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Inoculation of Fava Bean Plants (*Vicia faba* L.) with a PGPR Combination Suppresses the Impact of Root-Knot Nematode and Promotes Plant Growth

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ABSTRACT

Background: Plant-growth-promoting rhizobacteria (PGPR) are a class of beneficial bacteria that colonise the roots of plants and improve projected plant growth through a multivariate process. PGPR application is increasing steadily in the field of agriculture, providing an attractive mechanism to replace pesticides, chemical fertilisers and additional supplements.

Methods: In this study, PGPR from rhizospheric soil collected from Abha, a city located in the southern region of the Kingdom of Saudi Arabia (KSA), were isolated and characterised to facilitate verifying the efficacy of using PGPR as a biological control to improve fava bean growth. Four bacterial isolates from the nodules of fava bean plants, S4, S5, S6 and S17, were isolated and characterised. Consequently, an investigation highlighting the impact of PGPR isolates on the preservation of fava bean plants from the root-knot nematode was conducted through a pot culture experiment. Soil infested with the nematode was added to the pots. Two commercial types of fava bean plants (*Vicia faba* L.) were co-inoculated with PGPR 15 days after planting.

Result: The results reveal that the nodule-forming bacteria interact synergistically; this was evidenced by a prominent increase in the shoot length and dry weight of the fava bean plants that had been cultivated in the nematode-infested soil. The results also demonstrate that the overall treatment of the plants did not lead to nodule formation. The results indicate that nodule-forming bacteria could be utilised in leguminous crops to facilitate biocontrol of the root-knot nematode.

Key words: Biocontrol, Fava bean, PGPR, Root-knot nematode.

INTRODUCTION

Fava beans (*Vicia faba* L.) are essential grain legumes that are cultivated on a large scale in various countries for feed and food purposes (Sillero *et al.,* 2010). The chief benefit of cultivating this crop is agricultural diversification, diminishing disease, pest and weed build-up and thereby ameliorating biodiversity in the area. Fava bean production also reduces fossil fuel consumption by generating food that is rich in protein and nutrients and improving the supply of biologically fixed nitrogen (N) to the agricultural system (Erik *et al.,* 2010). The cultivation of *Vicia faba* also enhances soil nitrogen content through symbiotic rhizome association (Bhardwaj *et al*., 2022).

Meloidogyne spp., or the root-knot nematode, is a plant-parasitic nematode that attacks multiple crops. Root-knot nematode species predominantly limit the leguminous yield of crops grown in infested soils (Montasser *et al.,* 2017). *M. incognita*, an economically damaging species of root-knot nematode, significantly limits plant growth and, therefore, yield, leading to an annual loss of approximately $100 billion (Mukhtar *et al.,* 2014). Root-knot nematodes are sedentary endoparasites that infect the root walls of plants (Bird, 1974), impairing the root system's ability to absorb water and minerals (Ghosh *et al.,* 2024). As the roots of leguminous plants are attacked by root-knot nematodes, rhizobial nodule growth is also affected (Taha, 1993). Hence, the plant's growth is affected both directly and indirectly by root nodule suppression.

The rhizosphere is comparatively richer in nutrients than other parts of the soil because 40 per cent of the total plant photosynthesis is lost from the plant (Nihorimbere *et al.,* 2011). The microorganisms in the rhizosphere use the materials and compounds excreted from crop roots for

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nutrition. Consequently, the rhizosphere sustains a large and active population of microbes that can have neutral, beneficial, or detrimental impacts on plant growth (Ali *et al.,* 2017). Plant-growth-promoting rhizobacteria (PGPR) are soil bacteria living in the rhizosphere that are involved in promoting plant growth and development, which is even more evident when plants are grown in challenging environmental conditions (Vocciante *et al.,* 2022). The beneficial effects of rhizobacteria are primarily their direct promotion of nutrient absorption through symbiotic nitrogen fixation and the solubilisation of inorganic phosphate, the synthesis of phytohormones (auxins, cytokinins and gibberellins) and siderophore production. Indirectly, rhizospheric bacteria inhibit or mitigate the negative effects of pathogenic organisms and play a major role in the biocontrol of plant pathogens. These indirect mechanisms include the production of antibiotics and enzymes that degrade the cell walls of pathogens, competition for nutrients and root colonisation (Vocciante *et al.,* 2022).

There has been an increased focus on PGPR recently due to its promotion of plant growth in various crops and its potential as a biological control agent (Sunkad *et al.,* 2023). However, there is a dearth of literature that discusses the utilisation of PGPR as a biocontrol agent in fava bean crops. This study, therefore, aims to determine which PGPR strains are most compatible with the fava bean crops found in the Kingdom of Saudi Arabia (KSA) and investigate the impact of the co-inoculation of fava bean plants with nodule-forming bacteria to suppress root-knot nematodes.

MATERIALS AND METHODS

Soil sampling, culture conditions and isolation of bacterial strains

A soil sample (500 g) was collected from a square area measuring 50 cm in length and width, with a depth ranging from 0 to 20 cm, in Abha, a city located in the southern region of the KSA. The following measurements were taken from the soil sample: pH (8.02), calcium ion content (1892.38 mg/Kg), K_2O content (155.35 mg/Kg), P_2O_5 content (29.60 mg/Kg), sodium ion content (96.45 mg/Kg), total nitrogen content (0.74 mg/Kg), magnesium content (122.71 mg/Kg), soil conductivity (0.27 mS/cm), C/N ratio (6.43) and organic particle content (0.81%). Surface-sterilised seeds of *Vicia faba* L. ('Bachar') were pre-germinated in agar media (7 g/l) and then each was planted in a single 300-ml pot filled with soil from the sample. The plants were grown under ambient conditions and watered when necessary. Eight weeks later, the roots were inspected for the presence of nodules. Bacteria were isolated from the nodules according to the procedure described by Vincent (1970). One isolate was obtained from each nodule. Yeast extract mannitol agar (YEMA) media was used to isolate, cultivate and purify these bacteria (Vincent, 1970). All isolates were stored in tubes at 4°C on YEMA medium (Vincent, 1970). Detailed information concerning the isolates is listed in Table 1.

Physiological testing

The tests were performed in triplicate on 24×24 cm Petri dishes prepared with YEMA plates, which were divided into 4 squares of equal size. Ten μL of the strain culture that had previously been grown on the YEMA liquid medium, with an exponential value of $\pm 10^9$ cells/mL, were spot-inoculated in each square. Each of the inoculated Petri dishes was incubated at 28°C for a total of 7 days, after which colonial growth was visually monitored.

Tolerance of salt

Salt (NaCl) tolerance was evaluated by determining bacterial growth rates in solid YEMA medium that had been supplemented with 3, 6, 9 and 12 g/L NaCl after the 7-day incubation period at 28°C. Bacteria whose growth was inhibited in these media were considered sensitive to salt.

Tolerance of pH

Acid and alkaline tolerance was assessed with the use of YEMA medium in which the pH was adjusted to 5, 6, 7, 8, or 9 by the addition of NaOH or HCl. The control culture was maintained at pH 7. The tolerance of the bacteria to these conditions was noted.

Impact of the strains on phosphate availability

Inorganic phosphate solubilisation was performed by determining the growth rates of multiple isolated strains in Pikovskaya medium (PVK) containing insoluble di-calcium phosphate precipitates as the chief source of phosphate for growth. The PVK medium was composed of 0.2 g NaCl, 10 g glucose, 0.295 KCl, 0.373 g NH_4NO_3, 0.41 g $MgSO_4$, $7H_2O$, 0.003 g $FeCl_3$, 0.7 g $CaHPO_4$, 20 g of agar and 3 g of extracted yeast in 1000 ml of distilled water (DW) (Pikovskaya, 1948). The bacterial cultures were streaked on the surface of the replicated box of agar. After the inoculation phase, the clear zone that was present around the bacterial colonies acted as the indicator for the solubilisation of the phosphate. Differences within the colonies were recognised and sub-cultured based on the halo zones. As plate assay is not regarded as a standard method for determining whether a strain is a phosphate solubiliser (Johri *et al*., 1999), pure cultures were further investigated using a liquid medium of $Ca_2(PO_4)$. The supernatant cultures were filtrated using a Millipore filter (Sartorius). The pH and phosphate content of the culture filtrate was set as described by Murphy and Riley (1962). The principle of phosphate solubilisation in a liquid medium is the reduction of the phosphomolybdate complex by ascorbic acid with an antimony catalyst, resulting in an intense blue colour that indicates the presence of soluble phosphorus in the medium. Bacteria that reduced the pH of the medium and generated a blue colouration were noted as phosphate-solubilising bacteria.

Culture conditions and plant material

The four bacterial isolates were tested to determine the extent of their ability to suppress the root-knot nematode

as well as promote fava bean plant growth. To do so, every isolate was grown in YEMA medium until a concentration of 10^9 cells/mL was achieved before inoculation of aseptically-germinated *Vicia faba* 'Bachar' and commercial seeds. Pre-germinated fava bean seeds were sown in pots filled with sample soil infested with the nematode. At the four-leaf stage, the seedlings were inoculated with nodule-forming bacteria. Each seedling received 20 ml of the liquid bacterial suspension. The inoculum was injected at a depth of 2 cm within the rhizosphere through three holes that were made around the base of each stem using a plastic rod. Four treatments were performed in the experiment: 1) only S6, 2) S6 + S4, 3) S6 + S5 and 4) S6 + S17. The plants were grown under favourable conditions and were watered as needed. The total number of leaves, total dry weight, number of root knots and shoot and root length of the plants were visually monitored for 45 days after the plants were transferred into the pots. The roots and shoots were then dehydrated for 3 days at 70°C.

Data analysis

Statistical analysis of the fava beans' biological parameters was conducted using a completely randomised experimental design with four replications. A one-way analysis of variance (ANOVA) test was performed using SPSS software. Duncan's multiple range test with a 5% significance level, along with the mean standard deviation, was used for data analysis. Only two outputs were obtained from the tests of the physiological parameters of the bacteria: positive (+) or negative (-) behaviour.

RESULTS AND DISCUSSION

The plant-growth-promoting activity of the rhizospheric bacteria was evaluated on aseptic media and by the inoculation of fava bean plants growing in pots filled with root-knot nematode-infested soil. Then, the isolates' performance was analysed by assessing their tolerance to different pH and salinity levels, their capacity to solubilise phosphorus and their beneficial effect on the dry weight and root and shoot length of the inoculated plants. Isolates that significantly increased the desired physiological characteristics of fava bean plants (p<0.05) were regarded as potentially useful bacteria.

pH and salinity tolerance

The pH tolerance was negative at a pH of 5 for all the isolates, while positive tolerance was detected at pH values of 6-9 for S4, S5, S6 and S17 (Table 1). The S^4 bacterial isolate from Abha city had no tolerance to any concentration of NaCl. On the other hand, the S5 isolate tolerated 3-9 g/L of NaCl. In addition, S6 tolerated all concentrations (3-12 g/L NaCl), while the S17 isolate tolerated NaCl concentrations of 3-6 g/L NaCl but not NaCl concentrations of 9-12 g/L.

The physiological characteristics of the four fava bean isolates indicate that all strains grew in the control culture (pH 7.0, 0.1% NaCl and 28°C) (Table 1). These physiological characteristics led to increased diversity among the nodule-forming bacteria. Most of the isolates demonstrated growth at pH levels of 5-9 and tolerated NaCl concentrations of 3-9 g/L.

Phosphate solubilisation ability

All four strains demonstrated a high potential for phosphate solubilisation. The four rhizobacteria produced clear zones around the colonies after six days of growth on the PVK plates. Moreover, they produced a blue colouration and reduced the pH of the inoculated liquid medium (Table 1).

Table 1: Habitats, accession numbers and physiological properties of the used microbial species.

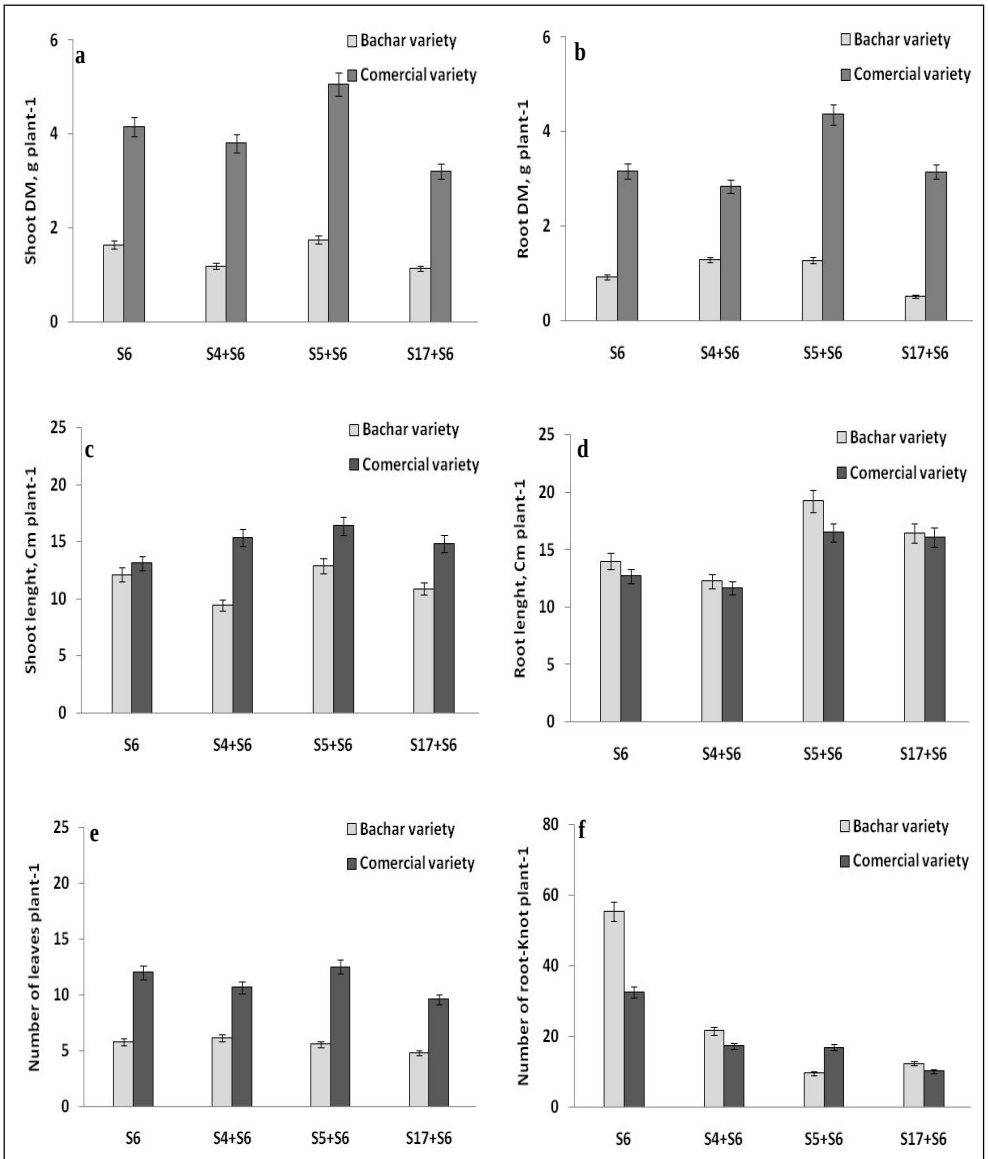
Isolate references	S4	S5	S6	S17
Habitat	Abha city Saudi arabia	Abha city Saudi arabia	Abha city Saudi arabia	Jendouba city Tunisia
pH tolerance				
5	-	-	-	-
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
9	+	+	+	+
NaCl tolerance				
3 g L-1	-	+	+	+
6 g L-1	-	+	+	+
9 g L-1	-	+	+	-
12 g L-1	-	-	+	-
Phosphate solubilisation on PVK medium after 6 days				
Halo zone *	+	+	+	+
Blue color **	+	+	+	+
pH decrease **	+	+	+	+

(-) negatie response, (+) positive response, * : Solid PVK medium, **: Liquid PVK medium.

All of these traits are indicative of dicalcium phosphate solubilisation.

Effect of co-inoculation with PGPR on fava bean growth and root knot suppression

Only S6 permitted the plants cultivated in the nematode-infested soil to develop a high number of root knots. Any combination of nodule-forming bacteria tended to reduce the number of root knots (Fig 1, 2). However, the S6 + S5 combination produced a pronounced increase in the shoot and root length of the fava bean plants, along with an increase in their dry weight. Duncan's multiple range test, along with the mean standard deviation, was used to analyse the data. This analysis showed that all results were statistically significant (p = 0.05). The plants that were exposed to the S6 + S5 combination exhibited a pronounced increase in the dry mass of plant shoots (Fig 1a). Similarly, the dry mass of plant roots was notably greater in the plants exposed to the S6 + S5 combination of isolates (Fig 1b). The isolate combinations S6 + S4, S6 + S17 and S6 + S5 also increased shoot length; the greatest increase in root length was recorded in plants exposed to the S6 + S5 isolate combination (Fig 1c, 1d). The number of leaves also increased the most in plants exposed to the S6 + S5 combination, with a significant increase in plants exposed to the S6, S6 + S4 and S6 + S17 isolates (Fig 1e). Lastly,



a) shoot dry weight, b) root dry weight, c) shoot length, d) root length, e) number of leaves, f) number of root-knot. All the data were significantly different (α=0.05) according to Duncan's multiple range test; bars mean standard deviation.

Fig 1: Effect of Co-inoculation with PGPR on faba bean (*Vicia faba L.*) plant after 45 days.

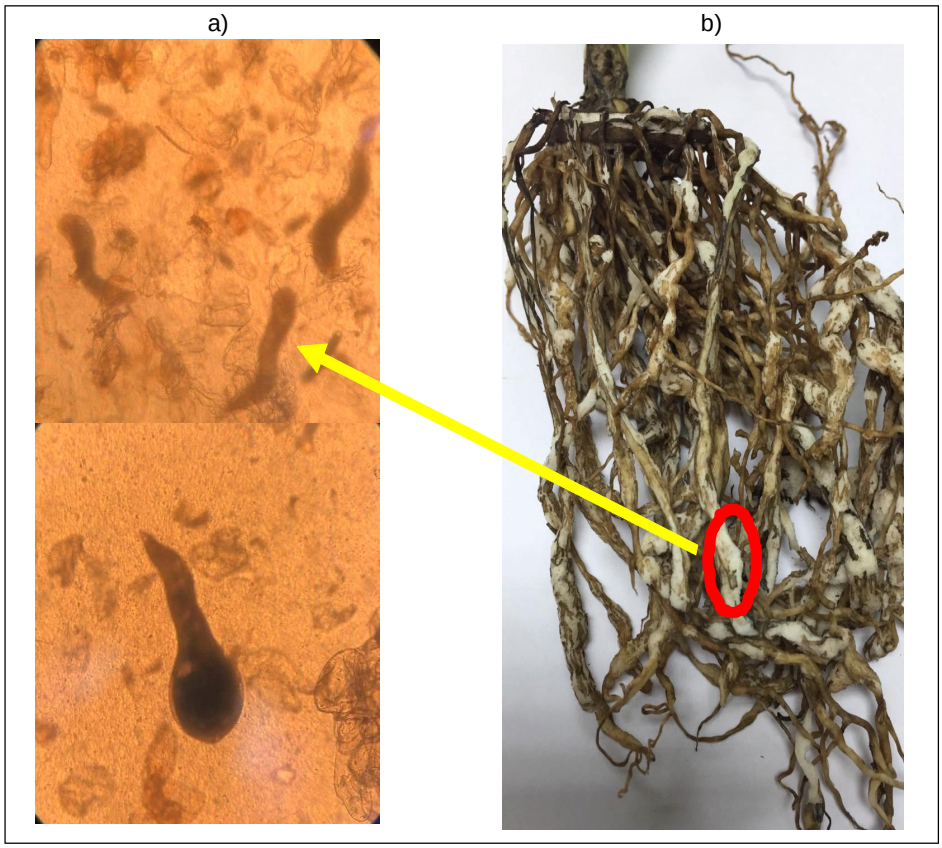


Fig 2: (a) microscopic image of the nematode inside of root-knot and (b) Image showed the formation of root-knot on the root of the plant cultivated in the soil infested with nematode.

the number of root knots in the plants was greater in those exposed to the S6 isolate than in those exposed to the other bacterial isolates (Fig 1f). No nodules were formed in any of the treated plants. No pronounced differences between any of the treated leaves were noted in the results (Fig 1).

This study has verified the efficacy of PGPR for the biocontrol of the root-knot nematode in fava bean crops, with a proposed hypothesis that the amalgamated use of the nodule-forming bacteria might offer improved protection against the pathogen.

The bacterial strains identified were able to solubilise inorganic phosphate, which enhanced the growth of the fava bean plants; several previous investigations have reported nodule-forming bacteria as among the most important phosphate-solubilising bacteria (L'taief *et al.,* 2019). The results of this study reveal that the four strains could also grow at high pH levels and high salinities. The nodule-forming bacteria were more tolerant of high salinity levels, consistent with L'taief *et al.* (2019) findings.

PGPR colonises plant roots; in addition, the effect of PGPR on pathogens is plant-mediated and, therefore, can be observed throughout the plant. Our findings show that the rhizobial bacteria responsible for nodule formation interact synergistically. A substantial increase in the root and shoot length of the fava bean plants, as well as their dry weight, was recorded. The enhanced growth of the plants may be due to PGPR's suppressing effect on both parasitic and non-parasitic root pathogens (Resmi *et al.,* 2024), which is caused by the synthesis of biologically active substances (Gamliel and Katan 1993). In addition, PGPR may directly affect plant growth by converting inaccessible organic compounds and minerals into forms that are readily available to plants (Siddiqui and Mahmood, 1999).

In this study, co-inoculation with phosphate-solubilising strains of PGPR reduced the number of root knots in fava bean varieties grown in soil infested with root-knot nematodes to a greater extent than simple inoculation did. This finding suggests that PGPR induce increased plant resistance to the root-knot pathogen. PGPR can act directly on the root-knot nematodes by limiting iron availability through the production of siderophore and can also kill the plant pathogen by synthesising antibiotics (2,4-diacetylphloroglucinol) and other molecular metabolites such as phytohormones (IAA), hydrogen cyanide (HCN) and biosurfactants.

Namita Bhutani *et al.* (2018) have asserted that nodule-forming bacteria (*Rhizobium*) help to fix atmospheric nitrogen and produce many toxic metabolites that inhibit several plant pathogens. In addition, Barker and Huisingh (1970) have found that necrosis in nodular tissues is

followed by the nematode invasion that reduces root nodule development. Chakraborty and Purkayastha (1984) found that rhizobitoxine is secreted by *Rhizobium,* whereas Chakraborty and Chakraborty (1989) have observed that *Rhizobium leguminosarum* produces high levels of phytoalexin (4-hydroxy-2, 3, 9, trimethoxypterocarpan) in pea plants. In a study conducted by Harshitha and Goudar (2021), rhizobia were found to produce antibiotic bacteriocin. The available evidence suggests that rhizobia are responsible for increasing nitrogen content and improving plant growth and can control the multiplication of nematodes (Siddiqui and Mahmood 1995). Notably, rhizobia use several mechanisms to suppress root-knot nematodes. Siddiqui (2006) has also mentioned that the combined use of these microorganisms does not necessarily lead to them harming each other.

Generally, a single biocontrol agent is utilised to biocontrol a single pathogen in diseased plants (Wilson and Backman, 1999). This sometimes leads to inconsistent plant performance, as a single agent may not be active against all the pathogens attacking the host plant, given the different soil environments and conditions that may apply. Alternatively, a combination of biocontrol agents with different colonisation patterns could be beneficial for the biocontrol of multiple plant pathogens through different disease-suppression mechanisms (Akhter and Siddiqui, 2008). Furthermore, the amalgamation of biocontrol agents demands optimal temperature, moisture and pH conditions to allow aggressive colonisation of the roots while improving plant growth and biocontrol efficacy (Siddiqui, 2006). Dual inoculation with different biocontrol agents with diverse mechanisms of action can offer a higher level of biocontrol against plant pathogens in multiple crops than inoculation with a single agent (Guetsky *et al*., 2002).

The plants in this study did not form root nodules under any of the treatments. Ali *et al.* (2010) have suggested that the suppression of nodulation that they observed in their study may have reflected the impact of *Meloidogyne incognita* inhibiting the development and formation of nodules, or may have been due to the premature conversion of functional nodules into non-functional nodules. Moreover, the damage that nematode larvae cause to root hairs is also likely to impact nodule formation (Khan *et al.,* 2002). The endoparasitic nematode *Meloidogyne,* as well as its larvae, may penetrate plants' stele tissue (Eisenback, 1985). Therefore, in cases of nodule invasion, root-knot nematodes may damage the vascular bundles and cortices of nodules, as well as the bacteroid zone (Taha and Kassab, 1979). A prominent decline in the bacteroid population, as well as the Lb nodule contents, suggests that nematodes' invasion of the nodule and feeding could impact the multiplication of the rhizobia, thereby affecting the normal development of nodules (Khan *et al*., 2016). The pathogenesis of the nematode on the nodule could also lead to an increase in O_2 concentration in the bacteroid zone, further aggravating nodule dysfunctionality (Khan *et al*., 2016). The larvae of nematodes attack young lateral roots and their hairs, limiting the specific root mass available for invasion by rhizobia. As a result of this damage, root nodulation was not found in the fava bean plants infested by the root-knot nematode.

CONCLUSION

This research has revealed that the treatment of fava bean plants with nodule-forming bacteria predominantly enhances the dry weight and the root and shoot length of the plants. These findings indicate that combinations of nodule-forming bacteria could be utilised in leguminous crops for the biocontrol of root-knot nematodes. Further research in similar field conditions is required to confirm our findings.

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Conflict of interest

All authors declare that they have no conflict of interest.

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