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Full Length Research Paper

Effects of some *RHIZOBIUM* strains on chickpea growth and biological control of *RHIZOCTONIA SOLANI*

Imen HEMISSI[1,2,3], Yassine MABROUK[3]*, Neila ABDI[1,4], Manel BOURAOUI[1,4], Mouldi SAIDI[3] and Bouaziz SIFI[1]

[1]Institut National de la Recherche Agronomique de Tunisie.
[2]Institut National d'Agronomie de Tunis, Tunisie.
[3]Unité d'Utilisation Médicale et Agricole des Techniques Nucléaires, Centre National des Sciences et Technologie Nucléaires, Sidi Thabet, 2020 Tunisie.
[4]Faculté des Sciences de Bizerte, Tunisie.

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***RHIZOCTONIA SOLANI* is one of the most important soil-borne fungal pathogens that attack the roots of plant and causes significant damage to different plants particularly to chickpeas (*CICER ARIETINUM* L.). The aim of this work is to study the antagonistic activity of different *RHIZOBIUM* strains against *R. SOLANI* in dual culture *IN VITRO* and under greenhouse conditions. The benefits of rhizobial inoculant in nitrogen fixation, phosphorous uptake and on plant growth promoting were demonstrated with 42 *RHIZOBIUM* strains. Among the 42 strains tested, 24 isolates had effective control on *R. SOLANI IN VITRO*. In order to study the biological control mechanisms, the *RHIZOBIUM* strains ability to produce volatile compounds and to solubilise phosphate were investigated. The results showed that 10 strains were able to solubilise phosphorus and 13 strains produced volatile compounds. In pot trials, the percentage of chickpea plants inoculated with different rhizobia showed significant reduce of root rot symptoms compared to the control growing in uninoculated soil. Among these rhizobiums, the strain S27 proved efficient against the soil borne pathogen *IN VITRO* and in pot experiments. Our study suggested that inoculation with specific *RHIZOBIUM* exerts significant disease suppress against *R. SOLANI* in controlled conditions.**

Key words: *Rhizobium*, *Rhizoctonia solani,* chickpea, plant growth promotion, biological control.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is one of the most popular vegetables in many regions of the world. In Tunisia, the cultivated area and production have significant instability and decrease, the chickpea crop was affected by biotic and abiotic constraints (Kharrat et al., 1997). The major diseases affecting chickpea are *Ascochyta rabiei, Fusarium oxysporum* f. sp. *ciceri, Botrytis cinerea* and *Rhizoctonia solani* (Stanisław et al., 2004).

R. solani is a soil-borne fungal pathogen, which causes worldwide serious losses in many different agricultural crops (Domsch et al., 2007). *R. solani* strains are ubiquitous and cosmopolitan as saprophytes in soil and as plant pathogens attacking over 500 host species (Ogoshi, 1996). The pathogen is a species complex composed of different genetic or anastomosis group (AGs) with a distinct degree of host specificity (Schneider et al., 1997; Carling et al., 2002). Strategies to control *Rhizoctonia* diseases are limited because cultivars with complete resistance are not available at present (Li et al., 1995). Control of the pathogen is difficult because of its ecological behaviour; it is extremely broad host range and the high survival rate of sclerotia under various environmental conditions. For this reason, efficient strategies to control the pathogen are urgently required.

The chemical use was considered as the most effective method of controlling *R. solani*. The use of standard fungicides to control this pathogen has been tested by Meyer et al. (2006). Antagonism and biological control

*Corresponding author. E-mail: yassine.mabrouk@fst.rnu.tn.

agents for plant diseases are currently being examined as alternatives to synthetic pesticides due to their perceived level of safety and minimal environmental impacts. It has been developed successfully during the lasted decade. It was based on the reduction of inoculant or of pathogenic activity due to the natural presence of one or more organisms, through the management of the environment, the host or antagonists.

Several bacterial strains 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 (Nelson, 2004; Siddiqui, 2006). The use of antagonistic microorganisms such *Rhizobium* sp. to control the chickpea diseases has been reported (Arfaoui et al., 2006). As compared to the other biocontrol agents, rhizobia play an important role in legume plant nutrition through their ability of symbiotic nitrogen fixation and phosphorous solubilization (Peoples et al., 1995; Halder et al., 1990a, b). Among the *Rhizobium* group, *Rhizobium leguminosarum, Sinorhizobium meliloti,* and *Bradyrhizobium japonicum*, rhizobia are also reported to significantly inhibit the growth of pathogenic fungi, that is, *Macrophomina phaseolina* (Tassi) Gold, *R. solani* Kuhn and *Fusarium* sp., in both leguminous and non leguminous plants (Esteshamul-Haque and Ghaffar, 1993).

The attempts of the present study were the evaluation of symbiotic effectiveness of *Rhizobium* strains nodulating chickpea and the screening of the most promising bacteria antagonist award *R. solani in vitro* and *in vivo* trials.

MATERIALS AND METHODS

Plant material

The seeds of chickpea variety Beja1 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 chickpea plants using the crushed nodule method (Vincent, 1970). All isolates were purified and tested for their ability to form nodules on chickpea as previously described (Beck and Materon, 1993). Forty two isolates were collected from different localities (Table 1). These strains were grown at 28°C (Vincent, 1970) 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.

Fungal isolate

Fragments of chickpea roots obtained from plants that showed *R. solani* disease symptoms were submerged in 5% sodium hypochlorite for five minutes. After this treatment, they were extensively washed with sterile distilled water and placed on Petri dishes containing potato-dextrose-agar (PDA, Difco) and incubated at 22°C for 48 h. The isolated *R. solani* strains were identified (Rieuf, 1985) and stored at 5°C in tubes containing PDA.

Chickpea inoculation by *RHIZOBIUM* strains and plant yield parameters control

To assess rhizobia infectivity and effectiveness in fixing atmospheric N_2, each strain was gown on YEM liquid medium containing 0.08% yeast extract (w/v), 0.02% $MgSO_4$ (w/v), 0.01% Nacl (w/v), 0.05 KH_2PO_4 (w/v) and 1% mannitol (w/v). The bacterial isolates were grown to a logarithmic phase (10^9 cells ml^{-1}). The inoculants were used to inoculate germinated seeds of chickpea (Béja1 variety) in aseptic conditions. The seeds were soaked for 30 min in 2% calcium hypochlorite solution, washed 5 times with sterile distilled water, and germinated in the dark at 28°C. Four-day-old seedlings were inoculated (soaked in YEM liquid medium containing approximately 10^9 cells.ml^{-1} of each strains) and then transplanted in plastic pots containing sterilized sand. Sand had been sterilized three times for 1 h at 120°C. The experiment was statistically laid out with four replications and a completely randomized design. Plants were provided with an N-free nutrient solution every 3 days (Broughton and Dilworth, 1971). Plants were cultivated in a light: 8 h dark cycle and 28°C day: 20°C night temperature.

Nodule number and biomass of shoot were recoded 45 days after planting .Shoot was dried at 60°C for 3 days. Plant nitrogen content was measured according to khjeldahl method (Parkinson and Allen, 1975). Phosphorous uptake by chickpea plants was measured according to Nitrovanadomolibdate method (Fleury and Leclerc, 1943).

Inhibition of mycelia growth

The growth inhibition of *R. solani* mycelium by the *Rhizobium* strains was tested *in vitro* using the dual culture technique as described by Landa et al. (1997). Three drops (50 µl) from the 10^8 cells ml^{-1} rhizobia suspension were equidistantly placed on the margins of potato dextrose agar (PDA) plates and incubated at 28°C for 24 h. A disc (Φ: 4 mm) from fresh PDA cultures of *R. solani* was placed at the centre of the PDA plate for each bacterial isolate and incubated at 25±1°C for seven days. The radius of the fungal colony towards and away from the bacterial colony was measured. Percent growth inhibition of *R. solani* after 7days was calculated using the formula of Whips (1987):

$$\% \text{ Inhibition} = (R-r) / R * 100$$

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

Volatile antifungal compounds and phosphate solubilization

The production of volatile antifungal compounds by the *Rhizobium* isolates was assayed by a sealed plate method as described by Fiddman and Rossal (1993). From the rhizobium culture in Yeast Extract Mannitol liquid media (Vincent, 1970) 72 h old, 200 µl were spread in a Petri dish on YEMA medium prepared as described above adding 1.5% Agar before autoclaving. After incubation at 37°C for 24 h, a second Petri dish containing PDA was inoculated with a plug (Φ: 6 mm) of the test fungus in the centre of the plate, inverted and placed over the bacterial culture. Each two plates,

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

Reference	Strains name	Localities	Years	Genotypic groups
S 1	Raiess5	Jendouba -Tunisie	2002	*Mesorhizobium ciceri*
S 2	M.Tmim1	Nabeul - Tunisie	1992	*Mesorhizobium ciceri*
S 3	Exirat	Nabeul - Tunisie	2002	*Mesorhizobium Loti*
S 4	M.Bou1	Bizerte - Tunisie	1992	*Mesor. Mediterranium*
S 5	Raiess7	Jendouba -Tunisie	2002	*Mesorhizobium Loti*
S 6	M.Tmim2	Nabeul - Tunisie	1992	*Mesor. Mediterranium*
S 7	Rah2	Nabeul - Tunisie	2002	*Mesorhizobium ciceri*
S 8	Mateur	Bizerte - Tunisie	2002	*Mesorhizobium ciceri*
S 9	Abidi	Siliana - Tunisie	2002	*Mesorhizobium ciceri*
S 10	Korba	Nabeul - Tunisie	2002	*Mesor. Mediterranium*
S 11	Av.fer2	Bizerte - Tunisie	2002	ND
S 12	Rah1	Nabeul - Tunisie	2002	*Mesorhizobium ciceri*
S 13	Test1	Béjà - Tunisie	1992	*Mesorhizobium ciceri*
S 14	Klibia	Nabeul - Tunisie	1998	ND
S 15	Raiess1	Jendouba -Tunisie	2002	*Mesorhizobium ciceri*
S 16	Mornag1	Ariana - Tunisie	1992	*Mesorhizobium ciceri*
S 17	Sidi.N2	Béjà - Tunisie	2002	*Mesorhizobium ciceri*
S 18	Test2	Béjà - Tunisie	1992	*Mesorhizobium ciceri*
S 19	SOM	Maroc	1988	*Mesor. Mediterranium*
S 20	Bouf3	Sousse - Tunisie	2002	*Mesorhizobium ciceri*
S 21	B. kh1	El kef - Tunisia	2002	*Mesorhizobium ciceri*
S 22	Sidi.N1	Béjà - Tunisie	2002	*Mesorhizobium ciceri*
S 23	Elwa1	Siliana - Tunisie	2002	*Mesorhizobium ciceri*
S 24	Elwa2	Siliana - Tunisie	1998	*Mesorhizobium ciceri*
S 25	Mornag2	Ariana - Tunisie	1992	*Mesorhizobium ciceri*
S 26	Béja	Béjà - Tunisie	2002	ND
S 27	Azmour	Nabeul - Tunisie	2002	*Mesorhizobium ciceri*
S 28	Kassar2	Béjà - Tunisie	1992	*Mesorhizobium ciceri*
S 29	Pch43	ICARDA -Syria	1988	ND
S 30	Om dhwill2	Nabeul - Tunisie	1996	*Mesorhizobium ciceri*
S 31	Ferm1	Bizerte - Tunisie	2002	*Mesorhizobium ciceri*
S 32	Pch35T	INRA Montpellier-Franc	1989	ND
S 33	Raiss 2	Jendouba -Tunisie	2002	*Mesorhizobium ciceri*
S 34	DMS	CIRAD Montpellier	1989	ND
S 35	Ferm2	Bizerte - Tunisie	2002	*Mesorhizobium ciceri*
S 36	Bouf2	Sousse - Tunisie	1998	*Mesorhizobium ciceri*
S 37	BelliNab	Nabeul - Tunisie	1998	*Mesor. Mediterranium*
S 38	Raiess6	Jendouba -Tunisie	2002	*Mesorhizobium ciceri*
S 39	Raiess4	Jendouba -Tunisie	2002	*Mesorhizobium ciceri*
S 40	Elwa 3	Siliana - Tunisie	2002	*Mesorhizobium Loti*
S 41	Raiess3	Jendouba -Tunisie	2002	*Mesorhizobium ciceri*
S 42	Ine sol tane	Béjà - Tunisie	2002	*Mesorhizobium ciceri*

ND= not determined.

containing pathogen and bacteria, were sealed together with Para film 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 there radial length after 5 days. Each test was replicated 3 times. The ability of rhizobium to solubilize inorganic phosphate was evaluated by using the Pikovskaya (1948) medium. Dicalcium phosphate agar plates were incubated with 24 h bacterial cultures and incubated at 28°C for 5 days. The colonies forming clarification halos were considered as phosphate solubilizers.

Germination of chickpea seeds inoculated with *RHIZOBIUM* and *RHIZOCTONIA SOLANI*

Chickpea seeds were surface sterilized with 2% sodium hypochlorite and washed five times with distilled sterile water. The seeds were thoroughly soaked in the bacterial suspension (10^8 cells ml^{-1}) to ensure uniform coating of the surface. These seeds were inoculated with 1 g of crushed fragment infected by *R. solani*. The seeds were aseptically plated in PDA medium. The plates were incubated at 27 ±1°C for 7 days. After germination, the number of necrosis root was measured.

Rhizobia antagonism to fungal pathogenesis under greenhouse conditions

R. solani inoculant production with oat seeds (120 g) were mixed with distilled water (200 ml) in polyethylene bag and autoclaved during 15 min at 120°C. Each bag was subsequently inoculated with 5 discs (Φ: 4 mm) deducted from a fresh PDA culture of *R. solani*. The seeds inoculant was incubated at 25 ± 1°C for 7 days. Bacterial inoculant was prepared in 250 ml flasks containing YEM. After inoculation with bacteria, the flasks were incubated on a rotator shaker at 150 rpm at 28°C during 72 h. Bacterial concentration was adjusted to 10^8 cells ml^{-1} (OD_{620} 0.8 to 0.9) before to be used as inoculant. *R. solani* isolate pathogenesis of chickpea was confirmed under glass house conditions. Control trials was realised in pot containing sterile medium and seeds contaminated with the pathogen. Fungus specific symptoms were observed as the rotting of seeds, root rot of seedlings, and later necrosis of root collar and suberization of infested tissues.

Chickpea inoculation with fungal and *RHIZOBIUM* isolates

All bacterial isolates which induced more than 50% inhibition of *R.solani* mycelia growth *in vitro* assay were selected for the *in vivo* evaluation in the glass house. Chickpea seeds surface were sterilized with 2% sodium hypochlorite for 1 min and rinsed five times with sterile distilled water. The seeds were then pre-germinated in sterile vermiculite for three days at 30°C in the glass house. Prior to seedling transplanting, the oat seeds inoculant of *R. solani* was mixed into sterile soil in plastic pots. For the control treatments, autoclaved oat seeds inoculated with the pathogen were mixed into the soil at the same rate. Two chickpea seedlings were transplanted into each pot and the pots were maintained in the greenhouse at 25±2°C. After transplanting of seedlings, each pot was drenched with 5 ml of each of the bacterial inoculants (10^8 cells ml^{-1}). The treatments biocontrol experiments *in vivo* were: Plants inoculated with *R. solani* and rhizobia, Plants inoculated with *R. solani* (control a) and a non-inoculated control (Control b). The non-inoculated control was treated with sterile oat seed without fungal and bacterial inoculant. Plants were watered as needed. All the treatment *in vitro* and *in vivo* experiments were arranged in a randomized block design in three replications.

RHIZOBIUM* antagonism towards *RHIZOCTONIA SOLANI IN VIVO

Every lot of 4 chickpea plants (Béja1 variety) was inoculated with one *Rhizobium* strain. After planting, plants were inoculated with *R. solani* at level of 15 g of contained oat /kg of sterile soil. The virulence of pathogen was evaluated at a scale Indices from 0 to 4 according Tezcan and Yildiz (1991). The recorder data were based on mass disease index (MDI %) according to the formula:

$$MDI(\%) = \left[\frac{\sum_1^4 N\_xI}{4N} x100\right]$$

N: number of total plants; n: number of plant with Indices I.

Statistical analysis

All experiments were replicated as completely randomised blocks. Consequently, the data are means ± confidence interval (n=3, α =0.05). Statistical analysis (ANOVA) was performed with SPSS 10.0 for Windows, followed by Duncan's multiple range test (P=0.05).

RESULTS

Effect of *RHIZOBIUM* strains on chickpea nutrient uptake and yield

Chickpea *Rhizobium* strains showed great difference in their capacity to infect the host plant and to fix atmospheric nitrogen. The mean nodule number per plant varied from 12 with S34 strain to 63 with S27, which is the more infective strain (Table 2). The data indicate a significant increase of most growth parameters of chickpea plants in this study, when the soil is inoculated with different *Rhizobium* strains compared to the control soil without inoculation (Table 2).

The highest values of controlled growth parameter were obtained with plants growing in soil inoculated with S27 strain compared to the soil without inoculation and the other treatments. Chickpea plants dry weight was significantly increased in a 158% (p: 0, 05) when the soil was inoculated with S27 compared to the control. Inoculation of chickpea by *Rhizobium* increase significantly N and P uptake compared to the control. Highest N (2.016 mg/g D.W) and P (0.352 µmol/g D.W) contents were recorded in plant inoculated with S27 strain.

Nitrogen content in shoot showed an important variation among plants inoculated with different *Rhizobium* strains (Table 2). The nitrogen content in plant inoculated with S27 strain was increased to 160% compared to the plant control. Thus, the results that the chickpea plants inoculated with S27 strain have a significantly higher nodule number, dry matter, phosphorus and nitrogen content compared to plants growing in uninoculated soil. It can be concluded from these results that if *Rhizobium* is used as microbial inoculants, nodulation is improved as well as N and P uptake by chickpea and hence the yields are also increased.

Rhizobium antagonism toward *RHIZOCTONIA SOLANI IN VITRO*

Among the 42 *Rhizobium* strains tested in dual culture

Table 2. Effect of inoculation of soil with *Rhizobium* on chickpea plants growth, nitrogen and Phosphorous contents.

Treatments	Nodule number	Dry weight (mg)	Total N/plant (mg)	Total P(µmol)/g MS
T	0.00 ± 0.00	823.40 ± 21.84	1.00 ± 0.18	0.027 ± 0.06
S2	25.00 ± 1.91	767.67 ± 45.82	1.28 ± 0.13	0.217 ± 0.03
S3	28.00 ± 2.16	834.67 ± 47.33	1.73 ± 0.03	0.191 ± 0.02
S4	32.00 ± 5.12	1301.00 ± 78.89	1.01 ± 0.04	0.271 ± 0.01
S5	33.33 ± 3.24	1163.00 ± 75.40	1.82 ± 0.05	0.271 ± 0.09
S6	33.67 ± 2.77	1138.67 ± 75.80	1.84 ± 0.03	0.244 ± 0.04
S1	24.00 ± 2.16	1130.67 ± 58.05	1.87 ± 0.08	0.163 ± 0.02
S7	35.00 ± 1.67	1310.00 ± 55.82	1.45 ± 0.41	0.191 ± 0.06
S8	35.33 ± 1.33	1227.67 ± 88.89	1.96 ± 0.03	0.163 ± 0.05
S9	35.67 ± 1.25	1100.33 ± 59.76	1.87 ± 0.03	0.271 ± 0.01
S10	37.00 ± 1.35	1222.67 ± 78.01	1.01 ± 0.04	0.217 ± 0.06
S11	37.67 ± 2.77	1116.67 ± 45.40	1.45 ± 0.02	0.271 ± 0.01
S12	39.00 ± 1.25	892.33 ± 59.22	1.59 ± 0.01	0.271 ± 0.12
S13	39.00 ± 3.22	950.00 ± 43.45	1.65 ± 0.04	0.271 ± 0.14
S14	39.67 ± 1.09	1121.33 ± 78.09	1.93 ± 0.03	0.290 ± 0.03
S15	40.67 ± 1.87	867.33 ± 44.09	1.42 ± 0.41	0.191 ± 0.14
S16	41.00 ± 1.91	1365.67 ± 47.33	1.62 ± 0.22	0.298 ± 0.12
S17	41.33 ± 5.25	1260.33 ± 23.56	1.01 ± 0.01	0.191 ± 0.01
S18	42.67 ± 1.22	1218.67 ± 22.12	1.01 ± 0.02	0.271 ± 0.02
S19	42.67 ± 2.25	1189.67 ± 44.09	1.76 ± 0.24	0.298 ± 0.15
S20	43.33 ± 1.91	1248.00 ± 47.54	1.98 ± 0.01	0.271 ± 0.03
S21	45.00 ± 3.25	1003.50 ± 32.62	1.01 ± 0.01	0.217 ± 0.02
S22	46.00 ± 1.87	1366.33 ± 45.32	1.01 ± 0.01	0.244 ± 0.08
S23	48.00 ± 1.25	1033.33 ± 11.98	1.59 ± 0.41	0.244 ± 0.02
S24	48.67 ± 7.24	1064.33 ± 15.07	1.65 ± 0.32	0.244 ± 0.12
S25	48.67 ± 5.67	1360.00 ± 43.37	1.54 ± 0.23	0.244 ± 0.04
S26	56.33 ± 8.16	1432.33 ± 25.21	1.87 ± 0.01	0.298 ± 0.0 6
S27	63.67 ± 5.24	2079.33 ± 21.23	2.01 ± 0.02	0.352 ± 0.02
S28	36.08 ± 6.43	1332.21 ± 14.23	1.08 ± 0.05	0.187 ± 0.01
S29	32.56 ± 3.35	1354.54 ± 23.21	1.05 ± 0.03	0.201 ± 0.06
S30	33.67 ± 1.54	1243.76 ± 11.65	1.98 ± 0.21	0.191 ± 0.01
S31	32.21 ± 2.87	1221.87 ± 12.33	1.43 ± 0.32	0.143 ± 0.05
S32	35.54 ± 4.14	1154.07 ± 34.21	1.76 ± 0.43	0.204 ± 0.03
S33	34.32 ± 6.57	1254.04 ± 32.34	1.34 ± 0.23	0.156 ± 0.67
S34	12.01 ± 1.54	965.92 ± 13.67	1.56 ± 0.54	0.103 ± 0.05
S35	32.45 ± 5.23	1235.21 ± 11.56	1.76 ± 0.13	0.189 ± 0.02
S36	42.21 ± 6.21	1124.11 ± 21.12	1.32 ± 0.21	0.123 ± 0.01
S37	34.21 ± 5.32	1243.12 ± 23.54	1.23 ± 0.12	0.204 ± 0.03
S38	35.78 ± 4.35	1233.12 ± 11.34	1.21 ± 0.45	0.243 ± 0.01
S39	32.56 ± 5.34	1165.21 ± 11.78	1.11 ± 0.04	0.143 ± 0.02
S40	41.56 ± 3.12	1243.41 ± 14.54	1.45 ± 0.02	0.207 ± 0.05
S41	32.63 ± 4.12	1432.31 ± 13.12	1.28 ± 0.01	0.301 ± 0.01
S42	63.33 ± 4.32	1756.33 ± 21.54	1.96 ± 0.32	0.271 ± 0.04

Each value is a mean of 3 replicates.

with *R. solani*, 27 inhibited the fungus growth (Table 3) and reduce their development more than 50%. Isolates S2, S1, S42 and S17 were the most preferment *in vitro* and caused growth inhibition of *R. solani* more than 70%. 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 (Table 3) revealed that the inhibition was highly significant (P= 0.05).

No physical contact was observed between any of the

Table 3. Effect of *Rhizobium* isolates on of *R. solani* growth *in vitro* and determination of the ability of the most effective bacterial isolates in dual culture to solubilise phosphate and to produce volatiles.

Treatments	% Growth Inhibition(+)	% Inhibition of fungal growth by volatiles	Phosphate solubilization
S12	41.65 ± 2.10	0.00 ± 0.00	+
S4	46.60 ± 1.75*	0.00 ± 0.00	+
S20	47.17 ± 3.33*	0.00 ± 0.00	+
S28	50.50 ± 3.80*	49.25 ± 1.75*	-
S27	50.52 ± 2.81*	38.25 ± 2.81*	+
S29	51.62 ± 2.80*	32.50 ± 3.80*	-
S30	52.17 ± 2.87*	37.50 ± 2.84*	+
S31	52.17 ± 2.87*	0.00 ± 0.00	-
S32	52.75 ± 1.79*	25.75 ± 1.84*	-
S33	52.75 ± 2.10*	0.00± 0.00	-
S34	53.27 ± 1.81*	38.25 ± 2.87*	+
S35	53.30 ± 1.79*	0.00± 0.00	-
S36	54.40 ± 1.27*	40.75 ± 1.54*	-
S22	54.40 ± 2.84*	0.00± 0.00	+
S37	52.20 ± 1.27*	0.00± 0.00	-
S38	57.72 ± 1.83*	25.00 ± 1.23*	-
S10	57.75 ± 1.79*	25.75 ± 2.87*	+
S16	58.85 ± 1.98*	43.25 ± 3.3*	+
S39	63.85 ± 3.30*	25.00 ± 1.43*	-
S15	67.70 ± 1.27*	37.50 ± 1.54*	+
S40	68.27 ± 1.81*	0.00± 0.00	-
S3	69.37 ± 2.89*	37.50 ±2.89*	-
S41	69.95 ± 2.89*	0.00± 0.00	-
S17	76.15 ± 1.93*	35.75 ± 1.93*	+
S42	77.72 ± 1.83*	40.75 ± 3.3*	-
S1	77.72 ± 2.84*	42.5 ± 2.89*	+
S2	79.95 ± 1.83*	0.00± 0.00	+
Control	0.00	0.000	-

(+) = Percent growth inhibition compared to uninoculated control was determined after 7 days of incubation using Whipps' (1987) formula. Each value is a mean of 3 replicates. Mean values followed by * were significant (P= 0.05), compared to the control, by Duncan's multiple range test.

antagonistic bacteria tested and *R. solani*; moreover, an inhibitory halo was observed suggesting the presence of fungistatic metabolites secreted by the bacteria. On the other hand, a change in mycelial colour was observed closed to the colony end of *R. solani*, being this one of a darker brown than the one observed at the center of colony (Figure 1). Microscopy observation of this zone, allowed detecting the cytoplasmic leakage that could be observed up to the hyphal septum, resulting in deformation and sliming of their apex up to 1/7 of its original size. Similar results were obtained by Montealegro et al. (2003).

RHIZOBIUM volatile substance effect on *RHIZOCTONIA SOLANI* growth *IN VITRO*

The results of the effect of *Rhizobium* volatiles substance on the pathogen growth (Table 3) indicate that among 24 isolates tested for volatiles activity; only 16 isolates were able to reduce the growth of the pathogen. The isolates S28, S16, S1, S36, and S42 are the most effective, inducing more than 40% of inhibition of fungus growth after 5 days of incubation.

RHIZOBIUM solubilization of inorganic phosphate

Most tested *Rhizobium* isolates were able to solubilize phosphate. Fourteen isolates produced a halo on dicalcium phosphate agar media plates (Table 3). Eight isolates, S27, S30, S34, S10, S16, S15, S17 and S1 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.

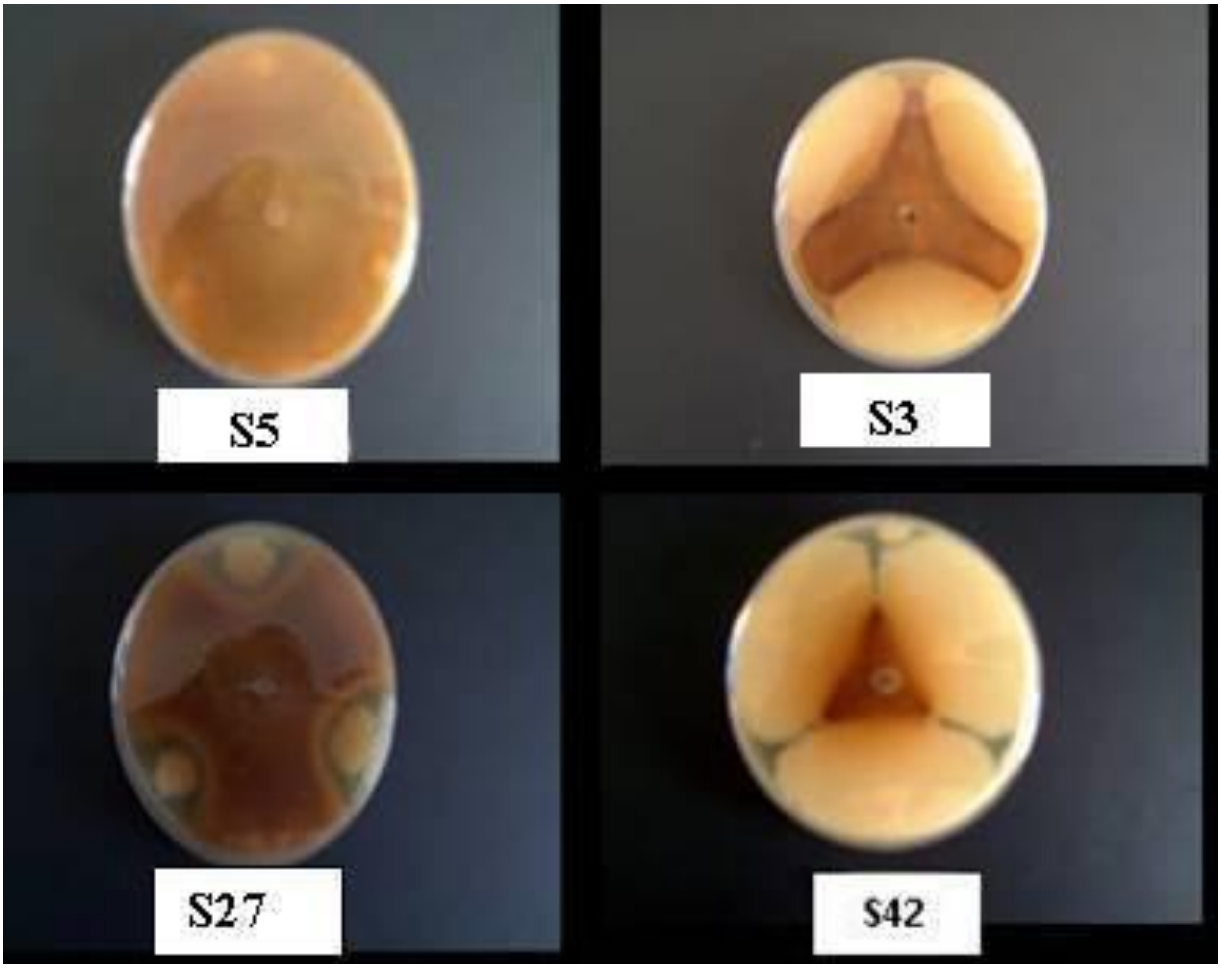

Figure 1. Dual culture of selected *Rhizobium* and *R. solani in vitro* showing variable inhibition of mycelium growth among the used strain and formation of visible clearly hallo on the plate with bacterial isolate S27 compared to the plate with S5 strain which resulted in no inhibition zones.

Antagonism of *RHIZOBIUM* towards *R. SOLANI IN VITRO*

Rhizobia isolates have a variable and significant effect on germination of chickpea seeds inoculated with *R. solani*. Germination of seeds contaminated with the pathogen was significantly ameliorated in presence of some *Rhizobium* strains and the percentage of germination was varied from 40 to 90 % (Figure 2). These rhizobia improve the chickpea seeds germination and reduce the necrotic root induced by the pathogen. The highest significant differences were obtained with inoculation by S33, S38 and S2 strains. Chickpea seeds germination were ameliorate and there root necrotic was reduced by 80%.

Antagonism of *RHIZOBIUM* towards *RHIZOCTONIA SOLANI IN VIVO*

To study the rhizobia antagonism activities, the previous trial was replicate in pot under glass house conditions.

The results indicate that S29, S17, S16, S15 and S39 isolates inhibited significantly crown rot of chickpea caused by *R. solani* (Figure 3). Mass Disease expression MDI (%) was calculated according to Tezcan and Yildiz (1991) methodology. These treated plants looked healthy showing no symptoms of crown rot. Inoculation with S27 and S17 isolates induced suppression of root rot more than 80%, while inoculation with S16, S15, S39, S38 isolates reduced the disease more than 60% (Figure 3).

In these tests, no bacteria protected the plants completely against *R. solani*, although all isolates increase significantly in fresh weight compared to the infested control. Plants inoculated with antagonistic *Rhizobium* induced a reduction on number of diseased plants when it's grown in soil artificially inoculated with *R solani* (Figure 3). The inhibition of the fungus growth by some of Rhizobium isolates are varied from 60 to 87% (Figure 3).

Control plants not treated with bacteria but inoculated with *R. solani* alone rendered up to 100% root rot incidence with the majority of plant completely stunted or

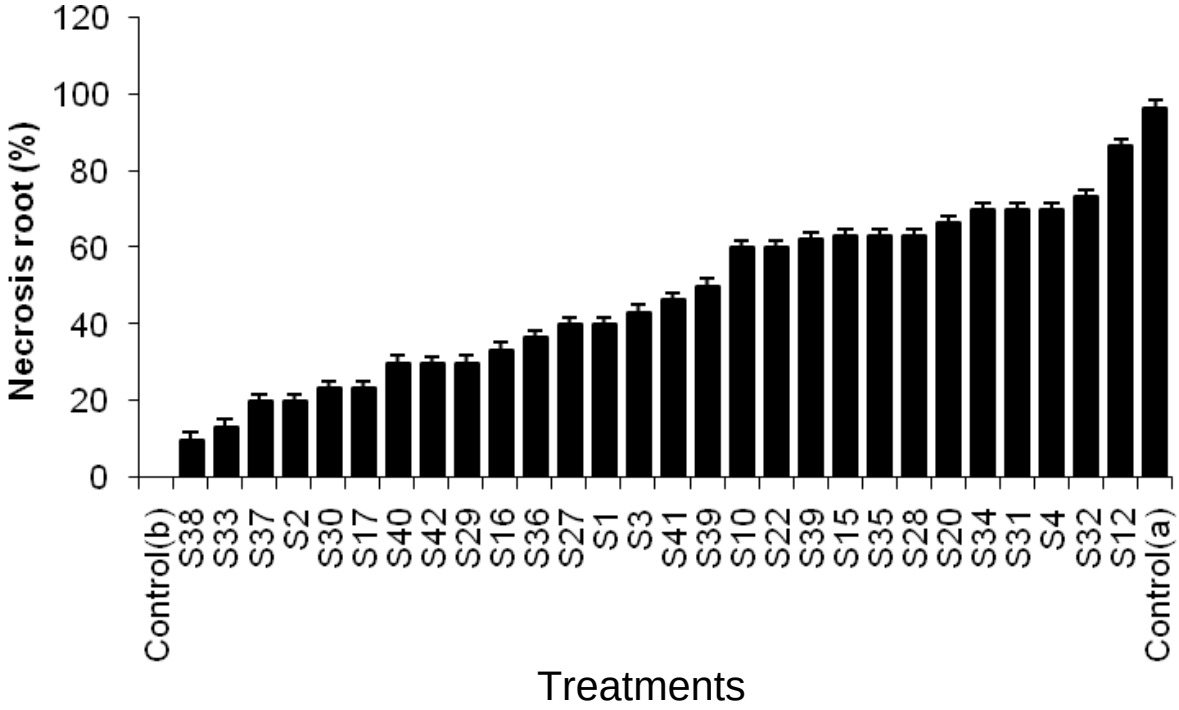

Figure 2. Antagonism of *Rhizobium* towards *R. solani* estimated by the percentage of necrotic roots of chickpea seeds inoculated by the pathogen and different *Rhizobium* strains *in vitro*. Each value is a mean of 3 replicates. (Control (b) represents un-inoculated chickpea plants and control (a) plants infested with *R.solani*).

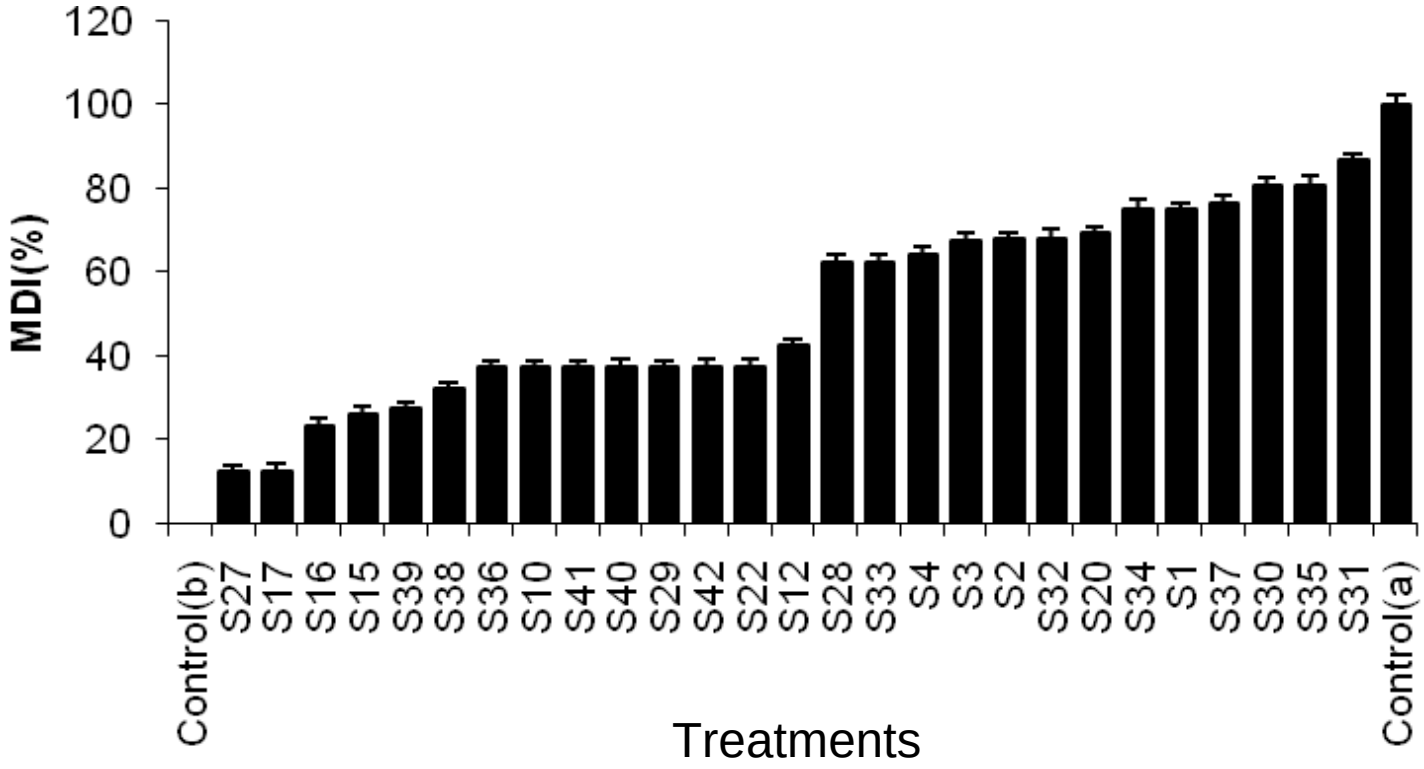

Figure 3. Antagonism of *Rhizobium* towards *R. solani* calculated according MDI (%) of necrotic roots of chickpea seeds inoculated by the pathogen and different *Rhizobium* strains *in vivo* under greenhouse conditions. (Control (b) represents un-inoculated chickpea plants and control (a) plants infested with *R. solani*).

Table 4. Effect of inoculation by *Rhizobium* strains on growth parameters of chickpea infected with *R. solani* under glass house conditions 8 weeks after sowing.

Treatments	Nodules number	Shoot height (cm)	Shoot dry weight (g)
S12	8	32.33 ± 1.15*	7.01 ± 0.40
S4	10	34.33 ± 0.57*	9.26 ± 0.14*
S20	15	35.00 ± 1.15 *	10.63 ± 0.20*
S37	5	21.67 ± 0.57	3.57 ± 0.14
R. solani	0	22.67 ± 0.57	3.62 ± 0.15
S31	10	28.67 ± 0.57	6.13 ± 0.76
S22	12	31.00 ± 0.57	8.74 ± 0.31*
S28	11	33.00 ± 0.57*	8.22 ± 0.45*
S1	15	33.00 ± 0.57*	5.10 ± 0.21
S30	9	33.33 ± 0.57*	5.41 ± 0.40
S39	15	34.00 ± 0.57*	10.07 ± 0.09*
S35	10	34.67 ± 0.57*	8.76 ± 0.17*
S15	19*	35.33 ± 0.57*	10.04 ± 0.55*
S38	13	35.33 ± 1.15 *	10.81 ± 0.17*
S33	8	35.33 ± 1.54*	8.59 ± 0.09*
S34	7	35.67 ± 0.57*	5.84 ± 0.31
S3	12	36.00 ± 0.57*	9.83 ± 0.76*
S32	7	36.33 ± 1.00*	7.01 ± 0.76
S41	11	36.33 ± 0.57*	8.26 ± 0.21*
S2	12	36.67 ± 0.57*	8.92 ± 0.21*
S16	13	38.67 ± 0.57*	9.86 ± 0.17*
S40	12	39.00 ± 0.57*	8.68 ± 0.31*
S10	20*	39.00 ± 0.57 *	11.02 ± 0.40*
S42	13	40.00 ± 0.57*	9.19 ± 0.55*
Control	0	41.00 ± 0.57*	8.76 ± 0.58*
S29	12	41.33 ± 0.57*	9.16 ± 0.20*
S36	14	42.67 ± 0.57*	11.36 ± 0.21*
S17	22*	43.00 ± 1.00*	10 .46 ± 0.55*
S27	25*	45.33 ± 0.15*	11.53 ± 0.45*

Each value is a mean of 3 replicates. Mean values followed by * were significantly different (P=0.05), compared to the positive control, by Duncan's multiple range test.

dead.

The fungus resulted in a pronounced decrease in the dry weight of the shoots compared to the uninoculated control and to some of the treatments with the most effective bacteria isolates. Shoots dry weight reduction was about 58, 64% in the plant control inoculated with *R. solani* alone. Whereas, shoot dry weight reduction, of plants inoculated with both the pathogen and *Rhizobium* strain S32 in was 20% recorded. However, a 33% increase of shoot dry weight was recorded with plant inoculated by strain S27, one of the effective isolates that prevented root and crown rot in this study (Table 4). Percentage of chickpea plants roots showing *R. solani* symptoms in the various treatments recorded a reduction of the fungus ranging variable from 60 to 87% related to the used isolates (Figure 3).

At flowering stage, plants none inoculated with *Rhizobium*, whether or not inoculated with *R. solani* showed no nodulation (Table 4). Inoculation with different *Rhizobium* strains induces a variable nodules formation in chickpea cultivars Béja1 (INRAT93-1). The three *Rhizobium* strains S27, S17 and S10 had increased significantly the nodules number in presence of *R. Solani.*

DISCUSSION

The aim of this study was the screening and selection of *Rhizobium* promoting the chickpea plants growth and determinate their antagonist potential against R*. solani* which caused root rot in chickpea. The beneficial effects of inoculation with rhizobia on nitrogen fixation, phosphate solubilising, plants nodulation, nitrogen content and grains legumes yield significant increase, have been reported by many investigators (Dubey, 1996; Khan et al., 1997). In our investigation, all tested strains were able to infect their host plant and to fix atmospheric N_2, leading

to more plant shoot production than in the control. Efficient strains (S27 and S26) can induce an increase in dry biomass production more than 50%, could be used in field inoculation trials. Rupela and Beck (1990) have interlined the beneficial effects of inoculation with selected rhizobia strains on chickpea yield, especially when it was cultivated on poor soils or those lacking specific rhizobia. In fact, beside the fixation of nitrogen, rhizobia are reported to produce plant growth regulators such as auxins, cytokinins and gibberellins like substances that stimulate and enhance plant growth (Sheng, 1993). Several other workers have noticed the beneficial effects of rhizobia on plant growth and reduction of diseases incidence (Hussain and Ghaffar, 1990). Since, the rhizosphere provides front line defence for roots against attack by pathogens, the rhizobia present in the rhizosphere are ideal for use as biocontrol agents.

The control of soil- borne pathogens is difficult because of their ecological behaviour, their extremely broad host range and the high survival rate of resistant forms such as chlamydospores and sclerotia under different environmental conditions. Many researchers have mentioned that biological control offers an environmentally friendly alternative to protect plants from soil borne pathogens (Whipps, 2001; Weller et al., 2002).

To find new biocontrol agents antagonistic to the soil-borne fungus (*R*. *solani*), 42 *Rhizobium* strains were evaluated using a combination of different screening steps. As a result of the primary screening in the plate assay, some bacterial isolates were found to be highly inhibitory of *R. solani* growth, whereas others showed only mild activity or no activity. This result is in agreement with Tjamos et al. (2004).

Reduction of fungal growth *in vitro* by some rhizobia and formation of inhibition zones were presumably due to the metabolites released by the bacteria into the culture medium. Chakraborty and Purkayastha (1984) reported that rhizobia produce toxic metabolites which have inhibitory effect on soil-borne plant pathogens.

In the dual culture assay, some of the isolates not only inhibited the mycelium growth but also changed the appearance of the mycelium from fate brown to dark brown as was evident for isolate S3 (Figure 1). This suggests that the fungal mycelia might have been inhibitted not only by antibiosis but also by other antifungal metabolites such as siderophores, hydrogen ions and gaseous products including ethylene, hydrogen cyanide and ammonia (Williams and Asher, 1996; Kumar et al., 2002). For some strains that caused prominent inhibition of fungal growth in the dual culture experiment, the inhibition zone formed was of such size that there was no physical contact with the pathogens (Figure 1),suggesting that the rhizobium could be producing certain antifungal metabolites (AFMs) (Montealegre et al., 2003). Moreover, as the PDA medium used for the dual culture assay is rich in nutrient, competition might be excluded as the mode of action for these isolates (Landa et al., 1997).

Different studies have implicated antifungal secondary metabolites produced by *Rhizobium* spp. in the control of plant disease caused by pathogenic fungi (Perdomo et al., 1995; Siddiqui et al., 2000). Further-more, the efficacy of a given biological control agent mostly results, not only from a single mechanism but from a combination of different modes of actions (Alabouvette et al., 1993). Our experiments revealed the effectiveness of the bacterial S17, S36 and S27. These significantly reduced the percentage of attacked plants. Based on *in vitro* dual culture experiments, most of this isolates were not the most effective. Despite their high effectiveness *in vitro*, isolates S2, S1 and S42 were partially ineffective under glass house conditions. According to Chérif et al. (2002) our results suggest that antagonistic micro-organisms performing best *in vivo* are not necessarily the most effective *in vitro*. Ownley et al. (2003) have indicated that such differences result from variability in the physical and chemical proprieties within niches occupied by bio-control agents which is turn affect both colonization and expression of bio-control mechanisms. This is particularly exemplified by *Rhizobium* strain S30, which caused more than 50% fungal growth inhibition in dual culture, produced volatiles and positive for phosphate solubilisation, but showed no effectiveness *in vitro* under glass house conditions. By contrast, the isolates S29 and S27 performed better *in vivo* than *in vitro*, resulting in giving the best levels of disease control under green-house conditions. These isolates were also effective in promoting chickpea growth, increasing shoot dry weights compared to the control. 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 (Algawadi and Gaur, 1988).

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.

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