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Research Article

Effect of Some *Rhizobium* Strains on Fenugreek Growth and Biological Control of Sclerotinia Stem Rot of Fenugreek Caused by *Sclerotinia trifoliorum*

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Abstract

Fenugreek is an annual leguminous crop grown for hay and grains in Tunisia. It is also considered as a valuable rotation crop with cereals. Sclerotinia rot was observed in production fields since 2010. The aim of this work is to study the antagonistic activity of different *Rhizobium* strains against *Sclerotinia trifoliorum* in dual culture *in vitro* and under greenhouse conditions. Among the 32 strains tested, 26 isolates had effective control on *Sclerotinia trifoliorum in vitro*. In order to study the biological control mechanisms, the Rhizobium strains ability to produce volatile compounds and to solubilize phosphate were investigated. The results showed that 18 strains were able to solubilize phosphorus and 19 strains produced volatile compounds. In pot trials, the percentage of fenugreek plants inoculated with different rhizobia showed significant reduce sclerotinia stem rot symptoms compared to the uninoculated plants. Among these rhizobiums, the strain Soli proved efficient against the pathogen in vitro and in pot experiments. The results promise the use of rhizobia for protection of fenugreek against *Sclerotinia trifoliorum*.

Keywords: Antagonism; Biocontrol; Rhizobium; Sclerotinia trifoliorum; Trigonella foenum graecum

Introduction

Fenugreek (*Trigonella foenum graecum*) is an annual plant belongs to the family *Leguminosae*. It is the famous spices in human food. The seeds and green leaves of fenugreek are used in food as well as in medicinal application that is the old practice of human history.

An increasing interest is given to this crop in Tunisia. The fenugreek is grown in rotation with cereals and used mainly for hay and grains. Many investigations have demonstrated that fenugreek seeds of Tunisian cultivars are a rich source of protein, fat, fiber and carbohydrates and suggested that it should be considered in the food and feed consumption [1,2]. Other research has indicated the medicinal benefits of this legume [3,4]. Haouala., *et al.* [5] and Omezzine., *et al.* [6] have reported the antifungal, insecticidal and allelopathic potential of fenugreek extracts from aerial parts of a Tunisian cultivar.

Fenugrec is subjected to attack by number of diseases. Among these diseases damping-off, root-rot caused by *Rhizoctonia solani* Kuhan, *Fusarium solani* Mart and *Macrophomia phaseolina* [7]. Recently, Gargouri., *et al.* [8] have reported the occurrence of sclerotinia stem rot of fenugreek caused by *Sclerotinia trifoliorum* and *S. sclerotiorum* in Tunisia.

Management of these diseases is achieved mainly by the use of resistant cultivars and fungicide application. But frequent application of fungicides to the soil has not only caused environmental hazards like water and soil pollution but also destruction of non-target beneficial microorganisms in soil. Recently, biocontrol approaches have been initiated by using antagonistic microorganisms to combat these diseases.

Biological control agents for plant diseases are currently being examined as alternatives to synthetic pesticides due to their perceived level of safety and minimal environmental impacts. Strains of several bacterial species such as *Bacillus*, *Pseudomonas* and recently the *Rhizobium* group were isolated and found to effectively control various soil-borne plant pathogenic fungi under green house and field conditions. As compared to the other biocontrol agents, Rhizobia offer the great advantage of symbiotic nitrogen fixation by association with legumes [9]. Rhizobia have several mechanisms of action that allow them to control pathogens. These mechanisms include competition for iron by production of siderophores [10,11], competition for nutrients [12], production of antibiotics [13,14], promotion of plant growth, in terms of better shoot height, root length, dry weight and root nodulation [15,16], and induction of plant de fence mechanisms [17]. The aim of the present work was to select *Rhizobium* isolates with antagonistic activity against *sclerotinia* stem rot of fenugreek caused by *Sclerotinia trifoliorum*. Isolates were purified from fenugreek nodules and their effects on fungal growth and disease development were assessed *in vitro*, in dual cultures, as well as *in vivo* under greenhouse conditions.

Materials and Methods

Plant material

The seeds of fenugreek variety 'Ryhana' used in this work were provided by the Legume Program, Field Crop Laboratoy, Institut National de la Recherche Agronomique de Tunisie (INRAT).

Bacterial isolates

Rhizobium isolates were obtained from nodules of 50 days old fenugreek plants using the crushed nodule method [18]. All isolates were purified and tested for their ability to form nodules on fenugreek as previously described [19]. Thirty two isolates were collected from different localities (Table 1). These strains were grown at 28°C [18] on a yeast extract mannitol medium containing 0.08% yeast extract (w/v) and 1% mannitol (w/v). Stocks of strains were prepared on yeast extract-mannitol agar and kept at −70°C (under 30% of glycerol) for long-term storage and at 4°C as source cultures. A culture was repeated every 6 months to have stocks of younger generations.

N°	*Rhizobium strains*	Localities	N°	*Rhizobium strains*	Localities
1	Mat 1/6	Mateur	17	Ari 2	Ariana
2	Mat 1/3	Mateur	18	Tak	Takelsa
3	Mat 1/10	Mateur	19	Jbn	Jbeniana
4	Mat 3/16	Mateur	20	Uti	Utique
5	Mat 3/26	Mateur	21	Mat M	Mateur
6	Mat 3/17	Mateur	22	Elj	Eljem
7	Mat 3/22	Mateur	23	INRAT	Ariana
8	Mat 3/15	Mateur	24	Soli	Soliman
9	Mat 1/11	Mateur	25	Morg2	Morneg
10	Mat 1/1	Mateur	26	Bej	Beja
11	Mat 1/9	Mateur	27	Sad	Saida
12	Mat 3/28	Mateur	28	Sbit	Sbeitla
13	Mat 3/25	Mateur	29	Mok	Mokenine
14	Morg1	Morneg	30	Morg3	Morneg
15	MenT	Menzel Temim	31	Han	Hancha
16	Ari 1	Ariana	32	Mid	Mida

Table 1: Rhizobium strains collected from different localities used in experiments.

Fungal isolate

Sclerotinia trifoliorum was originally isolated from fenugreek in naturally infested field in northern Tunisia, according to the method described [11]. A fungal culture of the pathogen was cultured on PDA in 9-cm-diameter Petri dishes at 25 °C in the dark for 10 d for the production of *sclerotia*.

Antagonism test in dual culture

The *in vitro* inhibition of mycelial growth of *Sclerotinia trifliorum* by the bacterial isolates was tested using the dual culture technique as described by Paulitz., *et al.* [20] and Landa., *et al.* [21]. Three 50 µl drops from the 10^8 CFU ml^{-1} bacterial suspension were equidistantly placed on the margins of PDA plates and incubated at 25°C for 24 h. A 4-mm agar disc from fresh PDA cultures of *Sclerotinia trifliorum* was placed at the center of the PDA plate for each

bacterial isolate and incubated at 28°C for 7 days. The radius of the fungal colony towards and away from the bacterial colony was measured. The percentage growth inhibition was calculated using the following formula:

% inhibition = (*R-r*)/*r* x 100

Where r is the radius of the fungal colony towards the bacterial colony and R is the maximum radius of the fungal colony away from the bacterial colony.

Eluciation of antagonistic traits : Volatile antifungal compounds

The production of volatile antifungal compounds by the *Rhizobium* isolates was assayed by a sealed plate method as described by Fiddman and Rossall [22]. From 72-h-old cultures of *Rhizobium* strains in yeast extract mannitol (YEM) liquid media [18], 200µl was spread on YEMA medium in a Petri dish. After incubation at 28°C for 24 h, a second Petri dish containing PDA was inoculated with a plug (Φ: 6 mm) of the test fungus in the center of the plate, inverted and placed over the bacterial culture. Each two plates, containing pathogen and bacteria, were sealed together with parafilm and incubated at 25°C. This ensured that both organisms were growing in the same atmosphere though physically separated. As a control, a Petri dish containing YEMA medium without bacteria was placed under the PDA medium inoculated with the fungal pathogen. Fungal growth was measured as increases in the radial length after 5 days. Each test was replicated three times

Phosphate solubilization efficiency

Bacterial isolates were then employed for phosphate solubilization by streaking them on the Pikovskaya's agar medium [23] and incubated for 7 days at 28±2 ºC. The phosphate solubilization was expressed as positive and negative depending on the halo zone formation. The size of the clear zone around the colonies showing phosphate solubilization was noted. The P solubilizing ability of bacteria can be assessed in terms of the solubilization index (SI). As described in several studies [24,25], phosphate SI can be determined using the following formula:

$$SI = \frac{Colony\ diameter\ +\ halo\ zone\ diametre}{Colony\ diameter}$$

Evaluation of antagonistic activity of rhizobacteria against pathogenic fungi

The study was conducted to determine the efficacy of *Rhizobium* strains to reduce the incidence of *sclerotinia* stem rot on fenugreek cultivar 'Ryhana'. Seeds were surface disinfected in 2% NaOCl for 3 min, washed three times with sterile distilled water and germinated on autoclaved layers of paper towels in moist chamber at 25°C for 3 days. Germinated seeds, selected for the uniformity (length of radicle), were sown in perlite at a depth of 2 cm. After transplanting of seedling, each pot was drenched with 5 ml of each of the bacterial suspension (10^8 cells/ml).

Preparation of the fungal inoculants

Two centimeters long surface sterilized fenugreek stem sections were colonized by placing them for two weeks on individual PDA cultures with *Sclerotinia trifliorum* isolate. A colonized section was placed against each of three stems per pot and maintained in position with a wooden toothpick. A sterile fenugreek stem section was placed against each of the control stems. Pots were placed in a green house with a temperature regime of approximately 25 °C during the day and 16 °C at night and were irrigated daily. The treatments in the *in vivo* biocontrol experiment were as follows: Plant inoculated with *Sclerotinia trifliorum* and rhizobial strains, plants inoculated only with *Sclerotinia trifliorum* (control +) and non-inoculated fenugreek (control -). The percentage of dead plants was assessed two weeks after inoculation. The experimental design was a randomized complete block design with 6 replicates (pots) consisting of three plants per pot per isolate. Diseased plants were collected, seedling length shoot dry weight were recorded at the end of the experiment. Samples were kept for 72 h at (60± 2) °C in an oven for measuring the dry weight of seedling.

The virulence of pathogen was evaluated at scale indices from 0 to 4 according Tezcan and Yildiz [26]. The recorder data were based on mass disease index (MDI %) according to the formula :

$$MDI\ (\%) = \sum_{4}^{1} (n\ x\ i/4N)x100$$

n: number of plant with indices i

N: umber of total plants.

Statistical analysis

All experiments were replicated as completely randomized blocks. Consequently, the data are means ± confidence interval (n = 3, α = 0.05). Statistical analysis (ANOVA) was performed with SPSS 20.0 for windows, followed by Duncan's multiple range test (P = 0.05).

Results and Discussion

In vitro experiments

Fungal inhibition assays

Among the 32 *Rhizobium* strains tested in dual culture with *Sclerotinia trifliorum*, 27 inhibited the fungus growth (Figure 1) and reduce their development more than 50%. Isolates Soli and Tak were the most preferment *in vitro* and caused growth inhibition of *R. solani* more than 90% Control plates without rhizobia were completely covered by the pathogen mycelium showing no growth inhibition of the fungus. The mean mycelium growth inhibition of the most effective bacterial isolates (Figure 1) revealed that the inhibition was highly significant (P = 0.05). No physical contact was observed between any of the antagonistic bacteria tested and *Sclerotinia trifliorum*; moreover, an inhibitory halo was observed suggesting the presence of fungistatic metabolites secreted by the bacteria. On the other hand, a change in mycelial color was observed closed to the colony end of *Sclerotinia trifliorum*, being this one of a darker brown than the one observed at the center of colony (Figure 2).

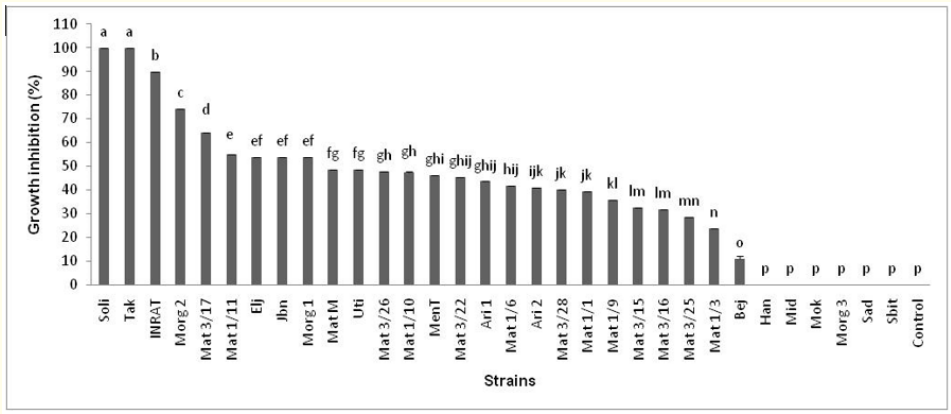

Figure 1: Effect of *Rhizobium* isolates on *Sclerotinia trifliorum* growth *in vitro.*

(*) = Percent growth inhibition compared to uninoculated control. Values followed by (*) were significant (P = 0.05) by Duncan's multiple range test.

Figure 2: Illustration of the *in vitro* effect of *Rhizobium* strains on the development of *Sclerotinia trifliorum.*

Rhizobium volatile effects on *Sclerotinia trifliorum* growth *in vitro* and Phosphorus solubilizing efficiency

As to the effect of *Rhizobium* volatiles on pathogen growth, among 19 isolates tested for volatiles activity, 18 were able to reduce the growth of pathogen. The isolates Tak, Soli, Jem, Morg 2 and Ari 2 seemed to be the most effectives, giving more than 40% inhibition after 96h of incubation (Figure 3).

Most tested *Rhizobium* isolates were able to solubilize phosphate. Eighteen isolates produced a halo on tricalcium phosphate agar media plates (Figures 4, 5). Four isolates, Tak, Soli, Morg 2 and Ari2 were positive for inorganic phosphate solubilization, volatiles substances production and generate more than 50% of pathogen inhibition. They were classified the most effectives isolates *in vitro* trials.

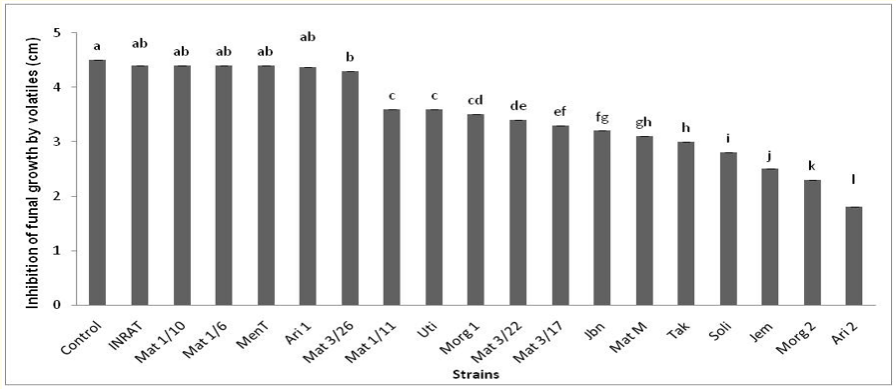

Figure 3: Ability of the most effective bacterial isolates in dual culture to produce volatiles.

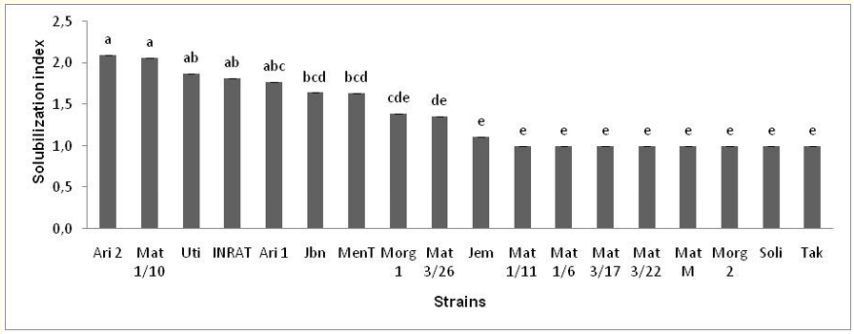

Figure 4: Ability of the most effective bacterial isolates in dual culture to solubilize inorganic phosphorus.

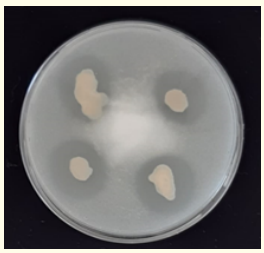

Figure 5: Phosphorus solubilization by *Rhizobium* strains.

Greenhouse experiments

To study the rhizobia antagonism activities, the previous trial was replicate in pot under glass house conditions. The results indicate that Soli, INRAT, Mid, Tak, and Morg2 isolates inhibited significantly sclerotinia stem rot of fenugreek caused by *Sclerotinia trifliorum* (Figure 5). Mass Disease expression MDI% was calculated according to Tezcan and Yildiz (26) methodology. Inoculation with Sol isolates induced suppression of sclerotinia stem rot more than 80%, while inoculation with Jbn, Mat1/11, Mat3/17, Ment and Morg1 isolates reduced the disease more than 60% (Figure 6). In these tests; no bacteria protected the plants completely against *Sclerotinia trifliorum*, although all isolates increase significantly in fresh weight compared to the infested control (Figure 7, 8). The inhibition of the fungus growth by some of Rhizobium isolates are varied from 60 to 80% (Figure 5). Control plants not treated with bacteria but inoculated with *Sclerotinia trifliorum* alone rendered up to 100% sclerotinia stem rot incidence with the majority of plant completely stunted or dead. The fungus resulted in a pronounced decrease in the fresh weight of the shoots compared to the uninoculated control and to some of the treatments with the most effective bacteria isolates (Figure 7).

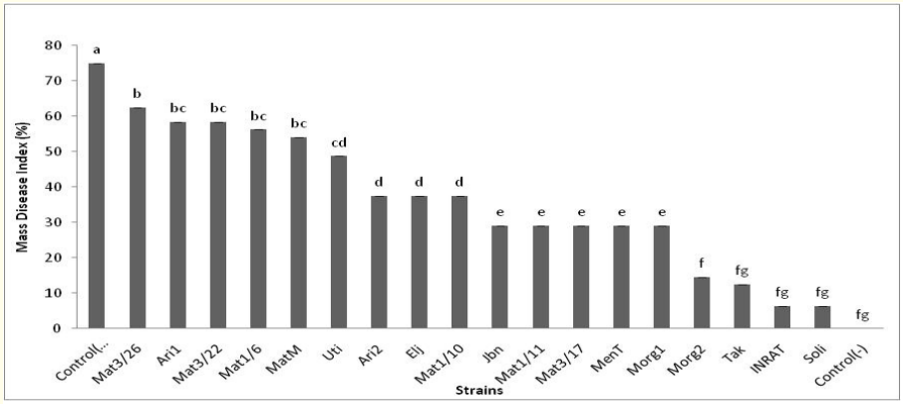

Figure 6: Antagonism of *Rhizobium* towards *Sclerotinia trifliorum* calculated according MDI (%) of sclerotinia stem rot of fenugreek inoculated by the pathogen and different.

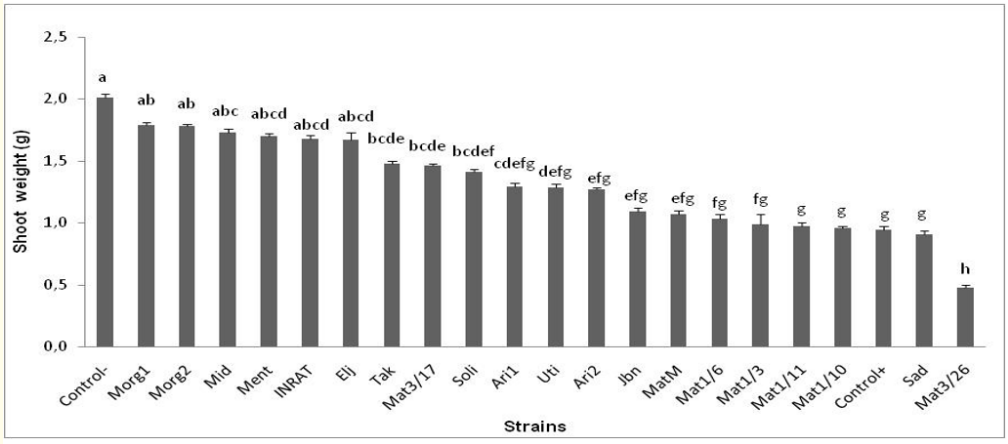

Figure 7: Effect of inoculation by *Rhizobium* strains on shoot weight of fenugreek infected with *Sclerotinia trifliorum* under glass house conditions 8 weeks after sowing.

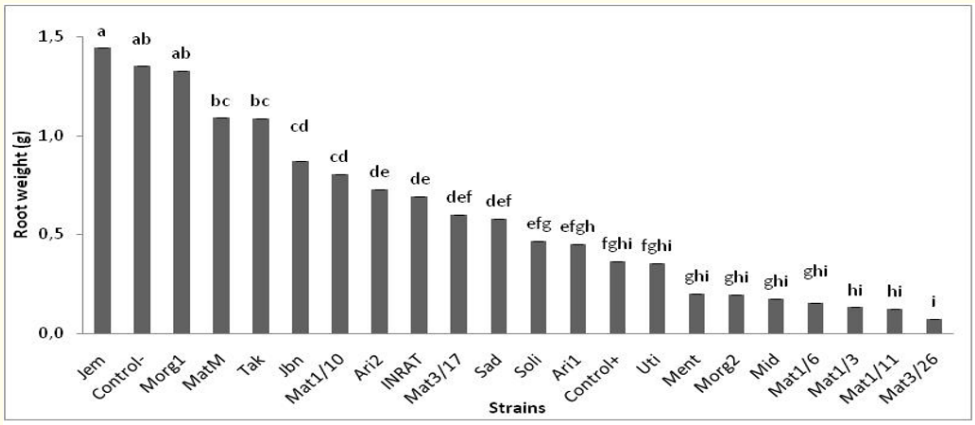

Figure 8: Effect of inoculation by *Rhizobium* strains on root weight of fenugreek infected with *Sclerotinia trifliorum* under glass house conditions 8 weeks after sowing.

Shoots fresh weight reduction was about 40% in the plant control inoculated with *Sclerotinia trifliorum*, alone. Whereas, shoot dry weight reduction, of plants inoculated with both the pathogen and *Rhizobium* strain Morg1 was 10% (Figure 7).

Rhizobium strains *in vivo* under greenhouse conditions. (Control (-) represents un-inoculated fenugreek plants and control (+) plants infested with *Sclerotinia trifliorum*)

Discussion

The ability of rhizobia to inhibit certain soil borne plant pathogens and plant parasites [27-29] has increased the importance of rhizobia besides their use in nitrogen fixation. In this study, an experiment was therefore carried out to study the antagonist effect of thirty two *Rhizobium* strains to *Sclerotinia trifliorum*. We tested rhizobia for volatiles and solubilizing phosphorus because previous studies suggested that bacteria possessing these traits can increase plant growth [30]. The reduction in fungal growth observed *in vitro*, by the selected *Rhizobium* isolates, was presumably due to metabolites being released from bacteria into the culture medium. These metabolites may include antibiotics and/or cell- wall degrading enzymes. Different studies have implicated antifungal secondary metabolites produced by *Rhizobium* spp. in the control of plant diseases caused by pathogenic fungi [15].

Our experiments showed that four isolates (Tak, Soli, Morg 2 and Ari2) solubilized inorganic phosphorus and significantly inhibited pathogen growth by producing volatiles.

Greenhouse experiments showed that inoculation of fenugreek with *Sclerotinia trifliorum* reduced plant growth and caused sclerotinia stem rot of hyhana variety. Several other workers have noticed the beneficial effects of rhizobia on plant growth and reduction of diseases incidence [29,31,32]. Interestingly, application of *Rhizobium* isolates significantly reduced the mass disease index and increased plant growth (Figure 5). These rhizobia also increased fresh weight of roots and shoots. These benefits may be attributed to better disease control in presence of the bacteria and /or to better nutrition, due especially to higher nodulation and phosphorus uptake [33]. In our study, the basic mechanisms behind such protection is not clearly defined, the possibility that competition, antibiosis, direct parasitism and induced resistance by the antagonistic bacteria, may operate synergistically after inoculation with effective *Rhizobium* strain cannot be ruled out. Currently investigations are being conducted to determine the modes of actions of all the promising isolates from this study. To determine whether these promising strains can be developed into commercial inoculants, their biocontrol efficacy must first be confirmed under field's conditions.

Conclusion

Generally, the use of Rhizobium have greatly reduced the sclerotinia stem rot incidence as well as enhanced the growth of fenugreek plants. The selected rhizobia could be used effectively as biocontrol agents of fenugreek against *Sclerotinia trifoliorum*. Nevertheless, further studies are needed to characterize the mechanisms involved in Rhizobium-inoculated fenugreek resistance to *S. trifoliorum* to improve *Rhizobium* strains uses as biofertilizer and biocontrol agents in fenugreek fields. The use of bioagents is highly beneficial as environmentally friendly application and can be used as an alternative for fungicides to enhance the plant growth and reduce disease incidence, resulting in higher yield.

Conflict of Interest

The authors declare that they have no financial interest and no conflict of interest.

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