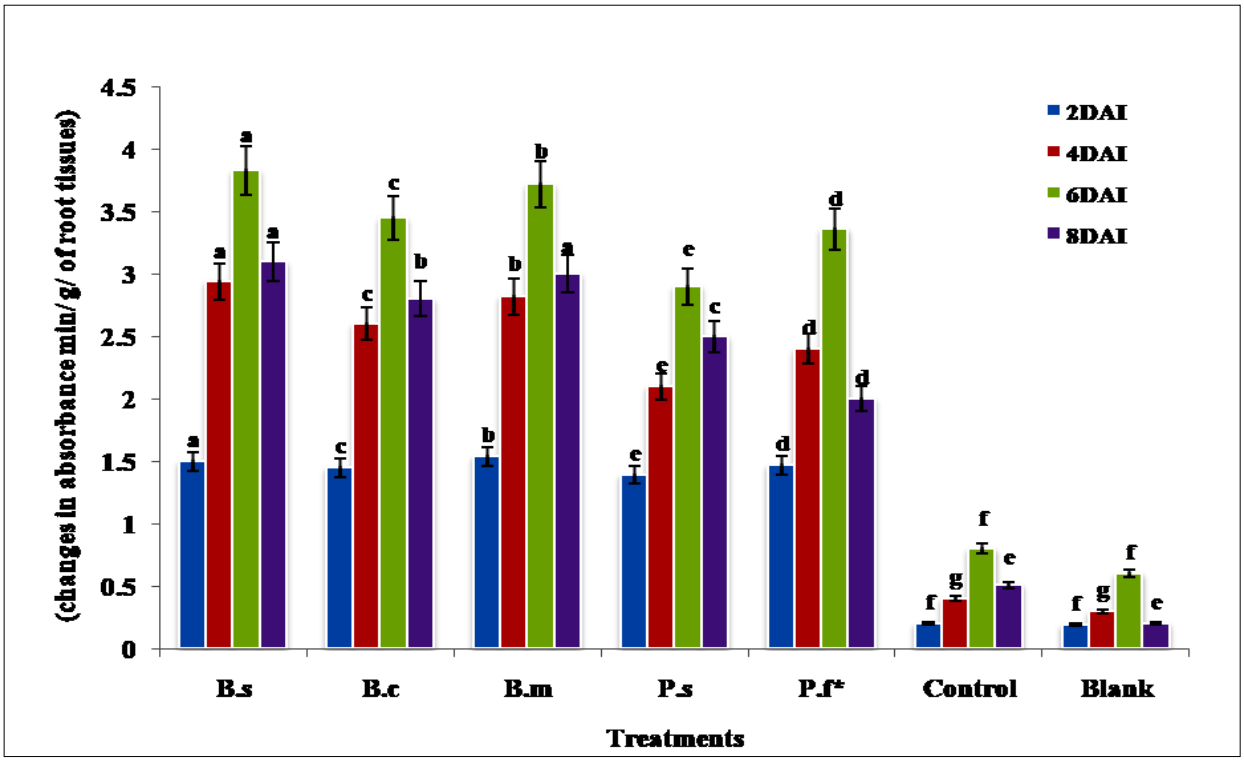

Fig. 6b. Changes in polyphenol oxidase (PPO) activity in rice root treated with bacterial isolates against *M. graminicola* juveniles under pot condition at 2 days intervals (2, 4, 6 and 8 DAI). This charts represent means ± SE; n = 3. (Note: DAI – Day of Inoculation, B.s - *Bacillus subtilis*, B.c- *Bacillus cereus*, B.m - *Bacillus megaterium*, P.s - *Pseudomonas stutzeri* and *P.f*- Pseudomonas fluorescence*). In each time interval, values marked with the same letters are not significantly different from each other (*P* <0.05: Tukeys' HSD post hoc test).

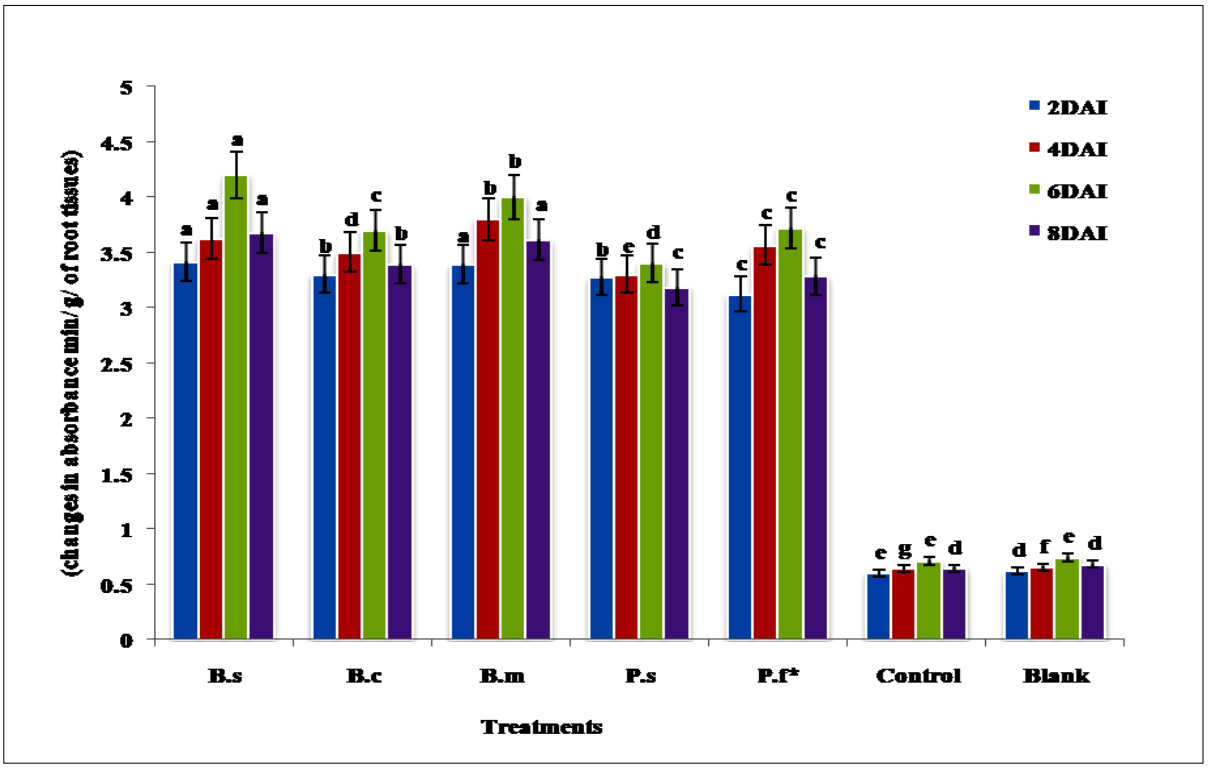

Fig. 6c. Changes in phenylalanine ammonia-lyase activity (PAL) activity in rice root treated with bacterial isolates against *M. graminicola* juveniles under pot conditions at 2-day intervals (2, 4, 6 and 8 DAI). This charts represent means ± SE; n = 3. (Note: DAI – Day of Inoculation, B.s - *Bacillus subtilis*, B.c- *Bacillus cereus*, B.m - *Bacillus megaterium*, P.s - *Pseudomonas stutzeri* and *P.f*- Pseudomonas fluorescence*). In each time interval, values marked with the same letters are not significantly different from each other (*P* <0.05: Tukeys' HSD post hoc test).

reducing nematode infestation.

The enzymes chitinases and proteases, which are found in the isolated bacterial strain that could hydrolyze N-acetyl-D-glucosamine polysaccharide chains and peptide bonds, respectively, are present in the chitin/ protein combination that composes the nematode eggs, lead to nematode mortality.

The increase in the abundance of bacterivores in the rice-wheat cropping system (RWCS), due to the incorporation of green manures contributed to the identification and molecular characterization of promising bacterial strains. For example, SRB7 showed 99 % similarity to *Bacillus subtilis* (OL716087), SRB9 had 98 % similarity with *Bacillus cereus* (OL716088), SRB13 was 99 % similar to *Bacillus megaterium* (OM816754) and SRBS6 showed 100 % similarity to *Pseudomonas stutzeri* (OL716089). In pot culture experiments, these bacterial isolates - *B. subtilis*, *B. cereus*, *B. megaterium* and *P. stutzeri*- were tested for their antagonistic effects against M. graminicola.

According to the study, *B. megaterium* demonstrated significant plant growth-promoting activities, enhancing germination, seedling vigour and dry weight (42). Similarly, the bacterial isolates in this study promoted plant growth by significantly increasing rice plant height, shoot weight and root weight, likely due to the secretion of IAA and phytohormones (43, 44). Many biocontrol agents can induce plant resistance by activating chemical signalling pathways, triggering the plants' defence mechanisms. This activation leads to the secretion of phenols and specific enzymes that reinforce the cell wall and directly inhibit nematode entry, a process known as the hypersensitive response (45). In this study, the biocontrol agents induced systemic resistance in rice roots against *M. graminicola* by stimulating the production of defence-related proteins and promoting lignification to strengthen cell walls and prevent further spread of the nematode (46).

In contrast to this study, the nematicidal efficacy of *B. subtilis* and carbofuran treatments against *M. incognita*, *M. javanica* and *Pratylenchus zeae* in sugarcane was equivalent. Additionally, evaluating the antagonistic activity of *Bacillus methylotrophicus* and *Lysobacter antibioticus* was more effective than the abamectin and carbofuran against *M. incognita* (47).

Compared with the peak defence activity in plants, it was observed on day 6 after nematode inoculation, after which it gradually decreased (48). A higher accumulation of defence enzymes, such as PPO, PO, PAL and SOD, was detected in rice roots inoculated with *P. fluorescens* (49) as well as in roots inoculated with arbuscular mycorrhizal fungi (50). These treatments enhanced plant growth and improved the root systems' resistance to nematode infection.

Conclusion

In conclusion, this study demonstrated that the isolate SRB7 (*Bacillus subtilis*) exhibited the highest antagonistic activity against *M. graminicola*, followed by SRB13 (*Bacillus megaterium*), SRB9 (*Bacillus cereus*) and SRBS6 (*Pseudomonas stutzeri*). These bacterial isolates, isolated from the rice-wheat cropping system, offer promising potential for managing *M. graminicola* infestations in rice fields.

Furthermore, applying these bacteria resulted in elevated levels of plant defence enzymes, such as peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase.

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Authors' contributions

SR helped in experimentation, data analysis, and manuscript drafting. PA helped in conceptualization, resources, manuscript editing and revision. DS helped in assistance in biocontrol experiments. SP helped in assistance in bacterial isolate characterization

Compliance with ethical standards

Conflict of interest: All authors declare that they have no conflicts of interest

Ethical issues: None.

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