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CHARACTERIZATION OF *RHIZOBIUM* ISOLATES AS BIOCONTROL AGENTS AGAINST *FUSARIUM SOLANI* AND *MACROPHOMINA PHASEOLINA*

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SUMMARY

This study recovered 15 *Rhizobium* and *Bradyrhizobium* isolates from nodules of different legume plants (Faba bean, Pea, Soybean, and Clover) grown in nine Egyptian governorates representing the delta region (Menoufia, Gharbia, Suez, Qalubia, Cairo, Sharkia, Behirah, Kafr El-sheikh, and Ismailia). The infectivity test proceeded with all isolates retaining and infecting their hosts, demonstrating that they are *Rhizobium* cultures. Molecular identification based on 16S rRNA gene sequencing revealed that the isolates belonged to two species of *Rhizobium*, namely *Rhizobium leguminosarum* (RL1, RL2, RL3, RL4, RL5, RL6, RL7, RL8, RL9, RL10, RL11, RL12, and RL13) and *Bradyrhizobium japonicum* (BJ1 and BJ2). Moreover, PCR based on specific primers was employed to detect *nifH* gene in the genomes of tested isolates. Most of the isolates exhibited sensitivity toward antibiotics, and were able to produce HCN. The isolates of RL4, RL6, RL9, and RL10 were positive for chitinase activity and exhibited clear zones ranging from weak to sturdy. The antagonism evaluation of *Rhizobium* isolates against *Fusarium solani and Macrophomina phaseolina* ensued under in vitro conditions. The isolates RL6 and RL9 of *R. leguminosarum* were the most effective, suppressing more than 50% growth of *M. phaseolina*. Similarly, the isolates RL4, RL6, RL9, and RL10 revealed the most effective and inhibited more than 50% growth of *F. solani.* Interestingly, the isolates RL6 and RL9, which exhibited high levels of suppression against *M. phaseolina,* also displayed high levels of suppression against *F. solani.*

Keywords: *Rhizobium*, 16S rRNA, antagonism, *Fusarium*, *Macrophomina*

Key findings: Some isolates were superior in inhibiting *M. phaseolina* and *F. solani* pathogens. These results indicate that *Rhizobia* can be used as an excellent biological control agent in root rot disease management.

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INTRODUCTION

The intensive application of pesticides for controlling plant pathogens has adverse effects on the soil, the environment, and living organisms, causing the development of new generations of resistant pathogens (Rush *et al*., 2021). A study stated that residuals of the synthetic pesticides in food are toxic and therefore harm all elements of the ecosystem (Islam *et al*., 2022). The annual loss of crop yield due to plant pathogen attacks is about USD 23.5 billion (Rossman, 2009).

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Biological control agents represent an inexpensive, eco-friendly, and safe alternative to widely used pesticides (Abdel-Lateif, 2017; Barratt *et al*., 2018). *Rhizobia* are well known for supplying plants with nitrogen and as an excellent biological control agent against soil-borne pathogens (Deshwal *et al*., 2003). *Rhizobia* can improve plant growth through N_2 fixation and the availability of nutrients and minerals. Similarly, *Rhizobia* have shown to increase plant growth via the secretion of secondary metabolites of alkaloids and flavonoids that improve plant defense against pathogens (Basu *et al*., 2021). Earlier studies stated that *Rhizobia* could stimulate auto defenses in legumes via hormone signaling and start programmed cell death in pathogens (Dodds and Rathjen, 2010; Kumar *et al*., 2011). *Fusarium solani* and *Macrophomina phaseolina* are crucial and dangerous pathogens that cause root rot disease in several economic crops (Al-Ani *et al*., 2012; Choudhary *et al*., 2013). *M. phaseolina* is the chief cause of root rot disease in essential plants, such as, corn and cotton (Aly *et al*., 2007; Khaledi and Taheri, 2016). *Fusarium* is one destructive fungus that causes fruit and vegetable crop rot and is responsible for about 60–70% loss of fruit yield (Darmadi *et al*., 2019; Srinivas *et al*., 2019; Hasan *et al*., 2020).

Ketta et al. (2021) investigated the antagonism of *R. leguminosarum* combined with certain *Trichoderma* species against *R. solani* and *F. solani,* the primary sources of damping-off diseases in peas. Used microbial combinations significantly reduced the disease symptoms caused by these pathogens. Moreover, other studies reported that *Rhizobium* isolates could inhibit the radial growth of *F. oxysporum* f. sp. *ciceris* and *M. phaseolina* in higher levels, as well as, improve each plant's growth and yield in vitro and the field (Arfaoui *et al*., 2006; Al-Ani *et al*., 2012; Hasan *et al*., 2020). The *nifH* is a well-known principal gene that encodes for one of the nitrogenase enzyme subunits (Rubio and Ludden, 2002). The study of the *nifH* gene is a point of interest for many researchers thanks to its conservation in *Rhizobia* (Laguerre *et al*., 2001; Dhole *et al*., 2016).

Studies asserted that discrimination against *Rhizobia* based on morphological and biochemical tests is inaccurate and can lead to false results (Wolde-Meskel *et al*., 2004; Hameed *et al*., 2004). Hence, identifying *Rhizobia* via 16S rRNA sequencing has proven an efficient tool for the discrimination among the different isolates (Silva *et al*., 2012; Ismail *et al*., 2013; Khalid *et al*., 2020 and Sijilmassi *et al*., 2021). In this study, collecting 15 isolates of *Rhizobium* and *Bradyrhizobium* took place from roots of different leguminous plants, identified based on 16S rRNA, and investigated for their biocontrol activity versus *M. phaseolina* and *F. solani.*

MATERIALS AND METHODS

Microorganisms

Rhizobial isolates

Recovery of *Rhizobia isolates* progressed from root nodules of various leguminous plants (faba bean, pea, soybean, and clover) grown in different governorates of Egypt (Table 1), as previously described by Gauri *et al.* (2011).

Infectivity test

Seeds of faba bean, pea, soybean, and clover underwent surface sterilization, according to Mansour *et al.* (2003). Sterilized seeds grown in Petri plates continued for two days at room temperature, and afterward aseptically moved to growth pouches made of transparent plastic. Inoculation of all acquired isolates into seedlings followed, with each seedling receiving 0.01 packed cell volume of each propagated isolate. Keeping the inoculated seedlings in a growth chamber went to confirm their ability to produce nodules.

Fungal isolates

The agricultural research center in Giza, Egypt, provided the pathogenic fungi cultures (*Fusarium solani* and *Macrophomina phaseolina*).

Molecular identification of *Rhizobial* isolates

The bacterial DNA isolation used Qiaprep Spin Miniprep Kit (QIAGEN). Achieving the 16S rRNA amplification employed the following primers purchased from Sigma, Egypt: P1 (AGAGTTTGATCCTGGCTCAGAACGAACGCT) and P6 (TACGGCTACCTTGTTACGACTTCACCCC), as shown by Khalid *et al.* (2020) using Master Mix (Thermo Scientific Fisher, USA). The PCR program proceeded with the following: denaturation at 94 °C for 10 min followed by 35 cycles for 1 min at 94 °C, 1 min at 58 °C, and 2 min at 72 °C. Sequencing followed with the Big Dye Terminator v3.1 Cycle Kit using

3500 Genetic Analyzer, Applied Biosystems (Daejeon, Korea). The nucleotide blast application matched *Rhizobial* isolates sequencing results with the NCBI database (http://www.ncbi.nlm.nih.gov/). The phylogenetic tree construction used MEGA version 6 (Tamura *et al*., 2013).

Amplification of *nifH gene*.

The *nifH* gene amplification advanced using the following primers purchased from Sigma, Egypt: NIFHF -5'TACGGCAACGGCGGCATCGG CAA3' and NIF-HR-5' AGCATGTCCTCGA GGTCCTCCA3' (Laguerre *et al*., 2001). Amplification conditions were: denaturation at 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing at 57 °C for 30 s, extension at 72 °C for 30 s for 35 cycles, and final extension at 72 °C for 7 min.

Evaluation of antibiotic sensitivity

Testing the resistance or sensitivity of bacterial isolates against 10 different antibiotics employed the antibiotic disc assay according to Eaglesham (1987) and Ahmad (2001). The following antibiotics purchased from Sigma, Egypt, included Streptomycin (S10), Amoxicillin (AMC 10), Cloxacillin (Cx1), Rifamycin (Rf30), Oxacillin (Ox1), Gentamycin (CN10), Chloramphenicol (C30), Tobramycin (TOB), Ampicillin (Am10), and Ofloxacin (OFX).

Production of HCN

Production of hydrogen cyanide by the bacterial isolates followed the procedure of Manasa *et al.* (2017).

Chitinase test

Preparation of colloidal chitin and testing of the chitinase activity for the tested isolates were done as previously described by Murthy and Bleakley (2012).

Antagonism of *Rhizobial* isolates against pathogenic fungi

Growing *Rhizobia* isolates in YEM medium at 30 °C in a rotary shaker at 120 rpm took two days. Two ml of the culture suspension (10^5 cell ml^{-1}) went into 20 ml of yeast glucose mineral agar (YGMA) (mannitol, 5g; glucose, 5 g; K_2HPO_4, 0.5 g; $MgSO_4.7H_2O$, 0.8 g; NaCI, 0.1 g; $ZnSO_4.7H_2O$, 0.1 mg; $CuSO_4.7H_2O$, 0.05 mg; $FeSO_4.7H_2O$, 0.2 mg; $MnSO_4.5H_2O$, 0.5 mg; Na_2MoO_4, 0.05 mg; H_3BO_3, 0.25 mg; yeast extract, 2.5 g, and 15 g agar in one-liter water). After the agar solidification, a mycelial plug was placed on the agar surface. Cultures without *Rhizobia,* inoculated only with fungus, served as control (Küçük, 2013). Moreover, *Rhizobia* isolates inoculated on the same media also served as a control. Every treatment was triplicate, with plates kept at 26 °C for seven days. The percentage of pathogen growth inhibition measurement used the formula of Whipps (1987): (R1-R2)/R1×100, where R1 is the radial growth of the fungal grown alone (measured in cm) and R2 is the radial growth toward the antagonist.

Data analysis

The data analyzed statistically used ANOVA to determine the standard error of means and comparisons of means at a 5% significance level, according to Duncan's multiple range test analysis, the software Costat version 6.3.

RESULTS

Isolation and identification of *Rhizobial* isolates

Fifteen *Rhizobial* isolates gained recovery from root nodules of different and essential leguminous plants (Faba bean, Pea, Clover, and Soybean) collected from nine governorates of Egypt (Menoufia, Gharbia, Suez, Qalubia, Cairo, Sharkia, Behyrah, Kafr El-sheikh, and Ismailia) (Table 1). This study recovered five isolates from Pea (RL3, RL6, RL8, RL9, and RL12), four from Faba bean (RL1, RL2, RL7, and RL11), four from Clover (RL4, RL5, RL10, and RL13), and two isolates from Soybean plants (BJ1 and BJ2).

The infectivity test went on by reinfection of host plants with the isolated *Rhizobial* cultures. After four weeks of inoculation, all seedlings derived from the parent host of each *Rhizobium* culture started forming nodules along their root system, demonstrating that they are *Rhizobium* cultures. The 16S rRNA employed identified these isolates on a molecular level. After a comparison of the sequenced data with those published on the NCBI website (http://www.ncbi.nlm.nih.gov/), it revealed that the tested isolates clustered into two species of *Rhizobium*: 13 isolates identified as *Rhizobium leguminosarum* (from RL1 to RL13) and two isolates as *Bradyrhizobium japonicum*

Table 1. Details of samples, collection sites, isolate codes, and blast identification.

Isolates	Isolation sites	Host	Sequence Similarity (%)	Species name
RL1	Menoufia	Faba bean	97	*Rhizobium leguminosarum bv. viciae*
RL2	Gharbia	Faba bean	98	*Rhizobium leguminosarum bv. viciae*
RL3	Menoufia	Pea	92	*Rhizobium leguminosarum bv. viciae*
RL4	Suez	Clover	96	*Rhizobium leguminosarum bv. trifolii*
RL5	Qalubia	Clover	97	*Rhizobium leguminosarum bv. trifolii*
RL6	Gharbia	Pea	94	*Rhizobium leguminosarum bv. viciae*
RL7	Cairo	Faba bean	98	*Rhizobium leguminosarum bv. viciae*
RL8	Sharkia	Pea	91	*Rhizobium leguminosarum bv. viciae*
RL9	Behirah	Pea	94	*Rhizobium leguminosarum bv. viciae*
RL10	Menoufia	Clover	98	*Rhizobium leguminosarum bv. trifolii*
RL11	Behirah	Faba bean	99	*Rhizobium leguminosarum bv. viciae*
RL12	Behirah	Pea	94	*Rhizobium leguminosarum bv. viciae*
RL13	Kafr El-sheikh	Clover	97	*Rhizobium leguminosarum bv. trifolii*
BJ1	Sharkia	Soybean	94	*Bradyrhizobium japonicum*
BJ2	Ismailia	Soybean	98	*Bradyrhizobium japonicum*

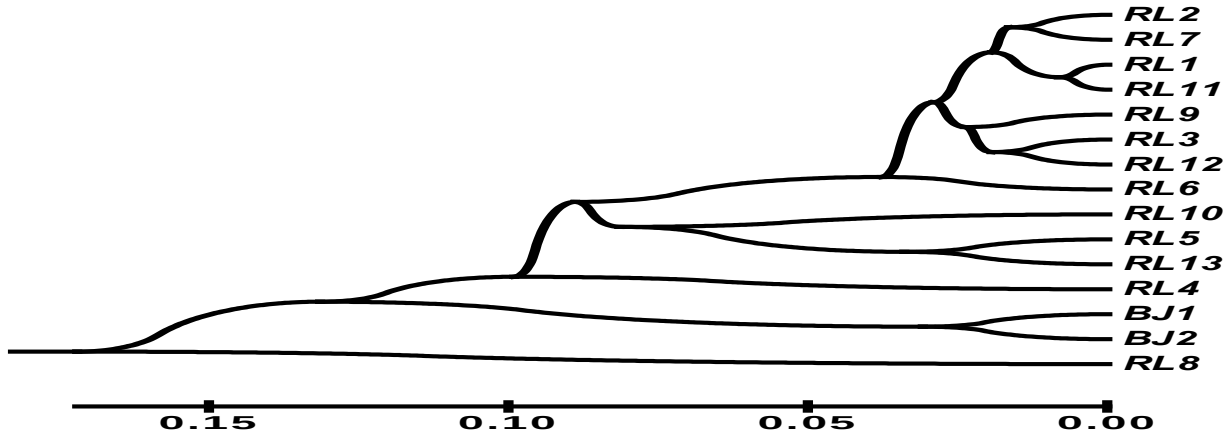

Figure 1. Dendrogram based on 16S rRNA sequences analysis using MEGA 6 software.

(BJ1 and BJ2) (Table 1). The sequenced data exhibited 91% to 99% similarity with the published sequences on the NCBI website. The cluster analysis classified the *Rhizobial* isolates into two main groups: one group included only isolate RL8, while the second group contained all the other isolates (Figure 1). Since *nifH* is a critical gene that encodes for nitrogenase enzyme, hence, PCR based on specific primers got employed to detect this gene in the genomes of tested isolates. One band of 500bp appeared in all isolates (Figure 2).

Antibiotics sensitivity and HCN production

Assessment of all tested isolates for their antibiotic sensitivity against 10 different antibiotics occurred (Table 2). Generally, most isolates exhibited sensitivity toward the antibiotics, with some exceptions. The isolate RL1 of the faba bean exhibited resistance against all the tested antibiotics. Moreover, the isolate RL4 of clover showed resistance against six antibiotics: Amoxicillin, Cloxacillin, Oxacillin, Chloramphenicol, Ampicillin, and Ofloxacin. Cloxacillin showed the lowest antibacterial effect, whereas Gentamycin, Rifamycin, Streptomycin, and Tobramycin exhibited the highest antibacterial effect.

Production of HCN tested in all isolates used the colorimetric method by giving colors from orange (weak) to red (strong). All isolates exhibited a weak (+) to moderate (++) HCN production. A moderate HCN production emerged for isolates RL2, RL5, RL7, RL9, RL10, and BJ2. On the other hand, a weak HCN production appeared for isolates RL1, RL3, RL4, RL6, RL8, RL11, RL12, RL13, and BJ1 (Table 3).

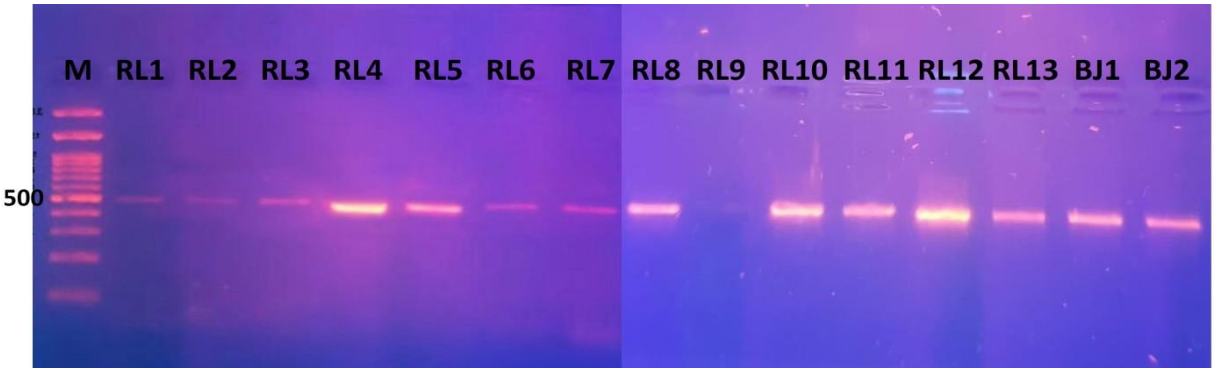

Figure 2. The *NifH* gene amplification in the *Rhizobial* isolates.

Table 2. Evaluation of antibiotic sensitivity against 10 different antibiotics: Chloramphenicol (C-30), Tobramycin (TOB-10 mcg), Ofloxacin (OFX-5 mcg), Ampicillin (Am-10 mcg), Streptomycin (S-10 mcg), Amoxicillin (AMC-10 mcg), Oxacillin (OX-1 mcg), Rifamycin (Rf-30 mcg), Cloxacillin (CX-1 mcg), Gentamycin (CN-10 mcg).

Isolates code	C-30	TOB-10	OFX-5	Am-10	S-10	AMC-10	OX-1	RF-30	CX-1	CN-10
RL1	R	R	R	R	R	R	R	R	R	R
RL2	S	S	S	S	S	S	R	S	R	S
RL3	S	S	S	S	S	S	S	S	R	S
RL4	R	S	R	R	S	R	R	S	R	S
RL5	S	S	S	R	S	S	S	S	R	S
RL6	S	S	S	S	S	S	S	S	R	S
RL7	S	S	S	S	S	S	S	S	R	S
RL8	S	S	S	S	S	S	S	S	R	S
RL9	S	S	S	S	S	S	S	S	R	S
RL10	S	S	S	S	S	S	S	S	S	S
RL11	S	S	S	S	S	S	S	S	S	S
RL12	S	S	S	S	S	S	S	S	R	S
RL13	S	S	S	S	S	S	S	S	S	S
BJ1	S	S	S	S	S	S	S	S	R	S
BJ2	S	S	S	S	S	S	S	S	R	S

Where: R (Resistance) and S (Sensitive).
The concentration of antibiotics is in micrograms (mcg).

Table 3. The ability of isolates to produce HCN and their chitinase activity.

Isolates code	HCN production	Chitinase activity
RL1	+	
RL2	++	–
RL3	+	–
RL4	+	+
RL5	++	–
RL6	+	++
RL7	++	–
RL8	+	–
RL9	++	+++
RL10	++	++
RL11	+	–
RL12	+	–
RL13	+	–
BJ1	+	–
BJ2	++	–

Where: negative (–), weak (+), moderate (++), or strong (+++) production.

Chitinase activity

Chitinase enzyme plays a critical role in degrading the pathogenic fungi cell wall. Hence, the chitinase activity of the tested isolates underwent evaluation by measuring the hydrolysis zones formed around the colonies grown on a medium containing chitin as the sole carbon source.

The large chitin-clearing zones around the colonies indicate the excellent production of chitinase. Most of the isolates have no chitinase activity but the isolates of RL9, RL6, RL10, and RL4 displayed positive for chitinase activity and exhibited weak (**+**) to strong clear zones (+++) as presented in Table 3. The isolate RL9 gave the largest clear zones, followed by RL6 and RL10 isolates, then RL4 isolate.

Antagonism of *Rhizobial* isolates against the pathogenic fungi

The investigated virulence of *Rhizobial* isolates versus *F. solani* and *M. phaseolina* proceeded in vitro (Figure 3). The isolates RL9, RL6, RL7, RL1, BJ2, and RL4 showed the highest percentages of *M. phaseolina* radial growth inhibition, with 64.83%, 64.10%, 45.38%, 43.34%, 42.23%, and 39.92%, respectively (Figure 4). On the contrary, isolate BJ1 exhibited the lowest radial growth inhibition against *M. phaseolina* at 4.76%. Moreover, the isolates RL4, RL6, RL9, and RL10 demonstrated the highest percentages of inhibition of *F. solani* radial growth with 76%, 68.30%, 67.50%, and 51.86%, respectively (Figure 5). In contrast, the isolates RL11, RL12, and RL13 did not show any antagonism against *F. solani.*

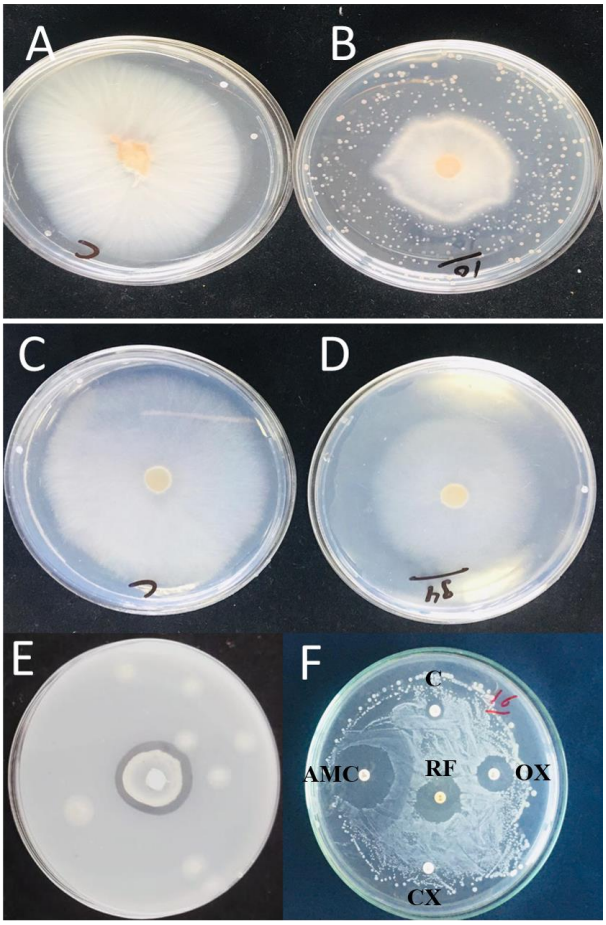

Figure 3. Antagonism of *Rhizobium* isolates against *M. phaseolina* and *A. niger*.
(A) *F. Solani* (B) *F. Solani* and *Rhizobium* (C) *M. phaseolina* (D) *M. phaseolina* and *Rhizobium.* (E) Chitinase activity on medium supplemented with colloidal chitin *(*F) Resistance and sensitivity of *Rhizobium* isolates on different antibiotics; AMC (Amoxicillin), RF (Rifamycin), C (Chloramphenicol), CX (Cloxacillin), and OX (Oxacillin).

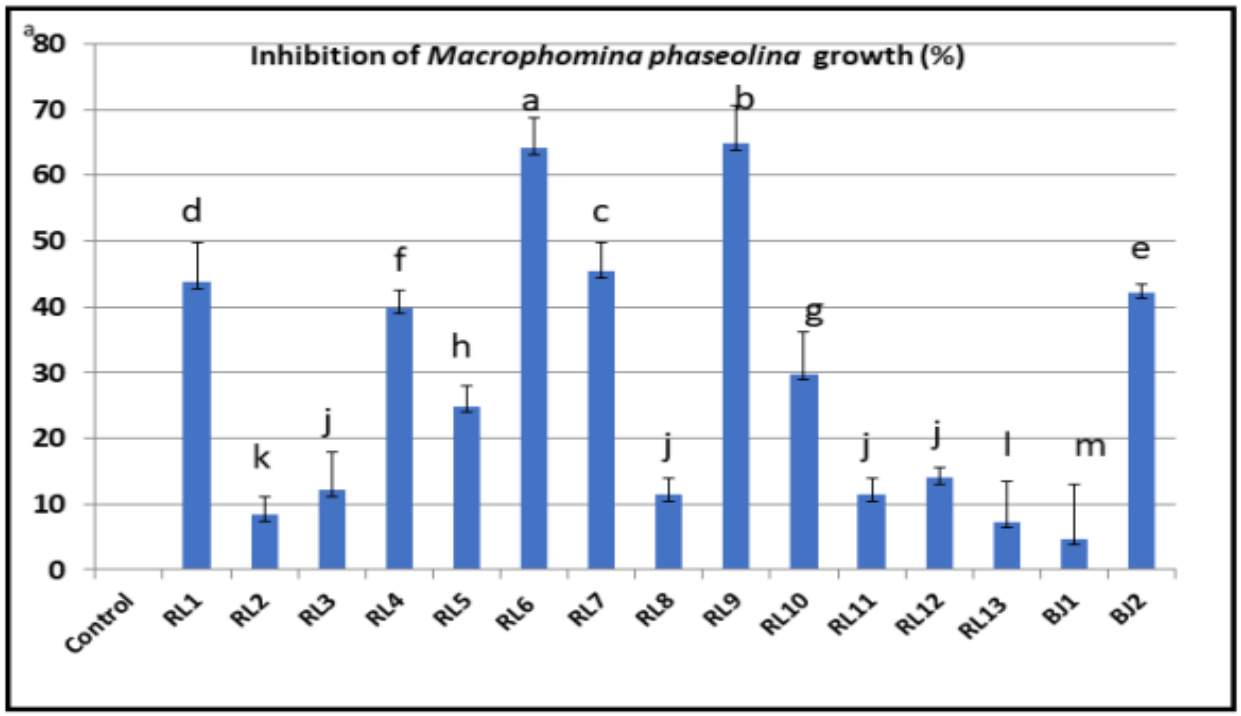

Figure 4. Antagonism of *Rhizobium* isolates against *M. phaseolina.*
Bars represent the standard deviation of means, and the means with the different letters are significantly different.

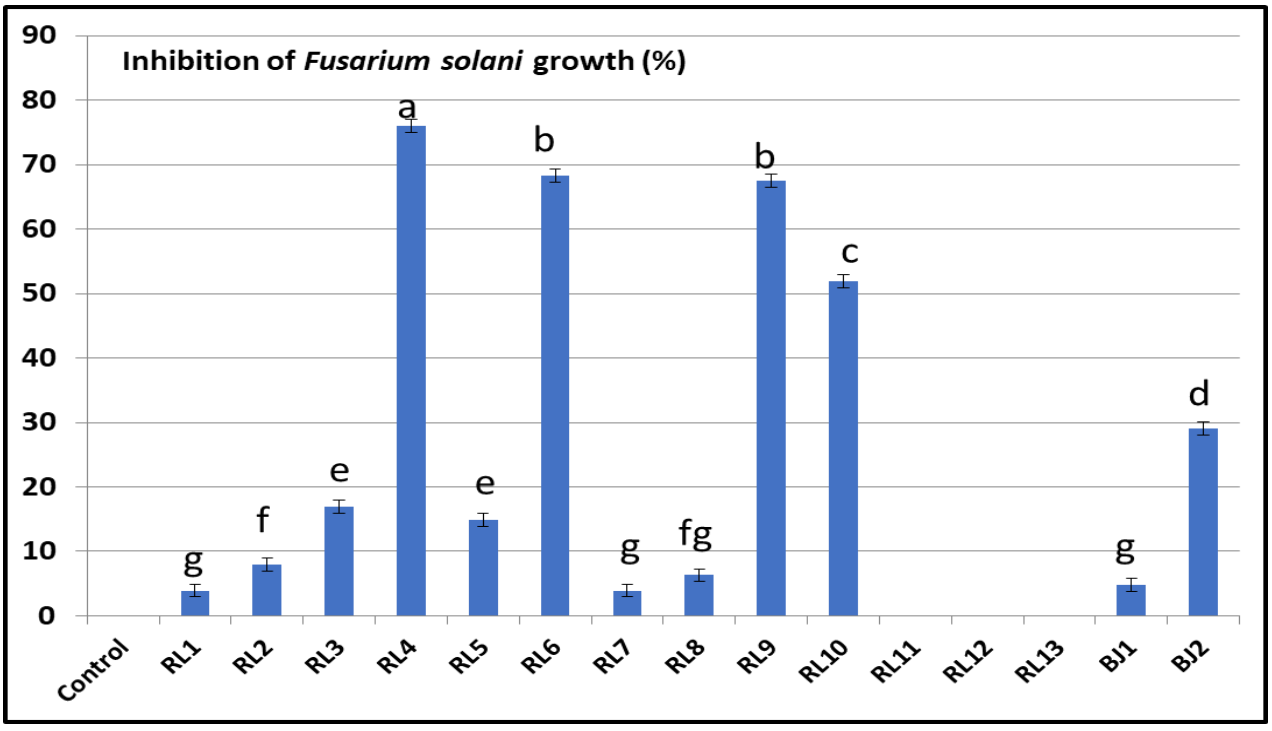

Figure 5. Antagonism of *Rhizobium* isolates against *F. solani.*
Bars represent the standard deviation of means, and the means with the different letters are significantly different.

DISCUSSION

In this study, the infectivity test proved that the tested isolates belonged to *Rhizobium*. Similarly, the identification of *Rhizobial* isolates relying on 16S rRNA sequencing exhibited that the isolates belong to two species of *Rhizobia*: *R. leguminosarum* and *B. japonicum.* These results indicate the efficiency of 16S rRNA as a smart tool to discriminate among the different isolates of *Rhizobia* (Khalid *et al*., 2020, Sijilmassi *et al*., 2021; Ismail *et al*., 2022). Moreover, the detection of the *nifH* gene in all isolates concurs with the previous studies showing that the *nifH* gene is conserved within *Rhizobia* (Laguerre *et al*., 2001; Dhole *et al*., 2016).

Evaluation of antibiotic resistance revealed that most isolates exhibited sensitivity toward the antibiotics. Resistance of some isolates, such as RL1 and RL4, to an antibiotic is due to the presence of plasmids carrying resistance genes that mediate the detoxification of drugs (Gauri *et al*., 2011). The study results agreed with previous studies that stated resistance of *Rhizobia* against streptomycin, chloramphenicol, erythromycin, kanamycin, and penicillin (Kucuk and Kivnac, 2008; Elboutahiri et al., 2010). Screening of isolates for their ability to produce HCN is an essential concern due to their important role as a toxic substance in controlling plant pathogens (Haas and Défago 2005; Heydari and Pessarakli, 2010; Rijavec 2016; Tomaz, 2016). A report stated that *Rhizobia* could produce HCN (Alemu, 2016). The study's isolates exhibited a weak (+) to moderate (++) HCN production, and these results strengthen the importance of rhizobia as a bio-control agent.

Only the isolates of RL9, RL6, RL10, and RL4 showed positive chitinase activity and exhibited strong to weak clear zones, respectively. Previous studies indicated that most of the cell wall composition of pathogenic fungi intensely consisted of chitin, and some *Rhizobium* isolates were positive for chitinase (Tamiru and Muleta, 2018).

The *Rhizobial* isolates showed high efficacy in inhibiting *M. phaseolina* and *F. solani* in vitro. The isolates RL6 and RL9 of *R. leguminosarum* confirmed the most effective and suppressed more than 50% growth of *M. phaseolina*. Similarly, the isolates RL4, RL6, RL9, and RL10 revealed the most effective, restraining more than 50% growth of *F. solani.* Interestingly, the isolates RL6 and RL9, which exhibited high levels of suppression against *M. phaseolina,* also exhibited high levels of suppression against *F. solani.* Al-Ani et al. (2012) reported that the filtrates of *Rhizobial* cultures suppressed the radial growth of *F. solani* and *M. phaseolina* and improved soybean seed germination. Tamiru and Muleta (2018) tested the antagonism of 27 *Rhizobium* isolates against *F. solani,* and they found that some of the isolates inhibited the fungal radial growth by 70.5%. Arfaoui et al. (2006) tested the antagonism of *Rhizobium* isolates against *F. oxysporum*. The tested isolates inhibited the mycelial growth of the pathogen at a higher level and improved each plant's growth and yield.

Rhizobia have many weapons against pathogens, antibiotics like trifolitoxin, metabolites such as riboflavin, and vitamins such as ascorbic acid (Deshwal *et al*., 2003; Mehboob *et al*., 2009; Ahemad and Kibret 2014; Palacios *et al*., 2014). In addition, *Rhizobia* can produce siderophores (iron-chelating substances) able to suppress *M. phaseolina,* the leading cause of charcoal rot in groundnut (Arora *et al*., 2001; Ferreira *et al*., 2019).

The diversity in the antagonistic ability of *Rhizobial* isolates against *M. phaseolina* and *F. solani* is a return to the variability in their genetic component, pathogen cell wall structure, types of enzymes, volatiles, and antibiotics produced by these isolates. For example, the isolates RL6 and RL9, which exhibited superior antagonism against *M. phaseolina* and *F. solani*, were positive for each chitinase and HCN production. Moreover, *R. leguminosarum* bv. *Trifolii* can produce antibiotic trifolitoxin, while *B. japonicum* secrete the antibiotic rhizobitoxine and uses it to suppress *M. phaseolina* (Chakraborty and Purkayastha, 1984; Breil *et al.*, 1996).

CONCLUSIONS

The tested isolates varied in their chitinase, HCN production, and antibiotic resistance. Some isolates were superior in inhibiting *M. phaseolina* and *F. solani* pathogens. These results indicate that rhizobia can be an excellent biological control agent in root rot disease management. Additional experiments need an application in the field to select the best-promised ones.

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