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Characterization and evaluation of native rhizobacteria isolated from *Meloidogyne incognita*-infected tomato (*Solanum lycopersicum*)

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ABSTRACT

Three native rhizobacteria, IRh9, IRh10 and IRh15, were isolated from nematode-infected tomato (*Solanum lycopersicum* L.) rhizosphere were characterized and evaluated for their efficacy against root-knot nematode (RKN), *Meloidogyne incognita* infecting tomato crop under pot conditions in the protected structure environment during 2020–21 at Centre for Protected Cultivation technology (CPCT), ICAR-Indian Agricultural Research Institute, New Delhi. Based on 16S rRNA sequences, IRh9, IRh10, and IRh15 showed > 98% homology with *Bacillus licheniformis, Priestia megaterium* and *Pseudomonas putida*, respectively. Compared to the control, the penetration of root-knot nematode in tomato roots was reduced by >50% under pot conditions through inoculation of these bacteria. Characterization through gas chromatography-mass spectrometry analysis revealed 30 different volatile organic compounds from these rhizobacteria. Among them, 19 compounds were identified from *P. megaterium* IRh10, followed by 16 compounds each from *B. licheniformis* IRh9 and *P. putida* IRh15. Furthermore, among the detected volatile organic compounds, acetic acid, hexadecane, hexadecanoic acid, octadecanoic acid, 1-decene and 9-octadeconoic acid are reported to possess nematicidal properties. Additional characterization of plant growth promotion traits associated with these rhizobacteria revealed that *P. megaterium* IRh10 and *P. putida* IRh15 produced indole acetic acid, while *B. licheniformis* IRh9 and *P. megaterium* IRh10 produced ammonia, but only *P. putida* IRh15 could solubilize the tricalcium phosphate under laboratory conditions. Among the 35 substrates studied, *P. megaterium* IRh10 utilized most (12), followed by *B. licheniformis* IRh9 and *P. putida* IRh15, which were able to use 11 and 8 substrates, respectively. The study showed that *B. licheniformis* IRh9*, P. megaterium* IRh10 and *P. putida* IRh15 are potential candidates for combating *M. incognita* infestation in tomato crops.

Keywords: *Meloidogyne incognita*, Nematicidal activity, Rhizobacteria, Tomato, Volatile organic compounds

Tomato (*Solanum lycopersicum* L.) is among the important vegetable crops susceptible to root-knot nematode (RKN) infection in open fields and protected cultivation. Tomato suffers about 23% of annual yield loss with an estimated monetary loss of up to ₹6035.20 million rupees (Kumar *et al.* 2020). The severity of RKNs infestation is rapidly increasing under protected cultivation due to the availability of favourable temperatures, relative humidity and hosts. The massive buildup of the RKNs population (sometimes 12–15 times more than in open field conditions) inflicts severe yield loss (Phani *et al.* 2021), leading to poor crop growth and making cultivation under a protected structure uneconomical. Since plant parasitic nematodes (PPNs) are soil-borne and generally attack underground parts of the plant, their management is difficult and complex.

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Management of RKNs problem is done through cultural practices, use of resistant cultivars and chemical nematicides. Though effective, chemical nematicides are discouraged due to human health and environmental safety concerns, particularly in light of the massive demand for organic produce, which has spurred research towards eco-friendly pest management alternatives. Several studies demonstrated that *Meloidogyne* spp. could be managed through biocontrol agents (Holajjer *et al.* 2018, Forghani and Hajihassani 2020). Many fungal bioagents have been extensively investigated and successfully utilized for RKN management (Abd-Elgawad and Askary 2018). Likewise, some plant growth-promoting rhizobacteria (PGPR) are gaining significance as nematode antagonists. Among PGPR, *Bacillus* and *Pseudomonas* genera are widely exploited for managing *Meloidogyne* spp. on various crops. They exhibit nematode antagonism directly by producing toxins/ antimicrobial compounds or indirectly by activating plant defense mechanisms (Mhatre *et al.* 2019).

In this context, native rhizobacteria isolated from RKN-

infected tomato rhizosphere having *in vitro* nematicidal activity were selected in the present study for characterization and evaluation against *M. incognita* penetration in tomato roots under pot conditions. Volatile organic compounds (VOCs) produced by these rhizobacteria were identified using gas chromatography-mass spectrometry (GC-MS) analysis. In addition, plant growth promotion traits of these rhizobacterial isolates were identified.

MATERIALS AND METHODS

The present study was conducted at the Centre for Protected Cultivation technology (CPCT), ICAR-Indian Agricultural Research Institute (IARI), New Delhi during 2020–21 for characterization and evaluation of native rhizobacteria isolated from *Meloidogyne incognita* -infected tomato (*Solanum lycopersicum*).

Selection of rhizobacterial isolates: Nematode antagonistic rhizobacterial isolates IRh9, IRh10, and IRh15 were isolated during 2020 from *M. incognita* infected tomato plants grown in protected structure (28˚62' N, 77˚15' E) at Centre for Protected Cultivation technology (CPCT), ICAR-Indian Agricultural Research Institute (IARI), New Delhi. The pure cultures of these rhizobacteria were maintained at the Division of Nematology, ICAR-Indian Agricultural Research Institute, New Delhi and used for the present study.

Morphological characterization of rhizobacterial isolates: Gram's staining technique was employed following the manufacturer's protocol (Gram's stain-kit, Himedia Laboratories Pvt. Ltd., India). To visualize colonies of nematode-antagonistic rhizobacteria, each isolate was grown on a conical flask containing 25 ml of nutrient broth media for 48 h. Then, one ml of each broth culture was transferred to 1.5 ml eppendorf tubes and centrifuged for 2 min at 500 rpm. After discarding the supernatant, the pellet was fixed with 3% glutaraldehyde solution for 12 h at 25 ± 2°C. After removing the fixative, pellets were washed four times with phosphate buffer solution (*p*H 7.2). Further, the pellet was post-fixed with a 2% osmium tetroxide solution for 6 h at 25 ± 2°C. Post fixed pellet was again washed with phosphate buffer solution four times. Then, 10 µl of pellet suspension was placed on a sterile cellulose nitrate membrane (pore size: 0.22 µ, Himedia Laboratories Pvt. Ltd., India) and air dried for one h. Air-dried cellulose nitrate membrane sections containing bacterial pellets were mounted on SEM stubs and coated with gold-palladium (SC7620 sputter coater, Quorum). Mounts were examined with a TESCON (Tescan Orsay Holding, Czech Republic) Vega 3 LMU scanning electron microscope.

Molecular characterization of rhizobacterial isolates: Genomic DNA of freshly grown (24 h) rhizobacterial isolates was extracted by using HiPurA rhizobacterial genomic DNA purification kit (Himedia Laboratories Pvt. Ltd., India) following the manufacturer's protocol. The polymerase chain reaction was performed in a thermocycler using primer pairs 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT). The 50µl PCR reaction mixture consisting of 2.0 µl of 20 µM forward primer, 2.0 µl of 20 µM reverse primer, 2.0 µl of DNA template 25 µl of GoTaq® green master mix (Promega), and 19 µl of nuclease-free water and the PCR conditions are as follows: Initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 90 s with a final extension at 72°C for 10 min. Further, PCR products were electrophoresed (90 V for 45 min) and visualized under UV transilluminator (Wealtec Bioscience Co., Ltd. Taiwan). The PCR products were purified and sequenced (M/s. Sequencher Tech, Ahmedabad, India). The resultant 16S rRNA gene sequences were subjected to BLAST in NCBI to search for similar sequences in the database. Similarity of ≥98% with the sequences available in the GenBank was used for the species-level identification. Further, the nucleotide sequences of these rhizobacterial isolates were deposited at GenBank, National Center for Biotechnology Information (NCBI).

Nematode inoculum: Root-knot nematode (*M. incognita*) pure culture was maintained on the susceptible tomato (cv. NS-4266) plants under greenhouse conditions. Infected roots were rinsed with sterile water, and egg masses were collected. Further, eggs were incubated for hatching up to 120 h at 25 ± 2°C under laboratory conditions. Juveniles were collected every 24 h up to 120 h, concentrated and used for penetration assay.

Evaluation of bio-efficacy of rhizobacterial isolates on Meloidogyne incognita *penetration in tomato roots*: The experiment was conducted in plastic pots (5 cm bottom diameter, 6 cm height and 7.5 cm top diameter) under protected structure condition during the year 2021. Each plastic pot was filled with 200 g sterilized soil and sand mixture and transplanted with tomato (cv. NS-4266) healthy seedling (25 days old seedlings grown on portrays having sterilized mixture of cocopeat:vermiculite:perlite (3:1:1 ratio)). Further, the rhizosphere region of the tomato plant was drenched with respective treatments after plant establishment. The details of treatments are as follows: T_1, Control (6 ml of distilled water)/plant; T_2, Nutrient broth 6 ml/plant; T_3, Chemical control (Fluopyram 34.48% SC) 0.5 ml/plant; T_4, *Bacillus licheniformis* IRh9 @6 ml (2.20×10^9 CFU/ml)/plant; T_5, *Priestia megaterium* IRh10 @6 ml (2.24×10^9 CFU/ml)/ plant; T_6, *Pseudomonas putida* IRh15 @6 ml (2.15×10^9 CFU/ml)/plant. The experiment was conducted in a completely random design (CRD) with 5 replicates. Four hundred second-stage juveniles (J_2) of *M. incognita* were inoculated after seven days of rhizobacterial treatment. After 10 days of J_2 inoculation, plants were uprooted, roots stained with acid fuchsin and observed under a stereo zoom microscope. The number of nematodes penetrated per plant root system was recorded.

Characterization of volatile organic compounds from rhizobacterial isolates: A single colony of each isolate was inoculated in 500 ml nutrient broth media and incubated for 48 h. After incubation, the culture broth was centrifuged for 20 min at 8000 rpm. Further, clear supernatant was collected and transferred to a separating funnel. Then, ethyl

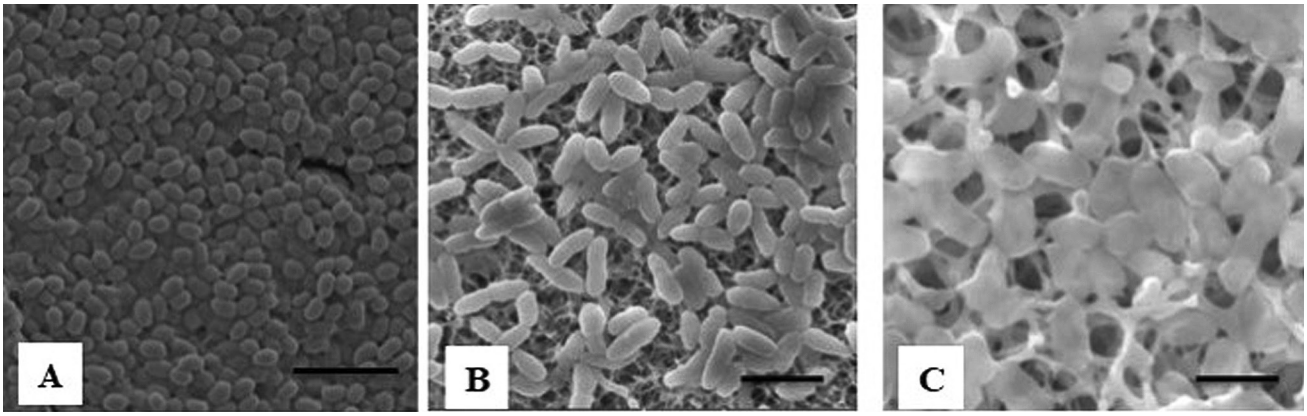

Fig 1 Scanning electronic microscopic images of nematode antagonistic rhizobacterial isolates A. *Bacillus licheniformis* IRh9, B. *Priestia megaterium* IRh10, C. *Pseudomonas putida* IRh15 (Scale bars = 5 µm).

acetate (100 ml × 3) was added to the separating funnel and thoroughly shaken. Liquid-liquid partitioning method was employed to separate VOCs. Then, the ethyl acetate layer suspension was collected in a conical flask and added with 10 g of anhydrous sodium sulphate to remove traces of moisture, if any. The solution was vacuum dried using a rotary evaporator (Heidolph, Germany), and the residue was redissolved in GC-MS grade diethyl ether (2 ml). Compounds were analyzed in GC-MS by following the standard protocol (Kundu *et al.* 2021), and were identified by matching their mass spectra and fragmentation pattern using the NIST (National Institute of Standards and Technologies) Mass Spectra Library.

Characterization of rhizobacterial isolates for plant growth promotion traits and substrate utilization: Plant growth promotion traits such as phosphate solubilization (Malboobi *et al.* 2009), ammonia production (Dunca *et al.* 2007) and IAA production (Islam *et al.* 2016) of rhizobacterial isolates were characterized by following standard protocols. A substrate utilization study was performed with ready-to-use strips having 35 different substrates (Hi-Carbohydrate Kit, Himedia Laboratories pvt. Ltd., India). Each strip well was inoculated with 10 µl of 24 h grown fresh broth culture of rhizobacterial isolate and incubated for 24 h at 28 ± 2 °C. Further, the ability of each isolate to utilize available substrate in strips was recorded after 24 h of exposure based on a colour change in each substrate by comparing it with the control strip well.

Statistical analysis: The numerical data of the pot experiment was square-root transformed before analysis. Analysis of variance (ANOVA) using PROC GLM SAS (version 9.3; SAS institute 2011, Cary, NC, USA) was performed on the transformed data, and back-transformed data only were presented. Comparisons of relevant means were made using Tukey's significance test values at the 5% significance level.

RESULTS AND DISCUSSION

The rhizobacterial isolates IRh9, IRh10 and IRh15, exhibited nematicidal activity against *M. incognita* by reducing 74.6%, 77.9% and 64.3% of egg hatching inhibition and causing 82.8%,73.1% and 71.8% of juvenile's mortality (Gowda *et al.* unpublished) were further characterized in the present study. Nematode antagonistic rhizobacterial isolates IRh9 and IRh10, reacted positively to Gram's reaction, and IRh15 reacted negatively (Table 1). All the isolates had rod-shaped cell morphology as observed under the scanning electron microscope (Fig 1).

At the molecular level, 16S rRNA sequences of the rhizobacteria, IRh9, IRh10 and IRh15, showed >98% homology with *Bacillus licheniformis, Priestia megaterium* (earlier name *Bacillus megaterium*) and *Pseudomonas putida*, respectively. The 16S rRNA nucleotide sequences were submitted in NCBI, and their accession number is given in Table 1. These three rhizobacteria predominantly belong to the genera *Bacillus* and *Pseudomonas,* which are recognized as potential nematode antagonists, including their PGPR activity (Li *et al.* 2015).

When these rhizobacteria were inoculated to tomato plants challenged with *M. incognita* under pot conditions in a protected structure environment, nematode (J_2) penetration was reduced significantly ($P<0.05$). Among rhizobacterial isolates, maximum reduction (57.9%) in nematode penetration was recorded in *P. megaterium* IRh10, while *B. licheniformis* IRh9 and *P. putida* IRh15 reduced nematode penetration in tomato roots by 54.2%

Table 1 Characterization of nematode antagonistic rhizobacterial isolates

Rhizobacterial isolates	Gram's reaction	Name	% Similarity	NCBI accession number
IRh9	+	*Bacillus licheniformis*	99.08	OM463444
IRh10	+	*Priestia megaterium*	98.56	OM818655
IRh15	-	*Pseudomonas putida*	100.0	OM468182

(+), positive; and (–), negative.

and 52.2% respectively, compared to the control. However, no significant ($P<0.05$) difference was observed among the rhizobacterial isolates, while they significantly differed from chemical control (Fig 2). The analysis of variance revealed a significant reduction of nematode penetration in tomato roots treated with rhizobacterial isolates (df: 5, 24, F: 324.47, $P<0.0001$) compared to control.

The reduction of nematode invasion in tomato roots indicated that rhizobacterial isolates exhibited nematicidal activity against *M. incognita*. *Bacillus* species are known to exhibit diverse antagonistic mechanisms against PPNs. They produce antimicrobial compounds, show strong competition with nematodes for food and space, and induce systemic resistance in plants (Engelbrecht *et al.* 2018, Caulier *et al.* 2019). Similarly, *Pseudomonas* species have been reported to inhibit nematode egg hatch, cause juvenile mortality and reduce nematode invasion to roots by producing hydrogen cyanide, phenazine, pyoluteorin, pyrrolnitrin and siderophores (Khan *et al.* 2016).

Besides, VOCs produced by the rhizobacteria play a vital role in nematode antagonism (Bui and Desaeger 2021). In the present study, the total ion chromatogram (TIC) in GC-MS analysis showed several peaks corresponding to 30 VOCs. These compounds were identified based on their mass spectral matching with the NIST library and respective mass fragmentation patterns. The major compounds belonged to alkanes (Hexadecane, Octadecane, Docosane, Triacontane, Cyclohexadecane, Hexacosane, Cyclotetracosane, Tritriacontane, Nonacosane, Pentacosane), alkenes (1-Tetradecene. 1-Decene, 1-Hexadecene, 1-Octadecene, 1-Docosene, 1-Eicosene, 1-Hexacosene, 9-Hexacosene, 1-Nonadecene), acids (Acetic acid, Butanoic acid, 2-Propenoic acid, Tetradecanoic acid, Dodecanoic acid, Octadecanoic acid, 9-Octadecenoic acid, Hexadecanoic acid), pyrazine (Pyrrolo-pyrazine-1,4-dione), ketone (Methylpyridazinone), and alcohol (Benzenemethanol) groups (Table 2 and Fig 2).

Among the rhizobacterial isolates, 19 VOCs were identified from *P. megaterium* IRh10 and 16 compounds each from *B. licheniformis* IRh9 and *P. putida* IRh15. Among VOCs, hexadecane, cyclohexadecane and cyclotetracosane were exclusively identified in *B. licheniformis* IRh9. Similarly, Butanoic acid, 9-Octadecenoic acid, 1-Eicosene, Docosane, Triacontane, 1-Nonadecane, Tritiacontane and Nonacosane were identified in *P. megaterium* IRh10. In addition, characteristic peaks corresponding to Dodecanoic acid, Hexacosane, Hexadeconoic acid, Pentacosane and Methylpyridazinone appeared in *P. putida* IRh15 (Table 2).

The maximum number of VOCs detected in *B. licheniformis* IRh9*, P. megaterium* IRh10 and *P. putida* IRh15 are known to have antimicrobial activity against many plant pathogens, including nematodes. Among them, acetic acid, hexadecane, hexadecanoic acid, octadecanoic acid, 1-decene and 9-octadeconoic acid were reported to have nematicidal activity (Caulier *et al.* 2019, Wolfgang *et al.* 2019, Tadigiri *et al.* 2020, Bui and Desaeger 2021). The VOCs in rhizobacteria exhibit antagonistic activity against PPNs by acting as a fumigant, repellent or attractant and as contact nematicide. Many studies hypothesized that VOCs might target the nematode cuticle surface coat, intestine, oesophagus and nervous system, thereby it destroys the nematode (Cheng *et al.* 2017). Likewise, in the present study, the rhizobacterial isolates VOCs facilitated more potent nematicidal activity against *M. incognita*.

Similar results were reported by Huang *et al.* (2010), who demonstrated that VOCs emitted by *Bacillus megaterium* YFM3.25 inhibited egg-hatch and reduced the *M. incognita* infection in a pot experiment. In addition,

Table 2 Volatile organic compounds identified from nematode antagonistic rhizobacterial isolates

Volatile organic compound (VOC)	*Bacillus licheniformis* IRh9	*Priestia megaterium* IRh10	*Pseudomonas putida* IRh15
Acetic acid	+	+	+
Butanoic acid	-	+	-
Benzenemethanol	-		+
1-Tetradecene	+	-	+
1-Decene	+	+	-
1-Hexadecene	+	+	+
Hexadecane	+	-	-
2-Propenoic acid	+	+	-
Tetradecanoic acid	+	+	+
1-Octadecene	+	+	+
Octadecane	+	+	-
Dodecanoic acid	-	-	+
Octadecanoic acid	+	+	+
1-Docosene	+	+	+
9-Octadecenoic acid	-	+	+
1-Eicosene	-	+	-
Docosane	-	+	-
Triacontane	-	+	-
Cyclohexadecane	+	-	-
1-Hexacosene	+	+	-
Hexacosane	-	-	+
Cyclotetracosane	+	-	-
Pyrrolo-pyrazine-1,4-dione	+	+	+
9-Hexacosene	+	-	+
1-Nonadecene	-	+	-
Tritriacontane	-	+	-
Nonacosane	-	+	-
Hexadecanoic acid	-	-	+
Pentacosane			+
Methylpyridazinone			+
Total	16	19	16

(+), presence; and (–), absence.

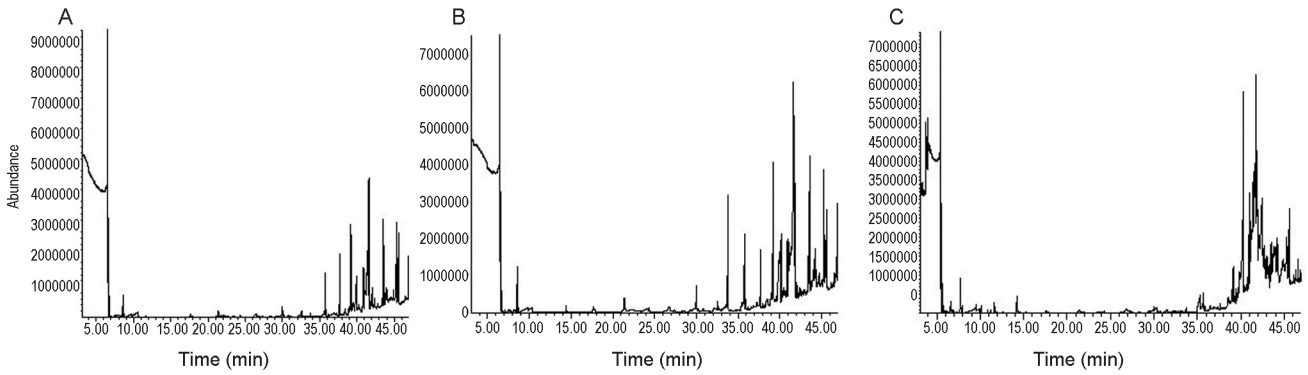

Fig 2 GC–MS chromatogram of A. *Bacillus licheniformis* IRh9, B. *Priestia megaterium* IRh10, C. *Pseudomonas putida* IRh15.

VOCs from *P. putida* strain 1A00316 showed diverse modes of action (fumigant, repellent, and nematicidal activities) against different stages of *M. incognita* (Zhai *et al.* 2018).

Furthermore, plant growth promotion traits associated with rhizobacteria can play a crucial role in stimulating plant growth by increasing the availability of essential nutrients (nitrogen, phosphorus, iron), phytohormones and ethylene levels by ACC-deaminase activity (Olenska *et al.* 2020), thereby indirectly helping to protect the plants from biotic stresses. In the present study, the rhizobacterial isolates showed varied plant growth promotion traits. They produced IAA, ammonia and solubilized tricalcium phosphate. Among three rhizobacteria, *P. megaterium* IRh10 and *P. putida* IRh15 were found to synthesize IAA in the presence of tryptophan. Indole acetic acid is a member of the phytohormones group that enhances plant growth and acts as a key signalling molecule for plant-microbe interactions (Matsuda *et al.* 2018). Similarly, *B. licheniformis* IRh9 and *P. megaterium* IRh10 showed the ability to produce ammonia which acts as a natural biofertilizer for plants (Zulfarina *et al.* 2017). However, among three rhizobacteria, only *P. putida* IRh15 could solubilize the tricalcium phosphate, which helps to increase phosphorus availability to plants (Table 3).

In addition, rhizobacterial isolates also showed a diverse range of substrate utilization profiles. *P. megaterium* IRh10 utilized maximum substrates (12), viz. maltose, fructose, dextrose, lactose, galactose, trehalose, melibiose, L-arabinose, mannose, mannitol, esculin and D-arabinose.

Table 3 Characterization of plant growth promotion traits associated with rhizobacterial isolates

Rhizobacterial isolate	P solubilization	IAA production	Ammonia production
Bacillus licheniformis IRh9	-	-	+
Priestia megaterium IRh10	-	+	+
Pseudomonas putida IRh15	+	+	-

(+), positive; and (–), negative.

B. licheniformis IRh9 utilized 11 substrates, viz. xylose, maltose, fructose, galactose, melibiose, glycerol, salicin, melizitose, mannoside, ONPG and sorbose and *P. putida* IRh15, eight substrates (xylose, dextrose, lactose, melibiose, ONPG, esculin, citrate and malonate) under laboratory conditions. The production of antimicrobial compounds is closely related to cell metabolic status and is highly influenced by the type of substrates available at the particular habitat (Raaijmakers *et al.* 2002). Therefore, multiple substrate utilization abilities of rhizobacterial isolate ensure its better survival and establishment at distinct habitats and exhibit greater nematode antagonistic activity against PPNs. Hence, the present study indicates that these three rhizobacteria could be potential candidates for combating *M. incognita* infestation in tomato crops. Nevertheless, their plant-growth-promoting and biocontrol potential need to be evaluated against *M. incognita* under field conditions using different application methods for developing a viable option for nematode management.

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