Research Article

Effect of Sole and Consortium Application of Endophytic Bacteria on Plant Growth Promotion and Inhibition of *Meloidogyne incognita* Infection in Okra

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Abstract | Plant-parasitic nematodes (PPNs) are a serious threat to food security. Root-knot nematodes (RKNs) are important plant parasitic nematodes that affect vegetable crops worldwide including okra. Among the RKNs, *Meloidogyne incognita* [(Kofold and White) Chitwood] is one of the major constraints to okra production. In this study, the effect of different bacterial strains i.e., *Bacillus* sp. MN54, *Enterobacter* sp. MN17 and *Burkholderia. phytofirmans* PsJN alone and in different combinations was assessed on plant growth promotion and inhibition of *M. incognita* infection on okra in a greenhouse experiment under completely randomized design (CRD). The results revealed that application of *Enterobacter* sp. MN17 significantly enhanced the root length (19.0), root weight (8.7), and shoot dry weight (19.6) as compared to other treatments. However, the combined treatment of *Bacillus* sp. MN54 + *Enterobacter* sp. MN17 + *B. phytofirmans* PsJN has successfully reduced the number of galls (10.5), number of females (23.2), egg masses (19.5), egg mass index (4.1), and galling index (2.1) against the RKNs. Conclusively, the combined application of all the bacterial strains was more effective in causing the suppression of RKNs and promotion of plant growth. This study illustrates the role of endophytic bacteria in controlling root knot nematode infection through the different changes in plants.

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Keywords | Okra, *Meloidogyne incognita*, Growth promotion, Root knot nematode, *Bacillus*, *Enterobacter*

Introduction

Okra (*Abelmoschus* and *esculentus*) belongs to the family *Malvaceae* is a cost-effective vegetable crop cultivated worldwide (András *et al.*, 2005; Saifullah and Rabbani, 2009). Unfortunately, okra is vulnerable to attack by several pathogens including plant-parasitic nematodes (Arain *et al.*, 2012; Iqbal *et*

al., 2012). PPNs are some of the most significant and numerous creatures in the animal kingdom, and they can live in any habitat (Aleuy and Kutz, 2020). PPNs that parasitize plants are one of the main sources of biotic stress in agricultural production (Miller *et al*., 2017). PPNs are so-called plant parasites as they feed on plant nutrients. They have a stylet, which is a needle-like shape that aids them in puncturing plant cell walls and obtaining the juicy substances on which they feed (Bernard *et al*., 2017). Plant-parasitic nematodes are a worldwide food security threat. Globally, plant-parasitic nematodes cause up to 5% losses (Poveda *et al*., 2020). Among the PPN nematodes, root-knot nematodes (RKNs) are the most dangerous and sedentary endoparasites with a wide host range (Mukhtar *et al*., 2017). Annual global yield losses of more than US$400 million were caused by PPN nematodes (Huang *et al*., 2014). Within the genus, *Meloidogyne incognita* is the most prodigious and devastating species causing yield losses in diverse crops and vegetables around the world (Sikandar *et al*., 2020). RKNs are widespread in Spain's cultivation fields (Archidona-Yuste *et al*., 2018), resulting in crop failures especially in important vegetable crops includes cucumber (85%), tomato (59%), watermelon (36%), and lettuce (29%) (Gullino *et al*., 2019). Similarly, RKNs are ubiquitously distributed in different types of soil and the infestation of RKN *Meloidogyne* species has been reported in 85% of okra fields across Punjab province of Pakistan's with an average incidence of 38% (Hussain *et al*., 2012).

Various management strategies such as crop rotation, resistant cultivars, and soil treatment in addition to the application of chemical nematicides have been commonly used to control RKNs (Collange *et al*., 2011; Ali *et al*., 2017). The management of nematode infection in plants demands the development of new, cost-effective, and eco-friendly solutions (Samada and Tambunan, 2020). Biological control with microbial antagonists is considered a safe and environment-friendly approach (Tariq *et al*., 2020). Among microbial antagonists, endophytic bacteria play an important role (Xiong *et al*., 2015; Tran *et al*., 2019) in suppressing RKN populations and improving plant growth (Vetrivelkalai *et al*., 2010). However, endophytic bacteria compete for nutrients and space and this interaction happens in the rhizosphere to facilitate the endophytic bacteria, which leads to the reduction in the RKN populations (Siddiqui and Shaukat, 2003). Endophytic bacteria improve the availability of nitrogen and phosphorus, eliminate the toxins, and improve resistance against the pathogens. Likewise, cyclic lipopeptides, lytic enzymes, and other secondary metabolites are produced by endophytic bacteria (Farzand *et al*., 2019, 2020), play an essential role in suppression of *Meloidogyne* sp. A few studies have reported the inhibitory effect of plant growth-promoting rhizobacteria (PGPR) on nematodes (López-Bucio *et al*., 2007). Similarly, in several crops, including tomato and brinjal, Pseudomonas fluorescens was found to be inhibitory against *M. incognita* (Anita and Rajendran, 2002) in chickpea (Khan *et al*., 2001), and turmeric (Srinivasan *et al*., 2001). Also, the impacts of inoculating two maize cultivars with the bacterial endophytes *Burkholderia phytofirmans* strain PsJN and *Enterobacter* sp. FD17 was studied on growth, hydration status, and photosynthetic activity under drought stress (Naveed *et al*., 2014). Further, PsJN is one of the well-studied bacterial endophytes, capable of establishing rhizosphere and endophytic populations in a wide range of plants. It has been observed colonizing in potatoes, tomatoes, peat moss, and grapevines (Naveed *et al*., 2014). Moreover, they boost plants to develop induced systemic resistance, which defends them from nematodes attack (Siddiqui and Mahmood, 1999). Many PGPR, including *Agrobacterium, Bacillus, Pseudomonas, Rhizobium*, and *Burkholderia*, have been demonstrated to reduce *M. incognita* by decreasing gall formation, regulating nematode reproduction, and hatching and killing juveniles by releasing toxins (Davies *et al*., 2001; Khanna *et al*., 2019). To gather information that may be beneficial in enhancing the current vegetable RKN control, it is important to identify RKN nematodes and management in the examined soil and cultivated Okra crop. Therefore, the present study was aimed to assess the inhibitory effect of three bacterial strains *Bacillus* sp. MN54, *Enterobacter* sp. MN17 and *B. phytofirmans* PsJN on *M. incognita* infection in okra. This outcome will help the development of effective and sustainable management strategies against RKNs.

Materials and Methods

Nematode inoculum

To prepare inoculum, *M. incognita* was multiplied on eggplant (*Solanum melongena*) in the greenhouse of Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan. Heavily infected eggplant roots were gently shaken to remove the

adhering soil and slightly washed under running water. The egg masses of *M. incognita* were picked by using surface sterilized tweezers and used for egg hatching to attain second-stage juveniles (J2s). The egg masses were surface sterilized with 1% NaOCl and rinsed with sterilized distilled water thrice and placed in Petri dishes containing 20 mL sterilized distilled water and incubated at 28 °C. J2s were collected after 24 hours for further experiments (Huang *et al*., 2014).

Bacterial cultures

Pre-isolated cultures of antagonistic bacteria i.e., *Bacillus* sp. MN54, *Enterobacter* sp. MN17 and *Burkholderia phytofirmans* PsJN were obtained from the Institute of Soil and Environmental Sciences UAF, Faisalabad, Pakistan. All culture were maintained and preserved at -80°C in Luria Bertani (LB) broth with 60% glycerol. Fresh bacterial cultures were retrieved time to time before conducting the experiment and grown on LB agar medium. Prior to conducting greenhouse experiments, endophytic bacterial strains were grown in nutrient broth and incubated at 28°C for 48 h. The density of bacterial cultures was adjusted to 10^7 CFU/mL to prepare inoculum of the endophytic strains.

Greenhouse experiment

The greenhouse experiment conducted at the Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan followed a completely randomized design (CRD). Seeds of okra cv. Sabzpari, were obtained from the Vegetable Research Institute, Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan. Seeds were surface sterilized with 5% NaOCl and subsequently washed thrice with sterilized distilled water. The surface sterilized seeds were sown in earthen pots (9 × 9 × 10 cm) containing sterilized soil (Arshad *et al*., 2022). At four leaf stage, the plants were treated with bacterial strains by applying 10 mL of culture (10^7 CFU/mL) to each pot by soil drenching method according to Arshad *et al.* (2022). Endophytic bacterial strains were applied as water suspensions as stand-alone treatments and in different combinations. Control plants were treated with sterilized water only. Each treatment was replicated with 10 biological replicates of the experimental units under same conditions. The pots were kept in the greenhouse at 28°C with 8 h dark and 16 h daylight. Twenty-four hours after applying the bacterial cultures, the plants were inoculated with 1000 J2's of *M. incognita* in the soil. After two months of inoculation, okra plants were uprooted and rinsed with running water.

Counting of galls, females, and egg masses on okra roots

For the assessment of root galling a 0-5 scale given by Taylor *et al.* (1985) was used, where 0 = no gall, 1 = 1-2 galls, 2 = 3-10 galls, 3 = 11-30 galls, 4 = 31 galls, 5 = more than 31 galls. To count the number of females and egg masses of *M. incognita* infected roots were dipped in sterilized distilled water to wash the debris and soil. After rinsing with water, the roots were excised and dipped in (15 mg/L) Phloxine B staining solution for 15 to 20 minutes to count the number of egg masses (Sasser *et al*., 1984). The gelatinous matrix of egg masses on some parts of the roots which were stained while other parts remained unstained. The extra stain was removed by dipping roots in 1L distilled water consistently three times in a clean beaker. To count egg masses and pear-shaped females a dissecting microscope (Nikon Instruments Inc., Tokyo, Japan) was used.

Endophytic bacterial impacts on growth parameters of okra plants

To assess the effect of endophytic strains on plant growth parameters, plant height (cm) was measured, number of fruits per plant were counted, followed by measuring the fruit weight (g) after every week and sum up at the end, chlorophyl contents (SPAD) were measured with the help of SPAD (Soil plant analysis development) meter, for dry shoot weight (g), the shoots were dried in an oven and weighed, root fresh weight (g), root dry weight (g), dry shoot weight (g) were taken as mentioned above, fruit length (cm), number of seeds per fruit were recorded. All this data was taken after sixty days post-inoculation.

Statistical analysis

The data were subjected to the analysis of variance (ANOVA) under completely randomized design (CRD) using statistical package Statistix v. 8.1. The treatment means were compared using least significant difference test (LSD) with a 95% level of confidence.

Results and Discussion

Effect of bacterial strains on growth parameters of okra plants

The results revealed that the tested bacterial strains showed a significant growth promoting

effect on okra plants (Figure 1). Under greenhouse conditions, *Bacillus* sp. MN54 as single application or in combination with other strains improved the growth parameters of okra plants. The means of the fruit weight (FW) (18.0g), seed per fruit (SPF) (35.2g) of okra plants were maximum in *Bacillus* sp. MN54 as compared to positive (10.5g) and negative control (9.2g), respectively. *Enterobacter* sp. MN17 significantly enhanced the root length (19.0cm) of okra plants, followed by root weight (RW), shoot dry weight (SDW) with the values of 8.7g, 19.6g, respectively. Similarly, the application of *Bacillus* sp. MN54 + *B. phytofirmans* PsJN significantly increased the average plant height (PH) (15.3cm), followed by *Enterobacter* sp. MN17 and *Enterobacter* sp. MN17 + *B. phytofirmans* PsJN, respectively. Findings showed that root dry weight (RDW) was maximum with mean of (1.7g) by the application of *Enterobacter* sp. MN17 + B. *phytofirmans* PsJN. The combination of *Bacillus* sp. MN54 + *Enterobacter* sp. MN17 + *B. phytofirmans* PsJN significantly increased the shoot weight (SW) with a value of (13.2g) in addition to the highest value of chlorophyll contents (48.2).

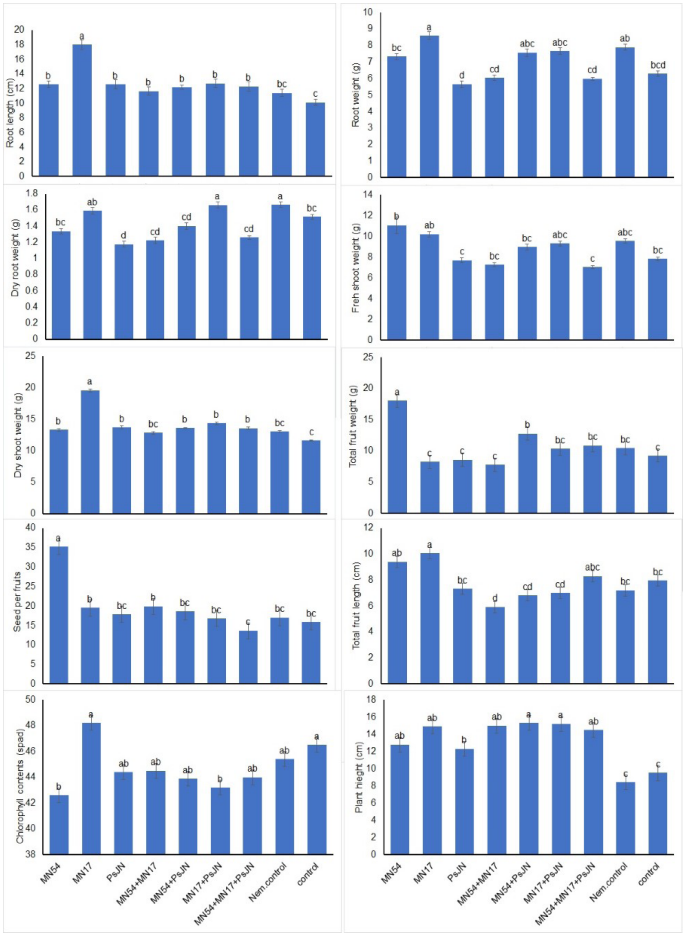

Figure 1: *Graphical representation using different treatments for growth parameters in Okra.*

Effect of bacterial strains on M. incognita infection and reproductive parameters

It was observed that endophytic strains as single treatments and their combination caused a significant reduction in number of galls, number of females, egg masses (EM), egg mass index (EMI), and galling index (GI). The treatment with consortium application of *Bacillus* sp. MN54 + *Enterobacter* sp. MN17 + *B. phytofirmans* PsJN showed the highest reduction in number of galls (10.5), number of females (23.2), egg masses (19.5), egg mass index (4.1), and galling index (2.1) (Figure 2). *B. phytofirmans* PsJN significantly decreased the number of galls in sole application. The combination of *Bacillus* sp. MN54+ *Enterobacter* sp. MN17 also reduced the number of egg masses with the mean of (22.1). The application of *Enterobacter* sp. MN17 + *B. phytofirmans* PsJN reduced the number of females in the range of (27.5) as compared to only nematode control. The outcomes revealed that strains in combination had more inhibitory effect on *M. incognita* infection and reproductive parameters. The untreated negative control showed the highest number of galls (26.2), number of females (68.0), egg masses (66.9), egg mass index (7.1), and galling index (4.2), respectively.

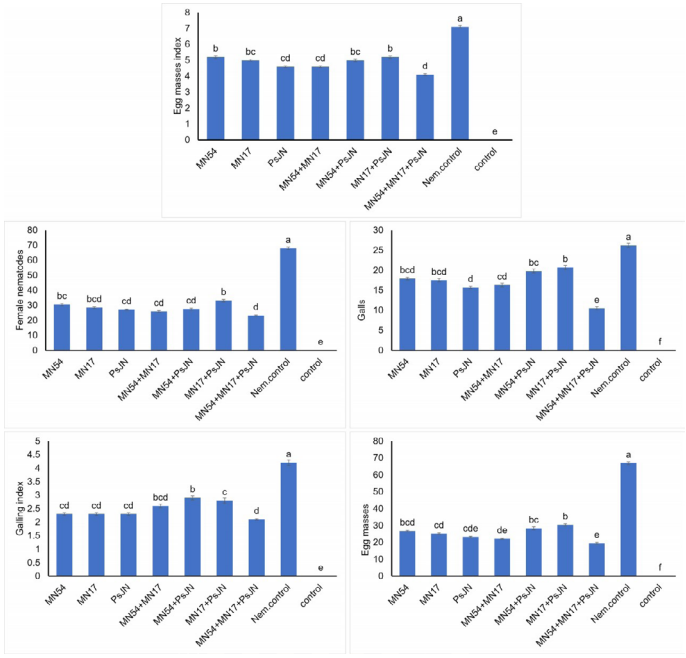

Figure 2: *Graphical representation using different treatments for M. incognita infection and reproductive parameters.*

Effects of endophytic strains (Bacillus sp. MN54, Enterobacter sp. MN17 and B. phytofirmans PsJN) on okra roots in response to M. incognita infection

Photographs showing that, combined application

of *Bacillus* sp. MN54 + *Enterobacter* sp. MN17 + *B. phytofirmans* PsJN observed the highest reduction in number of galls, number of females, egg masses, egg mass index, and galling index (Figure 3).

Figure 3: *Pictorial presentation of nematode galling in response to M. incognita infection in okra roots under different treatments (Bacillus sp. MN54, Enterobacter sp. MN17 and B. phytofirmans PsJN).*

PCA based correlation between the parameters under different endophytic strains treatments

The principal component analysis (PCA) showed the correlation between the plant growth and nematode reproductive parameters in Okra under different endophytic treatments (Figures 4 and 5). Results revealed that all the growth parameters were positively correlated except chlorophyll contents while root length and dry shoot weight were closely correlated to each other (Figure 4).

For nematode reproductive parameters, it was assessed that all the reproductive parameters of nematodes were positively correlated with each other under all endophytic different treatments (*Bacillus* sp. MN54, *Enterobacter* sp. MN17 and *B. phytofirmans* PsJN) (Figure 5). While no. of eggs was weakly correlated to other parameters like no. of females, and no. egg masses (Figure 5).

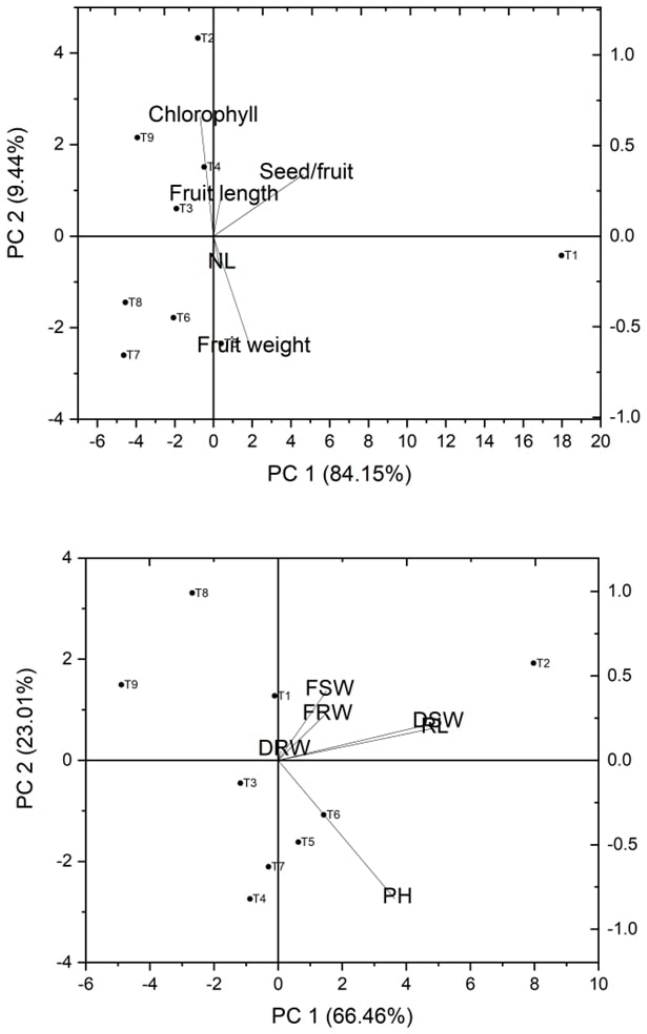

Figure 4: *Graphic illustration of PCA analysis among the endophytic treatments and growth parameters in Okra.*

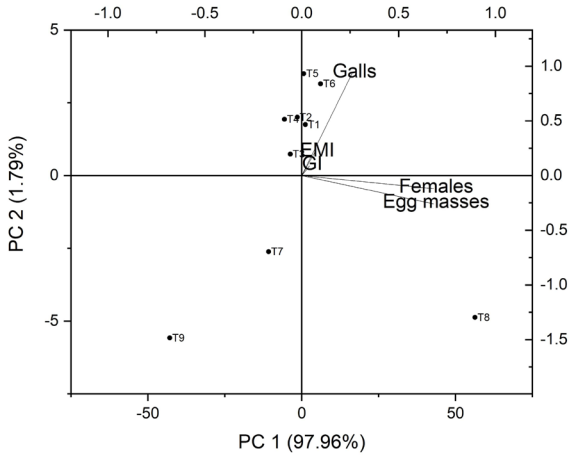

Figure 5: *Graphic illustration of PCA analysis among the endophytic treatments and nematode reproductive growth parameters in Okra.*

Plant-parasitic nematodes are a global threat to agricultural production. *M. incognita*, is one of the most dangerous soil-borne pathogens that damages a variety of crop plants (Kavitha *et al.*, 2012). The use of biological control agents is a safe strategy to suppress soil-borne plant pathogens. Several bacterial isolates, including *Bacillus* sp., *Pseudomonas* sp. and *Pasteuria* sp., have shown nematocidal effect against root knot nematodes (Chinheya *et al.*, 2017). Endophytic microorganisms (i.e., *Bacillus* sp.) have been used against *M. incognita* for decades. Some reports have indicated that bacterial endophytes promote the growth and yield of crop plants (Sturz *et al.*, 2000). Similarly, *Bacillus* spp. have remarkable bio-nematicide activity against root knot disease in tomato crop. However, in the present study, nematicidal potential of different endophytic bacterial strains against *M. incognita* was evaluated, which may open new areas for studies on bacterial biocontrol agents against *M. incognita*. We have used Bacillus sp. MN54 successfully to manage root knot nematodes in tomato and wheat (Arshad *et al.*, 2021, 2022). Similarly, this strain was used to suppress leaf rust of wheat (Din *et al.*, 2018).

In the current research, results revealed that the highest fruit weight (FW), seed per fruit (SPF), and number of leaves (NOL) were by *Bacillus* sp. MN54. Likewise, *Bacillus* sp. MN54 shows significant increase in fresh shoot weight, fruit weight, seed per fruit and no. of leaves. The endophytic bacteria may promote plant growth and suppress plant diseases probably by means like PGPR (Feng *et al.*, 2006). Similarly, Hallmann *et al.* (1998) used endophytic bacteria for the management of *M. incognita* in cucumber and cotton roots and observed a fewer number of galls on both crops as compared to the infected control. The inhibition of egg hatching and increased juvenile mortality of *M. javanica* was observed after treatment with *Bacillus* sp. Our study reveals that *Bacillus* sp. are promising antagonistic agents against RKNs. Application of endophytic bacterial strains in combination is a new strategy in the development of biocontrol agents against plant parasitic nematodes. Biological control agents are extensive colonizers of the plant rhizosphere, they suppress diseases through a variety of mechanisms, including antibiosis, competition, myco-parasitism, and degradation of cell wall, induce resistance, and promote plant growth (Junaid *et al.*, 2013). Therefore, the use of bacterial endophytes in controlling RKN is a promising approach. In our work the combined application of *Enterobacter* sp. MN17 and *B. phytofirmans* PsJN showed enhanced dry root weight and plant height. The combine application of *Bacillus* sp. MN54, *Enterobacter* sp. MN17 and *B. phytofirmans* PsJN caused a significant suppression of no. of egg masses, females, galls and galling, egg masses index. The combined treatment of *Bacillus* sp. MN54 + *Enterobacter* sp. MN17 + *B. phytofirmans* PsJN showed the highest reduction in number of galls (10.5), number of females (23.2), egg masses (19.5), egg mass index (4.1), and galling index (2.1). In a previous study, *Bacillus alvei* strains displayed antagonistic activity against nematode eggs and the antagonistic ability was attributed to the production of hydrolytic enzymes. Our findings suggest that using *Bacillus* sp. in a combination was more effective in causing the suppression of RKNs. In line with our findings, Choudhary and Johri (2009) reported that root-knot nematodes are significantly reduced by *B. subtilis*, *B. pasteurii, B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus*. It is suggested that the tested antagonistic bacteria are promising candidates for biological control of *M. incognita.* The use of antagonistic bacterial strains in combination may have a great opportunity to find potential application in the biological control of *M. incognita*.

Conclusions and Recommendations

In conclusion, the administration of all the bacterial strains simultaneously was more successful in suppressing RKNs and promoting plant growth. The use of mixture combination of *Bacillus* sp. was more efficient in suppressing RKNs. The strong nematocidal potential and growth promoting effect of the tested bacterial strains against *M. incognita* may open new areas for research on biocontrol agents against plant parasitic nematodes. This biocontrol strategy may make it possible to reduce the use of chemical nematicides for farmers and contribute to the development of sustainable farming practices.

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Novelty Statement

The study highlights the important role of endophytic

bacteria to reduce the invasion of root knot nematodes in okra. These bacteria and similar BCAs are regarded as the eco-friendly approach for nematode management. These biocontrol agents (BCAs) have dual role in the growth promotion of the host plants as well as suppression of RKNs. In this study, we have used a combination of different endophytic bacteria which reduced the nematode infection significantly as compared to the sole application. This is tangible research that could be translated as a way forward for organic vegetable production in the peri-urban areas.

Author's Contribution

HA and MJ: Collected the samples and conducted the experiment.
AM and UA: Did PCA analysis and prepared graphs.
AH and AJ: Contributed to statistical analysis.
MB: Provided literature and participated in review process.
MN: Review the paper and provided the bacterial strains.
MAA and AA: Contributed to the research and prepared manuscript and reviewed the submission.

Conflict of interest
The authors have declared no conflict of interest.

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